MAKING SENSE OF MIXTURES:

CHROMATOGRAPHIC SEPARATIONS

OF PLANT, INSECT AND MICROBIAL

BIOMOLECULES

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Acknowledgements

CONTENTS

1.   Introduction

Most studies aimed at determining the structure of natural products begins with a mixture of substances from which the compound of interest must be purified. Chromatography, in its various forms, is used primarily for the separation of classes of compounds and, in a more discriminating way, for the resolution of individual compounds in a mixture. The central theme of the research described here concerns the chromatographic separation of biomolecules from a mixture, and their subsequent identification by other means, usually mass spectrometry.

Volatile compounds are often best separated by gas chromatography (GC), and this technique is used in most of the studies. With this technique, the resolution of compounds takes place in a column, and in the earlier studies, the compound had to be physically collected while eluting from the column for subsequent mass spectral analysis of its structure. With the use of combined gas chromatography-mass spectrometry (GC-MS) in most of the later structural studies described, no actual collection of resolved compounds is needed. With this technique, many more compounds can be separated and identified in much less time.

As many natural products are chiral, and as the biological activity or usefulness to synthetic chemists of a molecule is often limited to only one of a pair of enantiomers, recent work has concentrated on the resolution of certain enantiomers by gas chromatography and high performance liquid chromatography (HPLC).

The list of publications is numbered and given in chronological order, and each copy of each publication has this number on its title page for easy reference. This presentation generally follows the same sequence but is divided into sections with each section emphasizing the publications arising from a period in a department at a particular institution. An attempt is made to group the papers on similar topics within each period, and in each section the main points of interest and most important research results are emphasized under 'Highlights'. In this way it is hoped that a reader will be able to follow the presentation in a meaningful manner reading only this description of the work, together with the abstracts of the papers.

2. Department of Biochemistry
University of Natal (Pmb)
1962 - 1970

2.1 Grass Lignin

My Masters Degree studies began at the beginning of 1962. An existing departmental project concerned the digestibility of grass hay in an artificial rumen. Observed variations in the *in vitro* digestibility of certain grass species were thought to be due in part to the solubility and structure of the lignin. A project aimed at attempting to assess the organic solvent solubility of lignin isolated from various grass species was chosen for a thesis topic. Other studies on the solubility of lignin had used conifer wood as the source of lignified material, so initial studies were conducted on a good quality veld hay comprised largely of *Themeda triandra*. In spite of reported differences in the monomeric components of the hardwood, conifer, and grass lignin, the solubility properties of isolated soda lignins from these plant materials, as well as the veld hay, were remarkably similar (1,2).

Any comparative study on the structural similarities and differences of lignin from various grass species would need to determine the ratio of the various monomers with their differing substitution patterns on the aromatic ring. This broad topic was chosen for a PhD program early in 1963 and it was recognised that it would require the use of the latest and most appropriate separation techniques available. The Organic Chemistry department, whose advice was sought, used adsorption column chromatography as their main separation technique for their natural product research.

It also was realised that one would need to determine quantitatively various compounds in a mixture and adsorption column chromatography did not seem capable of offering the required resolution. At that time no one in the Biochemistry department, or the University as a whole, had ever used thin layer chromatography (TLC), and gas chromatography was only just becoming commercially available, but no department had one.

It was well established that oxidation of conifer and hardwood lignin with either alkaline nitrobenzene or alkaline cupric hydroxide gave rise to the aromatic aldehydes *p*-hydroxybenzaldehyde (P), vanillin (V), and syringaldehyde (S).

Therefore, initial studies aimed at the determination of the monomeric ratio of the three aromatic aldehydes from grass lignin began with the development of a low-cost TLC method for the separation and quantitative estimation of the standard compounds, as no published method had been reported.

No apparatus for the spreading of adsorbent on TLC plates was available, and a commercially available one was beyond the budget of the department. Therefore, a home-made plate-spreading apparatus was designed and constructed for the preparation of TLC plates on pre-cut plate glass sheets (8" x 8" and 2" x 8"). Many plates were coated and many solvent systems tried before a very successful system was obtained (3, see Fig. 1).

Soon after the development of a suitable TLC method a group in Canada published the first GC method for the quantitative determination of P, V, and S from the alkaline nitrobenzene oxidation of lignin. Motivations were therefore submitted for the purchase of a GC and an isothermal one was obtained in 1964, which was upgraded to be temperature programmed in 1965.

No suitable apparatus was available for either the alkaline nitrobenzene or alkaline cupric hydroxide oxidation of lignified material. Stainless steel 'bombs', with a specially designed sealing system that did not need O-rings, were made and six of them accommodated in an oscillating heated aluminium block, which also had to be designed and built. About 2 years was spent making the equipment and developing suitable separation conditions with TLC and GC before any real work on lignin oxidation products could begin.

The separation and quantitative estimation of P, V, and S from alkaline nitrobenzene or alkaline cupric hydroxide oxidation of grass lignin by TLC did not give sufficiently reproducible results, even though the TLC method for their determination from mixtures of standards gave excellent results. Also, TLC and GC now showed that the alkaline cupric hydroxide oxidation procedure produced additional compounds to the above three. The major compounds were P, *p*-hydroxy acetophenone (Po), V, acetovanillone (Vo), S and acetosyringone (So) and a GC method for their determination was developed. The relative proportions of these compounds was determined for a variety of different grass species. These results were designed to provide a major part of the PhD thesis.

Recent literature of the time illustrated that certain lignin precursors such as *p*-coumaric acid and ferulic acid were present in grasses and could be liberated by mild alkaline hydrolysis. The study was extended to determine the proportions of these acids with the idea of comparing their ratios with the ratio of oxidation products of similar substitution pattern. It was found that a substantial amount of P and V were obtained by the alkaline cupric hydroxide oxidation of the alkali-soluble fraction. This fraction was now known to contain *p*-coumaric and ferulic acids and therefore ferulic acid itself was oxidised by the alkaline cupric hydroxide procedure and found to yield appreciable quantities of vanillin. This result devastated 3 years of work attempting to determine V thought to arise only from oxidation of the grass lignin. The implications of this finding illustrated that the yield of supposed lignin oxidation products is not independent of non-lignin substances in grass hay.

The only publications arising from this study were the TLC method for the quantitative estimation of P, V, and S (3) and the GC method for the estimation of lignin oxidation products (4). The oxidation of ferulic acid to vanillin, and therefore the difficulty of the interpretation of results on the oxidation of grass lignins, was presented only in a thesis (5). With the benefit of hindsight it would have been an appropriate publication at the time and was in fact the most important finding of the PhD study.

2.2   Highlights

This study on grass lignin was the first research topic to be undertaken and had a number of significant points.

TLC as a technique had not been used before by anyone at the University. Therefore, no advice or instruction was available. The only information available was early references and commercial catalogues.

With only this information a simple plate-spreading apparatus, different from any I have ever see since, was designed and constructed that made excellent chromatoplates for many years.

A GC instrument was purchased and again no advice or instruction was available on the suitability of various columns or the coating of solid supports and the packing of

columns. Copper petrol pipe purchased from a local garage was used for many successful columns and efficient packed columns were produced consistently.

In order to change the relative retention of certain compounds, mixed stationary phase supports were coated with successful results.

Two papers were published in the *Journal of Chromatography*. Each of these papers was a single author paper as my supervisor said the work was entirely my own.

The significance of the presence of hydrolysable *p*-coumaric and ferulic acids in grass material was realised as possible lignin precursors and their relative proportions should therefore be compared to appropriate oxidation products.

The oxidation of ferulic acid to vanillin clearly established the folly of assuming that vanillin from the alkaline cupric hydroxide oxidation of grass material, arose only from polymeric lignin.

A strong interest in the separation and identification of compounds from a biological source was firmly established. The need to learn practically everything about TLC and GC from scratch provided an excellent grounding in thoroughly understanding the fundamentals and basic principles of these two important techniques.

2.3   Early Pheromone Studies

The expertise developed with TLC and GC on lignin made me realise that I could contribute to the interests of two entomologists, D J C Fletcher and T Bosman. These interests were ant trail pheromones and lepidopteran sex attractants. Column chromatography and preparative TLC provided rapid procedures for the separation and recovery of microgram or sub-microgram quantities of biologically active compounds for bioassay purposes. In addition, GC would provide evidence of separated volatile compounds and contribute to their possible identification, and preparative GC could provide material for bioassays.

2.4   Ant Trail and Alarm Pheromones

Published work on ant trail pheromones showed that they came either from certain abdominal glands or the hind gut. However, observations on the trail-laying behaviour of the cocktail ant, *Crematogaster peringueyi*, showed that these ants laid trails with the tip of the gaster well elevated (6, see Fig. 1). It was determined finally that the trail of this genus is located in the distal segments of the metathoracic legs and is deposited from the terminal tarsal segment (6). This glandular source for trail pheromones is unique among ants. The active components were partially purified by adsorption column chromatography. It is pertinent to point out that in spite of recent attempts by E D Morgan, University of Keele, to identify the trail pheromone of *Crematogaster* species supplied by myself, it still remains unidentified.

When heads of *C. peringueyi* were crushed, a distinct odour was apparent that appeared to excite worker ants. GC analysis of solvent extracts of workers obtained by steam distillation with continuous liquid-liquid extraction, as well as head space analysis, indicated the presence of one major component and several minor components. A sufficient number of ants were extracted to allow the collection of approximately 100 µl of the major component and a smaller amount of the minor component. The IR spectra of these compounds indicated a carbonyl function in the major compound and a hydroxyl group in a minor compound. The first attempt at obtaining a mass spectrum on the drop-sized amounts of each compound collected over a period of eight months resulted in all the material being pumped out of the liquid vessel before a scan was obtained.

After a further two months work, an additional approximately 30 µl of the major peak and a few µl of the minor peak were collected by preparative GC. This time the mass spectrometrist adsorbed some of the material on graphite, which was then introduced into the ion source with the solid probe. This method of obtaining mass spectra allowed the identification of 3-octanone and 3-octanol (7). The combination of preparative GC and adsorption on graphite allowed the relatively easy obtaining of mass spectra on a few µg of material isolated from a number of *Crematogaster* species (8).

After completion of this MSc project of R M Crewe at the end of 1968 on the *Crematogaster* species alarm pheromones, an investigation was started in 1969 on the Dufours gland substances of the formicine ant, *Anoplolepis custodiens*, with another MSc student, G D Schreuder. Again, eluting gas chromatographic fractions

were adsorbed on powdered graphite for mass spectral analysis. This time however, the more volatile GC fractions were allowed to pass directly through the dry powdered graphite, tumbling it in the gas stream, while trapping the eluting compound. In this manner µg amounts of a relatively volatile compound could be trapped directly and sealed in a glass capillary and kept. The amount trapped from reasonable sized packed column GC peaks proved sufficient for recording mass spectra with the solid probe. A series of *n*-alkanes and *n*-alkenes was identified (15) which has recently been supported by capillary GC-MS analysis of individual Dufours glands (69).

Termites have different castes and a MSc project was started with J A Williams, also in 1969, with the aim of looking at the electrophoretic protein patterns of the various castes. Disc gel electrophoresis had just been developed using cylindrical gels. The scanning of cylindrical gels by a scanning densitometer required sophisticated instrumentation that was unavailable to us. The scanning of a flat gel was possible with apparatus we had available, but no apparatus was available to run flat electrophoretic gels. Therefore, an appropriate apparatus was designed and built (23). This general type of apparatus was soon available commercially and has been the most widely used form of disc gel electrophoresis for the past 20 years or so.

2.5   Lepidopteran Sex Attractants

At the same time as the work on the trail and alarm pheromones of *Crematogaster* was undertaken with D J C Fletcher and R M Crewe, the attempted purification and identification of two lepidopteran sex pheromones was also in progress with T Bosman. Fractions for electroantennogram bioassays were prepared by TLC and preparative GC. Studies on the wattle bagworm led to its partial purification (9). A project on the sex attractant of the Pine Emperor moth led to the trapping on graphite of an active GC peak and the obtaining of a rather poor mass spectrum. No more material was available to us and when this sex attractant was identified some years later by others, the interpretation of our mass spectrum was clear. We had in fact purified the sex attractant but were unable to interpret it correctly at the time.

Two techniques were attempted in the mid to late 1960's on sex pheromones which were possibly a little before their time. It appeared that many lepidopteran sex pheromones were acetate esters of long chain (C_{12} - C_{14}) alcohols. Our idea was to acetylate crude extracts on the assumption that the alcohol precursor might be

present, thereby increasing the behavioural activity of the extract. Any subsequent purification would be easier. It is now known that this approach could have been successful for certain sex pheromones as some species do have significant amounts of the alcohol precursor.

The second technique was an attempt to use a short column, packed with a GC stationary phase at room temperature, to trap volatiles in air drawn over live insects. Various purge and trap methods (on a variety of adsorbents) have been used widely with varying degrees of success ever since and solid phase extraction is also a commonly used method nowadays.

2.6   Highlights

This period was marked by many imaginative discussions with all those involved. Items of particular note would include.

The successful use of preparative GC for the collection of eluting compounds for adsorption on powdered graphite and mass spectral analysis.

The modification of the procedure to allow trapping of more volatile eluting compounds directly from the gas phase onto powdered graphite for mass spectral analysis of μg amounts.

The construction of a simple and successful isothermal preparative GC from an old stove purchased for R 5.00.

The design and building of a successful flat bed disc gel electrophoresis apparatus with rapid transverse destaining using a car battery charger as the power supply.

The relatively successful early studies on lepidopteran sex pheromones with no exposure to any other groups working in this area.

The successful completion of projects on the method of trail-laying in *Crematogaster* species and its alarm pheromones, and the successful analysis of the Dufours gland compounds of *A. custodiens*, again with no exposure to any other workers in the area.

This was really a time of developing techniques to separate, collect and identify small amounts of volatile compounds. Due to the need to develop most methodology in house, imagination was allowed to run free.

3.   Department of Entomology
University of Georgia
Laboratory of M S Blum
1970 - 1978

3.1   Fire Ant Venoms

In September 1970 I joined M S Blum in the Entomology Department of the University of Georgia. The chemistry of the major venom alkaloids of the imported fire ant had just been established in this laboratory. A project on the comparison of the structures of the venom alkaloids in the two imported species and the two main local species was begun. It was established that each species contained 2,6-disubstituted piperidines that were distinctive of the species, and that *S. xyloni* contained a possible precursor of certain of the alkaloids (10). In this study, the high-boiling eluting GC fractions were trapped in glass tubes, eluted in solvent, and adsorbed on graphite for mass spectral analysis. In addition, this was the first co-operative project with H M Fales of the NIH and his LKB-9000 GC-MS.

In all the *Solenopsis* species studied, the venom alkaloids in pooled samples were found to be characteristic of the species (22, 26). Even though the venom alkaloids of workers of the four species were characteristic of the species (10) it was observed that the venom of alate females of each of these species was similar (24). From the relative proportions of the alkaloids in alate females and workers of these species, a probable ancestral alkaloidal state is suggested (24). This exercise in biochemical evolution was initiated by discussions on the implications of the various findings with the well-known biosystematist, H H Ross. As *S. geminata* was available locally, had the simplest venom chemistry, and had recognisable soldier castes, it was chosen for the analysis of individual variation and the variation between soldier and worker castes (25). The results obtained thus far on fire ant venoms were integrated into a coherent whole on the topic of the contribution of fire ant venom alkaloids to chemosystematics and biochemical evolution (40).

3.2   Ant Alarm Pheromones

While studies on fire ant venoms were in progress, volatiles from glandular sources of other ants were also investigated. This series of papers is a mixed bag of reports

of various kinds and will therefore be presented briefly in chronological order of publication.

The identification of manicone (4,6-dimethyl-4-octene-3-one) in the mandibular glands of two *Manica* species (12) supported the removal of these two species from the genus *Myrmica* which does not have this compound. In collaboration with R H Wright, the molecular vibrations of a variety of compounds were correlated with their alarm activity on *Iridomyrmex pruinosus* (17).

The major components identified from various alate males of *Camponotus* species were identified as mellein (3,4-dihydro-8-hydroxy-3-methylisocoumarin), methyl anthranilate, and methyl 6-methylsalicylate, and 2,4-dimethyl-2-hexenoic acid (20,21). However, no definitive behavioural work has been done on these compounds.

Monoterpene hydrocarbons, identified in the poison gland of *Myrmicaria natalensis*, are unique to this myrmicine genus (27) and apparently function in an alarm-defence context. 6-Methyl-5-heptene-2-one occurs as the major component of 8 species of *Formica* in the subgenera *Neoformica* and *Proformica* (34). Again, this compound, as a major component, appears to be unique to these *Formica* species.

The primitive fungus-growing ant, *Mycocepurus goeldii*, produces the novel aromatic ketone *o*-aminoacetophenone in the mandibular gland (46). This compound was easy to identify as it has a grape-like odour and reminded me of methyl anthranilate identified earlier from carpenter ants (21).

3.3   Ant Trail Pheromones

The source of the trail pheromone and method of trail laying in the myrmicine ant, *Crematogaster peringueyi* (6) has already been mentioned. While work on fire ant venoms was in progress, the isolation and identification of the trail pheromones of the two imported species was attempted. Single compounds were isolated from each species and the mass spectrum and NMR spectrum of each suggested a farnesene and a homofarnesene as likely substances. On leaving the University of Georgia the data was passed on to workers in the USDA who later completed the study and confirmed our tentative identifications. Species specificity studies of active fractions,

after GC fractionation, established that the trail pheromones of the two imported were species specific, and that the two main local species appeared to have a common trail pheromone (31).

Worker ants of the formicine genus, *Camponotus*, lay trails from the hindgut. The trail pheromone of *C. pennsylvanicus* appeared to be a single gas chromatographic peak. Hindgut extracts of other *Camponotus* species showed that *C. pennsylvanicus* could follow the trail of other species (33). No active compound was identified but an acidic compound was suspected.

3.4 Beetle, Bee and Millipede Defensive Secretions and Lepidopteran Pheromones

A few other arthropod secretions received attention. Larvae of the leaf beetle, *Chrysomela interrupta*, produce a defensive secretion in their eversible thoracic and abdominal glands. Two esters, unknown from animal sources, were identified in this secretion (11). Another chrysomelid leaf beetle, *Gastrophysa cyanea*, has a secretion composed primarily of chrysomelidial (2-(2-formyl-3-methyl-2-cyclopentenyl)propanal) and its enol lactone (39). This secretion is particularly effective in repelling ants, including fire ants.

The free-living staphylinid beetle, *Drusilla canaliculata*, scavenges on dead insects and has an effective defensive secretion. Fifteen compounds were identified from the tergal gland exudate (18) and this remains one of the most complex secretions analysed from any coleopteran species. It contains alkanes, alkenes, aldehydes and quinones. The structural relationship between the alkanes, alkenes and aldehydes suggests a biosynthetic pathway linking these chemical classes.

Certain carpenter bees in the genus *Ceratina* have mandibular glands that contain terpenoid compounds and *n*-pentadecane (37). These compounds act in a defensive context.

The millipede, *Oxidus gracilis*, was collected from leaf litter of hardwood forests near Athens, Georgia. While bending over with a small dish in one hand containing a number of millipedes and scratching around in the leaf litter, I found that I became distinctly faint on a number of occasions. These millipedes were found to release benzaldehyde and phenol (19), the former compound arising from the release of

cyanide from mandelonitrile. Another polydesmid millipede was found to release guaiacol, in addition to benzaldehyde and phenol (28).

Many male Lepidoptera possess modified scales which release sex pheromones to promote successful male courtship. Male army worm scent brush secretion was extracted in solvent, separated by GC and trapped on graphite for MS analysis. Benzaldehyde, benzyl alcohol and benzoic acid were identified and their activity bioassayed by means of electroantennograms (16).

3.5   Reviews

Two review articles were co-authored. The one on Social Insect Pheromones (14) was extremely well received with over 1000 reprint requests being mailed out. My contribution to the other on Electron Impact Mass Spectrometry (13) consisted of H M Fales including some of the data we had both worked on.

3.6   Highlights

The three years spent in Athens, Georgia, were both fun and particularly productive due largely to the competence and enthusiasm of M S Blum of the Entomology Department and the mass spectrometrist, H M Fales, of the NIH.

The comparison of the venom chemistry of certain fire ants and the use of chemical compounds as characteristics for a contribution to biochemical evolution.

The identification of a variety of compounds from ants, many of them new to our knowledge of myrmicine and formicine glandular products.

The opportunity to exploit the technique of preparative GC and adsorption of eluting substances on graphite for mass spectral analysis.

The introduction to GC-MS with H M Fales at the NIH.

The realisation that the natural world of arthropods offers one the opportunity to discover and study the broad topic of chemical ecology.

4. Department of Microbiology University of Iowa Laboratory of A J Markovetz 1973 - 1988

I started in the Microbiology Department of the University of Iowa in August 1973 in the laboratory of A J Markovetz. This laboratory had worked on the subterminal degradation of alkanes and alkenes of C_{13} and C_{14} chain length by bacteria and fungi. 2-Ketones and acetate esters were confirmed intermediates in the degradative pathway. My having been involved in the isolation and identification of small amounts of similar compounds was a major reason for joining this laboratory. We planned to use mass spectrometry to prove the bacterial Baeyer Villiger reaction for the conversion of 2-ketones to acetate esters, and intended to initiate a study on the possible synthesis of insect pheromones by micro-organisms.

4.1 Microbial Degradation of Alkanes

Certain bacteria and fungi were known to degrade alkanes subterminally. A major identified product from n-C_{13} was 2-tridecanone and it was thought that this ketone was converted to an acetate ester by the direct insertion of molecular oxygen in the carbon-carbon bond adjacent to the carbonyl group. Proof of this reaction would require mass spectrometry. Cell-free extracts of Pseudomonas cepacia were supplied with 2-tridecanone, NADPH, ^{18}O, and an esterase inhibitor. The accumulated undecyl acetate was separated by preparative GC and trapped on graphite for mass spectral analysis. Appropriate ions of the ^{18}O-containing ester confirmed the incorporation of molecular oxygen into the carbon-carbon bond to form an acetate ester (29).

The Dufours gland of certain formicines contains alkanes, 2-ketones and acetate esters (C_{13} chain length). Formica schaufussi, a formicine ant collected Iowa, contains large amounts of undecyl acetate. Feeding ^{14}C labelled acetate to these ants showed that acetate is used in the formation of the ester, and that a reaction similar to the microbial Baeyer Villiger reaction does not take place (42).

As many alkanes actually end up in an anaerobic environment, A J Markovetz and myself discussed the possibility of the anaerobic degradation of alkane

hydrocarbons. For a chemical reaction to take place, certain thermodynamic conditions must be met. We attempted to set up conditions that might be favourable but were never able to establish the presence of any intermediates. A theoretical presentation of the problem was published (49) and this brief paper must surely have taken more thought than just about any other published work of mine.

4.2 Micro-organisms and Bark Beetle Pheromones

Many bark beetles produce aggregation pheromones in their hindgut. As the hindgut contains a large variety of diverse micro-organisms it was considered that the production of bark beetle pheromones would be an ideal topic to study the possible involvement of micro-organisms in insect pheromone synthesis. Pine bark beetles are exposed to α-pinene vapours in their galleries and many use *cis-* and *trans-*verbenol for aggregation, and later use verbenone for the termination of aggregation.

Ips paraconfusus feeding in the phloem of Ponderosa pine were obtained and a variety of micro-organisms isolated from the hindgut contents. One of these, a strain of *Bacillus cereus*, was found capable of producing *cis-* and *trans-*verbenol from α-pinene (30). Females of the southern pine beetle, *Dendroctonus frontalis*, have a mycangium in which they carry certain specific micro-organisms. These micro-organisms develop in the phloem galleries during the attack phase of these beetles. It was shown that exposure of one of the mycangial fungi to *trans-*verbenol converted it rapidly to verbenone (32). It was proposed that growth of this fungus in phloem galleries could be directly involved in the termination of the aggregation and attack phase of these beetles, a process known to be mediated by increasing amounts of verbenone.

A mycangial fungus (SJB-133) of *D. frontalis*, when grown in both liquid and solid culture, had a distinct fruity odour. GC-MS analysis identified isoamyl alcohol, a typical fermentation product, as well as 6-methyl-5-hepten-2-one and 6-methyl-5-hepten-2-ol (35). It was never established whether these compounds are involved in the behavioural responses of the southern pine beetle.

However, the involvement of metabolites of yeasts associated with *D. frontalis* in the enhancement of the aggregation pheromone was clearly established (36). Two major yeasts associated with the southern pine beetle produced a variety of fairly

typical fermentation products (isoamyl alcohol, isoamyl acetate, 2-phenyl ethanol and 2-phenylethyl acetate). The esters in particular greatly enhanced the attractiveness of a typical 'pheromone' blend of frontalin - *trans*-verbenol - turpentine (1:1:12) to walking beetles in laboratory bioassay. These bioassays were conducted by well-known bark beetle experts at Texas A&M as part of their routine bioassays. However, I could never convince them that microbial metabolites should be a part of their field bioassay program as it did not fit in with the specific aims of their funded research programs. While certain microbial products appear to be involved in the overall attractiveness of the 'pheromone blend' of the southern pine beetle, it has never been proved under field conditions.

This study on the southern pine beetle and its associated mycangial fungi and yeasts ended with a brief look at dimorphism in *Ceratocystis minor* var. *barrasii* (38). Unfortunately, the galley proof of this paper was never received and it appeared with a number of errors.

4.3   Review

R M Silverstein had been approached by Springer Verlag to write a review on insect pheromones for publication in 'Progress in the Chemistry of Organic Natural Products'and he wanted to include something a little different from the usual reviews that had appeared recently. As I had shown an interest in the biosynthesis of certain insect pheromones I was asked to collaborate on the writing of the review article with biosynthesis as one of the main topics (43). This review proved to be a major effort and has been cited widely.

4.4   Co-operation with Other University of Iowa Departments

Department of Medicinal Chemistry
College of Pharmacy

It is well established that certain enzymes and biological receptors recognise compounds with similar structural features. Quantum chemists have attempted to predict the structures of potentially active drugs from calculations alone i.e. prior to the synthesis and testing of a compound. J F Caputo and myself decided to attempt quantum chemical calculations on a series of compounds that were structurally

related and had been bioassayed independently as trail pheromones of an ant (41). It was found that the most active compounds all had a similar charge on the N-atom of the pyrrolic ring. This was as far as we got and to my knowledge no one has picked up on this topic since this paper was published.

Department of Obstetrics and Gynecology
University Hospitals and Clinics

R P Galask of this department had a joint appointment in the Microbiology department. We had had a number of discussions on the identification of odours and the fact that many patients with bacterial vaginosis had a discharge with a fishy odour led to a co-operative research project. I considered that the odour might be trimethylamine and developed a GC method with packed columns that analysed the headspace above a sample of discharge made basic with alkali. The presence of trimethylamine only in those discharges with a fishy odour was proved by GC-MS, again on a packed column, The source of the compound and the organism(s) responsible have not been conclusively established (58).

4.5   Highlights

The first project concerned the source of oxygen in undecyl acetate formed by what we suspected to be an enzymatic Baeyer Villiger reaction. Resolving the various compounds by packed column GC was easy. The only mass spectrometer available was an old Hitachi RMU-6 with a solid probe. This proved to be ideal for introducing a sample trapped on graphite. A sample was also run by H M Fales at NIH on his LKB-9000, which confirmed our results, and the problem was soon solved. This was the culmination of a project started by A J Markovetz some years earlier and was at an ideal stage for the trapping on graphite of an eluting compound for mass spectral analysis.

This project also led me to understand more about thermodynamics than I had ever known when we considered the anaerobic degradation of highly reduced compounds.

The synthesis of insect pheromones by microorganisms seemed most feasible for those pheromones known to be produced in the gut, especially those that could be

produced by the modification of ingested substances. Bark beetles were chosen for this study and the results obtained strongly indicate that certain bark beetle pheromone blends might well include compounds produced by associated microorganisms.

The writing of a review with R M Silverstein and J C Young indicated that some of my thoughts and data were taken seriously by some people. This long review has been well received and cited widely.

The brief co-operative projects with other departments looked at interesting diversions from the usual insect and microbial work.

5. Department of Biochemistry
University of Fort Hare
1978 - 1995

At the end of 1978 I accepted the position of Professor of Biochemistry at the University of Fort Hare. The only two lecturing staff at that time had Masters degrees and were grossly overworked. There was little time for any in-depth research projects and post graduate students were few. In the past 15 years I have supervised 2 Masters students and very recently co-supervised 1 Masters and 1 Doctoral student. Any research must therefore be done largely by oneself. In addition, the infrastructure of Fort Hare is not supportive of departments and most are understaffed. Also, a GC-MS was not available to me for the first 11 years.

Bearing these and other factors in mind I needed to get staff members involved in research and also needed to impress upon them the need to publish the results of research. Therefore, some of the published output from this department over the past 16 years has involved teaching topics, research training, as well as my own personal research.

5.1 Teaching

W A Lindner and myself found that certain topics in Physical Biochemistry were not presented satisfactorily in text books. Our attempted teaching of Activation Energy led us to write a coherent approach to this fundamental topic (50). Teaching the relevance of Thermodynamics to second year Biochemistry students is also not a simple matter as living systems are open systems with many reactions in a steady state. A short paper was published on predicting the reaction direction of steady state reactions (56). Finally, the Svedberg equation presented an opportunity to write a short account of a more coherent approach to its understanding (62).

5.2 Research Training

The Biochemistry department had started a project on cellulase production with the final intention being the microbial degradation of sugar cane bagasse to fermentable sugars. This was part of a National program on this topic. In addition, the only

microbiologist at Fort Hare had a research program investigating soft rot of maize by the bacterium, *Erwinia chrysanthemi*. A number of short papers were written on these topics and published in *Fort Hare Papers* (44,45,48,51) and presented at meetings and published in the proceedings of the meeting (47).

The cellulase program was soon terminated as it was realised that, while J S S Gray had a number of good ideas on the topic, we did not have the facilities to carry them out and thereby justify any research funding. Certain of these short papers culminated in the publication by J S S Gray and others of an in depth study in the *Journal of Bacteriology* on *E. chrysanthemi* (60), and later an excellent study on the structure of a polysaccharide from *E. chrysanthemi* in collaboration with R Montgomery and T C Koerner of the University of Iowa (70).

The desperate need for some dry silica gel one evening led us to put a beaker of it in the microwave to see if it would dry it. Ten minutes later we had it and this led to the short note published in *Journal of Chemical Education* (57).

W A Lindner was encouraged to begin a project on enzymes in guttation fluid of maize (63) and later *Helianthus annuus* (67). The *Helianthus* study has recently involved me in the co-supervision of a Doctoral student.

5.3 Personal Research

My own research continued to be involved with GC, insect pheromones and the microbial degradation of hydrocarbons. From 1978 to 1988, no GC-MS was available to me at Fort Hare. Therefore, insect pheromone work had to be done with GC alone and for much of this time no reliable GC was available. Before leaving the University of Iowa in 1978 I had developed an interest in the GC resolution of certain enantiomeric alcohols such as 2-heptanol and 3-octanol known to be part of various ant alarm blends. During 1977 and 1978 an attempt to resolve the diasteromeric esters of these two alcohols with *R*-(+)-*trans*-chrysanthemic acid produced strange results. It was finally established the the purchased chrysanthemic acid, which was claimed to be 99% pure, had been supplied as a racemate. It was only some years later that this project could be completed (52). This particular study should be repeated using chiral β-cyclodextrin based capillary columns which are now available.

The finding that two species of *Crematogaster* produce S-(+)-3-octanol (52) supplied the need to obtain this enantiomer in pure form for bioassay studies. It was not commercially available so the stereospecific enzymatic reduction of the appropriate ketones was undertaken. However, 2-heptanol and 3-octanol of greater than 95% ee could not be obtained (61) and bioassay studies still remain to be done.

Many ant alarm pheromones are a blend of relatively simple ketones and their alcohols. These blends generally have been analysed by the GC separation of solvent extracts of many ants and the ratio of the various compounds often appears to be species specific. From this type of data it was widely assumed that differing ratios of these compounds would be perceived differently by different species. As it is the gas phase ratio of compounds that is actually perceived, it was decided to analyse by GC the equilibrated gas phase ratio of ketone to alcohol above varying liquid phase ratios of the ketone and alcohol. The results obtained showed that the gas phase composition could be remarkably similar above widely differing liquid phase ratios (53). This finding led to the suggestion that in a pheromonal blend certain compounds may only be capable of conveying gross information.

A natural follow on to this study was the analysis of the ketone and alcohol ratio of individual ants of two related sympatric species (59). Even though the mean ratio of 3-octanone to 3-octanol in the mandibular glands varied between the two *Crematogaster* species, it was concluded, from the results of the previous study, that the gas phase composition would be fairly similar. Therefore, a slight change in the liquid ratios of these two compounds between the two species is unlikely to convey subtlety of information such as species recognition.

Some thoughts worthy of further study were published on enzymatic stereospecificity and the synthesis of certain hydroxylated products from the enantiomers of α-pinene (54). This short paper illustrates a continuing interest in chirality and the biosynthesis of various enantiomers. Also, there was a continuing interest in attempting to analyse smaller and smaller quantities of compounds from biological sources and the need to minimise the degree to which solvent extracts needed to be concentrated with the resultant loss of the more volatile fraction. Splitless injection and the solvent effect were well established and it was shown that large volumes of solvent containing very small amounts of a compound could be injected onto a packed column (55). This technique allowed the entire solvent extract of a single *Crematogaster* worker head to be analysed for the study on individual variation mentioned previously (59).

The purchase of a GC-MS in 1988 allowed the detailed analysis of the head extract and Dufours gland components of a local formicine ant (64). The occurrence of 4-heptanone and 4-heptanol in the head extract is rather unusual.

The remarkable similarity between certain inhibitors of seed germination and ant alarm pheromones led to the hypothesis that these ant secretions might possess the additional property of inhibiting seed germination in the nests of granivorous species. Since myrmicine ants are the most markedly granivorous, a selection of compounds that occur in certain myrmicine species was tested for inhibition of germination. Varying degrees of inhibition were observed ranging from as little as 30% to as much as 85% (65). Clearly, this is a topic for ant behaviourists to take further.

The Dufours gland and poison gland chemistry of another local ant was studied. The Dufours gland revealed many of the usual alkanes while the poison gland contained one main alkaloid (68).

At this stage it had been attempted to introduce single ant glands into the GC inlet, firstly as described by E D Morgan and more simply as described by B V Burger and others. The simpler method, slightly modified, has been used most successfully. Because of the simplicity and success of this solventless introduction of single ant glands, a reinvestigation of the Dufours gland components of *A. custodiens* (15) was carried out (69). The initial investigation had used packed columns and the trapping of the major compounds on graphite for mass spectral analysis (15). In addition, retention data had been used to establish the identity of minor components. This most successful ant is extremely widespread and of considerable economic importance in certain agricultural crops. This recent study using capillary GC-MS has indicated the presence of a variety of high boiling polar compounds which were not be seen in the original packed column work. A topic for further study would be the synthesis and possible trail-following behaviour of these various identified substances.

The poison gland of formicines has been known to contain formic acid for many years. The ability to introduce single glands into the GC inlet allowed me to analyse the poison gland of certain local formicines. Large amounts of formic acid and sometimes a small amount of acetic acid was seen. When the temperature of the column was programmed in the same manner as for Dufours gland analyses, certain

high boiling compounds were observed. The identification of hexadecanol and hexadecyl formate led to co-operation with E D Morgan. The presence of these compounds in five formicine genera was established and it was shown that their location is the convoluted gland and not the venom reservoir (71).

5.4   Highlights

The period at UFH has had its ups and downs. For a considerable period between 1978 and 1988 no reliable GC was available, and until the end of 1988 no GC-MS was available. The most was made of this period in my own research with GC alone. It is also relevant to point out that I have been Dean of Research since 1984 and around 1987 I was also Dean of Science for a peroid. These duties have taken a considerable amount of time, and as I have had very few graduate students, most of my research has had to be done entirely by myself.

The adaptaion of established methods to introduce single ant glands into the inlet of the GC and GC-MS has opened an interesting area. It is planned to exploit this technique more in the future. In addition, the recent introduction and use of solid phase microextraction should be useful. It is interesting to me that, on recently reading the original literature on the development of this now commercially available technique, I had in fact used a broken $1\,\mu l$ Hamilton syringe exactly as described in the early references.

The encouragement of staff to write up results, no matter how insignificant they might have thought them to be, has been a significant contribution to staff development.

6. Department of Microbiology
University of Iowa
Laboratory of D T Gibson
1989 - 1995

6.1 Aromatic Hydrocarbon Degradation

Two six month periods were spent with D T Gibson in 1989 and again in 1993. In addition, at least 4 weeks were spent in this laboratory during 1990, 1991, 1992, 1994 and 1995. This association arose from the fact that I had spent many years with A J Markovetz in Iowa and he was partly responsible for D T Gibson coming to Iowa. Furthermore, I had some experience with GC-MS and was particularly interested in the separation of enantiomers by either GC or HPLC. This was an area that D T Gibson's group had begun to pursue with certain mono-ols and diols produced by the microbial hydroxylation of some aromatic hydrocarbons.

The first project began with the re-investigation of the hydroxylation of indan (66). This investigation included the use of *E. coli* containing the cloned toluene dioxygenase genes from *Pseudomonas putida* F1. The enantiomers of 1-indanol were separated as their isopropyl urethane derivatives by capillary GC on a chiral column. Preliminary studies with whole cells of *Ps. putida* F1 and *Ps. putida* 39/D indicated that the ee of the 1-indanol produced from indan was dependent on the incubation time. It was finally shown that the cells contain a 1-indanol dehydrogenase that preferentially oxidises (+)-(1S)-indanol (66). However, the results obtained with the clone supported previously reported data. Late in 1989, at the end of this project, a chiral HPLC column was purchased (Chiralcel OB) and was shown to resolve the enantiomers of 1-indanol by many minutes. The later introduction of this HPLC technique has proved to be extremely effective in subsequent studies with D T Gibson's group.

In addition, this study on indan revealed the presence of additional compounds produced during the incubation of indan with whole cells of *Ps. putida* F1. These additional compounds appeared to be hydroxylated products of 1-indanone. This topic was pursued and solved some years later (73). The hydroxylation of 1-indanone and 2-indanone was examined with pseudomonad strains expressing naphthalene dioxygenase and toluene dioxygenase, as well as with purified enzyme components. In this study, preparative TLC, radial dispersion TLC, GC-MS, chiral

stationary phase HPLC, and proton and ^{13}C NMR were all used in a most successful manner to purify, isolate and prove the structures of the various metabolites (73).

During one 4 week stay I ran the GC separation and electron capture detection of a variety of oxidation products of polychlorinated biphenyls by two *Pseudomonas* strains (72).

Results obtained by myself and others in D T Gibson's group with *Ps.* strain 9816 containing naphthalene dioxygenase indicated that this enzyme was capable of monooxygenation, dioxygenase and desaturation reactions. Data collected over a period of time by a number of members of this group was put together in an excellent manner establishing the versatility of this enzyme (74).

CSP-HPLC was again used with success in the separation and ee determination of sulfoxidation products of certain alkyl aryl sulfides produced by purified toluene and naphthalene dioxygenases (75).

The sequence of the reactions involved in the oxidation of 1,2-dihydronaphthalene by toluene and naphthalene dioxygenases was studied (76). Bacterial strains and recombinant *E. coli* expressing the structural genes for toluene and naphthalene dioxygenase confirmed a pathway which includes desaturation in the case of naphthalene dioxygenase.

6.2 Highlights

The time spent in the laboratory of D T Gibson has been particularly exciting and productive. The initial work on the GC resolution of enantiomers was rather difficult and did not lend itself to be pursued. However, the development of a simple procedure for the collection of sub-microgram amounts of material using a melting point capillary and a foam coffee cup with a little dry ice has been useful.

The usefulness of the Chiralcel OB and OJ HPLC columns for the resolutions of aromatic alcohols and aromatic diols was first illustrated by myself in this laboratory and has now been adopted as a regular method for the resolution of many of these compounds produced in a number of projects. This, and some expertise with GC-MS, would be my most significant contributions to this laboratory.

The exposure to co-workers in this laboartory, with their expertise in microbial physiology and genetic engineering, has been an important contribution to my own development.

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ORGANIC SOLVENT EXTRACTION OF GRASS LIGNINS

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Submitted in partial fulfilment of the requirements

for the degree of

MASTER OF SCIENCE IN AGRICULTURE

in the

Department of Biochemistry

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SUMMARY

Since the variations in palatability and digestibility between grass species and within the same grass species on maturation cannot be explained by lignin content alone an attempt was made to extract lignin from veld hay under relatively mild conditions. The methods of Moon and Abou-Raya for the determination of acid-insoluble (3) and total lignin (4) were used for the estimation of the amount of lignin extracted by the various methods employed and the following findings have arisen from the investigations.

1. No extraction of lignin from veld hay was achieved by treatment with water, ethanol, methyl cellosolve or dioxan. Furthermore, no lignin could be extracted by dioxan from desiccated hay ground in anhydrous toluene in a centrifugal wet mill in an attempt to approximate the conditions recommended by Björkman (6).

2. The solubility in various solvents of an isolated soda lignin from veld hay was studied and the best solvents were found to be butyl cellosolve, dioxan, acetone, pyridine, methyl cellosolve and ethanol. Further solubility tests in organic solvents containing conc. HCl gave the same results but in this case ethyl acetate and ethyl acetoacetate, which were

not effective in the absence of conc. HCl, were also found to dissolve the isolated soda lignin.

3. The extraction of lignin from veld hay by organic solvents containing conc. HCl was effected by all solvents which brought about the partial or complete dissolution of the isolated soda lignin in the presence of conc. HCl.

4. The best lignin extractants were those solvents which were immiscible with conc. HCl and in which the isolated soda lignin was soluble in the presence of. conc. HCl.

5. Treatment of either the veld hay or the isolated soda lignin with conc. HCl alone for 2 hr. or longer caused an increase in the value of the acid-insoluble lignin. This increase suggests that the degradation products can repolymerise into a more insoluble form of lignin. However, if a solvent capable of dissolving the isolated soda lignin in the presence of conc. HCl is present the degradation products are dissolved by the solvent thereby effecting the extraction of part of the lignin.

6. Lignin was extracted with dioxan and conc. HCl from Themeda triandra, collected at different times of the year. The extent to which the acid-insoluble fraction was extracted decreased as the grass matured.

SOLUBILITY STUDIES ON A SODA LIGNIN ISOLATED FROM GRASS HAY*

(*Met opsomming in Afrikaans*)
(*Avec résumé en français*)

———◆———

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ABSTRACT

The solubility, in twelve solvents, of a soda lignin isolated from grass hay, was investigated and the solubility pattern was found to be the same as that reported by Schuerch (1952) for a hardwood soda lignin. The soda lignin was not soluble in ethyl acetate or ethyl acetoacetate but complete dissolution was obtained when conc. HCl was added to these solvents. Solubility was not markedly affected by the addition of acid to the other solvents tested. The absorption spectrum of the soda lignin in methyl cellosolve was similar to the absorption spectrum of conifer lignin obtained by Pew (1962).

INTRODUCTION

Most workers studying the solubility of isolated lignins have used either pine wood or spruce wood as their source of lignified material. Schuerch (1952) has tabulated the solubility, in numerous single solvents, of various isolated lignins, amongst them a soda lignin, " Meadol ". He concluded that the ability of solvents to dissolve an isolated lignin increases as the hydrogen bonding capacities of the solvents increase and as their solubility parameters approach a value of about eleven. The solubility parameter of a solvent, as defined by Hildebrand & Scott (1951), equals $(-U/v)^{\frac{1}{2}}$, where $-U = -E =$ the energy of vaporization to the gas at zero pressure (or heat of evaporation at constant volume) and V is the molal volume of the liquid. It has been stated (Schuerch, 1952) that polymers generally exhibit a maximum solubility in solvents with solubility parameters closest to their own.

Although much attention has been focused on the chemistry of grass lignins, no data, similar to those of Schuerch for wood lignins, have been reported. The investigation reported here was undertaken in order to test the applicability of Schuerch's findings to grass lignins.

A soda lignin was isolated from veld hay comprised largely of *Themeda triandra*, and its solubility tested in organic solvents of varying solubility parameters and hydrogen bonding capacities. The solubility of the isolated soda lignin in the same organic solvents containing concentrated hydrochloric acid was also investigated as most workers have found it necessary to add mineral acid to organic solvents in order to achieve extraction of lignin from plant material. The absorption spectra of the isolated lignin in some of the organic solvents were also determined.

EXPERIMENTAL PROCEDURE AND RESULTS

Isolation of soda lignin from veld hay

The method of Bondi & Meyer (1948) for the isolation of lignin from young grass was adopted for the isolation of a soda lignin from veld hay, except that the pre-extraction procedure was altered. Coarsely ground veld hay was extracted with an azeotropic mixture of ethanol-benzene (1:2) for 12 hours (siphoning every 20 minutes) in place of ether, the former solvent being widely used for the extraction of material prior to the isolation or determination of lignin. One kilogram of ethanol-benzene extracted material yielded 57 gm of soda lignin.

TABLE 1.—Solubility of soda lignin (0·1 gm) in various solvents (10 ml) (Ins=insoluble; Par=partially soluble; Sol=completely soluble; L=low; H=high)

TABEL 1.—*Oplosbaarheid van sodalignien (0·1 gm) in verskillende oplosmiddels (10 ml) (Ins=onoplosbaar; Par=gedeeltelik oplosbaar; Sol=volledig oplosbaar; L=laag; H=hoog)*

Solvent *Oplosmiddel*	Solubility of lignin *Oplosbaarheid van lignien*		Solubility para-meter* *Oplosbaarheids-parameter*	$\triangle \mu^*$
	in absence of *in afwesigheid van* HCl	in presence of *in aanwesigheid van* HCl		
				micron *mikron*
Ether/*Eter*	Ins	Ins	7·5	L
Ethyl acetoacetate/*Etielasetoasetaat*	Ins	Sol	8·1†	—
Carbon tetrachloride/*Koolstoftetrachloried*	Ins	Ins	8·6	L
Butyl cellosolve/*Butiel ,cellosolve'*	Par	Par	8·9	H
Ethyl acetate/*Etielasetaat*	Ins	Sol	9·1	0·12
Benzene/*Benseen*	Ins	Ins	9·15	L
Dioxan/*Dioksaan*	Par	Sol	10·0	0·14
Acetone/*Asetoon*	Par	Sol	10·0	0·14
Pyridine/*Piridien*	Sol	Sol	10·7	0·27
Methyl cellosolve/*Metiel 'cellosolve'*	Sol	Sol	10·8	H
Ethanol/*Etanol*	Par	Par	12·7	H
Water	Ins	Ins	23·4	H

* Solubility parameters and $\triangle \mu$ values according to Schuerch (1952)
  Oplosbaarheidsparameters en $\triangle \mu$ waardes volgens Schuerch (1952)

† Calculated from the formula: Solubility parameter $= \sqrt{\dfrac{(\triangle H - RT)\, S.G.}{M.W.}}$
  Bepaal volgens die formule: Oplosbaarheidsparameter

Solubility tests

Small portions (0·1 gm) of soda lignin were shaken at room temperature with 10 ml of individual solvents and observed for depth of colour of solution and for the presence of undissolved material. Further 0·1 gm samples of soda lignin were

shaken at room temperature with 9 ml of solvent and 1 ml of concentrated hydrochloric acid. Again the solutions were observed for depth of colour and for the presence of undissolved material. No further observable dissolution of the lignin in any of the solvents could be observed after a period of 5 minutes.

The results of both series of tests are presented in Table 1, the solvents being arranged in order of increasing solubility parameters.

Absorption spectra

Absorption spectra were determined for solutions of lignin in those solvents which do not absorb strongly below 300 mμ. Soda lignin (0·1 gm) was treated with 50 ml of solvent and allowed to stand for 5 hours with periodic shaking. An aliquot (0·1 ml) of solution was then diluted with 4 ml of the solvent and the absorption spectrum between 240 and 400 mμ determined with a spectrophotometer (Zeiss PMQ II). In each case the respective solvent was used as blank. The absorption curves are given in Fig. 1.
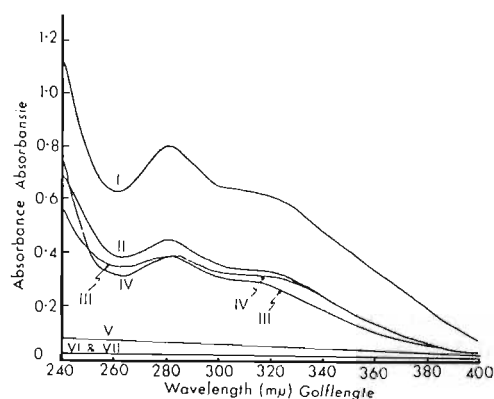
Fig. 1.—Absorption spectra of solutions of soda lignin (0·1 gm soda lignin treated with 50 ml solvent, resulting solutions diluted 1:40 and read in 1 cm cuvettes)

Fig. 1.—*Absorpsiespektra van oplossings van sodalignien (0·1 gm soda-lignien, behandel met 50 ml oplosmiddel, die gevormde oplossings verdun 1:40 en gelees in 1 cm kuvette)*

I = Methyl cellosolve/*Metiel ,cellosolve*'
II = Dioxan/*Dioksaan*
III = Ethanol/*Etanol*
IV = Butyl cellosolve/*Butiel ,cellosolve*'
V = Water
VI = Ether/*Eter*
VII = Carbon tetrachloride/*Koolstoftetrachloried*

The absorption spectrum of the lignin in methyl cellosolve containing 10 per cent conc. HCl was also determined in order to ascertain the effect, if any, of the acid. The presence of conc. HCl caused no change in the absortion of the soda lignin.

Discussion

The procedure adopted for the solubility tests conforms to that described by Schuerch (1952). Although qualitative, it is well established that lignin solutions are dark in colour, and for the present purpose this criterion is adequate. The observational results are substantiated by the absorbance data presented in Fig 1. The ether, carbon tetrachloride and water solutions remained colourless and their absorbance at all wavelengths between 240 and 400 mμ was very low, indicating that extremely little lignin was dissolved by these solvents. The darkness of colour of the butyl cellosolve, dioxan and ethanol solutions was of similar intensity and the absorbance data show that the concentration of lignin in solution in these solvents

763

was of a similar order. The methyl cellosolve solution was darkest in colour and the correspondingly higher absorbance relative to that of the other solutions confirms that methyl cellosolve was the best lignin solvent.

The addition of conc. HCl did not bring about any appreciable increase in the solubility of lignin in those solvents which effected partial or complete dissolution of the lignin in the absence of conc. HCl. In contrast, the solubility of lignin in ethyl acetate and ethyl acetoacetate was markedly affected by the addition of conc. HCl. Neither of these solvents dissolved lignin in the absence of the concentrated acid whereas both solvents effect complete dissolution in the presence of the acid.

This effect of HCl in altering the solubility of lignin in esters has not been reported previously. Several factors may play a role in this regard. The increased solubility may reside in the formation of hydrolytic products and the partial solubility of the lignin in ethanol and acetone is therefore relevant (ethyl acetate yields ethanol and acetic acid, while ethyl acetoacetate yields ethanol and acetoacetic acid which is decarboxylated to acetone with release of CO_2 in the presence of HCl). Unpublished data on the extraction of lignin from grass by various acidified solvents, also suggest that the miscibility of the solvents with conc. HCl is a further contributory factor.

The results obtained in this investigation on a soda lignin, isolated from grass, agree well with the observations by Schuerch (1952) that solvents with a high hydrogen bonding capacity and a solubility parameter about eleven are good solvents for a soda lignin. Schuerch (1952), using $\triangle \mu$ values obtained by Gordy (1941), showed that solvents having $\triangle \mu$ values of less than 0.14μ, e.g. esters, were poor to mediocre lignin solvents, while those having $\triangle \mu$ values of 0.14μ or higher, and solubility parameters about eleven, were good lignin solvents.

Pew (1962) has recently described the ultra-violet absorption spectra of two isolated lignins and various guaiacylpropane compounds. These compounds and related structures, which do not have a $C=C$ or $C=O$ group conjugated with the aromatic ring, give absorption spectra with a deep trough at 250 mμ, and a maximum at 280 mμ with the curve falling abruptly to zero at 300 mμ. The formation of a biphenyl derivative from two guaiacylpropane structures causes the absorption maximum to shift from 280 mμ to 290 mμ. The absorption at 250 mμ is extremely high, thereby eliminating the trough and, more important, absorption decreases rapidly at 320 mμ rather than at 300 mμ. The absorption curve for isolated conifer lignin reported by Pew (1962) has a slight trough at 260 mμ, a maximum at 280 mμ, and a shoulder at 320 to 330 mμ. He suggested that biphenyl structures in lignin could well contribute to a filling in of the trough at 260 mμ as shown by simple model compounds. The absorption spectrum for the isolated grass soda lignin dissolved in methyl cellosolve, conforms very favourably to that for conifer lignin and if Pew's deductions are correct it may be assumed that the lignin isolated from the grass hay also contains biphenyl groups.

It is widely believed that the composition of the monomeric components of grass lignin differs from both that of hardwood lignin and of conifer lignin. Recent gas chromatographic analyses conducted by Pepper, Manolopoulo & Burton (1962) have confirmed that wheat straw lignin, on alkaline nitrobenzene oxidation, yields p-hydroxybenzaldehyde, vanillin and syringaldehyde while aspen lignin yields mainly vanillin and syringaldehyde and spruce lignin yields mainly vanillin. In spite of an apparent basic difference between grass lignin, hardwood lignin, and conifer lignin, it appears that the solubility properties and absorption spectra of soda lignin isolated from these different plant types are remarkably similar.

764

Opsomming

OPLOSBAARHEIDSTUDIES OP 'N SODALIGNIEN GEÏSOLEER UIT GRAS-HOOI

Dit is gevind dat die oplosbaarheid van 'n sodalignien, geïsoleer uit veldhooi (hoof-saaklik Themeda triandra), *in verskeie organiese oplosmiddels, besonder goed ooreen-stem met die resultate verkry deur Schuerch (1952) vir die oplosbaarheid van 'n sodalig-nien verkry uit harde houtsoorte, in dieselfde organiese oplosmiddels. Die toevoeging van gekonsentreerde HCl tot die oplosmiddels het nie die oplosbaarheid van die sodalig-nien in hierdie oplosmiddels verander nie, behalwe in die geval van etielasetaat en etielasetoasetaat. Die toevoeging van gekonsentreerde HCl tot hierdie twee esters het tot gevolg gehad dat die sodalignien meer oplosbaar geword het. Die absorpsiespek-trum van die geïsoleerde sodalignien het goed ooreengestem met dié verkry deur Pew (1962) vir kegeldraerlignien.*

Résumé

ETUDES SUR LA SOLUBILITE D'UNE LIGNINE DE SOUDE, ISOLEE DE FOIN

On a constaté que la solubilité, dans plusieurs solvants organiques, d'une lignine de soude, isolée de foin (principalement de Themeda triandra), *correspond particulière-ment bien aux résultats obtenus par Schuerch (1952) pour la solubilité, dans les mêmes solvants organiques, d'une lignine de soude extraite d'espèces de bois durs. L'addition de HCl concentré à ces solvants n'a pas modifié la solubilité de la lignine de soude dans ces solvants, sauf dans le cas d'acétate éthylique et d'acéto-acétate éthylique. L'addition de HCl concentré à ces deux esters a eu comme résultat que la lignine de soude est devenue plus soluble. Le spectre d'absorption de la lignine de soude isolée correspondait bien à celui obtenu par Pew (1962) pour la lignine de conifères.*

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STUDIES ON GRASS LIGNINS

I. SEPARATION AND QUANTITATIVE DETERMINATION OF p-HYDROXY-BENZALDEHYDE, VANILLIN AND SYRINGALDEHYDE BY THIN-LAYER CHROMATOGRAPHY

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INTRODUCTION

In the past much attention has been paid to the development of a rapid quantitative method for the estimation of the three phenolic aldehydes, p-hydroxybenzaldehyde (P), vanillin (V) and syringaldehyde (S), obtained by the oxidation of isolated lignins or lignified material. Many methods for the separation and estimation of these and other lignin derivatives, obtained by oxidation procedures, have been reviewed by PEPPER AND SIDDIQUEULLAH[1].

In 1951, STONE AND BLUNDELL[2] reported a rapid micromethod for the quantitative determination of P, V and S formed by the alkaline nitrobenzene oxidation of lignin. These three substances were separated by paper chromatography, eluted, and assayed spectrophotometrically. Recently, PEPPER, MANOLOPOULO AND BURTON[3] have described a gas–liquid chromatographic technique for the separation and quantitative estimation of P, V and S.

The application of thin-layer chromatography (TLC) to the quantitative estimation of P, V and S appears to have been neglected. KRATZL AND PUSCHMANN[4] have described the preparative separation of P, V and S by TLC using silica gel G as adsorbent and di-isoamyl ether (water saturated)–n-butanol (3:1) as the developing solvent. Various TLC methods have been described for the detection of flavouring compounds, particularly vanillin, in vanilla extract, but most of these methods are not adapted for routine quantitative work. The numerous solvent systems mentioned by SUNDT AND SACCARDI[5] and by KAHAN AND FITELSON[6] for the separation and detection of vanilla flavouring compounds are only suitable for qualitative work with P, V and S.

The TLC separation of the 2,4-dinitrophenylhydrazones of many aromatic aldehydes, including P, V and S, has been reported by RUFFINI[7]. TOWERS AND MAASS[8] have also described the separation of P, V, S and other phenolic substances in silica gel G. A number of these substances have been extracted as such from the *Lycopodiales* while others have been obtained by the alkaline copper oxidation of the lignified material. This paper describes the quantitative determination of P, V and S after TLC separation on silica gel G by a new solvent system. The rapid quantitative extraction of these three substances from aqueous solutions is also described.

EXPERIMENTAL

Phenolic aldehydes

 p-Hydroxybenzaldehyde (Fluka), vanillin (British Drug Houses) and syring-aldehyde (Fluka) were purified when necessary by repeated recrystallisation until chromatographically pure. Standard solutions of each aldehyde were prepared in 95 % ethanol in concentrations of 10, 25, 75 and 100 μg per 0.025 ml. These solutions were used for the preparation of standard curves. Further standard solutions were made from a mixture of all three aldehydes, each being present in 10, 25, 50, 75 and 100 μg per 0.025 ml. These solutions were used for the determination of percentage recovery after separating the three substances on chromatoplates.

Treatment of silica gel

 It was found that syringaldehyde could not be quantitatively extracted from silica gel G (Merck) by ethanol or ethanol acidified with dilute HCl. As this was apparently due to the presence of iron in the silica gel, the silica gel used in this investigation was treated with ethanol–conc. HCl (9:1) for 30 min, washed with ethanol, and dried at 110°. Syringaldehyde could then be quantitatively extracted by ethanol from silica gel treated in this manner.

Standard curves

 In the determination of the standard curves for P, V and S it was attempted to simulate the condition which would be encountered during the TLC separation. Approximately 0.4 g silica gel, 0.025 ml of standard aldehyde solution and 10 ml of 95 % ethanol were added to 15 ml centrifuge tubes. A blank tube contained silica gel and ethanol only. The tubes were shaken thoroughly and centrifuged at 1400 × g for 10 min. The clear supernatant was decanted into further 15 ml centrifuge tubes, each containing 0.05 ml 1.25 N KOH. Any precipitate which formed at this stage was removed by centrifugation.

 The absorbance of the supernatant solutions was determined in a spectrophoto-meter (Zeiss PMQII) at the following wavelengths, which correspond to the wave-length of maximum absorption of the ionized form of the three substances: *p*-hydroxy-benzaldehyde, 335 mμ; vanillin, 352 mμ; and syringaldehyde 368 mμ. Glass cuvettes were used and the standard curves were found to obey Beer's Law over the concentra-tion range used.

Preparation and development of chromatoplates

 The acid-washed silica gel was spread on glass plates (8 in. × 2 in.) to give layers of 0.3 to 0.4 mm thick. The plates were activated at 110° for 30 min.

 The solvent finally selected for the separation of P, V and S was *n*-hexane–isoamyl alcohol (B.D.H.-purified for mild testing)–acetic acid (100:16:0.25). The positions of P, V and S on the chromatoplates were determined by spraying one of the plates with 2,4-dinitrophenylhydrazine (1.0 g in 300 ml concentrated HCl diluted to 1 l with water).

Recoveries from plates

 Activated chromatoplates were spotted 2.0 cm from one end with 0.025 ml of

the standard mixtures to give spots approximately 6–8 mm in diameter. The ethanolic spots were allowed to dry and the plates were then dipped into the developing solvent to a depth of 5–7 mm. After development of 15 cm the plates were air dried and the positions of the aldehydes noted by spraying one plate with 2,4-dinitrophenyl-hydrazine solution. Corresponding zones of adsorbent were carefully scraped, across the complete width of the plate, from the replicate plates into centrifuge tubes. A zone of adsorbent above the solvent front was scraped into a tube to serve as blank. Ethanol (10 ml) was added to each tube, the tubes were thoroughly shaken and centrifuged at 1400 × g for 10 min. The supernatant was decanted into further centrifuge tubes containing 0.05 ml 1.25 N KOH and the tubes shaken. Any precipitate which formed at this stage was centrifuged down. Absorbance values of the solutions were recorded and the results are presented in Table I.

TABLE I

PERCENTAGE RECOVERY OF P, V AND S FROM CHROMATOPLATES

Concentration (μg)	Recovery (%)[*]		
	P	V	S
10	102.0	100.0	101.0
25	100.0	99.6	100.0
50	100.0	99.6	100.0
75	98.7	98.0	97.3
100	98.4	99.0	100.0
Mean recovery	99.84	99.24	99.66
S.D.	± 1.40	± 0.78	± 1.40

[*] Means of three determinations.

Extraction of P, V and S from aqueous solutions

Accurately measured volumes of 2 % aqueous standard aldehyde solutions were pipetted into 25 ml burettes and about 15 ml of saturated ammonium sulphate added. Chloroform (2 ml) was added, the burettes stoppered and thoroughly shaken. When the two phases had separated the chloroform was run into 5 ml volumetric flasks until the aqueous phase reached the bottom of the tip of the burette. The burettes were stoppered, inverted and an iced cloth clasped around the upper half of the burette. The tap was slowly opened and the solution in the tip of the burette sucked back into the burette. The aqueous solutions were again extracted with 2 ml chloroform. The extracts were combined and made up to 5 ml with chloroform. Aliquots of the extracts (0.05 ml) were added to alkaline ethanol and the absorbance read as previously described. The results are presented in Table II.

DISCUSSION

The conventional method of spectrophotometric analysis employing a blank which does not contain the substance to be assayed, was more practicable than the utilisation of difference spectra as suggested by LEMON[9] for the spectrophotometric

TABLE II

PERCENTAGE RECOVERY OF P, V AND S FROM AQUEOUS SOLUTIONS SATURATED WITH AMMONIUM SULPHATE

Concentration (μg)	Recovery (%) [*]		
	P	V	S
10	98.00	103.00	105.00
25	97.00	100.80	97.00
50	100.00	100.80	100.00
75	100.30	102.00	100.27
100		102.00	100.00
Mean recovery	98.82	101.72	100.45
S.E.	± 1.59	± 0.93	± 2.40

[*] Means of four separate extractions.

determination of P, V and S. Standard curves of P, V and S obeyed Beer's Law over the concentration range employed.

The solvent employed by KRATZL AND PUSCHMANN[4] for TLC, namely di-isoamyl ether (water saturated)–n-butanol (3:1), was found to cause P and V to run too close together for quantitative work. The approximate R_F values obtained with this solvent mixture were: P, 0.85; V, 0.74; and S, 0.35. Many solvents were found to effect a separation of the three substances but P and V generally ran too close together, while S tended to trail. These problems were overcome by using n-hexane–isoamyl alcohol–acetic acid (100:16:0.25) as the developing solvent. All three substances appeared as distinct well resolved spots when sprayed with 2,4-dinitrophenylhydrazine (see Fig. 1).

This solvent, however, exhibited the phenomenon known as demixion, or the formation of more than one liquid front[10]. The most rapidly moving part of the solvent was rich in hexane and evaporated readily when the plates were removed from the developing tank. This phase was separated from the slower moving part of the solvent by a faint, yet distinct, yellow line which did not fade on drying.

The R_F values of P, V and S, relative to the demixion line, were approximately 0.87, 0.61 and 0.34 respectively. These R_F values, and the R_F value of the demixion line relative to the solvent front, varied according to the degree of saturation of the atmosphere in the developing tank. In a saturated atmosphere the demixion line did not move far enough up the plate for effective separation of P, V and S, while in an unsaturated atmosphere P was at the demixion line. The degree of saturation required for optimal separation can be readily determined from a few trial runs.

STONE AND BLUNDELL[2] found that the pure compounds, after separation by paper chromatography, could be determined to within ± 3%, after correction for chromatographic losses. The gas chromatographic method of PEPPER et al.[3] gave agreement with the original composition to within 3%. The results presented in Table I indicate that P, V and S may be determined with a greater accuracy by TLC than by either of the above two methods, agreement with the original composition being within 2%.

Preliminary investigations showed that P, V and S could not be quantitatively extracted from an aqueous solution by two extractions with a small volume of chloro-

form. If, however, the aqueous phase is saturated with ammonium sulphate, all three phenolic aldehydes are readily removed. This method has been successfully employed for the quantitative extraction of P, V and S after the oxidation of lignified material.
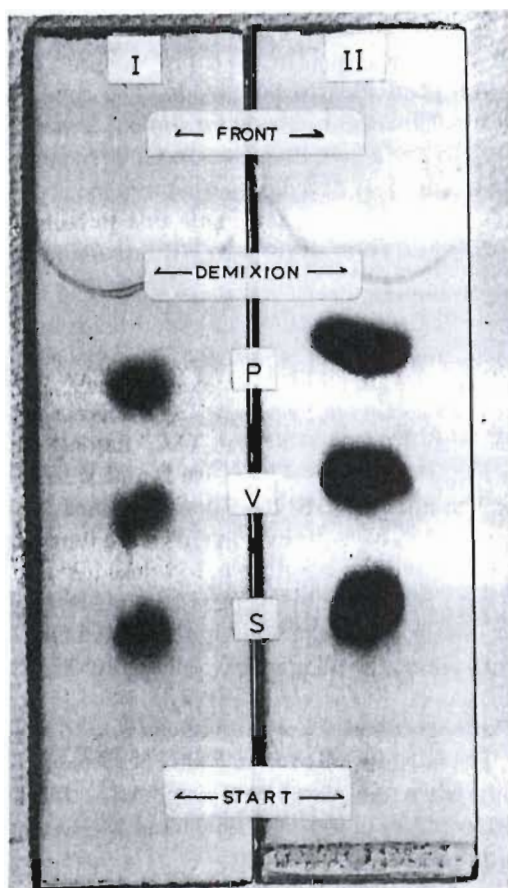

Fig. 1. Thin-layer chromatograms of p-hydroxybenzaldehyde (P), vanillin (V), and syringaldehyde (S) on silica gel G with n-hexane–isoamyl alcohol–acetic acid (100:16:0.25) as solvent. Plates sprayed with 2,4-dinitrophenylhydrazine. Plate I: 10 μg each of P, V and S. Plate II: 100 μg each of P, V and S.

The subsequent separation of these three substances by TLC provides a rapid and accurate method for their determination. It seems likely that this method could be adapted for the extraction of similar substances from a variety of sources.

SUMMARY

The separation of p-hydroxybenzaldehyde, vanillin and syringaldehyde by thin-layer chromatography on HCl-washed silica gel G using a new solvent system, n-hexane–isoamyl alcohol–acetic acid (100:16:0.25), is described. All three of these phenolic aldehydes can be quantitatively extracted by chloroform from saturated

ammonium sulphate solution and quantitatively determined after separation on chromatoplates.

NOTE ADDED IN PROOF

Since the preparation of this article the quantitative TLC separation of P, V and S has been described by REALE[11]. The slightly low recovery of S reported by REALE could possibly be improved by acid-washing the silica gel.

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4

STUDIES ON GRASS LIGNINS

II. THE ESTIMATION OF LIGNIN OXIDATION PRODUCTS BY GAS–LIQUID CHROMATOGRAPHY

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INTRODUCTION

Since the publication of the TLC method for the separation and estimation of *p*-hydroxybenzaldehyde (P), vanillin (V) and syringaldehyde (S)[1] it has become apparent that this method is not widely applicable for the quantitative estimation of these three phenolic aldehydes in a lignin oxidation mixture. The grass materials chosen for investigation by the TLC method were a particularly fortuitous choice as the oxidation mixtures were low in the phenolic ketones, *p*-hydroxyacetophenone (Po), acetovanillone (Vo) and acetosyringone (So). These ketones have R_F values very similar to their respective aldehydes in the solvent system previously described[1] and interfere with any quantitative work. A GLC method has now proved to be widely applicable for the quantitative estimation of these six products in a lignin oxidation mixture.

GLC was first used in lignin chemistry for the identification and separation of various phenols in the volatile fraction of white birch soda lignin by SOBOLEV AND SCHUERCH[2] in 1958. Many hydrogenolysis products of lignin have since been separated and identified by GLC[3–5].

The GLC separation of vanillin and related compounds, of interest to flavour chemists, has been described by a number of workers[6–12].

The two stationary phases most widely used for the separation of phenolic substances have been Carbowax 20 M[8–11] and SE-30[5,8,10,12–14]. It is generally accepted that polar compounds are best resolved by polar stationary phases, but in the author's experience, as well as that of VON RUDLOFF[14], Carbowax 20 M columns generally give broad tailing peaks for phenolic substances. SE-30 on the other hand gives sharp symmetrical peaks but resolution is often poor. PEPPER, MANOLOPOULO AND BURTON[15], using Apiezon N on Fluoropak, were able to determine quantitatively P, V, S and Vo in the oxidation products of the pre-extracted meals and the isolated lignins of aspen wood, spruce wood and wheat straw. Attempts by the author to use such a column did not prove successful as the peaks obtained were too broad.

This paper describes a temperature-programmed GLC method, using SE-30 as the stationary phase, which has been effective for the resolution of P, Po, V, Vo, S and So in a manner suitable for quantitative work.

EXPERIMENTAL

Packing the column

The column packing material was prepared by the evaporation technique. Chromport XXX (100/120 mesh) was added to a solution of SE-30 in chloroform in the ratio 100:30 (w/w) and the chloroform evaporated on a water-bath while the mixture was gently rotated. Equal weights (8.5 g) of the dried support were packed by suction into two coiled copper columns (6 ft. × 0.25 in. O.D.) with gentle tapping. Both columns were packed at the same time using the same vacuum source and were subsequently found to give equal flow rates under the same conditions.

GLC conditions

The following conditions gave satisfactory resolution of P, Po, V, Vo, S and So for quantitative work.

Instrument: Beckman GC-2A and Thermotrac temperature programmer.

Column: dual; 30 % SE-30 on Chromport XXX (100/120 mesh), copper 6 ft. × 0.25 in. O.D.

Carrier gas: helium.

Carrier gas inlet pressure: 10 p.s.i.g.

Carrier gas flow rate: 62 ml/min at 100°, 42 ml/min at 260°.

Thermal conductivity detector temperature: 190°.

Inlet and exhaust line temperature: 250° and 230°, respectively.

Filament current: 200 mA.

Chart speed: 1.5 in./min.

Program: 100° for 30 min; 100–180° in 2 min; 180–260° in 6 min.

Quantitative calibration

A standard solution containing 0.25 % of each of the components, P (recrystallized), Po (Koch-Light; pure), V (recrystallized), Vo (Koch-Light; pure), S (Fluka; purum CHR) and So (Koch-Light; pract), in ethanol was prepared. A number of separate injections of this solution, in the range 12.5–150 μg of each substance, were made, and calibration curves of peak height versus concentration were established.

Oxidation of grass hay

Grass hay (60 mesh) consisting mainly of *Themeda triandra* was extracted with ethanol–benzene (1:2) for 8 h. Air-dried samples (0.5 g) of the pre-extracted hay together with 1.7 g $CuSO_4 \cdot 5H_2O$, 10 ml 3 N NaOH and a helical copper rod were added to each of five stainless steel tubes (18 ml) equipped with stainless steel screw tops. The motion of the copper rod during the ensuing oxidation ensured rapid and complete mixing of the solution. The tubes were well shaken and heated in an oscillating aluminium block at 180° for 2.5 h. The warm-up time required to heat the tubes and block to 180° was 30 min.

After the heating period the tubes were cooled rapidly and the oxidation mixtures transferred to centrifuge tubes and spun at 1500 × g for 10 min in a refrigerated centrifuge. The chilled supernatant solutions were combined and acidified to pH 4 with conc. HCl, saturated with $(NH_4)_2SO_4$, and filtered on a Buchner funnel.

The filtrate was extracted three times with 5 ml chloroform and the extracts were combined. The chloroform extract was evaporated on a water-bath at approximately 50°, under a stream of air, until about 0.5 ml remained. This remaining solution was evaporated at room temperature so that the final residue was cold. This precaution was introduced to minimize any loss of the more volatile components. The residue was immediately taken up in 1 ml ethanol and 15 μl samples were injected into the gas-chromatograph.

TABLE I

YIELD OF ALKALINE CUPRIC HYDROXIDE OXIDATION PRODUCTS OF PRE-EXTRACTED GRASS HAY

Compound	Yield ($\mu g/15 \mu l$ injection)			Individual components as % of total		
	1	2	3	1	2	3
P	28	26	26	18	19	18
Po	15	13	14	9	9	10
V	71	62	63	45	45	43
Vo	13	11	13	8	8	9
S	21	18	19	13	13	13
So	10	9	10	6	6	7
Total	158	139	145			

Three separate samples of the pre-extracted grass hay were oxidised and duplicate injections of each sample were quantitated by peak height measurement. The results are presented in Table I and represent the means of duplicate injections.

Chromatograms of a standard solution of P, Po, V, Vo, S and So and of the lignin oxidation products from the grass hay are presented in Figs. 1 and 2.
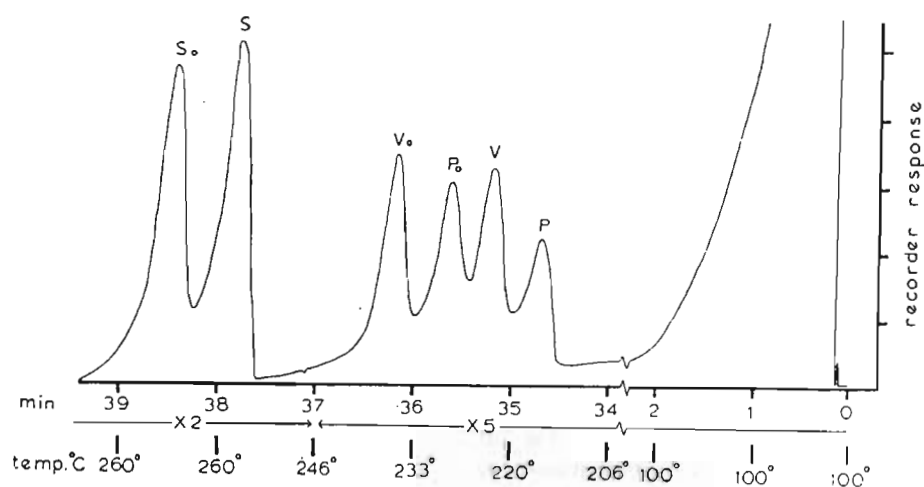
Fig. 1. Chromatogram of standard solution of p-hydroxybenzaldehyde (P), p-hydroxyaceto-phenone (Po), vanillin (V), acetovanillone (Vo), syringaldehyde (S) and acetosyringone (So): 50 μg each (conditions in text).
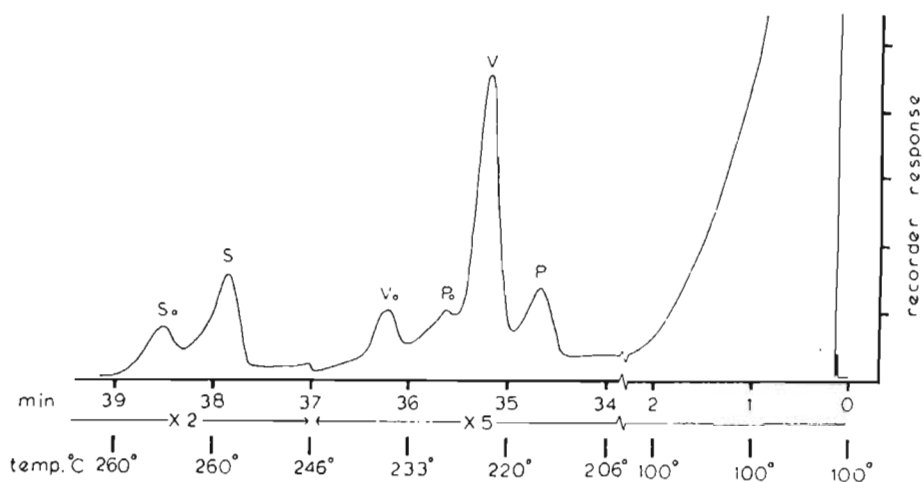
Fig. 2. Chromatogram of alkaline cupric hydroxide oxidation products of grass hay (conditions in text).

DISCUSSION

Attempts to use an Apiezon N on Fluoropak column[15], Carbowax 20 M columns, and columns packed with a mixture of Carbowax 20 M and SE-30 for the quantitative estimation of P, Po, V, Vo, S and So were unsuccessful. A column of 30 % SE-30 on Chromport XXX, packed with a support prepared by the evaporation technique, was found to be the most suitable for the separation of the six substances. A similar column, packed with a support prepared by the slurry filtration method[16] did not give as good a separation using the same GLC conditions. This is merely a statement of what was found and is not a criticism of the slurry filtration method.

Temperature programming was necessary for the production of a chromatogram in which all six substances had satisfactory retention times. The rather unusual program using a long, low-temperature, iso-thermal period was found to be necessary for the effective resolution of V and Po. A chromatogram showing the separation obtained with a standard mixture of the six compounds is presented in Fig. 1.

The six major peaks obtained in the chromatogram of the grass extract (Fig. 2) were identified on the basis of their retention times which were identical to those of the six pure compounds. Also, the major components of the ethanolic grass extract were resolved by two-dimensional TLC using hexane–isoamyl alcohol–acetic acid $(100:16:0.25)^1$ in the first dimension, and benzene–acetic acid $(9:1)^{17}$ in the second. The plates, when sprayed with 2,4-dinitrophenylhydrazine[1], gave six major spots corresponding in position and colour to a mixture of P, Po, V, Vo, S and So.

The extraction of P, V and S[1] and Po, Vo and So[18] from saturated $(NH_4)_2SO_4$ solution by chloroform, as described under Experimental, has been shown to be quantitative.

The results in Table I indicate that the total yield of P, Po, V, Vo, S and So from pre-extracted grass hay varies from one oxidation to another. However, when the yield of each substance is expressed as a percentage of the total yield, there is good

agreement between the results obtained with separate oxidations. The problem therefore lies in the non-reproducibility of the oxidation procedure rather than in the recovery of the resulting products. As the total yield of these substances, with the oxidation method employed, accounts for less than 4 % of the lignin present, the relative amounts obtained afford a better basis, than do the absolute amounts, for comparative studies.

After a number of analyses a black carbonaceous deposit was found at the entrance to the column and when the column was unpacked it was found that particles of the packing material had stuck together to form a thin film along its periphery. In spite of this condition, while the column was in constant use, resolution of the six compounds was satisfactory, but removal of the column from the instrument caused a marked deterioration in peak shape obtained in subsequent analyses, particularly of P, thereby rendering it unsuitable for re-use.

SUMMARY

A GLC method for the separation and quantitative estimation of p-hydroxybenzaldehyde, p-hydroxyacetophenone, vanillin, acetovanillone, syringaldehyde and acetosyringone, produced during the alkaline copper hydroxide oxidation of grass lignin is described. Using a six foot 30 % SE-30 column on Chromport XXX all six of the compounds can be quantitatively estimated in the range 12.5–150 μg with a thermal conductivity detector.

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A CONTRIBUTION

TO THE ENIGMA OF

GRASS LIGNIN

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M.Sc. Agric. (Natal)

Submitted in partial fulfilment of the

requirements for the degree of

D O C T O R   O F   P H I L O S O P H Y

in the

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UNIVERSITY OF NATAL

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S U M M A R Y

The separation of P, V and S by thin-layer chromato-graphy on HCl-washed silica gel G using a new solvent system, n-hexane - isoamyl alcohol - acetic acid (100:16:0.25), is described. All three of these phenolic aldehydes can be quantitatively determined by a spectrophotometric method after separation on chromatoplates. Furthermore, P, V and S can be rapidly and quantitatively extracted by chloroform from saturated ammonium sulphate solution.

The application of this TLC method to the estimation of P, V and S in an alkaline nitrobenzene or alkaline cupric hydroxide lignin oxidation mixture did not provide sufficient-ly reproducible results for its acceptance for general use on various grasses. Lack of reproducibility was ascribed to variations in the composition of replicate oxidation mixtures as well as interference from contaminating substances.

Two GLC methods, one for the quantitative estimation of P, V and S and the other for the quantitative estimation of P, Po, V, Vo, S and So, produced during the alkaline cupric hydroxide oxidation of grass lignin, are described. Using a 6 ft column of SE-30 on Chromport XXX (30% w/w) these

compounds can be quantitatively estimated in the range

12.5-150 µg with a thermal conductivity detector.

A comparison of the ratios of P, V and S and of

P, Po, V, Vo, S and So produced by alkaline cupric hydroxide

oxidation of various pre-extracted grasses shows that the

ratios depend upon both the species and the stage of maturity.

of the material. The leaf material of T. triandra, P. tuberosa

and B. balcooa as well as the young leaf material of various

grasses give a particularly high ratio of P.

The stem material of A. sativa, T. vulgare, H. hirta,

B. balcooa and Z. mays gave a higher ratio of S than the leaf

material of the same plant. No marked difference in the rela-

tive amounts of P, V and S could be established between xylem

and sclerenchyma of Z. mays stem.

An investigation of the relative proportions of

oxidation products obtained from various sub-families of

grasses failed to reveal any relationship of taxonomic value.

The ratios of Po, Vo and So from almost all the grasses were

found to be remarkably constant.

Analysis of alkaline $KMnO_4$ oxidation mixtures of

P. tuberosa, a mixed grass hay or a soda lignin isolated from

the hay, did not demonstrate the presence of 4-hydroxyisoph-

thalic acid, a product which would have been anticipated from

highly condensed p-hydroxyphenyl-residues.

Both p-coumaric and ferulic acid were liberated by alkali from all the grass species investigated and many of the grasses which gave high yields of P and/or V also gave high yields of p-coumaric and/or ferulic acid. However no consistent relationship could be established between either of the phenolic acids and their corresponding phenolic alde-hydes.

The influence of tyrosine on the yield of P obtained by oxidation was shown to be relatively unimportant. This effect, however, would be most noticeable in those grasses which contain a considerable amount of tyrosine and yield little P.

A substantial proportion of the P and V obtained by oxidation of B. balcooa leaf was shown to arise from the alkali-soluble fraction of the material. Two of the major components in this fraction were p-coumaric and ferulic acid. Oxidation of these acids produced P and V in yields of less than 5, and 40-50 per cent respectively. These findings, particularly the oxidation of ferulic acid to V, constitute a major difficulty in the interpretation of the oxidation data.

Recovery studies on P, Po, V and Vo showed that quantitative recovery of these compounds could not be obtained

after alkaline cupric hydroxide oxidation.

The implications of these findings are discussed in relation to current emphasis on lignin oxidation studies in the investigation of grass lignins. In particular, the demonstration that the yields of oxidation products are not independent of non-lignin components in the material under investigation suggests that some of our current views on the structure of grass lignin and its biosynthesis require careful reconsideration. It is concluded that a new approach is required if progress is to be achieved in this field.

SOURCE OF THE TRAIL PHEROMONE AND METHOD OF TRAIL LAYING IN THE ANT *CREMATOGASTER PERINGUEYI*

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Abstract—When laying odour trails, foragers of the black cocktail ant, *Crematogaster peringueyi* Emery, do not touch the tip of the gaster to the substrate, as do other trail-laying Formicidae. To determine the anatomical source of the trail pheromone of this species, solvent extracts were made of various parts of the body, partially purified on silicic acid chromatographic columns, and assayed by means of an artificial trail technique. The source of the pheromone was shown to be the distal segments of the metathoracic legs and this was confirmed by observations on trail-laying behaviour. Ants place their hind legs close together and drum vigorously on the substrate with the tips of the tarsi.

INTRODUCTION

THE HABIT of laying scent trails when foraging is widespread among the Formicidae, and SUDD (1967) lists 30 species in which it has been demonstrated experimentally. The glandular sources of the trail-marking pheromones have been shown to be variable in the range of species so far investigated. On the limited evidence available it appears that these sources correspond closely, although not absolutely, with existing taxonomic divisions at the subfamily level. In the Dolichoderinae the pheromones are secreted by Pavan's gland (WILSON, 1963) and in the Myrmicinae by the venom gland where the sting is functional (BLUM *et al.*, 1964; BLUM and ROSS, 1965) or by Dufour's gland where the sting is not as well developed (WILSON, 1959, 1963). The active principle is contained in the hind gut of the Formicinae (CARTHY, 1951; BLUM and WILSON, 1964) and in certain Dorylinae it is also derived from the gut (WATKINS, 1964; BLUM and PONTECARRERO, 1964), although in these two subfamilies it is not known whether the trail substances are glandular secretions or products of digestion.

All the known scent glands open at the posterior end of the ant's body and trails are laid by the common method of applying the tip of the gaster to the substrate. In the Myrmicinae the actual dispensing organ is the sting and in the Dolichoderinae it is the posterior border of the sixth abdominal sternite, whereas in the Formicinae a ring of hairs around the anus serves to streak out the gut contents (WILSON, 1963).

The black cocktail ant, *Crematogaster peringueyi* Emery, is a common species in South Africa where it is widely distributed (Skaife, 1961). It is usually arboreal in habit, building conspicuous carton nests in trees and shrubs, but it also frequently occupies suitable cavities such as rock crevices, hollow tree stumps and dead plant stems.

Foragers of this species follow well defined scent trails, as may be demonstrated by means of classic 'interruption' and 'turntable' experiments. The present investigation was motivated by repeated observations that after a searcher ant has discovered a new source of food, it returns to the nest without once touching the gaster to the ground, while at the same time it lays a scent trail which is readily followed by alerted recruits. This clearly represents a radical departure from the general method of trail establishment as outlined above and suggests a novel source of a trail pheromone in the Formicidae.

MATERIALS AND METHODS

Observations on trail establishment

Several colonies of ants in carton nests were installed in the laboratory on tables measuring 84×74 cm and were fed on sucrose solution, dead insects and water. Trail establishment was observed by depriving a colony of food for a short period and erasing all existing trails with ethanol before replacing the food in a new position.

Extraction of the trail pheromone

In order to determine the anatomical source of the trail pheromone, solvent extracts were made of various parts of the bodies of worker ants and assayed using an artificial trail technique. Crude extracts in pentane and ethanol showed some activity, but they could not be assayed reliably as many of the ants showed alarm or arrest responses to trails made with them. A satisfactory degree of purification was achieved by homogenizing 500 whole ants in pentane, and fractionating the extract, after concentration and centrifugation, on a 10×1 cm silicic acid chromatographic column and eluting with 50 ml volumes of pentane, benzene, acetone, and ethanol, respectively. After establishing which of the fractions contained the active principle, different parts of the bodies of batches of 100 ants were similarly treated. For assay purposes all fractions were made up to contain 25 ant equivalents/ml of solution, while 50 ml of each solvent were also concentrated to the same extent for use as controls.

Bioassay technique

A small table with additional food was set up a short distance from one of the colonies and the ants were given access to it over a wide bridging platform with narrow approaches at either end. The permanent natural trail from the nest to the centre of the food table was 122 cm long and was marked with a faint pencil line. On the bridging platform it followed a slightly sinuous course down the

middle of a sheet of plain paper measuring 30×21 cm and placed lengthwise. This paper could be replaced by similar sheets with artificial trails streaked on them, the narrow approaches permitting accurate alignment of the ends of the artificial and natural trails. A standard pattern was used to trace three faint pencil lines on each sheet. One line duplicated this segment of the natural trail and the other two were curved deviations to either side of it so that each had a maximum deflexion of 20° away from a straight line joining the ends of the natural trail and 20° back to it. The length of each deviation was 31·5 cm.

To test an extract the ants were given a choice of three possible routes. A measured amount of the extract, representing either five ant equivalents or half of an ant equivalent, was streaked evenly along one of the deviations, while an equal volume of the relevant concentrated solvent was streaked along the other, leaving the central pencil line to serve as a purely visual control. The solvents were allowed to evaporate completely before the commencement of a test. Since any ant following an artificial trail might reinforce it and hence influence the behaviour of other ants (such reinforcement was in fact occasionally observed after it had been discovered how natural trails were laid) an element of independent choice was introduced. Each test sheet remained in position for a maximum of 1 min and for the trail made with an extract to be considered active one ant had to follow it in one or other direction within this time and without any response being shown to either of the controls. This sheet was then discarded and a new artificial trail prepared. Active trails were removed as soon as the first ant reached the far end, which frequently took a few seconds only. Each extract was tested twenty times with intervals of approximately 3 min between tests. During the intervals the natural trail was replaced in position to permit any ants that had accumulated at the ends of inactive trails to cross the bridge, thus ensuring that the same individuals were never used in consecutive tests.

RESULTS

When equivalent amounts of the different fractions obtained from the extract of whole ants were tested, normal trail following was elicited by the benzene fraction only (Table 1). The single positive response to the pentane fraction was given by an ant which moved very hesitantly along the line, stopping frequently to examine the deposit. A strong alarm reaction was shown to the acetone fraction, which caused the ants to run around excitedly with the gaster raised. Many orientated to the trail with zig-zag movements for short distances and occasionally one would run rapidly in this manner along its whole length. No interest was displayed in either the ethanol fraction or in any of the controls.

It was inferred from these results that the trail pheromone had probably eluted cleanly from the column in the benzene fraction, but since it was possible that the alarm response to the acetone fraction was obscuring a trail-following response, both these fractions from extracts of the head, thorax and abdomen were assayed in the next series of tests.

Table 1—Number of positive responses in twenty tests to fractions of an extract of whole ants

Fraction	No. of ant equivalents/test	No. of positive responses
Pentane	5	1
Benzene	5	20
Acetone	5	11
Ethanol	5	0

The higher degree of purity of the extracts obtained from the three body tagmata separately, permitted the quantity used in the tests to be considerably reduced (Table 2). The response to the benzene fraction from the thorax was very marked and it seemed highly probable that the trail pheromone was produced in this region. The level of response to the benzene fractions from the head and abdomen showed a slight increase at higher concentrations and it is possible that these regions became contaminated to some extent during dissection. Precautions were taken to ensure that contamination was minimal in subsequent dissections.

The acetone fraction from the head elicited alarm reaction similar to, but weaker than, this fraction from the extract of whole ants, while at the higher concentration of 5 ant equivalents it was as strong as it had been in the earlier tests. This result is consistent with the occurrence of a natural alarm pheromone in the head, since if the head of a worker is crushed with a pair of forceps and held at a distance of about 1 cm from a group of feeding ants the typical alarm response is elicited. The ants displayed very little reaction to the acetone fractions from either the thorax or the abdomen even at higher concentrations, neither did they respond to the controls.

Table 2—Number of positive responses in twenty tests to fractions of extracts of various parts of the body

Part of body	No. of ant equivalents/test	No. of positive responses	
		Benzene fraction	Acetone fraction
Head	0·5	1	1
Thorax with legs	0·5	20	0
Abdomen	0·5	2	0
Pro-legs	0·5	1	—
Meso-legs	0·5	1	—
Meta-legs	0·5	20	—
Thorax without legs	0·5	0	—
Metatibiae to pretarsi	0·5	20	—
Metacoxae to femora	0·5	0	—

FIG. 1. Workers of *Crematogaster peringueyi* Emery, laying an odour trial (A) and following an odour trial (B), both photographed from the same vantage point.

The results of the tests with the benzene fractions from the legs confirm that the trail pheromone can be extracted from the thorax and demonstrate that its source is undoubtedly the distal half of the metathoracic legs. It seems possible that some of the glandular cells at least may be located in the tibiae, although the tarsi would seem the more likely site, since if the legs are severed just below the femoro-tibial joint, weak activity can be demonstrated in extracts from the proximal half, whereas if they are severed just above the joint, as they were for the main tests, activity is confined to the distal half. A histological study of the metatibiae and tarsi is at present being undertaken.

The behaviour of searcher ants leaving a newly discovered source of food was finally re-examined, and the results of the observations made are fully consistent with the evidence obtained from the assay of the solvent extracts. When trail laying, an ant raises its gaster slightly and places the hind legs much closer together than during normal walking (Fig. 1). The tarsi are turned so that they are more or less parallel with the long axis of the body and are drummed on the substrate at a relatively high frequency while the ant moves slowly forward. The gait of a trail-layer is thus quite distinctive. Numerous photographs have shown that in the large majority of cases the terminal tarsal subsegment only of the hind leg touches the ground, indicating that the trail pheromone probably passes to the exterior in this region.

DISCUSSION

Although normal trail following is elicited only by an authentic trail pheromone, it is evident from the results that ants may orientate to some extent to other natural odours, which is only to be expected in view of the rôle of olfactory stimuli in locating food for example. Such orientation is particularly noticeable to the natural alarm pheromone which, in order to serve its function, must also act as an attractant. WILSON (1963) has previously noted that workers of *Iridomyrmex pruinosus* Roger, weakly follow trails of their natural alarm substance, 2-heptanone.

Since many insects employ secretions to increase the adhesive power of the tarsal pads, it is not surprising that a footprint odour should have been evolved as a specific trail pheromone in at least one group of social insects, amongst which it could serve as a useful means of co-ordinating certain social behaviour such as communal foraging. The occurrence of such a footprint odour is not in fact unique amongst social insects, as it has recently been shown (BUTLER *et al.*, 1966) that worker honey bees leave a discrete substance, which can be obtained in solution, around the entrance to their nest, where it functions as an entrance marker. It is, furthermore, not surprising that such a source of a trail pheromone amongst ants should have been found in the genus *Crematogaster*, in which the dorsal attachment of the gaster to the petiole is an adaptation which enables the gaster to be raised at an acute angle over the thorax. This is presumably a defence posture, since the anal gland is the source of a strong-smelling secretion released only when the gaster is raised in a typical attitude of alarm. Although the gaster of *C. peringueyi* can be flexed ventrally until the tip touches the ground it is clearly

not primarily adapted for this and these ants are not frequently seen to display such behaviour.

It is of further interest to note that it is only amongst members of the sub-family Myrmicinae, to which the genus *Crematogaster* belongs, that two distinct glandular sources of trail pheromones were previously known to occur (see Intro-duction). The discovery of a third source further emphasizes the heterogeneity of this group and raises the question of whether a taxonomic review of the sub-family might not be profitable.

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Identification of an Alarm Pheromone in the Ant *Crematogaster peringueyi*[1,2]

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Chemical alarm communication commonly occurs among the social Hymenoptera, particularly among the more highly organized species in which the number of workers per colony is large (Maschwitz 1964). Several of the pheromones concerned with this alarm communication have been chemically identified, but the number identified is small compared with the large number of social Hymenoptera in which they must occur, even after allowance is made for an evident lack of specificity between related species. We report the identification of octan-3-one as the major component of an alarm pheromone complex from the heads of *Crematogaster peringueyi* Emery. This ketone has not previously been identified as an alarm pheromone in any species of social insect.

A strong but fugitive odor was liberated when heads of *C. peringueyi* workers were crushed, causing ants to display an alarm reaction consisting of attraction, raising of the gaster, and release of a frothy secretion from the anal gland. Similar responses were not obtained from other parts of the body, and we concluded that the alarm pheromone of this species is secreted in the head, probably by the mandibular glands. This conclusion was confirmed by dissecting out the mandibular gland reservoirs of several individuals and assaying their contents.

High-sensitivity gas chromatography on headspace samples of crushed worker heads indicated the presence of 1 major and several minor components. The major component was established as a single compound by separation on both SE-30® and Carbowax 20M®. For the collection of the major compound, the workers from several colonies were extracted into methylene chloride by continuous steam distillation and liquid-liquid extraction (Vogel 1961). The concentrated extract was fractionated by preparative gas chromatography on a 10% Carbowax 20M column (6 ft × 0.25 in. OD; 90°; 40 ml N_2/min), and the peak, corresponding to the major component of the heads, was isolated. This substance was homogeneous when chromatographed on SE-30.

The IR spectrum of the isolated fraction showed a strong absorption band at 1700 cm^{-1} indicating a carbonyl group, the presence of which was confirmed by the formation of a 2,4-dinitrophenyl-hydrazine derivative. The mass spectrum gave a molecular ion at m/e 128 with an accurate mass of 128.1208 indicating an empirical formula of $C_8H_{16}O$. The fragmentation pattern established the carbonyl group to be in position three. These data together with that obtained on a catalytic column as described by Beroza and Sarmiento (1963) suggested the compound was octan-3-one.

Identification was confirmed by comparison of the compound's IR spectrum, mass spectrum, and retention times on both Carbowax 20M and SE-30 with those of a sample of synthetic octan-3-one, synthesized according to Percival et al. (1953) and purified by preparative gas chromatography.

Both the isolated component and the synthetic octan-3-one elicited a strong alarm reaction. Although this compound is quantitatively the most important volatile component in the heads of the ants, the alarm pheromone appears to be a mixture of 4 substances. Work is proceeding with the identification of the minor components, and a full report will be published later.

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THE MANDIBULAR GLAND CHEMISTRY OF SOME SOUTH AFRICAN SPECIES OF *CREMATOGASTER* (HYMENOPTERA: FORMICIDAE)[1]

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Abstract

The compounds, 3-octanone and 3-octanol, (the two major components in the mandibular glands of *Crematogaster peringueyi* Emery) were identified as alarm pheromones in this species. Two minor components were identified as branched 3-nonanones. Gas chromatographic patterns were obtained from the crushed heads of a number of different species in the genus *Crematogaster,* and the relationship of these patterns with the subgenera of *Crematogaster* is discussed.

Key Words: Mandibular gland chemistry, Myrmicinae, *Crematogaster,* pheromone, alarm.

Introduction

The investigation of the alarm pheromones of *Crematogaster peringueyi* Emery formed part of a program to study the various pheromonal secretions of this formicid species. It is a member of the peculiar cosmopolitan genus *Crematogaster,* whose members are unique in that they can swing their gasters up over their heads and thoraces, have a spatulate sting (Buren 1958), and produce a frothy secretion from the tips of their gasters. *C. peringueyi* occurs widely in South Africa and is well known for its ability to build large carton nests (Wheeler 1910; Skaife 1961).

The first phase of the work on the pheromonal secretions of *C. peringueyi* was concerned with its trail laying behavior, and it was found to have a unique method of laying trails with its metathoracic legs (Fletcher and Brand 1968). The second phase of the work was concerned with alarm pheromones which were found to emanate from the mandibular glands. The major alarm releasing compound in the mandibular gland extracts was found to be 3-octanone (Crewe et al. 1969). In this paper we wish to report the identification of the other components in the mandibular gland extract. In addition, the results of a survey of the mandibular gland contents of a number of other species of *Crematogaster* are reported.

Materials and Methods

The heads of workers of *C. peringueyi* were removed and placed in methylene chloride. The resulting solution was dried over anhydrous sodium sulfate, filtered and concentrated to a small volume by distillation.

Table 1. The retention times of the major components of samples from the mandibular glands of a number of species in the genus *Crematogaster.*

Species	Compound	Retention time (min.) Carbowax 20M	SE-30
C. *(Crematogaster)*	Peak 1	4.4	6.0
peringueyi Emery	Peak 2	6.2	
	Peak 3	7.6	
	Peak 4	9.2	
	3-octanone	4.4	
	3-octanol	9.2	
C. *(Crematogaster)*	Peak 1	4.4	6.0
castanea F. Smith	Peak 2	6.2	
	Peak 3	7.6	
	Peak 4	9.2	
C. *(Crematogaster)*	Peak 1	4.4	6.0
monticola (Arnold)	Peak 2	6.2	
	Peak 3	7.6	
	Peak 4	9.2	
C. *(Sphaerocrema)*	Peak 1	1.3	
sp. Mayr	Peak 2	2.5	
	Peak 3	3.2	
	Peak 4	4.4	6.0
	Peak 5	6.2	
	Peak 6	7.5	
	Peak 7	9.2	
C. *(Decacrema)*	Peak 1	4.4	6.0
liengmei (Forel)	Peak 2	6.2	
	Peak 3	7.6	
	Peak 4	9.2	
C. *(Oxygyne)*	Contains no detectable volatiles		
santschii (Forel)			

The components of the concentrated extract were separated and collected by means of preparative gas chromatography. Alarm pheromones of other species of *Crematogaster* (Table 1) were surveyed by means of headspace samples obtained by removing 30 worker heads and placing them in a glass vial. The heads in the vial were crushed and the vial sealed with a parafilm membrane. Headspace samples were then obtained by removing a sample of air from the sealed vial with a gas tight syringe, and then injecting the sample into the gas chromatograph.

In addition, the components of the mandibular glands of *C. peringueyi* male and female alates, and fertilized dealate females, were assayed for the presence of alarm pheromones by means of headspace samples.

A Beckman GC-4 gas chromatograph equipped with flame ionization detectors was used for the gas chromatographic determinations. For the headspace samples, 6 ft. x 0.125 in O.D. stainless steel columns were employed. The columns were packed either with 10% Carbowax 20M on Gas Chrom Q (100/120 mesh) and operated at a temperature of

90 C, or they were packed with 10% SE-30 on the same support as above and operated at 110 C. In both cases, the carrier gas was nitrogen, used at a flow rate of 40 ml/min. For the separation and collection of the components of the *C. peringueyi* extracts, a 6 ft. x 0.25 in O.D. stainless steel column was used. It was packed with 10% Carbowax 20M on Chromport XXX (60/80 mesh) and operated at 90 C with nitrogen as the carrier gas.

The mass spectra of the components collected by preparative gas chromatography were obtained on an A.E.I. MS-9 mass spectrometer. Spectra were obtained by adsorbing 1 μl of each substance onto 3 hg of dry spectroscopic graphite. The graphite was placed in the tip of a probe, lowered into the ion source, and a spectrum recorded.

Colonies of all the species of *Crematogaster* investigated were set up in their carton nests on glass covered tables. The substances to be bioassayed were taken up in the gaseous phase into a gas tight syringe and then tested by slowly releasing the vapors from the syringe toward the carton nests. The compounds were released from the syringe in such a way that the ants were not alarmed merely by the movement of air around them, and they were not alarmed when a syringe containing air was discharged toward the nests.

The various species of *Crematogaster* were identified according to Arnold (1920), and the identifications confirmed by comparison with the specimens in his collection in the Bulawayo Museum in Rhodesia.

RESULTS

The retention times of the four components obtained by preparative gas chromatography from the *C. peringueyi* extracts are shown in Table 1. The fraction corresponding to peak 1 is the fraction previously identified as 3-octanone. The fraction corresponding to peak 4, gave a mass spectrum with an M-1 ion at m/e 129, an M-18 ion at m/e 112 and a base peak at m/e 59. Other major peaks occurred at m/e 101, 83, 55, and 41. The mass spectrum of this component, and its retention times on both Carbowax 20M and SE-30, corresponded exactly with those of a sample of authentic 3-octanol.

Both peaks 2 and 3 gave molecular ions at m/e 142. The accurate mass of the molecular ion of peak 2 was 142.1361, indicating a molecular formula of $C_9H_{18}O$ (expected 142.1353). Peaks in both fractions at m/e 113, 85, 72, 57, 43, and 29 correspond to those obtained from a mass spectrum of a sample of authentic 3-nonanone. However, additional peaks at m/e 95 in component 2 and peaks at m/e 101, 95, and 83 in component 3 suggest that these two compounds have different, possibly branched, structures. The retention time of 3-nonanone onboth Carbowax 20M and SE-30 does not correspond with the retention time of either peak 2 or 3, thereby providing further evidence for branched structures.

Table 1 indicates the retention times of the various components obtained from headspace samples from the species of *Crematogaster*.

The headspace samples obtained from mandibular glands of the alates and fertilized queens of *C. peringueyi* all had both 3-octanone and 3-octanol present in them, although the substances were present in only very small amounts in the heads of the males.

All four of the components were found to release alarm behavior. However, 3-octanone, which is quantitatively the greatest fraction, is probably the most important releaser of alarm.

DISCUSSION

The mandibular gland components of *C. peringueyi* are interesting in that they are non-isoprenoid, and consequently their synthesis cannot be explained by means of the pathway suggested by Happ and Meinwald (1965). Furthermore, this is the second species of ant in which both a ketone and its related alcohol have been found in an alarm releasing secretion. The alcohol in *C. peringueyi,* unlike the alcohol found in *Pogonomyrmex barbatus* (F. Smith) (McGurk et al. 1966), was found to be active in releasing alarm behavior. The structures of the two minor components, and the significance of their presence in the mandibular gland secretions remains a matter for conjecture, although they may be involved in the biosynthesis of the major components. Subsequent to the work on the genus *Crematogaster,* the North American species *Myrmica brevinodis* Emery was found to have the same four mandibular gland components as *C. peringueyi* (Crewe and Blum 1969) and indicates once again that alarm pheromones are neither genus nor species specific.

Maschwitz (1964) showed that crushed heads of *Crematogaster* workers elicited an alarm reaction, thereby dispelling the belief that the frothy secretion, produced on the tip of the gaster when the ants were alarmed, was the site of the alarm producing chemical. We have taken headspace samples of crushed gasters to try to determine whether any volatile compounds were present in the froth which might cause alarm behavior, but were able to find none. As Maschwitz (1964) indicated in his paper, the queens as well as the workers have alarm pheromones in their heads, but he was not able to show the presence of alarm pheromones in the males of *Tapinoma.* In the dolichoderine *Tapinoma,* the anal glands, the site of the alarm pheromone, are only present in females and workers, so that one would not expect to find an alarm pheromone in the males. The crushed heads of males of *C. peringueyi* on the other hand, have the same odor as that of a worker, and on the basis of the retention times of the components from their mandibular glands, they have the same alarm pheromones. Where the site of the alarm pheromones is the mandibular glands, it is perhaps not surprising that all the castes have the alarm substances, since all have mandibular glands.

Extracts of the three species, *C. peringueyi, C. castanea,* and *C. monticola* belonging to the sub-genus *Crematogaster sensu stricto,* all produce the same chromatographic pattern on the Carbowax 20M column. Crushed heads from each of these species have the same odor. The crushed heads of workers from *C. (Sphaerocrema)* have an odor which is readily distinguishable from that of the three previous species. The chromatographic pattern indicates that it is indeed different, but on the basis of retention times on Carbowax 20M this species still appears to have both 3-octanone and 3-octanol present in its mandibular glands, in addition to a number of other major peaks. The species *C. liengmei,* investigated from the subgenus *Decacrema* produced a chromatographic pattern very similar to that of the species in *Crematogaster sensu stricto*

and they cannot be differentiated by means of odor. Volatile compounds were absent from the mandibular glands of the species *C. santschii* belonging to the subgenus *Oxygyne*. Crushed heads of workers of this species had no detectible odor, although the workers showed a slight alarm reaction to crushed heads of the other species. They were extremely sensitive to vibrations of the glass plate on which their nest was placed, and when stimulated by disturbances of the plate, they exhibited the typical alarm reaction of *Crematogaster*.

The alarm pheromones of an additional two species of *Crematogaster* from Nigeria have been studied recently (Blum et al. 1969). One of the species, *C. (Nematocrema) stadelmanni* Mayr., was found to conform to the pattern of this investigation, since head extracts appeared to contain 3-octanone and 3-octanol. The other species, from the subgenus *Atopogyne*, had the unique alarm component 2-hexenal. This substance was previously shown to be present in *C. africana* (Bevan et al. 1961), also a species in the subgenus *Atopogyne*.

Although alarm pheromones have been shown to lack species specificity, the chromatographic patterns obtained from the crushed heads both from the species in this investigation and those of the two species from Nigeria, tend to be uniform within a subgenus. Evidence of this kind may be of some chemotaxonomic significance; if it could be shown that the chromatographic pattern of the components from the mandibular glands of species within a subgenus was constant, then this evidence might be of some value in a revision of the genus *Crematogaster* with its 11 subgenera (Wheeler 1922).

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Biological studies of the sex pheromone of *Kotochalia junodi* Heyl. (Lepidoptera: Psychidae) and its partial purification

by

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The presence of a volatile sex pheromone in the female bagworm was demonstrated by means of a bioassay based on the male copulatory response. The species is unusual in that the site of production of the pheromone is located anteriorly, as shown by bioassay and supported by histological evidence. The pheromone was partially purified by means of thin-layer and gas-liquid chromatography.

INTRODUCTION

Kotochalia junodi Heyl. is an indigenous psychid, well known in South Africa as the wattle bagworm. Its native host plants are thorn trees (*Acacia* spp.), from which it spread to the introduced wattle *Acacia mollissima* Willd. Valuable accounts of the species have been published by Fuller (1913) and Hardenberg (1917). It was Fuller who first suggested the existence in *Kotochalia* of "a perfume acting magnetically upon any male that might be nearby." Subsequently, Ripley observed that after aerial spraying operations mating of the bagworm appeared to be adversely affected, a phenomenon which he attributed to the "deodorising" effect of the diesel fuel which was used as spray vehicle. The strongly pectinate antennae of the male, which, unlike the vermiform female, is a free-living alate insect, provide a further indication of a dependence upon an olfactory sense in premating behaviour. The present authors are unaware of any recent investigation of psychid sex pheromones.

EXPERIMENTAL

Pupae were obtained from infested wattle plantations in the New Hanover district of Natal. Female pupae were removed from the bags and stored in wooden trays pending emergence. Male pupae were stored in the bags under moist conditions because of their susceptibility to dessication and handling injury. Mature females were removed from their pupal shells and agitated briefly (2 minutes) in *n*-pentane to obtain a crude pheromone extract. The females survived this treatment and subsequently the reappearance of the pheromone on the body surface could be demonstrated by bioassay. Two more washings in pentane on successive days were therefore carried out to enhance the yield. The principal contaminants of the crude extract were thus cuticular lipids.

Development of a bioassay procedure

The general behaviour of the male moth and mating have been described by both Fuller and Hardenberg (*loc. cit.*). Fuller's apt description of the male as "a veritable bundle of nerves" is indicative of the extreme sensitivity of the insect to such stimuli as mechanical shock or the fluttering of other individuals, as well as to the presence of the pheromone itself. Males to be used for bioassay purposes were therefore confined singly in stoppered glass vials measuring 3×1 inches, and provided with a suitable foothold. Careful handling to avoid vibration was necessary. Initially, a two-year-old bag was used as a natural object for each moth to settle upon; each vial was fitted with a cork stopper from which a single bag was suspended. It was very soon found to be more convenient to use $1 \times \frac{3}{8}$ inch dental rolls as a standardised disposable substitute.

A quiescent male, from a position of rest upon a suspended empty bag or dental roll, performs the following sequence of movements upon exposure to air contaminated with the pheromone:

    i. extension of the antennae from the position of rest

    ii. forward locomotion with characteristic rolling gait

    iii. wing vibration

    iv. orientation such as to permit probing movements of the abdomen to be directed to the site of the lower bag opening through which copulation would normally be accomplished.

It was established that males readily exhibit mating behaviour during the daylight hours and that the greatest activity is apparent during the forenoon.

The following procedure was then adopted: Males were used only on the day of emergence. Hyperactive individuals were rejected. Suitable males were allocated singly to numbered holding vials, each equipped with a suspended dental roll. The fractions of the extract which were to be bioassayed were pipetted into a further series of 3×1 inch vials in an appropriate volume of *n*-pentane or acetone (20 μl). After complete evaporation of the solvent, the test vials were stoppered and brought into the bioassay room. At no time was more than one vial unstoppered, and this only for the purpose of introducing a test insect clinging to a dental roll. It was possible to transfer each test insect systematically from one test vial to the next until a positive result was observed. The stimulated male would then be returned to its holding vial to recover, and the test would then proceed with the next male in the series. Any desired degree of replication could thus be achieved. Because the concentration of pheromone in individual fractions was sometimes liminal, a scoring system based on the male response was used. The criteria were as follows:

    a. Extension of antennae . . . . Score 1

    b. Extension of antennae and wing vibration . . . . Score 2

    c. Full response culminating in thrusting of abdomen at lower end
       of the bag substitute . . . . Score 3

If no response occurred within 15 seconds of lowering the male into a test vial, zero activity was recorded.

To eliminate bias in the experimenter, the identity of the fractions was unknown to him and duplicate and blank samples were included on a variable basis.

Fig. 1. a (top left). Sagittal section through anterior region of *Kotochalia junodi* female, showing (arrowed) the three glandular hypodermal zones. b (top right). Detail of a glandular zone, showing the clear transition from the general attenuated hypodermis to the glandular cells. c (lower left). Transverse section, showing the lateral extent of the glandular zone. d (lower right). Diagram of a two-dimensional TLC plate. Pheromone activity was confined to the spot on the extreme right, marked +.

Source of the pheromone

The results of numerous bioassays showed that the pheromone was present over the entire female body surface, over the ental surface of the pupal shell and in the fluffy mass of detached setae and scales extruded from the neck of the pupal shell. The pheromone could not be detected in extracts of any body tissue.

After washing in *n*-pentane to remove superficial pheromone contamination, females were ligatured and divided into sections and left overnight. Detectable activity was regained only in the anterior, sclerotized section (head and "thorax"). This finding was confirmed by excising strips of cuticle from successive body zones and storing them separately on filter paper. All strips cut from the soft white abdominal cuticle lost their activity overnight, but those from the sclerotized "thoracic" area remained active for four or five days.

Cauterisation experiments to define the excretory area more precisely were frustrated by lack of live adults, which are available for only a matter of several weeks of the year.

Histological examination revealed the presence in the "thorax" of three dorsal saddle-like areas consisting of well developed hypodermal cells, standing out in sharp contrast to the attenuated cells of the general hypodermis (see fig. 1a, b, c). The fact that these cells appear to be physiologically active makes it seem likely that they are, in fact, the site of pheromone production, but conclusive proof is wanting.

Purification procedures

The sex pheromone was extracted from the female bagworms by washing them with either petroleum ether (30–60) or *n*-pentane. Elution of this extract through a column of silica gel removed the active principle and subsequent elution with either benzene or methylene chloride provided a fraction which, after concentration, was used for both thin-layer chromatography (TLC) and gas-liquid chromatography (GLC) separations.

Partial purification by two-dimensional TLC

The concentrated active extract was spotted on silica gel plates (Merck F 254; 20 × 20 cm) and developed in the first dimension with petroleum ether (30–60)-benzene-acetic acid (7:3:0·2) and in the second with petroleum ether (30–60)-acetone (10:1). By eluting regions from a number of plates with acetone, the activity was established at Rf 0·6 (I) and 0·9 (II) (see fig. 1d).

Partial purification by GLC

The concentrated active extract was separated on SE-30 at 160°C and the activity was demonstrated in the fraction eluting between 30 and 40 minutes. This region of the chromatogram contained two peaks but due to the absence of suitable males for bioassay purposes it was not established which of the two peaks was active.

DISCUSSION

Typically, adult female bagworms do not leave the pupal case after ecdysis, although, as recorded by Fuller (*loc. cit.*), an unmated *K. junodi* female will, within a few days, protrude its body further and further until it comes to lie completely free.

In the normal course of events, however, ecdysis results in the exposure of little more than the anterior quarter of the adult. Because the fully fed larva assumes a head-down position prior to pupating, the anterior end of the adult female lies immediately adjacent to the cylindrical opening at the lower extremity of the bag. An anterior site of secretion of the sex pheromone would thus appear to have selective advantages in the discharge of a volatile pheromone into the surrounding air. Furthermore, the female, in the course of frequent telescoping movements, expels a quantity of scales and setae, which are contaminated with the pheromone, from the neck of the pupal shell and out through the bag opening. The presence of this fluffy mass on the outside of the bag probably further increases the efficiency of dissemination of the attractant odour. In all cases of sex pheromone secretion in the Lepidoptera reviewed by Shorey, Gaston & Jefferson (1968) the glandular source is situated at the caudal extremity of the abdomen.

Hardenberg described how the male inserts its highly extensible abdomen into the female pupal case and suggested that the sperm are expelled into the annular space without actual engagement of the genitalia and then find their own way into the female reproductive tract. Borthwick (1966) has demonstrated conclusively that normal copulation does in fact take place. In the course of the histological investigation described in the present paper, the presence of a small group of hypertrophied hypodermal cells in the vicinity of the female genitalia was observed. It is possible that a secondary attractant is secreted here in order to orientate the male genitalia, but this was not investigated nor was the presence of caudal chemoreceptors in the male determined.

The rolling gait evinced by aroused males appeared to be part of an alternately left-and-right movement of the antennae, possibly a klinotactic orientation. A similar movement has been observed in males of *Bombyx mori*.

Wing vibration in males clinging to a female bag is such as to transmit an intense vibration to the substrate. It is possible that this constitutes a mechanical stimulus to the female which might facilitate copulation.

The behavioural bioassay of sex pheromones is beset by many difficulties of interpretation, from the definition of a positive response to the evaluation of interacting factors which may elicit or inhibit that response. The criteria frequently used, for example, antennal movements ("sniffing"), wing vibration, circus movements, upwind flight or clasping movements of the valvae, are not specific responses to the pheromone alone: a variety of physical, chemical and mechanical stimuli could be responsible. It follows that appropriate definition of the experimental conditions is necessary in order that the response may provide valid evidence of the presence of the pheromone. Similarly, the bioassay must contain safeguards if failure to respond is to constitute a reliable indication of the absence of the pheromone in detectable concentration.

Difficulties arising in the behavioural bioassay of the *K. junodi* sex pheromone originated in particular from the following:

i. low threshold of response to a number of stimuli (e.g., air movement, vibration, mechanical shock, inadequate foothold and vapour of organic solvents such as acetone and pentane) which provoke intense sustained flight activity;

ii. low threshold of response to traces of organic solvent vapour which elicit antennal movements, wing vibration and clasping movements of the valvae;

iii. failure to respond in the presence of the pheromone after an earlier period of hyperactivity, in individuals exposed to dessication while in the pupal stage, or in bioassays conducted in the afternoon;

iv. prior inadvertent exposure to the pheromone (which under our insectary conditions could not be consistently avoided) was probably responsible for instances both of unresponsiveness and of general hyperactivity.

A solution to the bioassay problem was thus sought in a procedure embodying, as its basic criterion, a co-ordinated sequence of behavioral elements. In general, such a response can only take place provided that the experimental conditions are essentially adequate. In particular, two essential elements for the execution of the behavioural sequence were necessary before the response could take place, viz., the provision of a female bag or an adequate substitute, and the releasing stimulus provided by the pheromone itself. Spurious stimuli, such as those provided by vibration or volatile organic solvents, might elicit one or two of the components of the sequence, but not the entire behavioural pattern. On the other hand, incomplete responses occurring systematically throughout an assay set might well be taken to indicate the presence of the pheromone in liminal concentration, a possibility which would then be open to direct testing.

Males which failed to respond throughout a test series were then exposed to an extract of known activity at the $0 \cdot 1$ moth equivalent level. Failure to respond was taken as final proof of non-reactivity and they were then discarded from further tests.

The partial purification of the pheromone by two-dimensional thin-layer chromatography constituted an important step in the preparation of the material for gas-liquid chromatography. The value of this elegant technique in pheromone isolation is readily apparent.

The gas-liquid chromatographic study had to be terminated before the 30–40 minute fraction could be thoroughly investigated. It remains unknown whether one or other of the components represented by the recorded peaks, the combination of the two or a possible undetected third component was responsible for the biological activity of the fraction.

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FIRE ANT VENOMS: COMPARATIVE ANALYSES OF ALKALOIDAL COMPONENTS

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Abstract—The chemistry of the venom alkaloids of *Solenopsis geminata*, *S. xyloni* and the red and black forms of *S. saevissima* was investigated. All four forms have a venom composition characterized by the presence of various 2,6-disubstituted piperidines in amounts distinctive of the form. The alkaloids of the red and black forms mainly contain an alkenyl group in position 6 and a *trans* configuration of the substituents on the piperidine ring, while those of the two indigenous species of North American fire ants contain alkyl substituents which are both *cis* and *trans* on the ring. In addition, the venom of *S. xyloni* contains detectable amounts of 2-methyl-6-*n*-undecyl-$\Delta^{1,2}$-piperideine, a possible precursor of certain of these alkaloids.

INTRODUCTION

IN THE U.S.A. *Solenopsis saevissima* is considered to consist of two subspecies which are generally referred to as the red and black forms of the imported fire ant. The red form of this ant now has a widespread distribution in nine States of the southeastern U.S.A. and is a subject of topical interest due to the stinging propensities that this formicid possesses. The black form of *S. saevissima*, which also inflicts a painful sting, is confined to an area of northeastern Mississippi and western Alabama where it occurs as the dominant species. *S. geminata* is found mainly in the central plateau of Texas and in those parts of peninsular Florida where the red form is absent, while, in the southeast, *S. xyloni* is currently restricted to areas on the fringe of that occupied by the red form.

The chemical composition of the venom of the red form of *S. saevissima* has been the subject of several investigations (ADROUNY, 1966; SONNET, 1967) but only recently has it been possible to adequately elucidate its basic chemistry (MACCONNELL *et al.*, 1971). Besides water, the major components were found to be various 2-methyl-6-*n*-alkyl-(or alkenyl) piperidines. The noticeable lack of proteins in this venom makes it unique among stinging ants as the venomous exudates of species in more than twenty other ant genera are characterized by the presence of proteins (BLUM, 1966). This paucity of complex macro-molecules makes fire ant venom an ideal secretion for a thorough study of the chemical nature of its various constituents.

A comparative investigation of the venom alkaloids in other forms of *Solenopsis* was undertaken in order to further assess the possible distribution of these 2,6-disubstituted piperidines, and to comprehend more fully the significance of this alkaloidal-rich venom.

259

This paper reports on the chemical nature of the alkaloids present in the venom of both the red and black forms of *S. saevissima*, of *S. geminata* and *S. xyloni*. Each of these forms of *Solenopsis* secretes a characteristic venom and speculations on the biological activities of the various venom components are presented.

MATERIALS AND METHODS

Collection of venom

Pure venom was collected in capillary tubes from worker ants of four species of *Solenopsis* as previously described (BLUM *et al.*, 1958) and kept in *n*-pentane at −10°C. Venom samples for both gas chromatographic and mass spectral analyses were obtained from *S. saevissima* (black form) collected at Starkville, Mississippi, *S. geminata* collected at Gainesville, Florida, and *S. xyloni* collected at Athens, Georgia. Additional venom samples, used for qualitative gas chromatographic analyses only, were obtained from *S. saevissima* (red form) collected at Griffin, Georgia, Winter Haven, Florida, Gulfport, Mississippi, and Columbus, Mississippi; *S. geminata* collected at Austin, Texas, Lake Travis, Texas, and La Feria, Texas; and *S. xyloni* collected at Tucson, Arizona.

Standard compounds

The syntheses of *cis*-2-methyl-6-*n*-undecylpiperidine (*cis* C_{11}), *trans*-2-methyl-6-*n*-undecylpiperidine (*trans* C_{11}), *cis*-2-methyl-6-*n*-tridecylpiperidine (*cis* C_{13}), *trans*-2-methyl-6-*n*-tridecylpiperidine (*trans* C_{13}), *cis*-2-methyl-6-(*cis*-4′-*n*-tridecenyl) piperidine (*cis* $C_{13:1}$), *trans*-2-methyl-6-(*cis*-4′-*n*-tridecenyl)piperidine (*trans* $C_{13:1}$), *cis*-2-methyl-6-*n*-pentadecyl piperidine (*cis* C_{15}), *trans*-2-methyl-6-*n*-pentadecylpiperidine (*trans* C_{15}), *cis*-2-methyl-6-(*cis*-6′-*n*-pentadecenyl)piperidine (*cis* $C_{15:1}$) and *trans*-2-methyl-6-(*cis*-6′-*n*-pentadecenyl)-piperidine (*trans* $C_{15:1}$) have been described previously (MACCONNELL *et al.*, 1971) and these compounds were used as standards.

Gas chromatography and mass spectrometry

Two stationary phases, OV-1 (3 per cent on Chromosorb W (AW-DMCS) 100/120 mesh, 6 ft. × 0·25 in. o.d.) and Carbowax 20 M (10 per cent on Chromosorb W (HP) 100/120 mesh, 6 ft. × 0·25 in. o.d.) were used, each at 180°. The OV-1 column was used for collection of fractions from *S. saevissima* (black form) and *S. geminata* while the Carbowax 20 M column was used for collection of *S. xyloni* venom fractions. Each fraction was collected in 30 cm capillary tubes placed in the stream-split outlet during elution of each peak. The condensate was rinsed out with approximately 0·5 ml CH_2Cl_2, 1 mg spectroscopic graphite added to the solution, and the solvent removed under a stream of nitrogen. Mass spectra were recorded from each fraction by direct insertion of the adsorbed sample into the ion source of a Bell and Howell 21-490 mass spectrometer.

Mass spectra were also obtained on the components of *S. saevissima* (black form) and one component of the *S. xyloni* venom by combined gas chromatography–mass spectrometry utilizing a LKB 9000 gas chromatograph–mass spectrometer. In the case of the *S. xyloni* venom this component was separated on SP-1000 (Supelco Inc., Bellafonte, Pa.) as the stationary phase.

A sample of the venom of *S. xyloni* was reduced with both $NaBH_4$ and $NaBD_4$ and the resulting solutions chromatographed on Carbowax 20 M and SP-1000 respectively. The mass spectra of both *cis* C_{11} and *trans* C_{11} were recorded after $NaBD_4$ reduction. Finally, approximately 10 mg of the *cis* C_{11} piperidine was treated with 1 μl *t*-butylhypochlorite

and allowed to stand for 1 hr. The mixture was diluted with 100 μl CH$_2$Cl$_2$, basified with one pellet of NaOH, and aliquots of this mixture chromatographed on the SP-1000 column. Mass spectra of the two peaks eluting after the *cis* C$_{11}$ were recorded.

RESULTS

Gas chromatographic data

Carbowax 20 M resolved both the major and the minor constituents of the various venom samples better than OV-1, and it is for this reason that the chromatograms illustrating the venom components are of separations on this polar phase (see Fig. 1).
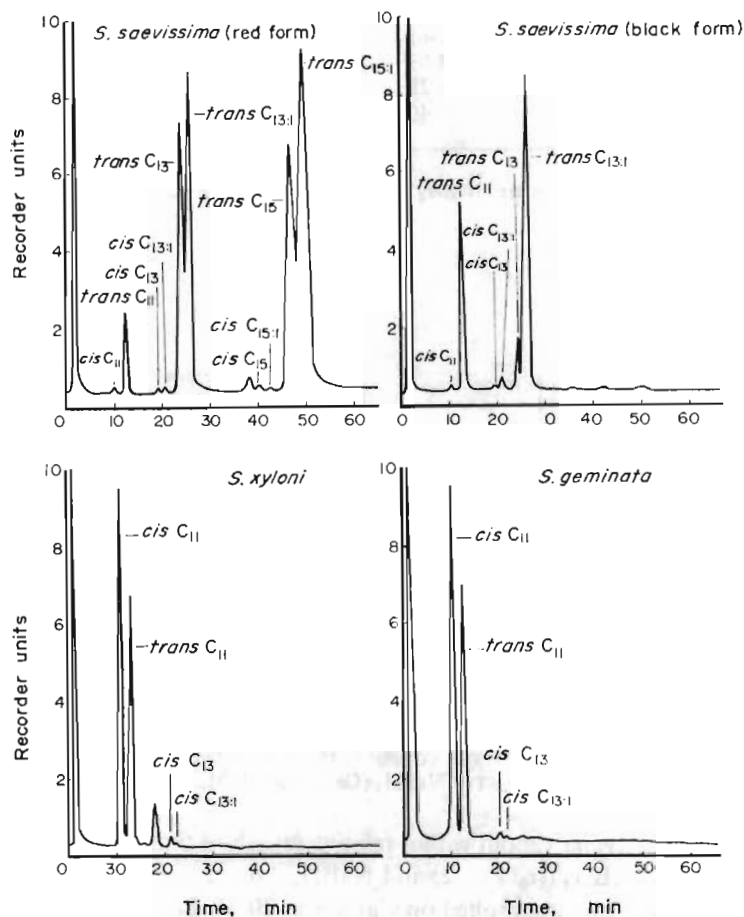
FIG. 1. GAS CHROMATOGRAMS OF THE VENOM COMPONENTS FROM FOUR FORMS OF FIRE ANT (Carbowax 20 M, 180°).

The identities of the various peaks in Fig. 1 are based on relative retention data on both OV-1 and Carbowax 20 M, together with mass spectral data of all major peaks and many of the minor peaks. The relative retention times, on Carbowax 20 M, of the various standard compounds, as well as those of the major and minor peaks in the venom of the four forms of fire ant shown in Fig. 1, are given in Table 1. Similar gas chromatographic data were obtained on OV-1 but are not presented.

TABLE 1. RELATIVE RETENTION TIMES OF THE STANDARD *cis*- AND *trans*-2-METHYL-6-*n*-ALKYL (OR ALKENYL) PIPERIDINES AND THE VENOM COMPONENTS OF FOUR SPECIES OF FIRE ANT (Carbowax 20 M, 180°)

Standard compound	Relative retention time	S. saevissima (red form)	S. saevissima (black form)	S. xyloni	S. geminata
cis C_{11}	0·82	0·82	0·82	0·83	0·82
trans C_{11}	1·00	1·03	1·04	1·04	1·03
				1·44	
cis C_{13}	1·69	1·69	1·70	1·69	1·69
cis $C_{13:1}$	1·79	1·80	1·83	1·82	1·82
trans C_{13}	2·13	2·12	2·12		
trans $C_{13:1}$	2·26	2·25	2·26		
		3·43			
cis C_{15}	3·69	3·61			
cis $C_{15:1}$	3·91	3·89			
trans C_{15}	4·26	4·25			
trans $C_{15:1}$	4·50	4·46			
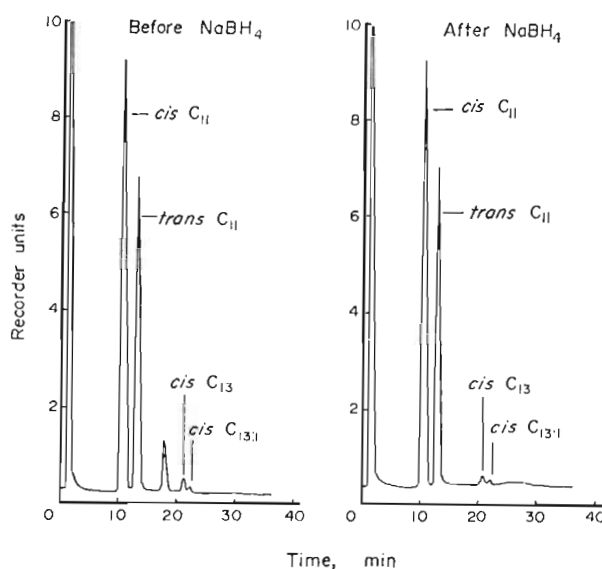
FIG. 2. GAS CHROMATOGRAMS OF THE COMPONENTS OF *S. xyloni* VENOM BEFORE AND AFTER REDUCTION WITH NaBH$_4$ (Carbowax 20 M, 180°).

The peak in the *S. xyloni* venom with a relative retention time of 1·44 (*trans* $C_{11}=1·00$) was reduced by both NaBH$_4$ (see Fig. 2) and NaBD$_4$. No new peak was observed when the reaction mixture was chromatographed on Carbowax 20 M, but a new peak eluting before *cis* C_{13} appeared on the SP-1000 column. The mass spectrum of this new peak indicated that it was not a piperidine and it was not investigated further.

Mass spectral data

The mass spectrum of *trans*-2-methyl-6-*n*-undecylpiperidine has been given in a previous report (MACCONNELL *et al.*, 1971). The spectra of *cis*-2-methyl-6-*n*-tridecylpiperidine and *cis*-2-methyl-6-(*cis*-4'-*n*-tridecenyl)piperidine, presented in Figs. 3 and 4, illustrate the variation in relative intensities of the high mass peaks, particularly the parent ion and P-15,
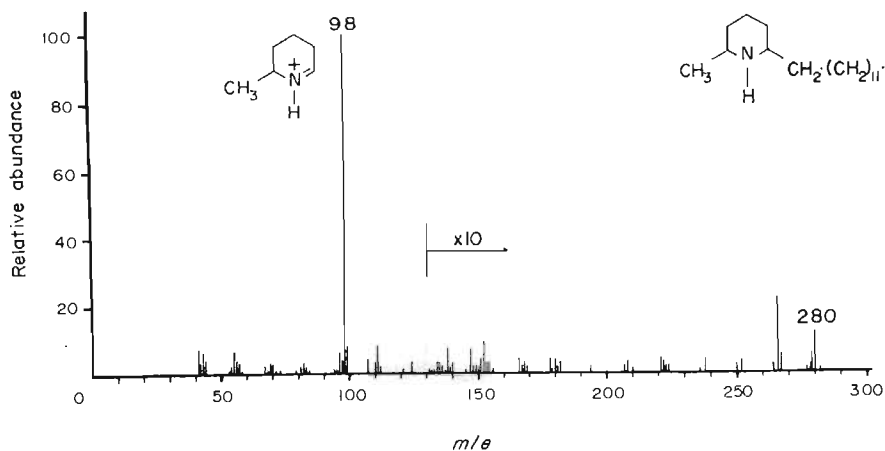
FIG. 3. MASS SPECTRUM OF cis-2-METHYL-6-n-TRIDECYLPIPERIDINE.
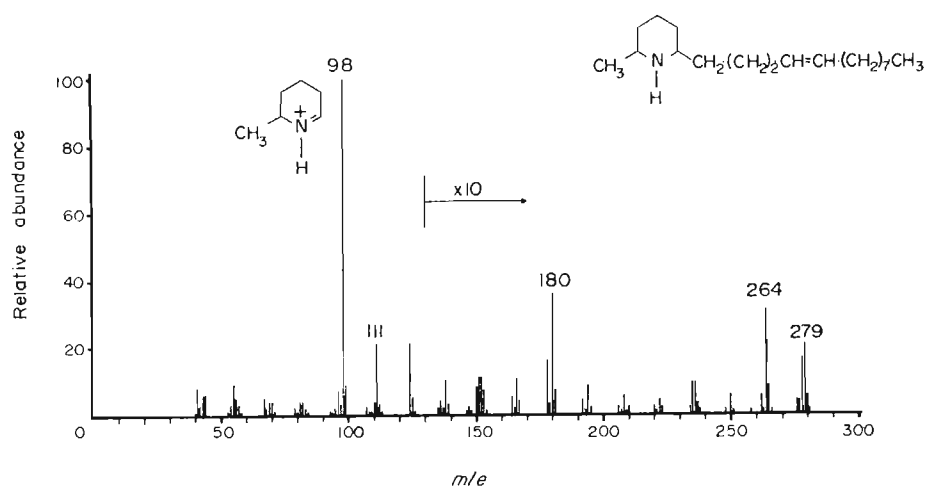
FIG. 4. MASS SPECTRUM OF cis-2-METHYL-6-(cis-4'-n-TRIDECENYL)PIPERIDINE

resulting from stabilization of the parent ion by the introduction of a double bond in the alkyl side chain. This difference also was observed between the spectra of cis-2-methyl-6-n-pentadecylpiperidine and cis-2-methyl-6-(cis-6'-n-pentadecenyl)piperidine. The spectra of the cis and trans isomers of each compound are essentially indistinguishable.

Samples of the following fractions were collected, adsorbed on graphite and spectra recorded: cis C_{11} and trans C_{11} from S. xyloni venom, cis C_{11}, trans C_{11} and cis $C_{13:1}$ from S. geminata venom, trans C_{11}, cis $C_{13:1}$, trans $C_{13:1}$, trans C_{13}, cis $C_{15:1}$ and trans $C_{15:1}$ from S. saevissima (black form) venom. Spectra of the above compounds in the S. saevissima (black form) venom, as well as cis C_{11} and the S. xyloni peak with a relative retention time of 1·44, were recorded on a LKB 9000 GC–MS. All these spectra confirmed the identifications for the various compounds presented in Table 1.

The mass spectrum of the odd peak in the *S. xyloni* venom was identical to that given in Fig. 5. The important peaks at m/e 96, 97 and 110 indicate that this compound is not a 2,6-dialkylpiperidine, but is a 2,6-dialkylpiperideine. The mass spectra of both the *cis* C_{11} and the *trans* C_{11} were recorded after $NaBD_4$ reduction of this peak and the ratios of m/e 99/98 and m/e 239/238 before and after deuteration are given in Table 2. These ratios show that about 6 per cent of the *cis* C_{11} has been produced by deuteration of the Schiff's base while no more than 0.2 per cent of the *trans* C_{11} is from this source. The identity of the compound in the red form venom with a relative retention time of 3.43 has not been established.
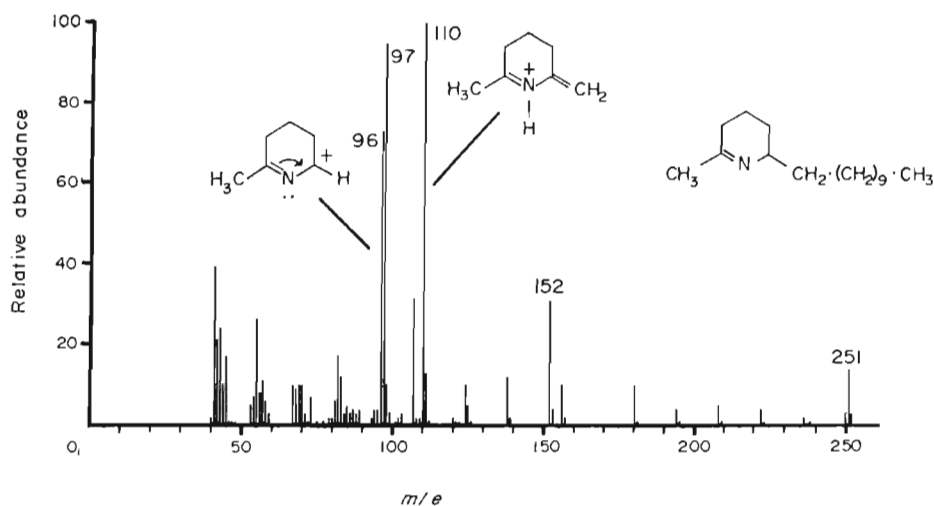
FIG. 5. MASS SPECTRUM OF 2-METHYL-6-*n*-UNDECYL-$\Delta^{1,2}$-PIPERIDEINE.

TABLE 2. RATIOS OF CERTAIN IONS OBTAINED FROM *cis* C_{11} AND *trans* C_{11} AFTER $NaBD_4$ REDUCTION OF THE *S. xyloni* VENOM

Mass No.	*cis* C_{11} (normal)	*cis* C_{11} (deuterated)	*trans* C_{11} (deuterated)
99/98	8.8	14.4	9.1
239/238 (P-15)	20.0	26.5	20.2

Treatment of *cis* C_{11} with *t*-butylhypochlorite resulted in a mixture containing two peaks eluting after the unreacted *cis* C_{11} corresponding to 2-methyl-6-*n*-undecyl-$\Delta^{1,6}$-piperideine and 2-methyl-6-*n*-undecyl-$\Delta^{1,2}$-piperideine. Each of these peaks amounted to about 25 per cent of the starting material and their retention times on SP-1000, relative to *cis* C_{11}, were 1.30 and 1.66 respectively. The mass spectra of the $\Delta^{1,2}$-isomer and the $\Delta^{1,6}$-isomer are presented in Fig. 5 and Fig. 6 respectively. The latter of the two peaks gave an equivalent relative retention time and identical mass spectrum (see Fig. 5) to the odd peak in the *S. xyloni* venom. Reduction of this reaction mixture with $NaBD_4$ caused the disappearance of both peaks and deuterium was found in the parent ion and m/e 98 of the *cis* C_{11} in amounts
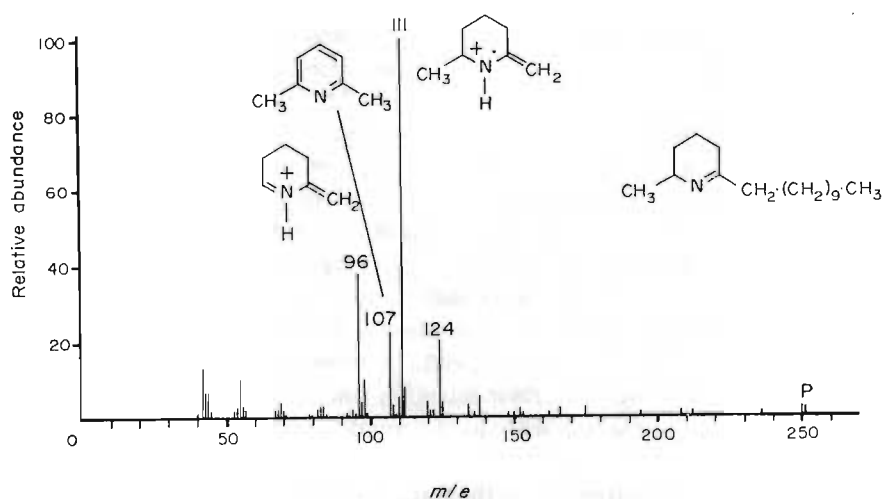
FIG. 6. MASS SPECTRUM OF 2-METHYL-6-n-UNDECYL-$\Delta^{1,6}$-PIPERIDEINE.

indicating that about 25 per cent of each piperideine ($\Delta^{1,2}$ and $\Delta^{1,6}$) had been present. Furthermore, only a few per cent of the *trans* C_{11} was formed in the reduction, which is in agreement with the results of the same reaction performed on the *S. xyloni* venom.

DISCUSSION

The venom of each of the four forms of *Solenopsis*, namely *S. saevissima* (red form), *S. saevissima* (black form), *S. geminata* and *S. xyloni*, contains distinctive amounts of certain 2,6-disubstituted piperidines. These alkaloids are characteristic of the form in so far as each venom has groups of similar compounds either dominating or almost completely lacking. This distinguishing quality is clearly evident from the four chromatograms presented in Fig. 1. It is particularly satisfying to obtain explicit chemical differences between the venoms of these forms, especially as these differences are consistent with current views of the taxonomy of this genus.

The venom of the imported fire ant, *S. saevissima* (red form), has five major components all of which are *trans*-2-methyl-6-n-alkyl (or n-alkenyl)piperidines. These compounds were identified by their mass spectra, by their chemical ionization (erroneously referred to previously as 'chemionization') mass spectra, and by synthesis (MACCONNELL *et al.*, 1971). The chromatogram in Fig. 1 of the venom of this ant shows that, while the *trans* form of these five compounds predominates, the corresponding *cis* form of all five compounds is present in trace amounts. The non-polar OV-1 stationary phase does not resolve all of these substances satisfactorily, e.g. *cis* C_{13} and *trans* $C_{13:1}$, and only on the polar Carbowax 20 M phase are the *cis* forms adequately resolved and clearly evident. This occurrence of *cis* isomers of the piperidine alkaloids in the venom of the red form of *S. saevissima* has not been indicated previously.

The position and conformation of the double bond in the side chain of the two unsaturated *cis* isomers has not been unequivocally established in this investigation. However, the mass spectrum of *cis* $C_{13:1}$ isolated from the venom of the black form of *S. saevissima* is congruent with that of the synthetic *cis* $C_{13:1}$, and the gas chromatographic behaviour

of the two unsaturated isomers obtained from these ants is identical to that of synthetic *cis* $C_{13:1}$ and *cis* $C_{15:1}$. We therefore assume that the double bond in the *cis* form of the natural products is in the same position and has the same stereochemistry (*cis*) as the double bond previously identified in *trans* $C_{13:1}$ and *trans* $C_{15:1}$ by MacCONNELL *et al.* (1971).

The black form of the imported fire ant has long been considered to be a colour variation of the red form of *S. saevissima*. It has been collected on a number of separate occasions at Starkville and Columbus, Mississippi, and has consistently given ratios of the components similar to those shown in Fig. 1, confirming that its venom has a characteristic composition distinct from that of the red form. The lack of any significant quantity of pentadecyl- or pentadecenylpiperidines is a dramatic deviation from the venom components of the red form in which the *trans*-2-methyl-6-*n*-pentadecenylpiperidine is quantitatively the most important constituent. However, mass spectra obtained on fractions collected from the C_{15} and $C_{15:1}$ region, together with their retention times, do indicate the presence of trace amounts of both *cis*- and *trans*-2-methyl-6-*n*-pentadecenylpiperidine in the venom of this form.

The ratio of *trans* $C_{13:1}$ to *trans* C_{13} in the black form venom also exhibits a variation from that usually obtained for these compounds in the red form venom. Although the *trans* compounds predominate in both forms, the amount of *trans* $C_{13:1}$ in the black form venom is very much greater than the amount of *trans* C_{13}. In the red form venom both of these compounds usually are present in approximately equal amounts. However, on occasions we have obtained venom from the red form which contains appreciably more of the *trans* $C_{13:1}$ component than *trans* C_{13}.

The mass spectra of the *cis* and *trans* ring isomers of these dialkyl piperidines are identical and the spectrum of *trans* C_{11} has been presented previously (MacCONNELL *et al.*, 1971). As the mass spectra of the piperidines containing saturated side chains differ slightly from those in which the side chain has a double bond, the mass spectra of *cis* C_{13} and *cis* $C_{13:1}$ are presented in Figs. 3 and 4 to illustrate this difference. The parent and P-15 ions are much more distinct in the spectrum of *cis* $C_{13:1}$ than in that of *cis* C_{13} and it is to be expected that olefinic linkages will stablilize molecular ions in general. Similar differences were obtained between the mass spectra of *cis* C_{15} and *cis* $C_{15:1}$.

The venom of the indigenous species of fire ant, *S. geminata* and *S. xyloni*, differs from those of the two colour forms of *S. saevissima* in that the C_{11} components are quantitatively the most important. This is in contrast to the abundance of the *trans* $C_{13:1}$ component of the black form and the *trans* C_{13}, *trans* $C_{13:1}$, *trans* C_{15} and *trans* $C_{15:1}$ components of the red form. All components containing C_{15} alkyl or alkenyl side chains are essentially lacking in these venoms and only trace amounts of the *cis* C_{13} and *cis* $C_{13:1}$ are detectable.

With the exception of the peak in the venom of *S. xyloni* eluting at 17 min (see Fig. 1), the venoms of *S. geminata* and *S. xyloni* are rather similar. This peak is not detectable at the sensitivities we have used in analysing the venoms of either the red or the black forms, and may be present in trace amounts in the venom of *S. geminata*. The reaction of a few micrograms of the venom of *S. xyloni* with either $NaBH_4$ or $NaBD_4$ caused the disappearance of this peak, and, on Carbowax 20 M, no new peak was observed. The data presented in Table 2 illustrate that $NaBD_4$ reduction of this peak brings about the introduction of deuterium into *cis* C_{11}. By peak height comparison on gas chromatograms, this peak usually corresponds to 5–8 per cent of the *cis* C_{11}, present in the venom. The incorporation of deuterium into *cis* C_{11}, as determined from the mass spectrum of the *cis* C_{11} after deuteration, is of a similar order. These results show fairly convincingly that reduction of this peak leads to the forma-

tion of *cis* C_{11}. As *cis* C_{11} is the more stable isomer, it is of interest to note the low incorporation of deuterium into *trans* C_{11}.

The mass spectrum of this peak is particularly interesting and is congruent with the spectrum given in Fig. 5 for 2-methyl-6-*n*-undecyl-$\Delta^{1,2}$-piperideine. The usual base peak at *m/e* 98 is missing and important peaks appear at *m/e* 96, 97 and 110. The parent ion at *m/e* 251, by analogy to the parent ions of C_{13} and $C_{13:1}$, suggests that this compound may be related to *cis* and *trans* C_{11} in the same way. However, a much more prominent parent ion and less important a-cleavage ions point to the fact that this compound is not a simple piperidine. The stabilizing influence of the double bond produces a more prominent parent ion, and, as it is reduced by $NaBH_4$ and $NaBD_4$, this double bond must be attached to the nitrogen atom. The peak at *m/e* 96 is presumably due to a-cleavage with the double bond retained by the ring.

m/e 96
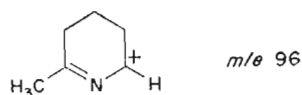
The fact that an ion at P-15 is not present supports placement of the double bond in this position.

The base peak at *m/e* 110 is apparently due to proton transfer to nitrogen with the 6-H migrating to the alkyl side chain followed by rupture of this chain, considerable stability being produced by the conjugation in the ion as shown.

m/e 110
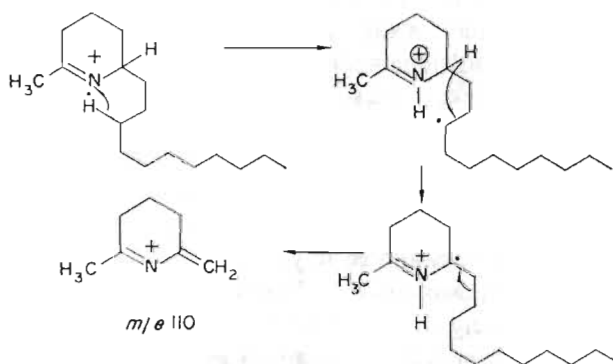
Mass 97 is unusual, in that it is an odd-electron species, and it may involve transfer of an H from the alkyl chain:

m/e 97   (or olefin)
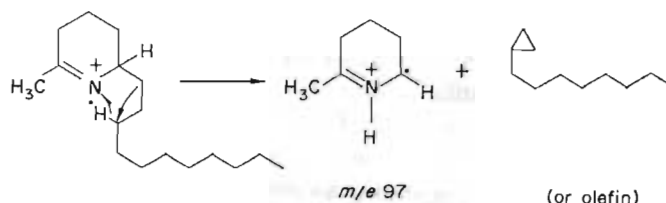
The reduction of *cis* C_{11} by *t*-butylhypochlorite to give two compounds, one of which is identical to the peak in the *S. xyloni* venom, confirms that this compound is 2-methyl-6-*n*-undecyl-$\Delta^{1,2}$-piperideine. A compound with this structure would be a likely candidate as a precursor of the 2-methyl-6-*n*-undecylpiperidines or as an intermediate in the interconversion of the *cis* and *trans* ring isomers.

The mass spectrum of the $\Delta^{1,6}$-isomer given in Fig. 6 is more straightforward, since the double bond is in a position to allow a McLafferty-type rearrangement of a proton from the side chain to produce the base peak at m/e 111. This then loses the 2-methyl group (proven by a metastable ion at m/e 83·0) to produce the ion at m/e 96. Apparently the possibility of a McLafferty-type rearrangement also destabilizes the molecular ion since it is much less intense in this isomer. Finally, both isomers show an ion at m/e 107 which may well be the 2,6-dimethylpyridine ion formed by loss of hydrogen from either m/e 110 or m/e 111.

Besides the decrease in the number of venom components of the two indigenous species of fire ants, a major change in the stereochemistry of the 2,6-dialkylpiperidines is apparent, especially of the 2-methyl-6-n-undecylpiperidine. The venom of both the red and the black forms essentially contains only the *trans* isomers, while both isomeric forms of this compound are present in the indigenous species with the *cis* form usually having the greater abundance. It would seem therefore, that while the imported forms have the biosynthetic capacity to synthesize, almost exclusively, the *trans* forms of the alkaloids from the appropriate precursors, there is no such biosynthetic bias in the two indigenous species. Alternatively, it is tempting to suggest that an oxidative enzyme is present which can convert either the *cis* or the *trans* ring isomer to the $\Delta^{1,2}$-piperideine and that this compound may then undergo biochemical reduction. In this way the $\Delta^{1,2}$-piperideine would act as an intermediate in the interconversion of *cis* and *trans* ring isomers. However, the actual biochemical rôle of the $\Delta^{1,2}$-piperideine is as yet unknown.

It must be emphasized that all the venom samples analysed in this investigation were obtained from numerous individuals and that the chromatograms in Fig. 1 show a mean ratio of the components present in the venom produced by many ants. Variation of the venom components between individuals cannot be excluded. In fact, we have obtained data on the venoms of individual workers and soldiers of *S. geminata* which show that the soldiers in a nest may consistently produce three to four times more *cis* C_{11} than *trans* C_{11} while the workers produce these two substances in approximately equal amounts. These and other data will be presented fully elsewhere. This finding, together with the variation in the red form mentioned previously, establishes that the ratios of the components in the venom of individuals of a species can vary appreciably.

The possibility of variation was considered between pooled samples of venom from the same species, collected in widely separated geographical areas. Worker ants of each of the four forms were collected from various localities (see Materials and Methods) and all chromatograms of the venoms of any one species were remarkably similar and consistent with the taxonomic classification of these forms of *Solenopsis*. Ants collected at Austin, Texas, from the population described as *S. geminata* var. *diabola*, gave a chromatogram of their venom similar to that presented in Fig. 1 for *S. geminata*, while ants collected at Tucson, Arizona, and identified by Dr. W. F. Buren as belonging to *S. xyloni* group, gave a chromatogram of their venom similar to that of *S. xyloni*.

A comparison of human skin responses to the stings of the red form and of *S. xyloni* has been made (BLUM *et al.*, 1961). The sting of the red form results in an inflamed pustule at the sting site while that of *S. xyloni* usually results in only mild irritation. In a comparison of the amounts of the *cis* C_{11} and *trans* C_{11} in the excised poison glands of equivalent sized workers of *S. xyloni* and *S. geminata*, we have found that *S. geminata* contains about five times more of these compounds than does the *S. xyloni*. The poison glands of both the red and the black forms appear to contain equal or greater amounts of their unsaturated *trans* compounds than the *S. geminata* poison gland contains of the C_{11} compounds. It therefore

seems reasonable to assume that during the process of stinging by these four species, *S. xyloni* workers will inject less venom than any of the other three forms of the fire ant.

It has been pointed out previously that the *trans*-2,6-disubstituted piperidines of the red form are the first of such alkaloids to be obtained from an animal source (MACCONNELL *et al.*, 1971). Indeed, only one *trans*-2,6-dialkylpiperidine has been isolated from plants, whereas a number of *cis*-2,6-disubstituted piperidines have been identified (HILL, 1970). The *cis* isomers, with the 2,6-substituents oriented diequatorially, are the more stable as this is the preferred conformation for substituents in these positions. Both the black and the red forms apparently can maintain a more selective control over this facet of their biosynthesis than either *S. geminata* or *S. xyloni*.

None of the 2,6-disubstituted piperidines identified from plant sources have alkyl chains with eleven, thirteen and fifteen carbon atoms. The two alkaloids, carpaine and cassine, have substituents in the 6-position containing 8 and 12 carbon atoms respectively, but neither of these two substituents is a simple hydrocarbon. It should be noted that the 6-alkyl substituents on the piperidine ring of the venom components correspond to *n*-hydrocarbons commonly found associated with defensive secretions of ants (BERGSTRÖM and LOFQVIST, 1968), hemipterans (BLUM *et al.*, 1960; WATERHOUSE *et al.*, 1961), and beetles (MEINWALD and EISNER, 1964). The glandular source of aliphatic hydrocarbons such as *n*-undecane and *n*-tridecane, in ants of the subfamily Formicinae, appears to be the Dufour's gland. As we have found *n*-heptadecane, together with other substances, in the Dufour's gland of both the red and black forms of *S. saevissima* and of *S. geminata*, it is possible that the presence of *n*-hydrocarbons in this gland may also be common to many myrmicine ants.

To our knowledge, all insects which sting, with the exception of species in the genus *Solenopsis*, utilize a venom rich in protein (BLUM, 1966). The occurrence of proteins in insect venoms seems almost a characteristic quality of these toxins. Recently, a trail substance produced by the ant *Atta texana*, methyl-4-methylpyrrole-2-carboxylate, has been isolated and occurs as a trace constituent together with the proteins in the poison gland of this myrmicine species (TUMLINSON *et al.*, 1971). Based on our present knowledge of animal venoms, the venom of *Solenopsis* is unique, as it comprises an aqueous suspension of 2,6-disubstituted piperidine alkaloids and contains no detectable amount of protein.

Workers of species in the genera *Solenopsis* and *Atta* are the only hymenopterons known to produce alkaloids in their poison glands. We wish to suggest that the proteinaceous venom of other stinging ants may contain trace quantities of biologically active alkaloids that may be utilized as trail substances or, more pertinent to this discussion, have toxic properties which contribute to the effectiveness of the venoms. In the process of the evolution of the myrmicine genus *Solenopsis*, selection may have emphasized the production of 2,6-disubstituted piperidine alkaloids as the main toxins in their venoms, at the expense of protein. The validity of this hypothesis could be tested by investigating carefully the proteinaceous venoms of other ants for the presence of trace quantities of alkaloids. In addition, investigations are being undertaken in collaboration with other laboratories to ascertain the toxicity of these piperidine alkaloids.

The various findings discussed in this paper lead us to speculate on the relative effectiveness of the venoms of these four forms of fire ants. We have observed that, during attack and defense, the venom of a fire ant will be injected into certain animals, whereas those insects with a rather hard exoskeleton often will have venom applied topically while the ants are attempting to sting. The major venom components of the red and the black forms are characterized by the unsaturated alkenyl side chain and their almost exclusive *trans* configuration.

A double bond in the side chain of these components will confer an added degree of polarity on the molecules as compared to their saturated homologues, and the *cis* configuration of this double bond will produce a marked deviation from linearity in the molecular shape of the side chain since one hydrogen on each α-carbon eclipses the double bond. Therefore, the overall shape of the two alkenyl substituted piperidines will be noticeably different from that of the two corresponding alkyl substituted homologues.

The venom components of *S. geminata* and *S. xyloni* are characterized by a shorter, saturated side chain, and appreciable quantities of both the *cis* and *trans* piperidine ring isomers. Molecular models of these *cis* and *trans* isomers show very clearly that the 2,6-disubstituted piperidine ring will have the hydrogen atom and electron lone pair of the nitrogen atom considerably more exposed when the substituents are *trans* (i.e. equatorial-axial). Diequatorial substituents in these positions tend to shield both the hydrogen atom and the electron lone pair of the nitrogen to a considerable extent. An illustration of this difference in polarity of a *cis* and *trans* pair is given by the longer retention time of the *trans* isomer on the polar Carbowax 20 M column and especially by the greater degree of retention of the *trans* relative to the *cis* isomer when chromatographed on alumina (MacCONNELL *et al.*, 1971).

It is reasonable to assume that the nitrogen atom plays a critical role in the biological activity of these alkaloids. If it were replaced by carbon it is likely that much of the activity of these compounds would be lost. The two imported forms of *Solenopsis* have a *cis* double bond in the alkenyl side chain and the substituents in positions 2 and 6 on the ring in the *trans* configuration. A side chain with a slightly polar character due to a double bond, along with the increase in the polarity due to the availability of the nitrogen atom in the *trans* configuration, should make these compounds better able to dissolve in lipophilic substances of varying polarity such as the insect cuticle and inter- and intra-cellular membranes. If, after penetration, the toxicity of these venoms is dependent to any degree on the availability of the nitrogen atom, then it is likely that the *trans* form will be more active than the *cis* form. The two imported forms of *S. saevissima* have achieved unquestionable success over the two indigenous species, and, it is interesting to speculate whether this success may be correlated, at least to some degree, with the differences in chemical structure of their venom alkaloids.

Addendum. We now wish to add that recent careful studies have shown the presence of a small amount of polypeptide material in the venom of the red form of the fire ant. We stress the importance of this finding and are attempting to establish the nature and amount of this material in the venoms.

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CHEMICAL CHARACTERIZATION OF THE DEFENSIVE
SECRETION OF A CHRYSOMELID LARVA

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Summary

    Larvae of the beetle Chrysomela interrupta produce
a defensive secretion in their eversible thoracic and
abdominal glands which is utilized to repel small
insect predators. This secretion is comprised pri-
marily of β-phenylethyl isobutyrate and β-phenylethyl
2-methyl butyrate, two esters which have not been ob-
tained previously from an animal source. This glandu-
lar exudate is the first defensive secretion of larvae
of Chrysomelinae reported to contain volatile compounds
other than salicylaldehyde.

Larvae of beetles in several genera in the subfamily

Chrysomelinae possess a series of paired defensive glands located

dorsally on the meso- and methathorax and the first 7 abdominal

segments (1,2). When disturbed, the larvae are capable of

evaginating these glandular reservoirs and exposing the defens-

ive secretion as a small drop on the tip of each projecting

glandular tubercle (3,4). Histological studies of the defensive

glands have indicated that they are evaginated by blood pressure

and withdrawn by special retractor muscles (1,2). Although the

larval integument may be slightly wetted by the defensive

secretion, the rapid retraction of the glandular reservoirs re-

sults in most of the exudate being withdrawn into the body after

a brief period of glandular eversion (1,2,3). This remarkable

mechanism of conservation insures that, notwithstanding the

limited capacity of the glandular reservoirs, the secretion will not be dissipated rapidly even if a larva has frequent defensive encounters.

Salicylaldehyde, an excellent defensive substance against small predators such as ants (4), is the primary constituent in the larval defensive secretion of Chrysomela scripta and of species in two other chrysomelid genera (4,5,6). We have now examined the defensive behavior and the chemistry of the defensive secretion of another chrysomelid species, Chrysomela interrupta, and report the identification of two new arthropod natural products in the glandular exudate. The biology of larvae of C. interrupta, which are commonly found feeding on hazel alder leaves (fam. Betulaceae: Alnus serrulata), is rather similar to that of other Chrysomela spp. (4,7).

Methods

The defensive secretion was collected in microcapillaries from the everted glands of tactually stimulated larvae and kept in n-hexane at -10°.

Gas chromatographic analyses were carried out on a Tracor MT-220 instrument equipped with flame ionization detectors and 183 x 0.6 cm o.d. glass columns. The following column packings and conditions were used: (1) 10 percent Carbowax 20 M on 100/120 mesh Chromosorb W, programmed from 80-150° at 1°/min; (2) 3 percent OV-1 on 100/120 mesh Chromosorb W, programmed from 70-160° at 1°/min; (3) 15 percent FFAP on 100/120 mesh Chromosorb W, isothermally at 130°. Nitrogen was used as a carrier gas at a flow rate of 30 ml/min.

Combined gas chromatographic-mass spectrometric analyses were carried out on an LKB 9000 instrument. Separations were

achieved on a 10 percent Carbowax 20 M column, programmed from
135-275° at 10°/min.

Results and Discussion

Clusters of larvae are conspicuous on hazel alder leaves
and with each succeeding molt the dark larvae become more apo-
sematically colored as the areas surrounding the tubercles,
especially on the thorax, become intensely yellow. Only the
thoracic defensive glands are functional in the gregarious first
instar larvae but the abdominal glands are utilized by subsequent
larval instars (3,4). Early instar larvae of C. interrupta, like
those of C. scripta and C. tremula (4,7), feed in clusters with
their heads projecting towards the margin of the leaf. This
feeding pattern results in most of the defensive glands being
positioned towards the base of the leaf, and in this way, these
glands are most favorably oriented to be utilized in the event
that a small terrestrial predator gains access to the leaf.
While the late larval instars of several other species of Chryso-
mela are solitary (3,4), those of C. interrupta larvae continue
to feed in aggregations which may persist through the terminal
larval instar. This prolongation of gregariousness in late
instar larvae of C. interrupta may result in an increased
defensive capability afforded by the combined action of the
defensive glands of several larvae.

When larvae were exposed to workers of the fire ant
Solenopsis invicta, the defensive value of the secretion was
evident. Contact between an ant and a Chrysomela larva resulted
in a sequential series of glandular eversions, usually originat-
ing with the glands closest to the point of contact stimulation.
Ants which were contaminated with the secretion immediately moved

away from the larva and began cleaning themselves, while at the same time exhibiting abnormal ambulatory movements.

Gas chromatographic analyses of the extract on 3 stationary phases demonstrated that 2 compounds constituted more than 90 percent of the observed volatiles. Combined gas chromatographic-mass spectrometric analyses established that both of these major constituents possessed a base peak at m/e 104 which appeared to be the molecular ion. The mass spectrum of the compound with the shorter retention time exhibited strong signals at m/e 43, 71, and 91 whereas the spectrum of the other main volatile contained major peaks at m/e 57, 85, and 91. The spectra of these two compounds indicated that they were members of a homologous series with the base peak at m/e 104 being most easily reconciled with a styrene ion (8). However, the signals at m/e 43 produced by one compound and m/e 57 by the other could not be associated with the fragmentation pattern of styrene ions, and these lower mass fragments suggest that the ion at m/e 104 is not the molecular ion. A base peak and apparent molecular ion at m/e 104 is also characteristic of certain β-phenylethyl esters. In this case the signal at m/e 43 could arise from an acetyl ion of an ester or a propyl ion which is generated from a C_4 acid of an ester. Similarly, the signal at m/e 57 could be identified with either a propionyl ion from an ester or a butyl ion generated from the C_5 acid of an ester (8).

Both the gas chromatographic and the mass spectrometric analyses of β-phenylethyl acetate and the propionate demonstrated that the retention times and mass spectra of these two esters were different from those of the 2 compounds in the C. interrupta defensive secretion. Comparison of the butyl and isobutyl esters of β-phenylethyl alcohol demonstrated that β-phenylethyl iso-

butyrate had an identical retention time to that of the lower-boiling C. interrupta volatile on all three stationary phases. Since the mass spectrum of this ester was completely congruent with that of the Chrysomela constituent, the latter is evidently β-phenylethyl isobutyrate. The higher boiling compound in the C. interrupta secretion had the same retention properties as β-phenylethyl 2-methylbutyrate and its gas chromatographic behavior on Carbowax 20 M clearly distinguished it from the other three pentyl esters of β-phenylethyl alcohol. The structure of this natural product was unequivocally established by the complete accordance of its mass spectrum with that of the β-phenylethyl ester of 2-methylbutyric acid.

The relative proportion of the esters in the secretion was established with samples collected over a 3 year period. The average ratio of β-phenylethyl isobutyrate to β-phenylethyl 2-methylbutyrate was about 1:4. A synthetic mixture of these two esters, made up in this proportion, appeared to be equivalent in repellency for fire ant workers to the natural secretion of a C. interrupta larva.

While certain β-phenylethyl esters are known as artificial flavor additives, neither of these two esters has been identified previously from animal natural products. As their only reported biological occurrence is in galangal oil (fam. Zingiberaceae: Alpinia officinarum) (9), it would appear that they are not common plant constituents, and it is interesting to note that, as in the secretion of C. interrupta, the 2-methylbutyrate ester greatly predominates in the ginger oil. Gas chromatographic analyses of headspace samples prepared from alder leaves did not reveal the presence of these two substances in this plant material.

Although this is the first report of these compounds from an animal source, it should be pointed out that both the acidic and alcoholic moieties of these esters have been obtained from arthropods. Isobutyric acid and 2-methylbutyric acid are the active principles in the defensive secretions of the larvae of swallowtail butterflies (10) and β-phenylethanol is a presumed aphrodisiac liberated from the scent hairs of male moths (11). While both portions of the C. interrupta esters can be synthesized by certain arthropods, the eversible gland products of other larval Chrysomelinae had been identified with only the aromatic compound, salicylaldehyde. The presence of two β-phenylethyl esters in this Chrysomela species demonstrates a greater diversity in the chemical nature of the defensive secretions produced by members of this arthropod taxon than was previously recognized.

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ALARM PHEROMONES IN THE GENUS *MANICA* DERIVED FROM THE MANDIBULAR GLAND

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Abstract—The alarm pheromones present in the mandibular glands of *Manica mutica* and *M. bradleyi* are dominated by a novel C_{10} ketone, 4,6-dimethyl-4-octene-3-one (manicone). Two other new insect pheromones, 4-methyl-3-hexanone and 3-decanone, are also present. In addition, two characteristic myrmicine alarm pheromones, 3-octanone and 4-methyl-3-heptanone, have been identified as mandibular gland constituents. While manicone functions as a powerful releaser of alarm behaviour for *Manica* workers a much weaker response was obtained to the other identified 3-alkanones. The significance of the occurrence of 3-ketones in members of the genus *Manica* and species in other genera of the Myrmicinae is analysed in terms of the accepted phylogeny of this subfamily.

INTRODUCTION

THE THOROUGH comparative investigation of European Hymenoptera by MASCHWITZ (1964) clearly established that alarm pheromones generally are not highly specific for species within a genus. However, in the formicid genus *Myrmica*, Maschwitz reported that the mandibular gland pheromone of one species, *M. rubida*, was not highly excitatory for three other species in this genus and vice versa. Furthermore, he noted that the odour of the mandibular gland products of *M. rubida* smelled differently from those of the other species of *Myrmica*.

The apparent singularity of the alarm-releasing specificity of the *Myrmica rubida* pheromone loses some of its significance when it is realized that in actuality this species is a member of another myrmicine genus. *M. rubida* and its four North American congeners have been removed from the genus *Myrmica* (WEBER, 1947; CREIGHTON, 1950) and assigned to the genus *Manica*, a taxonomic development which has been amply supported by a recent detailed investigation of this myrmicine taxon (WHEELER and WHEELER, 1970).

We have also noted that the odour of the mandibular gland secretion of North American species of *Manica* differed significantly from any species of *Myrmica* which we have examined. Because the chemistry of the alarm pheromone of *Myrmica* species has recently been described (CREWE and BLUM, 1970a, b),

species in the closely related genus *Manica* seemed ideally suited for a comparative investigation. The present paper forms the substance of this study.

MATERIALS AND METHODS

Sources of the pheromones

Workers of *Manica mutica* and *M. bradleyi* were employed as pheromone sources. Heads were removed from the workers and extracted in either Freon 113, CS_2, or *n*-pentane. These extracts were utilized for gas chromatographic analyses without further treatment. For mass spectral analyses, the extracts were obtained by macerating 10 worker heads in 25 μl of pentane. This procedure was repeated until a pentane extract with a total of 500 μl had been obtained. This extract was then concentrated and used in combined gas chromatographic–mass spectrometric (GC–MS) analyses.

Mandibular glands were dissected from workers into pentane in order to establish the exact source of the pheromones. This extract was analysed gas chromatographically.

Gas chromatography and mass spectrometry

Analyses were undertaken on a Tracor MT-220 gas chromatograph equipped with flame ionization detectors and 6 ft $\times$ 0·25 in. glass columns. The following stationary phases were employed: (1) 10% FFAP on Chromosorb W 100/120 mesh, (2) 10% diethylene glycol adipate (DEGA) on Chromosorb W 80/100 mesh, and (3) 10% Carbowax 20 M on Chromosorb W 100/120 mesh. The FFAP column was operated at 70°C and the other two at 80°C. Nitrogen was employed as the carrier gas at a flow rate of 30 ml/min.

Combined GC–MS analyses were performed using an LKB 9000 instrument containing columns of either 1% OV-17 or 10% SP-1000 on 80/100 mesh Supelcoport at 100°C.

Sodium borohydride reductions

Manica extracts and standard compounds were reduced with sodium borohydride by employing the method of Crewe and Blum (1970b).

Standard compounds

The following compounds used in this investigation were obtained from the Aldrich Chemical Company: 3-octanone, 3-octanol, 4-methyl-3-heptanone, 4-methyl-3-heptanol, and 4-methyl-3-hexanone. A sample of 3-decanone was obtained from P. G. Kletzke (Kletzke, 1964).

4,6-Dimethyl-4-octene-3-one was synthesized as follows. 2-Methylbutanal (6 ml) was combined with 12 ml of 3-pentanone and 12 ml of methanol in which was dissolved 3 g of potassium hydroxide. The solution was allowed to reflux overnight, cooled, and poured into water. The solution was extracted twice with ether, the extracts dried over sodium sulphate, and, after evaporation of solvents, distilled in the presence of 20 mg of I_2. The product distilled at 94°C (20 mm);

$v_{\text{max}} = 1680 \text{ cm}^{-1}$ [unsaturated carbonyl; δ 1·80 (doublet $J = 1 \cdot 37$ Hz*), δ 6·37 (broad doublet $J = 10$ Hz†)]; a red 2,4-dinitrophenylhydrazone, m.p. 129–131°C from ethanol. Analysis calculated for $C_{16}H_{22}N_4O_4$: C, 57·47; H, 6·63; N, 16·76. Found: C, 57·56; H, 6·75; N, 16·96.

Bioassay

All compounds, both singly and in mixtures, were tested on workers of *M. mutica* as described by CREWE and BLUM (1970a).

RESULTS

Gas chromatography and mass spectroscopy

Analyses of the head extracts on OV-17 demonstrated that a series of high boiling components was present which eluted between 170 and 225°C. Compounds definitely identified by their mass spectra were: a series of *n*-alkanes C_{23}–C_{29}, odd carbon members being more prominent and *n*-$C_{25}H_{52}$ the major component; di-(2-ethylhexyl) phthalate (probably from solvents, etc.); and cholesterol. These compounds are probably structural components which are derived from the cuticle and tissues in the head.

A typical chromatogram of the more volatile components, which accounted for only a few per cent of the total mass, is shown in Fig. 1. This sample was analysed on 10% SP-1000 at 100°C. Five components accounted for over 95 per cent of the observed low boiling volatiles and these compounds were all subsequently identified as ketones. In addition, the hydrocarbon *n*-C_9H_{20} was also identified in the extracts of *Manica* heads.

The mass spectrum of compound 1 (Fig. 1) had a molecular ion at m/e 114 with prominent peaks at m/e 85 and 57 and was congruent with that of 4-methyl-3-hexanone. The retention time of this compound and that of 4-methyl-3-hexanone were identical on the Carbowax 20 M, FFAP, SP-1000, and DEGA phases. In addition, reduction of the *Manica* extract removed this component and produced two compounds with retention times identical to those of the 4-methyl-3-hexanols (see below).

The component designated as 2 in Fig. 1 had a molecular ion at m/e 128 and possessed intense peaks at m/e 86, 71, 57, and 29. The mass spectrum of this compound was identical to that of 4-methyl-3-heptanone (McGURK *et al.*, 1966). The identity of this ketone was substantiated on the same four phases as before and, in addition, its reduction with $NaBH_4$ resulted in its disappearance and the production of compounds with a chromatographic behaviour identical to those of the 4-methyl-3-heptanols.
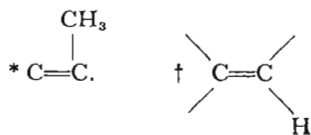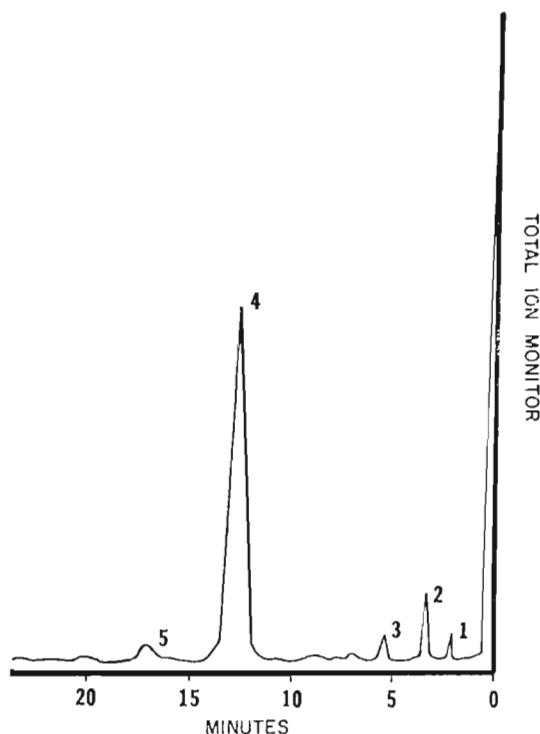
FIG. 1. Chromatogram of the low boiling volatile compounds in the mandibular glands of *M. mutica* workers: 1 = 4-methyl-3-hexanone, 2 = 4-methyl-3-heptanone, 3 = 3-octanone, 4 = 4,6-dimethyl-4-octene-3-one (manicone), and 5 = 3-decanone.

The compound represented as 3 (Fig. 1) gave a mass spectrum which was in complete accordance with that of 3-octanone. The chromatographic behaviour of compound 3 was also identical to that of 3-octanone.

The parent ion of compound 5 (Fig. 1) occurred at m/e 156. A peak at m/e 72, characteristic of β-cleavage of ethyl ketones, indicated that this compound was a 3-alkanone. Intense peaks at m/e 127 and 57 could then arise by α-cleavage on either side of the carbonyl group. The mass spectrum of this compound was completely congruent with that of 3-decanone. Evidence from gas chromatographic analyses and $NaBH_4$ reductions substantiated this identification.

Compound 4, the main component of the more volatile fraction (Fig. 1), proved to be less straightforward in structure than the above compounds. Thus, the mass spectrum (Fig. 2) showed a molecular ion at m/e 154 identified by its relative increase in intensity at 15 eV.

Loss of an ethyl group (29 amu) is followed by loss of water and either CO or ethylene. Metastables at 101·2, 91·6, and 75·3 (Fig. 2) support all of these processes. Since at least one oxygen is present (loss of water), the simplest required
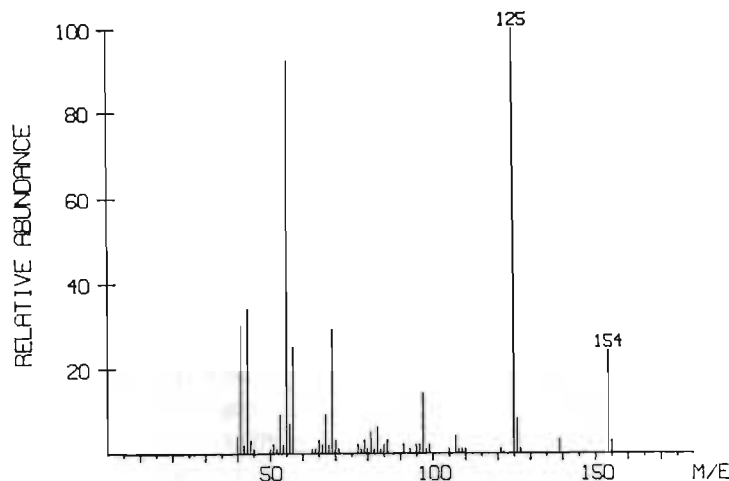
FIG. 2. Mass spectrum of manicone, 4,6-dimethyl-4-octene-3-one.

formula $C_{10}H_{18}O$ suggested two points of unsaturation or rings. Since the 3-ketones mentioned also showed pronounced loss of ethyl (due to α-cleavage adjacent to the ketone), proof that the compound in question was also a ketone was sought by its conversion to a methoxime by heating (60°C) with methoxyamine hydrochloride and pyridine. The molecular ion of this derivative (m/e 183) still exhibited loss of an ethyl group supporting the presence of a 3-keto function. On the other hand, in contrast to the other 3-ketones, this ketone showed no odd-electron ions (at m/e 72 or 86) due to the McLafferty rearrangement. For this reason the double bond was placed adjacent to the carbonyl group since it would be expected to suppress the rearrangement in this location. In support of this formulation the intensity of the ion at m/e 57 (CH_3CH_2CO) is very much lower than in the case of the aforementioned saturated 3-ketones. This ion also helps to rule out a possible alternative structure involving a cyclopropane ring adjacent to the ketone since cleavage between the propionyl group and ring with charge retention by this ion would be expected to be pronounced. Catalytic hydrogenation with PtO_2 in the presence of caproic acid (caproic acid was employed since acetic acid obscured the gas chromatogram and no reduction was observed in neutral solution) produced at least two (GLC) closely related isomeric 3-decanones whose mass spectra were identical. In contrast to the original ketone, these spectra now exhibited important McLafferty rearrangement ions at m/e 86 proving that a methyl group was present in the 4-position and the partial formula

$$CH_3CH_2-\overset{\displaystyle O}{\overset{\displaystyle \|}{C}}-\overset{\displaystyle CH_3}{\overset{\displaystyle |}{C}}=C\overset{\displaystyle X}{\underset{\displaystyle Y}{<}}$$
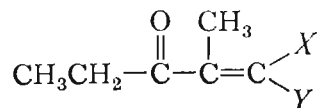
was proposed for the original compound. The simplest formulation, $X = H$ and $Y = n\text{-}C_4H_9$ did not seem likely since only one product ought to have been

produced by hydrogenation. Furthermore, the retention times of the saturated ketones were earlier than calculated for this compound on SP-1000 using 4-methyl-3-hexanone and 4-methyl-3-heptanone as models.

Reduction of the unsaturated ketone with sodium borohydride provided a valuable clue, since two alcohols whose mass spectra were identical (very weak molecular ion at m/e 156; loss of water and ethyl) were produced in equal amounts (GLC). (The 4-methyl-3-ketones, on reduction with sodium borohydride, also produce 2 diasteroisomeric alcohols that are separated by GLC on this phase, SP-1000, but when a double bond is present in the 4,5 position such isomerism is not possible.) This requires that $X = H$ and $Y = -CH(CH_3)C_2H_5$ since only this combination supplies the necessary assymetry to produce diastereoisomeric allylic alcohols. Furthermore, since the site of assymetry is remote from the ketone they should be produced in equal amounts as observed.

It is clear that this compound is an isomer of the recently discovered component of the defensive secretion of the opilionid *Leiobunum vittatum*, viz. E-4,6-dimethyl-6-octene-3-one (MEINWALD *et al.*, 1971). However, the reported mass spectrum of the latter compound is different in many important respects from that of compound 4, particularly with regard to the intensities of m/e 125 and 86. Furthermore, the infra-red and NMR spectra of the synthetic materials show the expected differences.

This compound, 4,6-dimethyl-4-octene-3-one, I, appears not to have been prepared previously, but the very closely related 4,6-dimethyl-4-heptene-3-one is

$$\underset{C_2H_5C}{\overset{O}{\underset{\|}{}}}\overset{CH_3}{\underset{|}{C}}=CH-\overset{CH_3}{\underset{\underset{H}{|}}{C}}\diagdown_{C_2H_5}$$
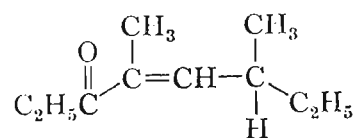
I

available by the base-catalysed condensation of isobutyraldehyde and 3-pentanone (FRANKE and KÖHLER, 1923; POWELL, 1924; POWELL *et al.*, 1933). A similar reaction using 2-methylbutanal produced I in good yield as the major product showing identical retention time and mass spectrum as I from *M. mutica*. A minor component of the synthetic mixture (3 per cent) of very slightly shorter retention time was probably the geometric isomer of I since its fragmentation pattern was very similar to that of I; differences were clear however, particularly in the relative intensities of the metastables involving loss of 18 and 28 mass units from m/e M-29.

The allylic coupling observed between the C_4 methyl substituent and the C_5 proton in the NMR spectrum of synthetic I is 1·37 Hz and suggests a *cis* (Z) relationship between these groups by analogy with 3-methyl-3-butene-2-one (JACKMAN and WILEY, 1960). However, this assignment must be viewed with caution since it was not possible to compare its NMR spectrum with the other isomer due to difficulties in isolating the latter. Compound I, given the trivial

name manicone, contains an assymetric carbon at C_6 but no information was obtained concerning its optical activity. The retention times of the low boiling compounds in the heads of *M. mutica* workers, as well as the reduced forms of manicone and its derivative, are presented in Table 1.

TABLE 1—RETENTION TIMES (RELATIVE TO 3-DECANONE, 12·5 min) OF THE MANDIBULAR GLAND LOW BOILING COMPOUNDS OF *M. mutica* ON SP-1000 AT 100°*

Compound	Relative time
n-C_9H_{20}	0·227
3-Octanone	0·348
4-Methyl-3-hexanone	0·141
4-Methyl-3-heptanone	0·212
Manicone	0·734
Manicone methoxime	0·600
Dihydromanicone	0·415, 0·433
$NaBH_4$-manicone	1·33, 1·45

* Retention times of reduced forms of manicone and a derivative are also included.

All ketones identified in the heads of *M. mutica* workers were established to be present in the mandibular glands. The results obtained with *M. bradleyi* extracts were the same.

Bioassay

Manicone released well-developed alarm behaviour in workers of *M. mutica*. This ketone was a potent attractant for the workers who approached the ketonic source with their mandibles spread while they moved frenetically over the nest surface. The other four ketones also functioned as alarm releasers but none of them appeared to be as active as manicone. A mixture of the five ketones made up in the same proportion as they were determined to be present in the mandibular gland did not appear to be any more active as an alarm releaser than manicone alone.

DISCUSSION

The diversity of ketones produced in the mandibular glands of *Manica* workers underscores the versatility of ants in biosynthesizing these short-chain oxygenated pheromones. *Manica* workers, it would appear, have carried this variation on a ketonic theme to an extreme, because their repertory of these carbonyl compounds is considerably greater than that of species in any other myrmicine genera. Indeed, *Manica* species share common ketones with species in all myrmicine genera in

which representatives of this class of compounds have been identified. Although the *Manica* mandibular gland is fortified with three ketones which have not been previously detected in ants, it would not be surprising if these unique compounds are subsequently found in species in other myrmicine genera.

4-Methyl-3-heptanone, which accounts for nearly 13 per cent of the ketonic mixture of *M. mutica*, is the only carbonyl-containing alarm pheromone produced by species in the myrmicine genus *Pogonomyrmex* (McGurk *et al.*, 1966). This compound also functions as a potent alarm releaser for *Atta* species which, in addition, produce a typical dolichoderine alarm pheromone, 2-heptanone, in their mandibular glands (Blum *et al.*, 1968; Moser *et al.*, 1968). Recently, workers of a species in another attine genus, *Trachymyrmex seminole*, have been demonstrated to produce 4-methyl- 3-heptanone in their mandibular glands but unlike *Atta*, the pheromone source of this species does not contain detectable quantities of 2-heptanone (Crewe and Blum, 1972). However, the methyl heptanone of *T. seminole* is accompanied by 3-octanone, another major compound found in the ketone-rich mandibular gland secretion of *Manica* species.

3-Octanone, like 4-methyl-3-heptanone, appears to be an alarm pheromone which is typical of species in genera of the subfamily Myrmicinae. 3-Octanone is the major volatile constituent found in the mandibular gland secretion of most species in the genus *Myrmica* (Crewe and Blum, 1970b) and it is also utilized as an alarm pheromone by some species in the myrmicine genus *Crematogaster* (Crewe *et al.*, 1970). This compound also serves as an alarm releaser for attine species in the genus *Acromyrmex* as well as species of *Trachymyrmex*, as indicated previously (Crewe and Blum, 1972). However, whereas 4-methyl-3-heptanone accompanies 3-octanone in *T. seminole*, this branched heptanone could not be detected in the 3-octanone-rich secretion of *T. septentrionalis*. Thus, in most of the myrmicine species which have been examined, 3-octanone and 4-methyl-3-heptanone do not occur together in the same glandular secretion.

The absence of detectable amounts of the alcohols corresponding to the ketones present in the mandibular secretion of *Manica* was rather unexpected. In workers in the genera *Myrmica* (Crewe and Blum, 1970b), *Crematogaster* (Crewe *et al.*, 1970), *Pogonomyrmex* (McGurk *et al.*, 1966), *Trachyrmyrmex*, and *Acromyrmex* (Crewe and Blum, 1972), the alcohol which corresponds to the 3-alkanone is a major volatile product in the mandibular gland secretion. Although it is attractive to speculate that 3-alkanols may be involved in the biosynthetic pathway for their ketonic counterparts, the apparent absence of these alcohols in the glandular secretion of *Manica* workers may raise doubts about the rôle of the carbinols as intermediates in the ketonic syntheses.

4-Methyl-3-hexanone and 3-decanone do not appear to have been previously identified in either plants or animals and their significance in the ketonic secretion of *Manica* workers is not clear. 4-Methyl-3-hexanone is not the first methyl hexanone to be identified in ants, since 4-methyl-2-hexanone has been shown to be an anal gland product of *Dolichoderus clarki* (Cavill and Hinterberger, 1962). *D. clarki* is characteristic of many dolichoderine species in producing a

2-alkanone which is probably of terpenoidal origin. Although *Atta* species also produce a 2-ketone in addition to 4-methyl-3-heptanone, the biosynthetic emphasis among species in the subfamily Myrmicinae is clearly identified with 3-ketones.

Seven different 3-ketones have been shown to be present in the mandibular gland secretions of myrmicine species and none of these compounds has possessed less than seven carbon atoms. The presence of 3-decanone in *Manica* species extends the unbranched 3-alkanone series in the Myrmicinae which was already identified with 3-octanone in species in many genera and 3-nonanone in *Myrmica* species (CREWE and BLUM, 1970b). The ability of many myrmicine species to produce 3-ketones which possess methyl branching on the C_4 carbon is well developed and it has recently been determined that some myrmicines synthesize 3-alkanones which are branched on the C_6 carbon (CREWE and BLUM, 1970b). In the case of manicone, 4,6-dimethyl-4-octene-3-one, the ability of myrmicine species to biosynthesize a 3-ketone which contains methyl branching on both the C_4 and C_6 atoms is evident.

Manicone is characteristic of myrmicine alarm pheromones in not being of apparent terpenoidal origin. Whereas species in the subfamilies Dolichoderinae and Formicinae produce many alarm pheromones which are terpenes, the alarm releasers utilized by species in the Myrmicinae are synthesized by metabolic pathways which have not been illuminated. However, the presence of the unsaturated ketone, manicone, together with its easy synthesis from the corresponding aldehyde and 3-pentanone suggest that a related process may be involved in its biosynthesis. The common occurrence in myrmicine species of 4-methyl-3-ketones and 3-ketones would then involve either 3-pentanone or 2-butanone condensing at either the methylene or methyl group respectively. The presence of the necessary aldehydes would not be too difficult to visualize especially in the light of the common occurrence of this class of compounds in insects.

The emphasis that myrmicine species have placed on the biogenesis of 3-ketones almost guarantees that these ants will possess olfactory receptors which exhibit maximal sensitivity to ethyl ketones. Thus, MOSER *et al.* (1968) reported that workers of *A. texana* were $100,000 \times$ more sensitive to their natural alarm pheromone, 4-methyl-3-heptanone, than they were to the closely related isomeric alkanone 4-methyl-2-heptanone. This result indicates that the myrmicine chemoreceptors are eminently capable of resolving between ethyl ketones and closely related methyl ketones. Although *A. texana* workers also produce 2-heptanone in their mandibular glands, their selective sensitivity to 3-ketones is illustrated by the fact that they are $1000 \times$ less sensitive to this methyl ketone than they are to 4-methyl-3-heptanone (MOSER *et al.*, 1968). BLUM *et al.* (1971) have reported that *P. badius* workers exhibit a much greater sensitivity to 4-methyl-3-heptanone and closely related ethyl ketones than they do to any methyl ketones. Indeed, it seems probable that myrmicine chemoreceptors are not only maximally responsive to ethyl ketones, but the receptors of each species are most sensitive to the specific ketones which are produced and utilized to generate an alarm signal.

Maschwitz (1964) studied the responsiveness of three *Myrmica* species to the alarm pheromone of *Manica rubida* and concluded that the *Myrmica* species were much less sensitive to the *M. rubida* secretion than they were to their own. Similarly, *M. rubida* workers did not respond nearly as well to the *Myrmica* pheromone as they did to their own mandibular gland secretion. The alarm pheromones of *Myrmica ruginodis* and *M. rubra* (= *laevinodis*), two of the species that Maschwitz studied, have been analysed and 3-octanone was shown to be the main volatile compound which was present (Crewe and Blum, 1970b). If it is assumed that *M. rubida* is typical of the North American species in producing an alarm pheromone dominated by manicone, then it is possible to analyse the results of Maschwitz in terms of specific chemical releasers of alarm behaviour. Since the mandibular glands of *M. rubida* probably contain 3-octanone, the responsiveness of *Myrmica* workers can reflect a reaction to this compound. Furthermore, we have determined that workers of *Myrmica americana* are not very responsive to manicone, and it is not unlikely that the European species of *Myrmica* which Maschwitz studied are similarly not very responsive to this ketone. *M. rubida* on the other hand probably exhibits maximal sensitivity to manicone as do North American *Manica* species and, as a consequence, the workers of this species would not be expected to respond as strongly to the *Myrmica* alarm pheromone which lacks this 3-ketone. The ability of *M. rubida* workers to react at all to the *Myrmica* pheromone is possibly a consequence of the presence of 3-octanone, a compound which *M. rubida* workers probably produce in their own mandibular glands and to which they are somewhat sensitive.

Although the genus *Manica* is very closely related to *Myrmica*, the differences in their alarm pheromones are striking. Our results offer strong biochemical support for the separation of *Manica* species from the genus *Myrmica* (Weber, 1947; Creighton, 1950; Wheeler and Wheeler, 1970). We have been unable to detect manicone in the mandibular glands of *Myrmica* workers and at this juncture we regard this 3-ketone as a compound which is characteristic of *Manica* species. The great diversity of ketones produced in the mandibular glands of *Manica* workers would seem to emphasize the critical phylogenetic position that this genus may occupy in terms of the biosynthetic capbilities of the species in myrmicine genera. *Manica* is considered to be among the least specialized genera in the tribe Myrmicini (Wheeler and Wheeler, 1970) and it may not be without significance that its species possess representatives of all the known 3-ketones which have been identified in species in more highly specialized myrmicine genera. If evolutionary reduction has characterized the qualitative composition of alarm pheromones in the genera of the subfamily Myrmicinae, then *Manica* must be regarded as one of the more primitive taxa from a biosynthetic standpoint. Loss of alkanones such as manicone, 3-decanone, and 4-methyl-3-hexanone would emphasize 4-methyl-3-heptanone and 3-octanone as alarm releasers in different species. The combination of both of these pheromones could characterize some species (e.g. *T. seminole*) whereas species in other genera would utilize either 4-methyl-3-heptanone (*Pogonomyrmex*) or 3-octanone (*Myrmica*) as alarm phero-

mones. The presence of 2-heptanone as a concomitant of 4-methyl-3-heptanone in *Atta* species would serve to emphasize the biosynthetic plasticity which characterizes this very specialized myrmicine genus.

WHEELER and WHEELER (1970) consider *Manica mutica* to be ancestral to the other North American species and to *M. rubida*. Thus, the identification of the alarm pheromones of *M. mutica* offers an excellent opportunity to analyse the evolution of this genus in terms of specific chemical constituents. It is not unlikely that the analyses of pheromones in species in other insect genera will have some substantive value as a systematic character with which specific relationships may be more meaningfully probed.

4-Methyl-3-heptanone, one of the major ketones in the *Manica* mandibular gland exudate, is not limited in its arthropod distribution to ants. This compound is also produced in the scent glands of opilionids in the genus *Leiobunum* and functions as an excellent defensive secretion, especially against small insect predators such as ants (BLUM and EDGAR, 1971). It may be reasoned, therefore, that the ability of species of social insects to biosynthesize many pheromones is an expression of a biochemical legacy inherited from non-social arthropods which had already developed the capacity to make the same substance for use in a defensive context. The similarity of these defensive secretions and pheromones has been suggested as indicative of the secondary and broader utilization of arthropod defensive substances by social insects to fill the additional rôle of volatile information-bearing substances played by pheromones (BLUM and EDGAR, 1971).

As mentioned previously, MEINWALD *et al.* (1971) recently identified 4,6-dimethyl-6-octene-3-one, an isomer of manicone, together with 4-methyl-3-heptanone, in the defensive secretion of another species of *Leiobunum*. Therefore, the occurrence of manicone in the mandibular glands of *Manica* species is also consistent with the conclusion that alarm pheromones have their biogenetic origins in the metabolic pathways for defensive compounds in solitary arthropods.

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Biological Applications of Electron Ionization and Chemical Ionization Mass Spectrometry

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I. Introduction

This paper deals with a subject that may have rather limited application to hormone research as it is practiced today. Still, when the occasion does arise that mass spectrometry can be used in a problem, there should be no hesitation in doing so since the technique is relatively simple experimentally. Furthermore, interpretation of the data is often straightforward in applications of this sort.

In order to be able to determine when and where the technique may be invoked, it is useful to have some basic understanding of the analytical process. Therefore, it may be helpful to run rapidly through the physics of the method, indicating present limitations and, hopefully, ways that some of these may be overcome in the future.

II. Methods

A basic mass spectrometer system is illustrated by Fig. 1. Essentially, it is composed of a curved, hollow pipe, evacuated to a very high degree, placed in the jaws of an electromagnet. Ions of unit charge formed by various processes at the entrance are accelerated to a constant energy during passage between two plates across which there is a fixed potential, usually of the order of thousands of volts. Although the energy ($mv^2/2$) of all the ions of unit charge is the same, ions of different mass will have different velocities and their momentum (mv) will also vary. A particle of unit charge is deflected in a magnetic field according to its momentum. Even intuitively it will be seen that the more momentum an ion has, the less it will be deflected by a magnetic field of a fixed strength. For this reason, ions of different mass will "fan out" in the magnetic field, and it might be possible to arrange for their detection by allowing them to fall on a photographic plate placed at an appropriate distance from the magnet. In fact, this method of detection is still

utilized today in some high resolution instruments where it has the advantage of detecting all ions simultaneously. Thus momentary fluctuations in ion beam intensity due to changes in sample pressure are integrated.

For low resolution work, it is more convenient to vary the strength of the magnetic field by using an electromagnet. The "fan" of ion beams of different mass will increase or decrease their arc as the field is changed, each ion beam falling in succession over a sensitive electrical detector fixed at a location in the ion trajectory. This results in the type of mass spectrum with which most are familiar; a series of peaks
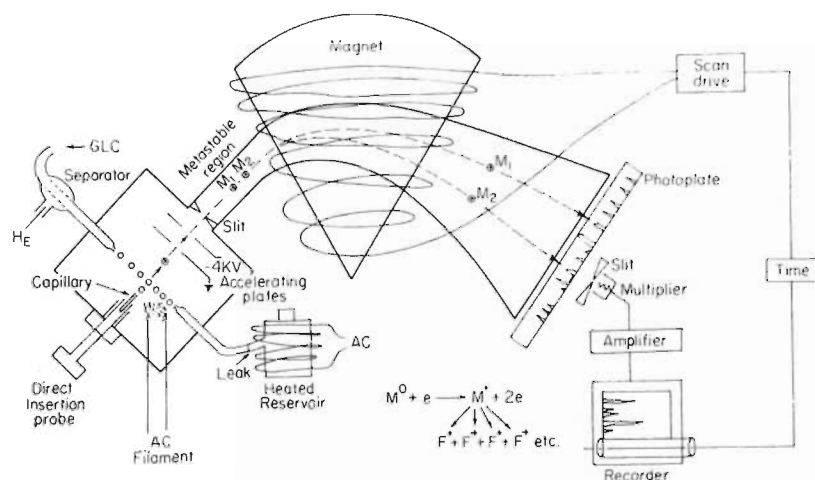
FIG. 1. Schematic diagram of a magnetic deflection mass spectrometer.

arranged according to mass whose height is proportional to intensity of the ion beam. Since the magnet may be scanned very rapidly (1–2 seconds), the entire spectrum is accumulated and a very rapid means of detection and display is required. Although expensive computer options are available today which present data in forms that are easy to comprehend, there are still very real advantages to direct recording, using an oscillograph with 3 beams, attenuated by factors of 1, 10, and 100. One of the strong points about mass spectrometry is the enormous dynamic range of the signal. I know of no other laboratory instrument that routinely uses three pens for recording. In conjunction with this system we use a photomultiplier detector which can detect a single pulse of a few ions. On the other hand, it may take many molecules to produce these ions because of inefficiency in the ionization process. It is in this area that we must look for improvements leading to greater

sensitivity. In fact, today all mass spectrometers are very similar in ionization efficiency, and claims of enhanced sensitivity by one manufacturer over another are to be viewed with caution. On the other hand, it is possible to enhance sensitivity by counting ions as we do β-particles. The only disadvantage of this lies in the time required for the process. Time is often very limited in mass spectrometry since it is difficult to predict the optimum temperature for sample volatilization. Choice of too high a temperature results in a strong signal for a very short time, as the sample rapidly evaporates from the probe tip. Gas chromatography aids in visualizing, via the total ion monitor or gas-liquid chromatographic (GLC) detector, the *rate* at which the sample is presented to the ionization chamber, but the typical GLC peak may last only a few seconds.
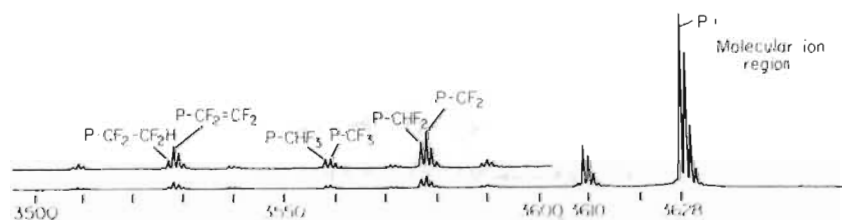
FIG. 2. The mass spectrum of a compound $C_{72}H_{24}O_8F_{128}N_4P_4$ of molecular weight 3628.

There is nothing inherent in the mass spectrometer that limits the molecular weight, or more correctly the ion weight, which may be investigated. The way to analyze higher masses is simply to turn the accelerating voltage down, imparting less momentum to the higher mass ions so that they may be deflected by whatever magnetic field is available. As this is done one tends to lose sensitivity, but it is not here that the true limitation of the mass spectrometer arises, and Fig. 2 shows how we were able to obtain a perfectly good spectrum on a compound $C_{72}H_{24}O_8F_{128}N_4P_4$ of molecular weight 3628 (Fales, 1966). Professor Lederer at Gif (for leading reference, see Vilkas and Lederer, 1968) has successfully analyzed derivatized peptides of well over 1500 molecular weight. Since so many of the conferees are involved in the field of high molecular weight substances such as peptides and proteins, it might be useful to pursue this question by discussing the means by which the molecules are converted to ions. The reason ionization must be brought about is so that the particles may be deflected and sorted according to mass by the various electric and magnetic fields within the spectrometer. Mass spectrometers today are in fact *ion* mass

spectrometers. Perhaps someday someone will discover a method for sorting molecules by mass alone, and this would be an authentic *mass spectrometer*.

At the present time, by far the most common method of producing ions from molecules is through electron ionization (Fig. 3). Thus, a high velocity electron approaching a molecule sets up force fields that are strong enough to cause the ejection of an electron from the molecule with the formation of a positive ion. Unfortunately, it is generally very difficult to carry out this process without imparting a good deal of energy (several electron volts) to the resulting ion. Each electron volt is equivalent to 22.4 kcal, and as little as ~ 60 kcal will cause rupture of any C—C bond. Figure 3 also shows some less familiar ways to make ions from molecules. Thus, if a very low velocity electron is used, collision may

$$M^0 + e \longrightarrow M^+ + 2e \text{ (Electron Ionization)}$$

$$M^0 + e \longrightarrow M^- \text{ (Electron Attachment)}$$

$$M^0 + A^* \longrightarrow M^+ + A + e \text{ (Penning)}$$

$$M^0 + A^* \longrightarrow MA^+ + e \text{ (Chemiionization)}$$
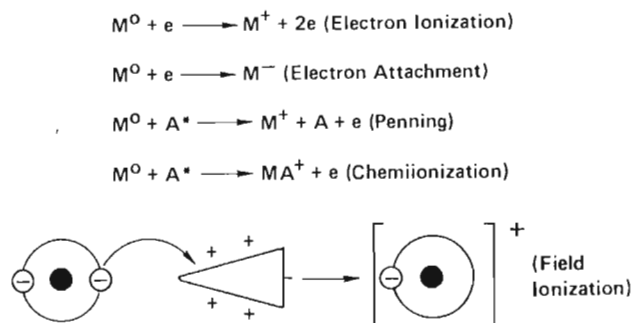
FIG. 3. Methods of ion formation.

result in attachment of an electron to the molecule resulting in a negative ion. Fragmentation may be minimized, but the technique is experimentally difficult. Or ionization may be brought about by allowing "soft" collisions with atoms in electronically metastable states, an electron being ejected in the process. Since these latter reactions involve only neutral reactants, the process has been called "chemiionization" to draw attention to its relation to condensed-phase chemical reactions. This is not, however, "chemical ionization," which will be discussed later.

Another way to produce ions is to approach the electrons of a molecule with a positively charged tip whose force field is as high as that surrounding an atomic nucleus within the molecule itself. Under these conditions an electron will be withdrawn from the molecule to the tip via an electronic tunneling process, leaving behind a positively charged ion. This is a very gentle process indeed, involving only one or two electron volts of energy, so that one may expect minimum fragmentation; often only the molecular ion is visible. Unfortunately, at present sources built

around this principle are difficult to operate and yield rather low intensity ion beams. Again we may look for great improvements in this area in the near future.

A fourth, and theoretically very simple, way to produce ions (Fig. 4), is through an exchange process with other ions formed independently by electron ionization. This is the area of ion molecule reactions that until recently has been the domain of physicists and physical chemists. Today, thanks to the work of Frank Field at Rockefeller University (for leading references, see Field, 1970), Burnaby Munson at the University of Delaware (Munson and Field, 1966), Jean Futrell at the University of Utah (Futrell and Tiernan, 1968), and V. A. Tal'roze in Russia (Tal'rose and Lyubimova, 1952), this method may be utilized by the mass spectrometrist in a very practical way. It is in this area

$$M^O + X^+ \longrightarrow M^+ + X^O \text{ (Charge Exchange)}$$
$$M^O + XH^+ \longrightarrow MH^+ + X^O \text{ (Proton Transfer)}$$
$$M^O + XR^+ \longrightarrow MR^+ + X^O \text{ (Alkyl Transfer)}$$

e.g.

$$CH_3-\underset{\underset{CH_3}{|}}{\overset{\overset{CH_3}{|}}{C}} \oplus + RNH_2 \longrightarrow RNH_3^+ + CH_3-\underset{\underset{CH_3}{|}}{C}=CH_2$$

t-butyl ion   isobutylene
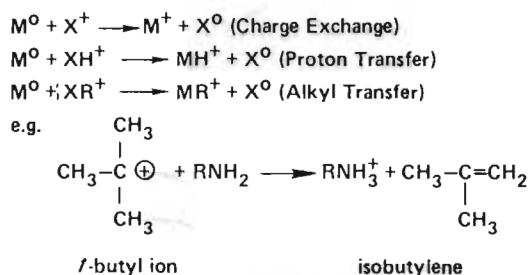
Fig. 4. Ion-molecule reactions.

that Dr. G. W. A. Milne and I have been working in recent years, since we feel that the method has particular promise for molecules of biological origin.

In this method an ion may be produced either by electron transfer from another ion or by a proton or alkyl group transfer. The most useful of these processes today is that of proton transfer as exemplified in Fig. 4 by the reaction of the *t*-butyl ion with an amine to form isobutylene and an ammonium ion. The proton derives from one of the methyl groups of the *t*-butyl ion. This reaction will occur with any base whose proton affinity is higher than that of isobutylene and therefore includes compounds containing nearly any heteroatom, aromatic ring, or conjugated olefin. This exemplifies the process of "chemical ionization" so named by Dr. Field to call attention to the essentially "chemical" nature of the bimolecular reactions taking place, and in this respect recalls, but is to be distinguished from, the term "chemiionization" referred to previously.

There are two ways to study ion-molecule reactions. The first (Fig.

5) is by a tandem arrangement of two mass spectrometers, one to prepare the reagent ion and one in which the actual reaction with another ion takes place. Such systems are expensive and complex, but the interpretation is straightforward since the reagent ion is precisely known. In the other system, developed by Field, only one spectrometer is employed. Here the bimolecular reactions required by the method are brought about by so constructing the source that pressures of 0.1–2 mm may be tolerated. In this case many sorts of ions may be present; interpretation

TANDEM

"CHEMICAL IONIZATION"
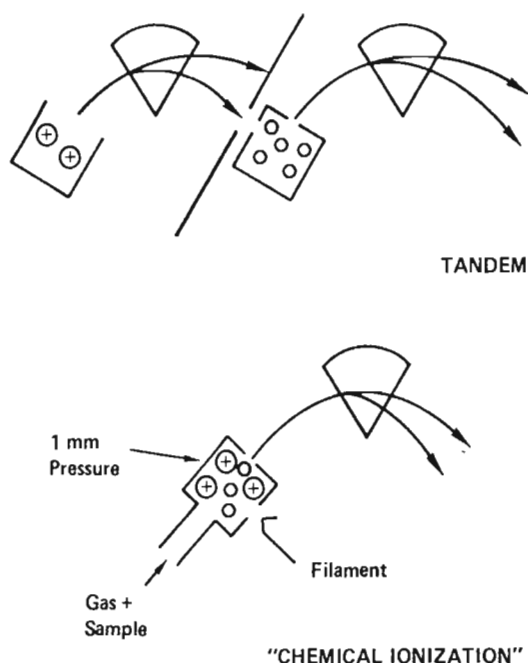
FIG. 5. Tandem versus chemical ionization spectrometer operation.

is correspondingly complicated, but construction and operation of the apparatus is much more practical.

From the foregoing discussion it will be noticed that the molecules have always been assumed to be in the vapor state prior to their conversion to ions. This is a mass spectrometric fact of life that seemingly bears no relation to the actual production or deflection of the ions. Nevertheless, at the present time it is absolutely essential that the material be vaporized prior to its conversion to ions. Therefore, in applications to peptide chemistry, much less protein chemistry, severe limitations are imposed. For example, the vapor pressure of polystyrene of molecular weight 14,000 atomic mass units has been calculated (Dole *et al.*, 1968)

to be 10^{-180} atm at room temperature. Nor is it merely a problem of molecular weight; we cannot obtain mass spectra of molecules as simple as the nucleotides because of their high degree of polarity. The situation reaches an extreme for compounds with formal positive and negative charges, such as sodium chloride, which may be held in crystal lattices with electrostatic bond energies at >200 kcal/mole. Furthermore, some polar groups (e.g., hydroxyls) tend to be thermally sensitive, so that decomposition occurs when attempts are made to heat the samples to the temperature required for volatilization. Sometimes we can work around the problem by chemically converting the compound to a less polar and more volatile derivative. The elegant work of Jim McCloskey and his co-workers at Baylor in the field of the nucleic acids (Desiderio et al., 1968) is a prime example. In this case compounds such as adenosine are converted to their polytrimethylsilyl ethers, masking hydroxyl groups and the phosphoric acid in one operation. Satisfactory spectra are obtained (Fig. 6) that should be of great assistance in elucidating the structures of new nucleosides.

Extreme examples of the derivatization technique are probably familiar to you in the work of E. Lederer and his co-workers in France (for leading references, see Vilkas and Lederer, 1968), and A. Kiryushkin and M. M. Shemyakin (Shemyakin, 1968) in Russia, where peptides (even decapeptides) are N-acylated and permethylated using methyl iodide and sodium hydride in dimethylsulfoxide to mask all polar amide NH groups as well as carboxyl groups. Arginine residues are tied up using acetylacetone, etc., and special derivatives must be made of other troublesome amino acids. These methods require a good deal of chemical expertise if the final material is still to reflect the original peptide. When a satisfactory derivative has been prepared, obtaining and interpreting the mass spectrum to yield the amino acid sequence may be relatively straightforward provided sufficient sequence peaks are visible. However, my recommendation to anyone contemplating the use of this technique is to seek firsthand advice from one of the aforementioned groups and to practice derivatization on a reference peptide as similar in amino acid composition as possible to the unknown peptide.

Although derivatization is today the most practical way to overcome lack of volatility, it has always struck me as unreasonable that we must start with a compound that often contains a positive or negative charge, such as a peptide or salt, then chemically modify it so as to *remove* the charge in order to vaporize it, only again to ionize it in the mass spectrometer. For that matter, it is really necessary to vaporize the sample at all? Two interesting experiments have been performed which offer some hope in this area. In the first, Dr. H. G. Beckey,
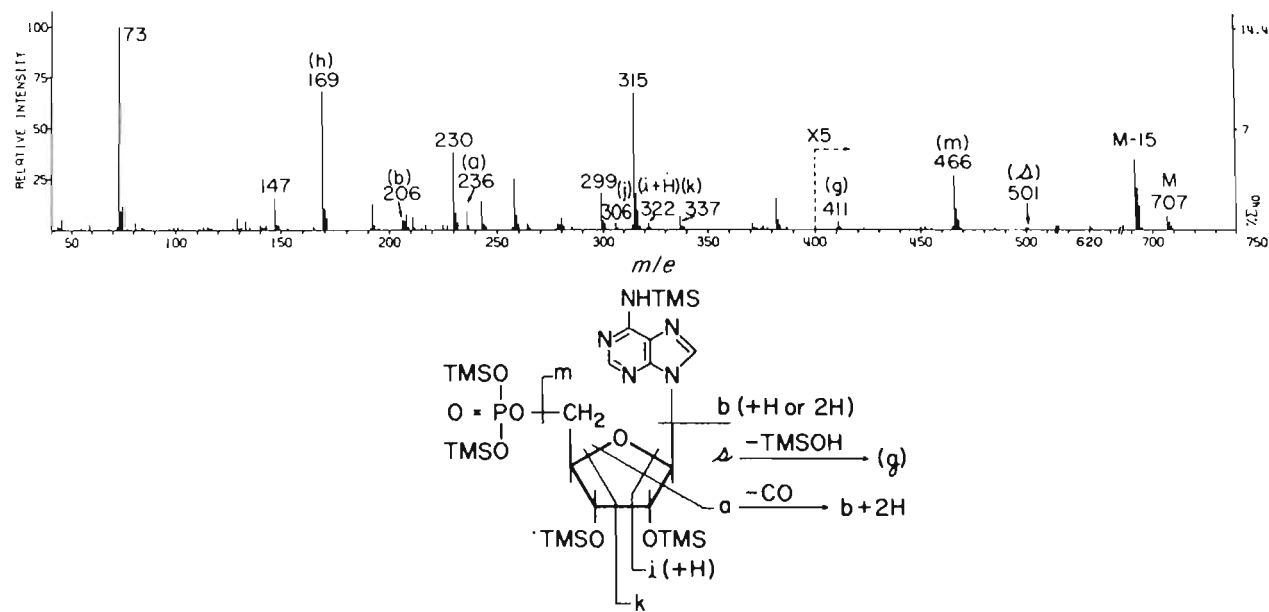
FIG. 6. Mass spectrum of a nucleoside trimethylsilyl ether.

a leader in the area of field ionization, has found that glucose deposited on the tip of a field ionization source may be caused to lose an electron to the tip. When this happens, the resulting positively charged glucose molecular ion will be desorbed from the tip by the mutual repulsion between it and the very highly positively charged tip. In this way he has obtained the very excellent spectrum of glucose (Beckey, 1969), shown in Fig. 7. As will be seen, under ordinary electron ionization conditions, glucose provides no molecular ion. If field desorption methods could be applied to more complex molecules this would be a breakthrough of the first magnitude.
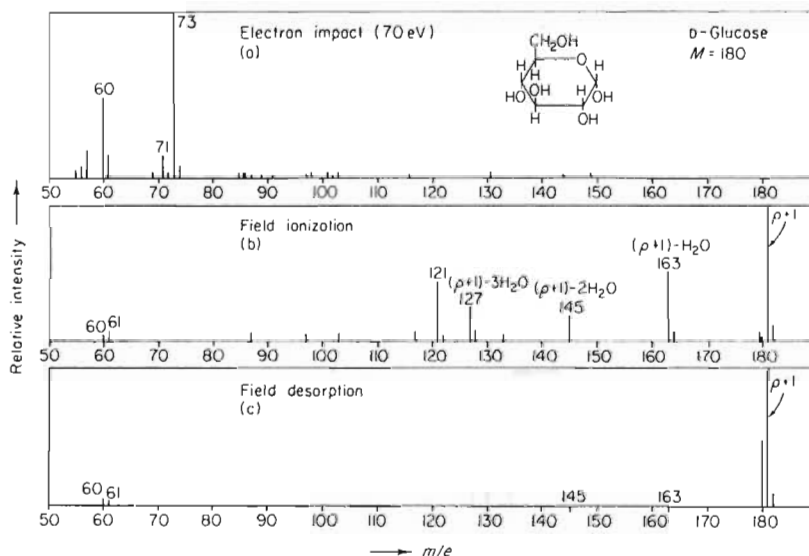
Fig. 7. Glucose mass spectrum via field desorption. From Beckey (1969).

In a second development, Professor M. Dole and his co-workers at Baylor (Dole *et al.*, 1968) have developed a novel mass spectrometer system applicable to compounds of very high molecular weight, such as polymers. The device is in a relatively crude form at present, but the preliminary results are exciting indeed. In this system (Fig. 8) a solution of a polymer is prepared in a solvent at a concentration such that, when it is sprayed in the form of fine droplets from a nebulizer tip, each droplet will contain on the average only one molecule of the polymer. As the droplets are formed at the tip of the nebulizer, they are charged by a potential applied to the tip. The charged, solvated droplet passes into a region where it is mixed with a carrier gas. It proceeds to lose its hydration sphere through collisions with this gas,

and the free macromolecular ion is then swept out of an orifice with
the gas at ultrasonic velocities. It is then directed toward a collector
plate in front of which is placed a charged grid whose potential is ad-
justed so that ions of increasing energy are selectively repelled. The
neat break observed in the resulting plot (Fig. 9) can be calculated
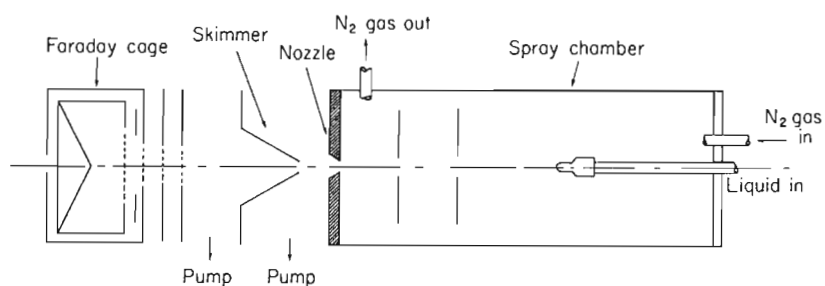
Fig. 8. Electrospray apparatus. From Dole *et al.* (1968).
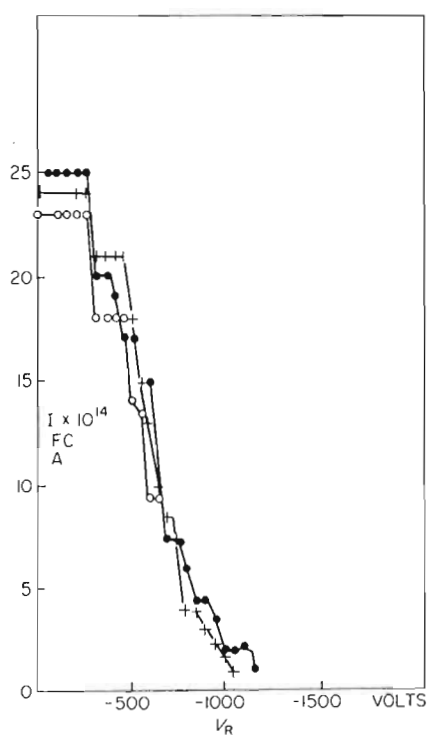
Fig. 9. Current-repeller grid plot from electrospray experiment. Voltage curves
for 0.01 wt% solution of 51,000 amu polystyrene; 2, 3, 3 inch spacings. From Dole
et al. (1968).

to result from a supersonic ion of m/e 50,000. If this eluting stream of macromolecules could be directed toward the entrance port of a specially constructed mass spectrometer, it should be possible to determine the molecular weight with a much higher degree of accuracy than is currently possible. It is my understanding that experiments along this line are already in progress.

III. Results and Discussion

A few examples will follow from current work in this laboratory that may help to indicate the range of problems that may be studied using currently available mass spectrometric systems. In the first place, it is clear that the combination of gas chromatography and mass spectrometry (GC-MS) has had an enormous impact in biochemistry in recent years. Gas chromatography of steroids was discussed some years ago at these sessions by Dr. Evan Horning at Baylor (Horning et al., 1963), and now all of this work has been extended in its utility by the use of GC-MS. The work on steroids by the Finnish and Swedish groups (Luukkainen, Aldercreutz, (1967) and others) demonstrates how valuable it can be in a clinical situation, and recent work of Dr. Engle emphasizes this fact. Recently Professor Röller in collaboration with Dr. C. C. Sweeley showed how the method was applied to the elucidation of the structure of the insect juvenile hormone (Röller and Dahm, 1968).

Entomologists and biochemists studying exocrine secretions and pheromones are indeed in a fascinating field, and I have been privileged to collaborate recently with Drs. Murray Blum, John MacConnell, and John Brand at the Entomology Department of the University of Georgia on related work. For example, we are studying the defensive secretions of the *Chrysomelae interrupta* beetle larvae shown in Fig. 10. This fellow is a local pest, dining on several species of alder trees. When he is attacked by a fire ant or other predator, as shown in Fig. 11, his blood pressure rises forcing inside out small sacs along his back which contain a liquid which is highly offensive to his attacker. When the danger has passed, he simply draws the sacs back along with their droplets, conserving the liquid for future use. (I am indebted to Dr. John Brand for this picture; I understand that such pictures are very difficult to obtain.) The gas chromatogram of extracts of these glands is shown in Fig. 11, and from their mass spectra we have been able to identify the structures of the compounds as β-phenylethyl isobutyrate and β-phenylethyl 3-methylbutyrate. It seems that these materials are unique in the insect world. The mass spectra of the various possible isomers are very similar, and retention time information, in addition to the mass spectra, was required to distinguish among various isomers. These ex-

Fig. 10. *Chrysomela* larvae with alarm gland verted being attacked by fire ant.

ocrine secretions are almost uniquely suited for study by GC-MS because such compounds are necessarily volatile so that their signaling action does not persist beyond a reasonable time. Were this not so, considerable confusion would reign in the insect world. The importance of the gas chromatograph cannot be overestimated in this application, since it permits mass spectra to be obtained on biological products without the need for extensive purification. Even more important, it allows diagnostic

chemical reactions to be carried out on the micro scale without prior isolation and purification of the product. It is only in the most simple cases such as that described above, that the structure can be ascertained by inspection of a single mass spectrum. For example, we have also been concerned recently with the structures of the compounds found in the venoms of fire ants (e.g., *Solenopsis xylonii*) in the South. The fire ant is a rather serious problem in the southern United States since it is the dominant species in many areas. American taxpayers have spent
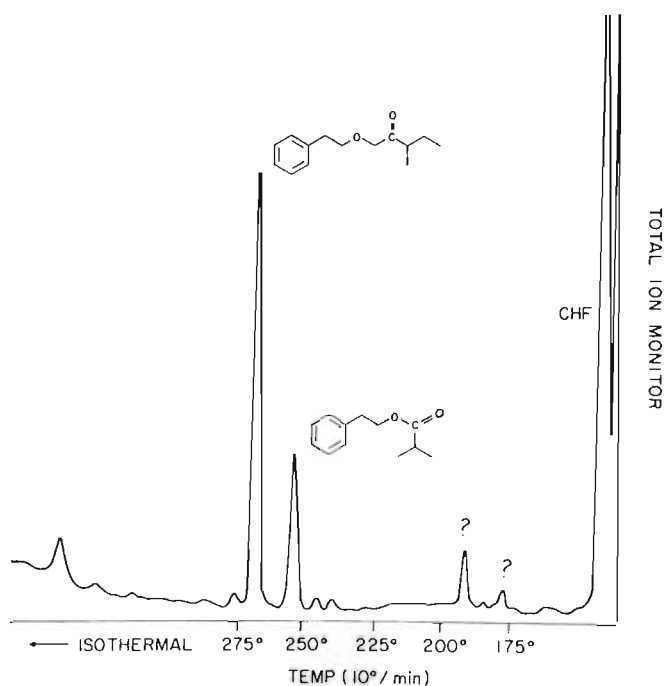
FIG. 11. Gas chromatogram of *Chrysomela* alarm substances (*Chrysomela* larvae; 10% Carbowax 20M).

around three hundred million dollars on this problem, and suggestions about spraying large areas of the South with insecticides are worrying the environmentalists.

It seems that there are two distinct groups of fire ants: the indigenous species which has been around for many years without causing any particular problem, and the more recently imported species which appears to be the real winner in the battle for survival. Dr. Brand was curious about the possible reasons for its success and felt that the components of its venom might supply a clue. Last year, utilizing GC-MS,

Drs. Blum and MacConnell and I were able to determine their structures (MacConnell *et al.*, 1970, 1971); as Fig. 12 shows, they are simple 2,6-dialkyl substituted piperidines. Now it is interesting to find that the imported fire ant contains almost exclusively the C_{13}- and C_{15}-side chain unsaturated homologs, whose substituents are arranged in a *trans* relation on the ring, whereas the *native* form contains the C_{11}-saturated homolog whose side chains are arranged *cis* as well as *trans* on the
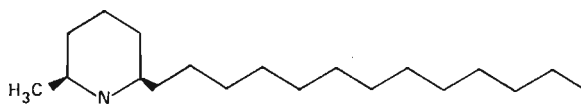
cis−2−methyl−6−n−tridecylpiperidine
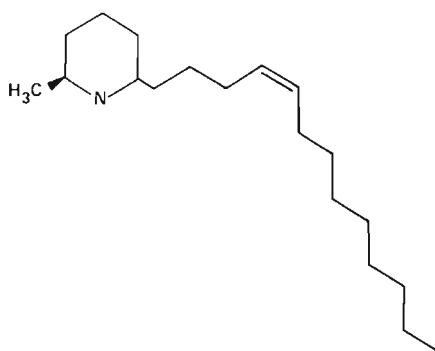
trans−2−methyl−6−n−(cis−4′−tridecylpiperidine)

Fig. 12. Structures of fire ant venom components.

ring. The physical properties of the *cis*- and *trans*-dialkyl piperidines are surprisingly different. In the *cis*-dialkyl piperidine the nitrogen electron pair is not nearly as accessible to various substrates (Fig. 13). For example, on alumina columns the *trans* form interacts and is absorbed much more firmly than the *cis* form.

It is tempting to speculate that the biological activity of the venoms is connected with this *cis-trans* difference, the *trans* being more active. But why is the *cis* form present only in the indigenous species? A recent analysis of this has disclosed the presence of a small peak in the gas chromatogram (Fig. 14) whose mass spectrum (Fig. 15) indicates
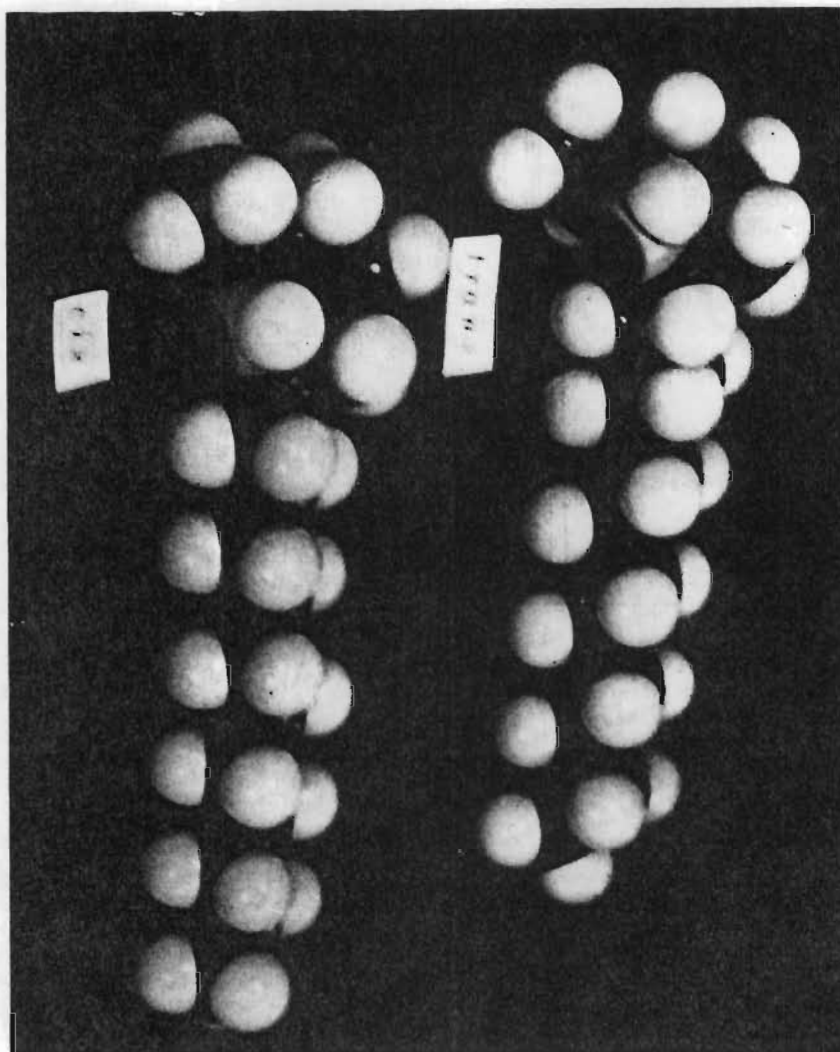
FIG. 13.   Models showing accessibility of electron pair on nitrogen.

it to be a $\Delta^{1,2}$-piperideine. Confirming this structure, it undergoes reduction with sodium borohydride, producing the *cis* form almost exclusively. We can then speculate that this $\Delta^{1,2}$-piperideine is a precursor which has been biologically reduced by the imported ant (NADH?) specifically to the *trans* form, while the indigenous ant has an enzyme capable of the borohydride-like *cis* reduction as well. Alternatively, the $\Delta^{1,2}$-piperideine may be an intermediate between the *cis* and *trans* isomers, in which case only the indigenous species has an enzyme capable of oxidiz-
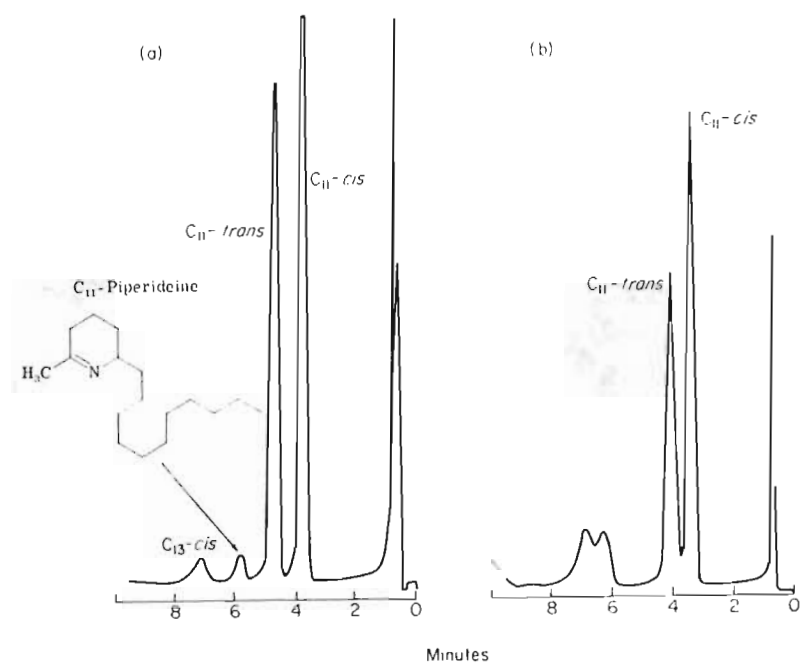
FIG. 14. Gas chromatogram of minor venom component. (*Solenopsis xylonii*; 10% SP-100; 180°C). (a) Before NaBD₄; (b) after NaBD₄.
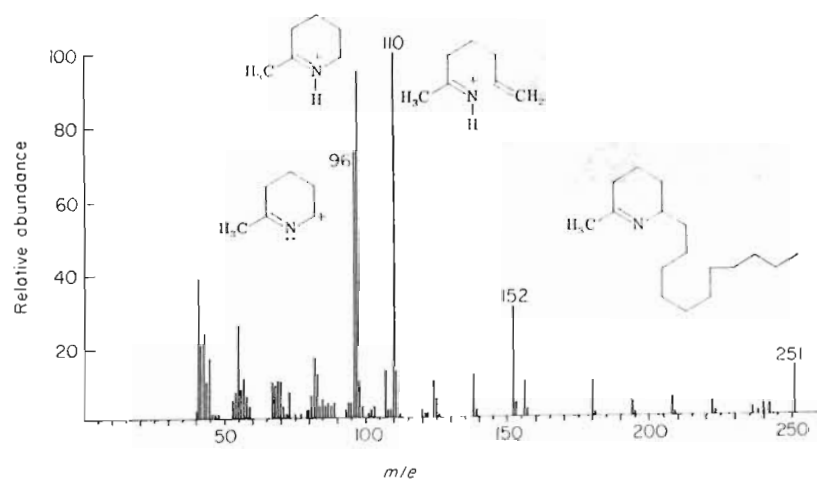
FIG. 15. Mass spectrum of minor venom component.

ing the *trans* form to the $\Delta^{1,2}$-piperideine. I must stress that all this is pure speculation, and biological assay of the various venoms are only now being undertaken. The success of studies such as this on trace amounts of material depends very much on the actual operating condition of the mass spectrometer itself. An instrument in poor condition due to misuse or neglect can be orders of magnitude less sensitive than one properly cared for. We are fortunate at NIH in having the services of Mr. William Comstock, whose expertise was essential to this work.

The fire ant problem was complicated by the fact that the molecular ions of the venom components were extremely weak (Fig. 16) and in fact, an ion that might be mistaken for the molecular ion
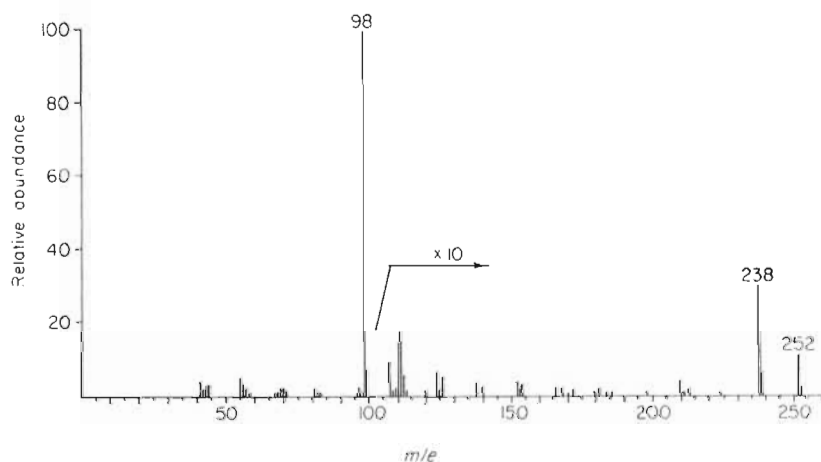
FIG. 16.   Electron ionization mass spectrum of *trans* fire ant venom piperidine.

is present at a location 1 mass unit less than expected. This is a common occurrence in the mass spectra of aliphatic amines, and if the compound was truly unknown, one could easily arrive at the erroneous conclusion that the substance was not an amine at all, because its molecular weight apparently would be even. This is a consequence of the so-called "nitrogen rule" in mass spectrometry, which is simply a statement of the fact that compounds with *no nitrogen* or an *even number* of nitrogens are *even* in mass, while those with *one nitrogen* or an *odd* number of nitrogens are *odd* in mass. Because it so often occurs in mass spectrometry that molecular ions (molecular weight ions) are either not present or of such low intensity as to be confused with impurities, we have investigated in some detail the process of "chemical ionization" mentioned previously. For this purpose we have had constructed a special ion source designed by M. Vestal of Scientific Research Instruments (Baltimore,

608   HENRY M. FALES ET AL.

Maryland) which allows the pressure in the spectrometer to rise to approximately 1 mm of mercury. These are the conditions required for a gas such as isobutane to form almost exclusively the tertiary butyl ion (Fig. 4) under electron bombardment. As mentioned previously, the t-butyl ion then transfers a proton to the compound under study, which has been added in trace amounts to the isobutane in the source. Some energy accompanies the proton, and this may result in some fragmentation of the $(M + H)^+$ ion, but in the case of simple bases the only ion observed is at $(M + 1)^+$, i.e., $(M + H)^+$. In the case of the fire ant venom, the intense $(M + H)^+$ ion which results (Fig. 17) has been very helpful in determining its structure since its molecular weight was determined unequivocally. On the other hand, if more fragmentation is desired for
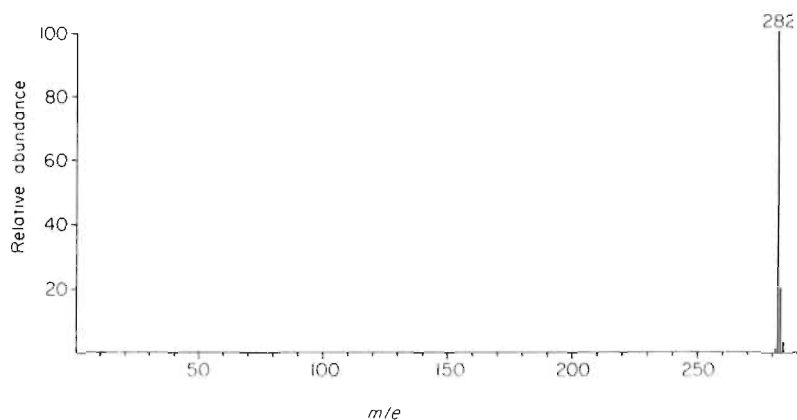
FIG. 17. Chemical ionization mass spectrum of *trans*-C_{13} fire ant venom piperidine.

structural purposes, one simply employs a more energetic source such as CH_5^+ (protonated methane) formed by electron bombardment of methane at these pressures. An extreme energy source is hydrogen which gives rise to the H_3^+ ion under these conditions. One may actually observe *more* fragmentation in this form of chemical ionization experiment than in electron impact, so the general statement that chemical ionization methods are milder should be slightly modified.

If instead of using a proton-donating reagent gas, we employ nitrogen, that under these conditions gives rise to N_2^+, *charge exchange* will occur and a molecular ion will be observed as in electron ionization rather than a molecular-weight-plus-1 ion. Subsequent fragmentation processes greatly resemble those occurring in electron ionization, since the recombination energy of this ion is similar to the energies used in electron ionization. In the case of simple amines, Field has shown recently that

the resulting spectra are virtually indistinguishable (Whitney *et al.*, 1971) from the usual electron ionization spectra. Although this method offers much promise, it is true today that when dealing with an unknown compound it is essential to have its electron ionization spectrum at hand since most of the rules relating fragmentation of the molecular ion to chemical structure have been evolved using this method; furthermore large libraries of such data exist for direct comparison with an unknown. Fortunately one can pass from the chemical ionization to the electron ionization mode at any time by merely turning off the reagent gas and waiting a few seconds for the pressure to drop.

When fragmentation is observed in chemical ionization how are the spectra rationalized as compared with electron ionization? In general,
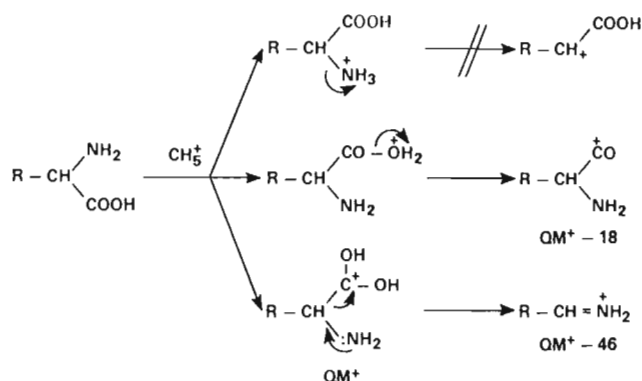
Fig. 18.   Rationalization of chemical ionization mass spectra of amino acids.

the fragmentation mechanisms are easier to understand than the relatively high-energy electron ionization spectra. Rearrangements are minimal and fragmentations generally go along pathways very familiar to anyone who has dealt with acid-catalyzed reactions in the condensed state in the laboratory. How simple this can be is illustrated by spectra of the amino acids (Milne *et al.*, 1970) (Fig. 18). In the simple amino acids one can visualize three sites for proton attachment: the amino-group, the hydroxyl, and the carbonyl of the carboxylic acid. Assuming, as seems reasonable, that some protons attach randomly at each possible site, fragmentation will be dictated by the nature of the resulting ion. If the proton attaches to the amino nitrogen it is possible that ammonia will be lost. However, the resulting carbonium ion, being adjacent to a carbonyl group, has no particular stability, especially since its generation is opposed by the dipole of the carbonyl group. On the other hand, if a proton attaches to the hydroxyl of the carboxyl group, loss of water

is accompanied by formation of the well-known and very stable acyl carbonium ion. Attachment of a proton to the carbonyl oxygen of the carboxyl group leads to an interesting cleavage with loss of the elements of formic acid ($COOH_2$) and stabilization of the resulting ion by participation of the lone electron pair on nitrogen. The resulting ion is the one which is generally observed at highest mass in electron ionization mass spectrometry of the amino acids and their esters. I think it is interesting to note in passing that all the amino acids except arginine and cystine have finite vapor pressures, so perhaps it is not surprising
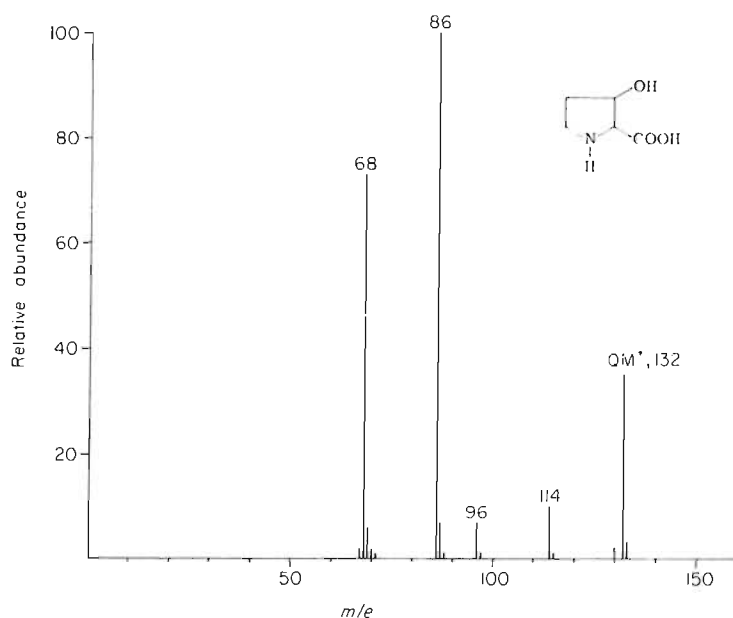
Fig. 19. Chemical ionization mass spectrum of 3-hydroxyproline.

that they all give satisfactory mass spectra under chemical ionization conditions. The advantage of chemical ionization spectra is that one can often discern the functionality of the amino acid in a very simple fashion. For example, 3-hydroxyproline (Fig. 19) displays, besides a peak at molecular weight-plus-1, loss from this ion of water, as well as loss of the elements of formic acid. Water is again lost as a secondary process *after* loss of the elements of formic acid, proving that an alcoholic hydroxyl group is present in the molecule in addition to the hydroxyl group of the carboxylic acid. The technique should be particularly valuable in elucidating the structures of new amino acids found in nature and even perhaps in identifying end-group residues in peptides when they can be removed as ether free or substituted amino acids.

The same technique can be used to elucidate the structures of small peptides (Kiryushkin *et al.*, 1971; Gray *et al.*, 1970) providing they can be volatilized through appropriate derivatization. The processes which occur in this case (Fig. 20) involve simple cleavage of the peptide bond with charge residing on the carbonyl oxygen. In this respect the process strongly resembles the analogous process in electron ionization spectrometry of the peptides. However, using CI it also happens that a proton rearrangement occurs so that the amino side of the peptide is also observed as an ion and one can check the sequence derived by analyzing for both fission products. We have worked out a simple com-

$$CH_3CO-NH-\underset{\underset{R_1}{|}}{CH}-CO-NH-\underset{\underset{R_2}{|}}{CH}-CO-OMe$$

$$CH_3CO^|$$

$$CH_3CO\cdots NH-\underset{\underset{R_1}{|}}{CH}-CO^|$$

$$CH_3CO-NH-\underset{\underset{R_1}{|}}{CH}-CO-NH-\underset{\underset{R_2}{|}}{CH}-CO^{L}$$

C-Sequencing

$$H_3N-\underset{\underset{R_2}{|}}{CH}-COOMe$$

$$H_3N-\underset{\underset{R_1}{|}}{CH}-CO-NH-\underset{\underset{R_2}{|}}{CH}-COOMe$$

N-Sequencing
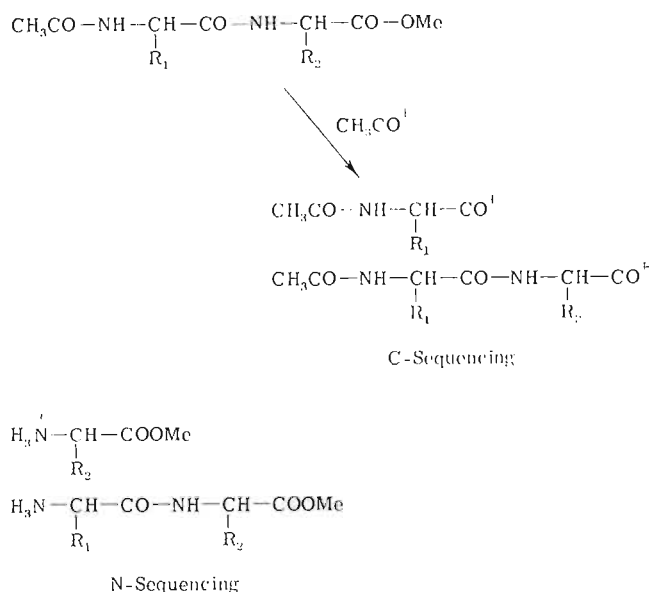
FIG. 20. Rationalization of chemical ionization mass spectrum of peptides.

puter program for performing this task (Kiryushkin *et al.*, 1971), but it can be done by hand without much difficulty. Figure 21 gives an example of such an analysis in an admittedly favorable case. As in most published peptide mass spectra, you will notice that the peptide is composed of relatively simple amino acids. Still, the improvement in the spectrum obtained using chemical ionization in this case is obvious. The enhanced intensity in the molecular weight region is important and you may notice that expansion factors of 10 have not been used to expand the higher mass end of the spectra. In all fairness, I must mention that in some other cases the electron ionization spectra have been more impressive than those obtained using chemical ionization.

In the case of the thyroid-releasing factor of Drs. Guillemin and Schally (Fig. 22) brought to our attention by Dr. Dominic Desiderio at Baylor (Desiderio *et al.*, 1971), nature has provided us with both blocking end groups and the spectrum (Fig. 23) obtained on less than 1 μg of material, provides an excellent example of the potential of the method in the area of small peptides that are difficult to analyze by the more usual methods.
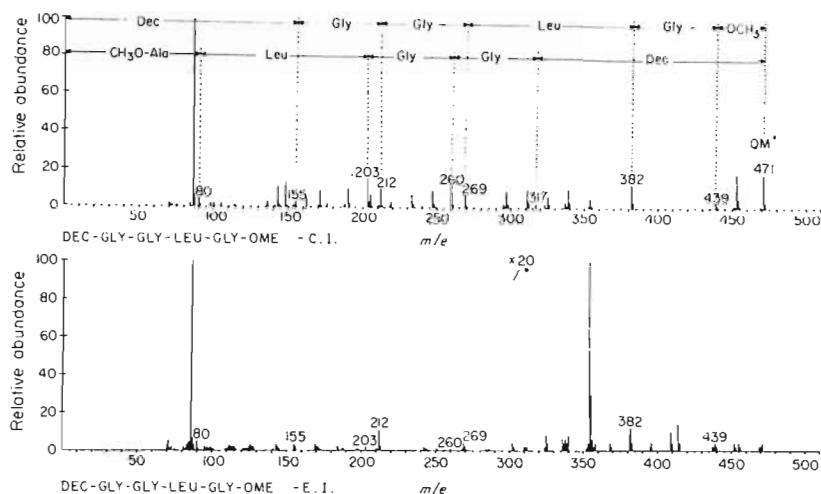

Fig. 21. Chemical ionziation mass spectrum of a typical small peptide.
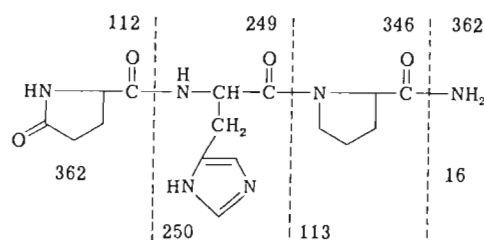
Fig. 22. Structure of the thyroid releasing factor.

From previous remarks, I think you may detect that I am somewhat less than enthusiastic about the general use of mass spectrometry today for the analysis of proteins and larger peptides. This comes about not only from the difficulties associated with the necessary chemical modification, but also from consideration of the very elegant technique of the Edman degradation. Using the automated versions of this method, combined with gas-liquid chromatographic analysis of the phenylthiohy-

dantoins, it is possible to degrade a peptide containing a sequence of 20 amino acid residues within 3–4 days. So rather than compete directly with this powerful method, we decided to see whether mass spectrometry had anything to offer in its behalf. In collaboration with Drs. Bryan Brewer, John Pisano, Yumiko Nagai, and Thomas Bronzert, we have used CI methods to identify and quantitate the phenylthiohydantoins as they are produced by the sequenator (Fales *et al.*, 1971). In chem-
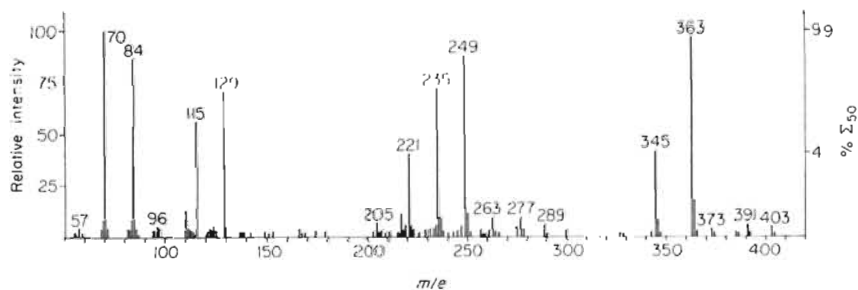
Fig. 23. Chemical ionization mass spectrum of the thyroid releasing factor.
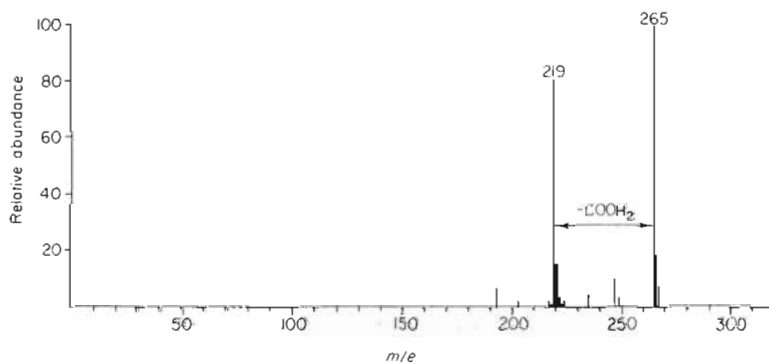
Fig. 24. Chemical ionization mass spectrum of glutamic acid phenylthiohydantoin.

ical ionization using isobutane, nearly all the phenylthiohydantoins provide either a single molecular weight-plus-1 ion or a very easily identified fragmentation peak, as Fig. 24 shows in the case of phenyl-thiohydantoin glutamic acid. A preliminary scan of the sample enables one to determine which phenylthiohydantoin is likely to be present and then addition of a known quantity of pentadeuterio-phenylthiohydantoin internal standard allows more exact quantitation (Fig. 25) by comparison of the ion with its neighbor at 5 mass units higher. Such quantitation is an essential feature late in the sequencing.

Figure 26 shows the results of our first attempt at myoglobin, and the results are seen to be quite satisfactory considering the somewhat impure nature of the myoglobin used in this experiment. We hope to simplify the process by scanning the sample many times, using a quadrupole mass spectrometer as the material evaporates from the direct insertion probe. The scans will be stored in a computer, and subsequently, each scan will be analyzed for the mass (or masses) pertinent to a given phenylthiohydantoin. The sum of these intensities is then an absolute
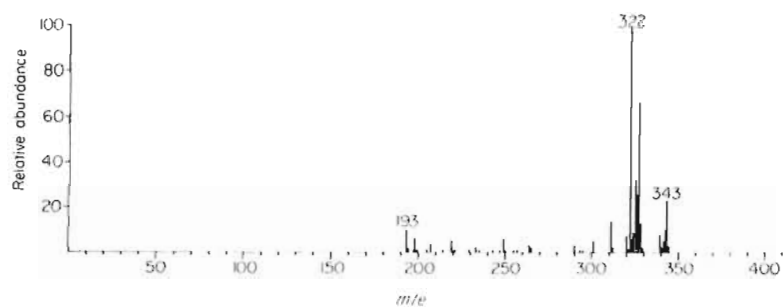
FIG. 25. Chemical ionization mass spectrum of a mixture of phenylthiohydantoins plus internal pentadeuterio standards.

	1	2	3	4	5	6	7	8	9	10
H₂N —	Val —	Leu —	Ser —	Glu —	Gly —	Glu —	Trp —	Gln —	Leu —	Val —

H_2N — Val — Leu — Ser — Glu — Gly — Glu — Trp — Gln — Leu — Val —

11   12   13   14   15   16   17   18   19   20   21   22
Leu — His — Val — Trp — Ala — His — Val — Glu — Ala — Asp — Val — Ala —

23   24   25   26
Gly — His — Gly — Gln
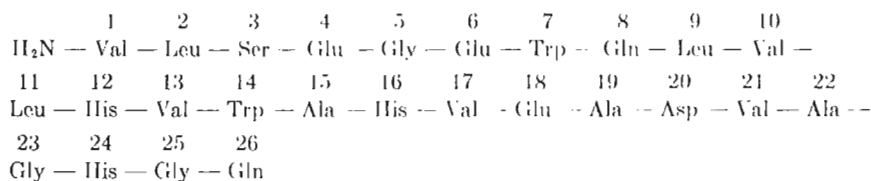
FIG. 26. Partial structure of sperm whale myoglobin.

measure of the quantity of phenylthiohydantoin present and the only number actually required. A similar system, using electron ionization methods, has been developed by Dr. R. Lovins (Lovins et al., 1971) at the University of Georgia and has proved most satisfactory.

Although alkaloids (Fales et al., 1970) and antibiotics are likely to be of little interest to this group, I cannot resist showing some scans of these basic materials since it is in this area that the chemical ionization method really excels. Figure 27 shows the difference in degree of fragmentation observed when narcotine is investigated by both methods, and it is clear that the molecular weight is much more easily determined from the chemical ionization scan. In the case of deoxyribose (Fales et al., 1969) (Fig. 28) loss of water is observed but the molecular weight + 1 peak is still distinct. Even a molecule such as erythromycin, which

contains hemiacetal linkages, survives on protonation (Fig. 29), probably because of the basic nitrogen atom. In general, if a molecule contains an amine function, this is a stabilizing influence in chemical ionization (in contrast to electron ionization) since protonation at this site seldom provides, on cleavage, a carbonium ion with any great stability.

Is chemical ionization useful in the steroid field? Perhaps occasionally, but it has been our experience that those processes which prevent observation of the molecular ion in electron ionization, such as loss of acetic
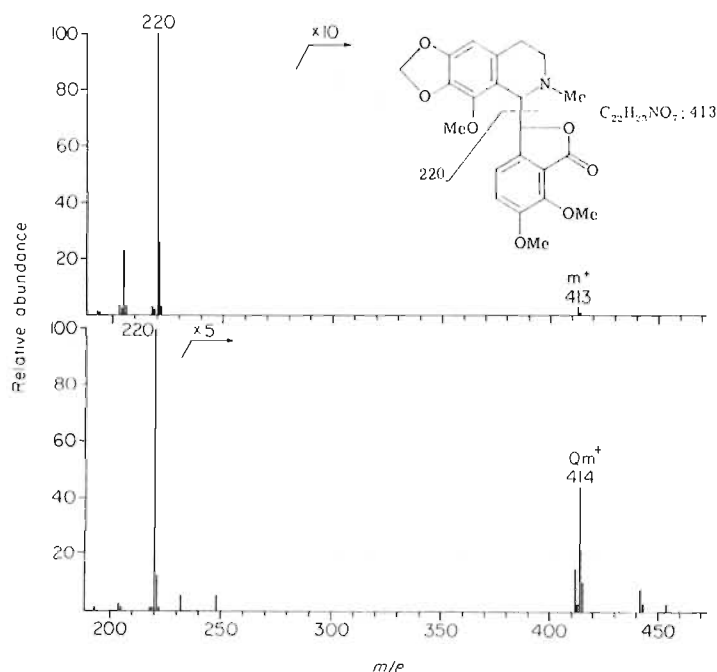
FIG. 27.   Chemical ionization mass spectrum (below) of narcotine compared with electron ionization spectrum (above).

acid and water, are even more pronounced in chemical ionization. In fact, it is not so much that chemical ionization is poor for the steroids, but that electron ionization is so very satisfactory, particularly since gas chromatography can also be invoked.

Finally, I would like to discuss a scientifically trivial, but very practical, use of both electron and chemical ionization mass spectrometry in the clinical area. In recent years, Dr. Milne, Virginia Aandahl, and I, in collaboration with Mr. Norman Law at nearby Surburban Hospital (Law et al., 1971, 1972), have found that by using GC-MS we are able
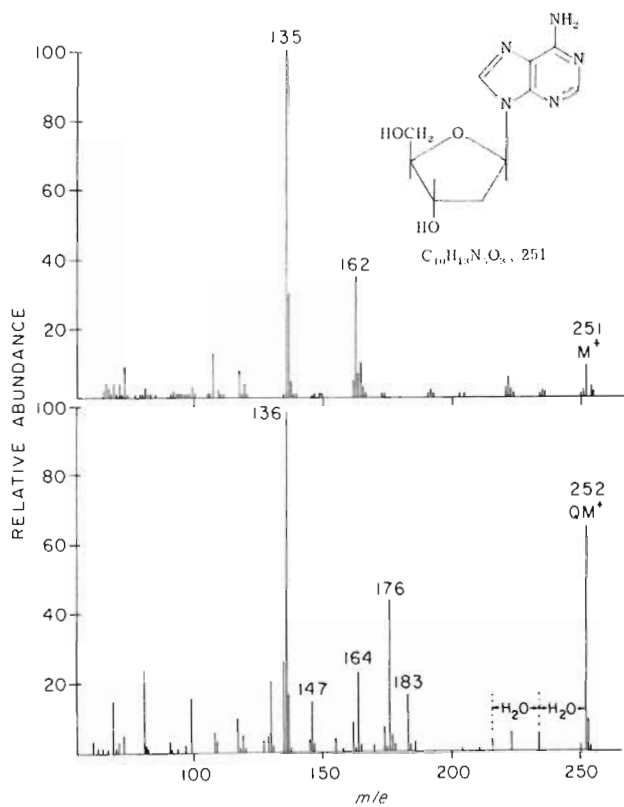
Fig. 28. Chemical ionization mass spectrum (below) of deoxyadenosine compared with electron ionization spectrum (above).
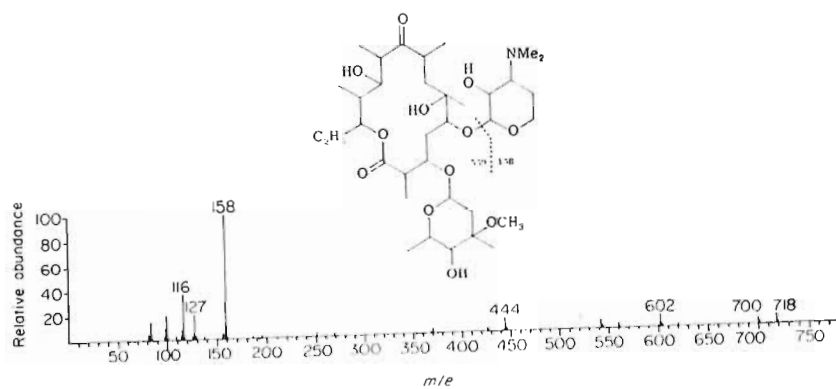

Fig. 29. Chemical ionization mass spectrum of erythromycin.

to identify quite easily the drug (or drugs) responsible for the comatose condition of individuals who had taken overdoses. [A similar technique was demonstrated earlier by Althaus *et al.* (1970) at M.I.T.] This involves a chloroform extraction of serum or gastric lavage, evaporation of the solvent, and GC-MS of the residue. All peaks are scanned as they elute from the gas chromatograph (Fig. 30), and comparison is made either manually or with a computer with a library of about 80 common drug spectra. Often the patient has ingested several compounds and, while this complicates the treatment, the mass spectral identification is straightforward. Even metabolites are picked up, and the potential
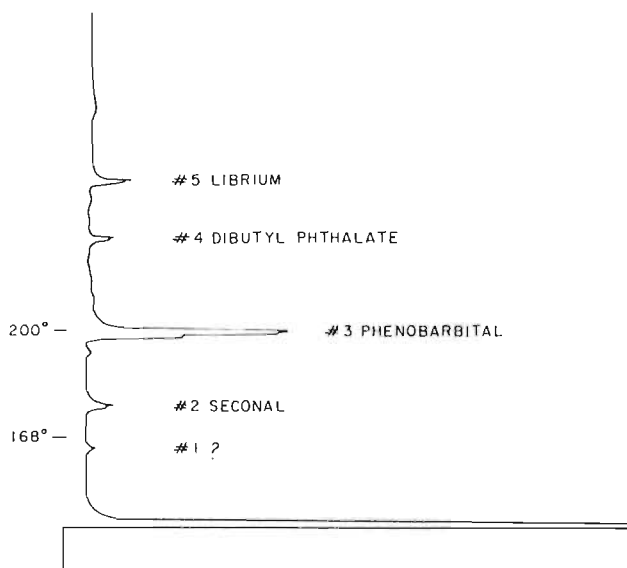
FIG. 30.   Gas chromatogram of drugs from gastric lavage in an overdose case.

for drug metabolism studies is obvious. Generally, we can have an unequivocal answer in 0.5–1 hour, and the physicians responsible for decisions about whether or not to dialyze seem to be very happy about the results. We have recently completed 100 cases, and Table I shows the drugs we have identified. Recently, we have wondered whether gas–liquid chromatography might be eliminated by using chemical ionization, as shown in Fig. 31. Most of these drugs (except for the barbiturate) were components of one pill, and even then we missed one trace component (homatropine). Such mixtures are not uncommon, and I think this scan dramatically illustrates the problems facing the clinical toxicologist as long as such combinations are commercially available and prescribed.

TABLE I
*Listing of Drugs Found in Overdose Cases
and Frequency of Occurrence*

Generic name of drug	Number of times identified
Phenobarbital	8
Barbital	1
Amobarbital	8
Hexobarbital	1
Secobarbital	8
Pentobarbital	8
Meprobamate	4
Carbromal	1
Glutethimide	8
Ethchlorvynol	5
Methapyrilene	2
Methaqualone	5
Primidone	1
Trifluoperazine	1
Amitriptylene	2
Chlordiazepoxide	1
Diazepam	6
Meperidine	2
Acetyl salicylate	4
Salicylamide	1
Phenacetin	1
Propoxyphene	2
Methyl salicylate	1
Caffeine	2
Pentazocine	1
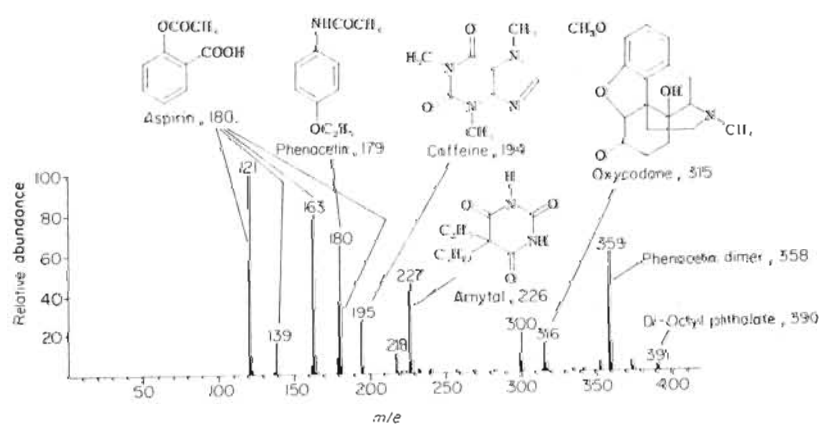
FIG. 31. Chemical ionization mass spectrum of drug sample from overdose case.

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DISCUSSION

J. R. Goding: What happens when you have a mixture—like prostaglandins? Are you able to tell exactly how much of each is present and is the specificity and so on agreeable to people used to working in other fields?

H. M. Fales: The question of quantitation in mass spectrometry is increasingly important, and I would like to answer in some detail.

The mass spectrometer has somehow acquired an undeservedly poor reputation for quantitation. In fact, the spectrometer itself is capable of very high precision and accuracy. There are several ways in which quantitation can be achieved. If the sample is a mixture of gases (or volatile, stable liquids which can be converted to gases below ~250°C), the vapor from a measured quantity may be admitted to the ion chamber through a fixed leak. Under these conditions, assuming trivial loss of sample during the analysis, a certain response will be obtained at each peak in the mass spectrum if all instrumental parameters are fixed. This response should remain constant within a few percent or better over a period of days. The mass spectrometer is very seldom used in this way today since considerable material (~1 mg) is required and few compounds have the required volatility and stability. On the other hand, if a known, small amount of compound is admitted via the direct insertion probe, the spectrum will obviously change intensity as the sample is consumed. Accuracy can still be achieved if the spectra are summed over the total lifetime of the sample, but this is tedious and done conveniently only with a rather expensive computer. This is the approach we are taking to the analysis of phenylthiohydantoin derivatives.

All the instrumental parameters can be eliminated as variables if the method of internal standards is employed. For this purpose, it is usually necessary to synthesize an analog of the material in question labeled with a heavy isotope (e.g., ^{15}N, ^{13}C, ^{18}O, ^{2}H). Because of the natural abundance of such isotopes, the labeling should be at least several percent excess at one location on the molecule. Lower values can be employed, but accurate measurement and subtraction of peak heights become necessary and render the whole procedure less reliable. It is even possible sometimes to substitute a closely related compound (e.g., a dihydro derivative) as an internal standard if it can be established that the volatility of the compound and standard are identical. Admixture of an amount of standard comparable (within a factor of 10–100) with the unknown and evaporation on the direct insertion probe is all that is required for analysis, but handling the volumes involved (1–3 μl) is not as easy as it may sound. In any case, repeated scanning of the spectrometer over the mass range containing the two isotopic species and comparison of the two peak heights gives directly their relative quantities in the sample.

Such analyses can be carried out with high accuracy well below the nanogram range, save for one factor: the instrumental background. A mass spectrometer in general laboratory use will usually show a small peak at every mass unit up to at least m/e 400 under high gain conditions.

Increased confidence in the reliability of a relationship observed between internal standard and unknown peaks can be obtained if their relative intensities remain constant well through the sample lifetime. Clearly, if we are able to compare not just one peak in the spectrum of each compound, but several (or even the whole spectrum), reliability increases still further.

Such comparisons can be done with a computer, but they suffer from the fact that time must be spent (3–10 seconds) in scanning through the whole spectrum. For maximum sensitivity we would like to present the sample to the mass spectrometer in a short burst of approximately the same time period so that only one or two scans could be obtained. This is particularly true when the sample elutes from the gas chromatograph as a narrow peak near the solvent front. One way around this problem involves high-speed switching between several pertinent m/e values by varying the accelerating voltage (the magnet cannot be switched rapidly because of hysteresis) [C. C. Sweeley, W. H. Elliott, I. Fries, and R. Ryhage, *Anal. Chem.* 38, 1549 (1966)]. Measurements are then made of the relative intensities of unknown and known mass peaks only over that period when their ratio is constant. Alternatively, this system can be used as a GLC detector for one compound without the use of internal standards by focusing on several of its important fragment ions. The total area of any one of the series of peaks is then used as a measure of the sample quantity only while its relationship to the other peaks remains constant [C.-G. Hammer, B. Holmstedt, and R. Ryhage, *Anal. Biochem.* 25, 532 (1968)]. Measurement in the 50–100 pg range have been claimed for this method [T. E. Gaffney, C.-G. Hammer, B. Holmstedt, and R. E. McMahon, *Anal. Chem.* 43, 307 (1971)], but it is clear that spectrometer and sample conditions must be ideal to realize this level of detection. When appropriate precautions are observed, there is little reason to doubt the specificity or accuracy of the method, and I am sure that this applies to the prostaglandins as well. It must always be borne in mind, however, that the mass spectrometer does not distinguish stereoisomers or geometric isomers very well, and confusion could arise from this source. Fortunately, GLC often supplies this information. In fact, it appears that most uses of the mass spectrometer in this regard have been to establish that a very weak GLC signal is in fact due to the suspected compound. Quantitation in such experiments is regarded as secondary. The use of mass spectrometers in following changes in biological systems over short time spans where many samples are involved has been minimal. I suppose this is due in some part to the fact that mass spectrometrists do not look kindly upon the required dedication of their instruments to a single project over a considerable period of time. As mass spectrometers become simpler and less expensive, we may expect that its use as a truly quantitative tool will increase.

F. C. Bartter: Have you been able to apply your phenylthiohydantoin method to sequencing of protein hormones?

H. M. Fales: By the time we worked out this method last summer, Dr. Bryan Brewer had already completed his sequencing of the parathyroid hormone [H. B. Brewer and R. Ronan, *Proc. Nat. Acad. Sci. U.S.* 67, 1862 (1970)], so we had no chance to test it in that case. Since that time we have run some samples for Dr. Brewer when the gas chromatographic analysis is equivocal. One case where it seems to be very useful is in deciding between phenylthiohydantoin-glutamine and -glutamic acid. It is not so useful with phenylthiohydantoin-serine or -threonine because of prior dehydration of the derivatives. We have decided to evaluate the method in depth using a dedicated chemical ionization–quadrupole

mass spectrometer-computer system, so we are obviously enthusiastic about its potential.

J. Rudinger: It may be useful in this connection to recall a trick which the late Professor Weygand introduced about three years ago. He proposed the use of variously substituted phenylisothiocyanates in the Edman degradation in such a way that a different isothiocyanate would be used in each of a sequence of degradation steps. The characteristic mass spectrometric peaks of the aryl groups should then make it possible to analyze the sum of the arylthiohydantoins formed in several degradation cycles by a single analysis, or alternatively to distinguish the arylthiohydantoins formed in a given step from those carried over from previous steps and thereby to reduce the "noise" of the determination [F. Weygand, *Fresenius' Z. Anal. Chem.* **243**, 2 (1968); F. Weygand and R. Obermeier, *Eur. J. Biochem.* **20**, 72 (1971)].

H. M. Fales: This is certainly a very valid approach. However, the automated sequenator removes the phenylthiohydantoin derivatives at each step, and there does not seem to be any real advantage in bypassing this feature. Furthermore, it is my understanding that problems do not arise from carrying over the phenylthiohydantoin derivatives from one step to the next, but rather from incomplete or nonselective degradation of protein. If this is true, varying the nature of the phenylisothiocyanate would be of no avail.

B. L. Rubin: I can see that you can tell whether there is a hydroxyl because you have lost that amount of weight, but how do you know where on the molecule it was? What do you do if you have no idea with which to start?

H. M. Fales: Structural information comes out of the mass spectrometer partly as a result of mechanistic considerations gleaned from solution phase organic carbonium ion chemistry [K. Biemann, "Mass Spectrometry, Organic Chemical Applications." McGraw-Hill, New York, 1962] but mostly via liberal use of analogy as in other fields, such as biochemistry and, I suppose, even medicine.

Specifically, it is sometimes quite easy to see where a hydroxyl group is in a steroid because steroids quite often undergo cleavage between the B and C rings localizing the functional groups in either portion of the molecule. At present it is true that some organic chemical training is nearly essential, and experience with many types of compounds greatly enhances one's chances for success. It is possible that in the future we will take much greater advantage of a naive but powerful approach known as the "learning machine." Drs. Isenhour and Jurs [T. L. Isenhour and P. C. Jurs, *Anal. Chem.* **43**, 20a (1971)] are the foremost proponents of the technique in which a computer is fed mass spectral data identified in simple dichotomic terms; i.e., "the molecule contains oxygen—yes, or no?" The computer searches for a "learning" surface in n-dimensions among a set of "training" spectra, which is then used to make decisions in unknown cases.

M. A. Kirschner: At the beginning of your talk you discussed various ways of "brushing the molecules lightly" with electrons or other means in order to delineate the parent ion. Further on you commented that hitting the molecule "hard" causes fragmentation into predictable patterns which can then be used to aid in structural identification. Do you envision a family of mass spectra in the analysis of a compound?

H. M. Fales: Yes; ideally, we would like to have high and low voltage electron ionization spectra, chemical ionization spectra using several reagent gases, and field ionization or field desorption spectra since data from one system often helps to explain unusual features of the other.

G. D. Aurbach: What about using a different type of detection system instead of an ionization chamber? Suppose one were looking for the biosynthesis of a particular compound, for example, from acetate. Would it be possible to hook the spectrometer up to a radiation detector and look only for compounds containing ^{14}C?

H. M. Fales: This has actually been accomplished in a very simple fashion using a graphite-covered photoplate. Thus ions containing ^{14}C will fall at $(M + 2)^+$ rather than M^+, and, since they are radioactive, an autoradiograph will abnormally blacken at this point [H. Knöppel, and W. Beyrich, *Tetrahedron Lett.* p. 291 (1968)]. Alternatively the ^{14}C can be measured directly if it is present at high enough concentration ($\sim 5\%$) [J. L. Occolowitz, *Chem. Commun.* p. 1226 (1968)]. The problem at present is that such detection is very insensitive compared to the usual scintillation counter methods. ^{14}C and other heavy isotopes are attractive alternatives for such studies, but, unfortunately, high-accuracy isotope ratio measurements on large molecules are difficult to obtain. In addition it must be remembered that there is considerable variation in the natural abundance of many of these isotopes. For the biochemist this means that he must achieve very high conversions of substrate. Fortunately, since the spectrometer sensitivity is very high, the experiment need only be performed on very small amounts of material.

J. C. Orr: In conjunction with Drs. Ofner and Engel, [L. L. Engel and J. C. Orr, *In* "Biomedical Applications of Mass Spectroscopy (G. Waller, ed.), Chapter 19, Wiley, New York (1972)] I have been doing some studies of biosynthetic transformation of steroids using gas–liquid chromatography and mass spectrometry. I can see that chemical ionization or other techniques which enhance the molecular ion contribution would be very useful. I took ordinary 17β-hydroxy-5α-androstan-3-one, which has a molecular weight of 290, and mixed it with precisely 43% of 7β-deuterio-17β-hydroxy-5α-androstan-3-one. Natural abundance ^{13}C and deuterium in the unlabeled compound contribute to the peak at m/e 291 $(M + 1)^+$; therefore less than an equimolar amount of the deuterated compound is added to make the ions at m/e 290 and 291 equal in size.

This dihydrotestosterone was incubated with a mince of canine perianal glands. Whatever biosynthetic transformation products you get from this dihydrotestosterone will again have the molecular ion and $(M + 1)^+$ of equal height, or approximately equal height.

In Fig. A on the bottom (merely for comparison) is authentic 5α-androstane-3β,7α,17β-triol. The upper mass spectrum is of the isolated metabolite which contains deuterium in 43% of the molecules. The molecular ions of the triol at m/e 308, 309 are very small; it would have been advantageous to use chemical ionization or some other such technique which causes less fragmentation of the molecule. However, the M-H$_2$O$^+$ ion at m/e 290 and 291 is a double ion and quite prominent enough to reveal this as a metabolite of the dihydrotestosterone. By using GC-MS one can scan a whole lot of mass spectra of the various different GLC peaks, and only those which have something which looks like a "double" molecular ion need you examine further.

In this case, the deuterium being at 7β was extremely fortunate because the 7α-hydroxy was introduced without loss of the label, but on oxidation to 5α-androstane-3,7,17-trione with chromium trioxide under mild conditions the deuterium was lost. This specifically identifies one transformation by the perianal gland as 7α hydroxylation.

H. M. Fales: This is an elegant example of the power of the internal standard

method in mass spectrometry. I might add that in my opinion chemical ionization would probably have given even less intensity in the $(M + H)^+$ ion than you observe in the M^+ ion by the electron impact approach since the only basic functions on the molecule are its hydroxyl groups. Probably only $(M + 1 - H_2O)^+$ ions would be visible.

J. Rudinger: Concerning the use of internal standards, ^{15}N is another case in point. By looking for twin peaks in peptides, etc., one can sometimes get the same results as with radioactive labeling since in this case it is much more difficult to localize the site of incorporation.
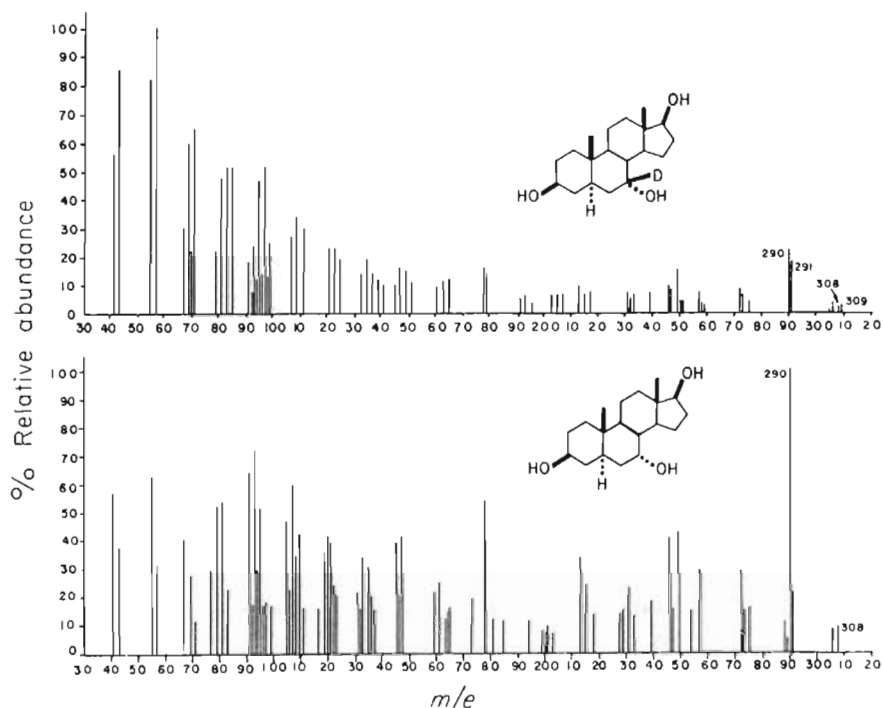
Fig. A. Mass spectra of 5α-androstane-3β,7α,17β-triol (bottom) and of isolated metabolite (top), which contains deuterium in 43% of the molecules.

J. T. Potts, Jr.: Could you amplify further the reasons for the slightly pessimistic scene with regard to peptide sequencing by mass spectrometry? You mentioned that the difficulties were in the derivatization. Is that because material is lost during derivatization or because it is a sort of uneven reaction and therefore the product being analyzed gives confusing results? Is it yield or control of derivatization that is the problem?

H. M. Fales: Most of the methods that have been used have involved prior acylation of the amino end group with something like acetic or propionic anhydride. This reaction is generally quite satisfactory and destroys the very polar zwitterionic character of the peptide providing no arginine or histidine residues are present. All active hydrogens can then be methylated, using methyl iodide and an appropriate

base. This is a somewhat unsatisfactory reaction, however, because of the strongly basic conditions that result in some hydrolysis of glutamine residues. Also, incomplete methylation is common. Furthermore, methyl iodide will quaternize any tertiary amines, such as histidine, rendering the protein totally involatile. So, to answer your question, it is a problem of low yield due to inadequate control of derivatization.

J. C. Orr: You mentioned that you got alkyl-substituted methyl piperidines. Were you able to identify which was *cis* and which was *trans* from mass spectrometry?

H. M. Fales: No. Their mass spectra were identical. This differentiation was done strictly on the basis of GLC and synthesis.

R. O. Greep: You have made it appear that these are very useful gadgets to have around and something that no good laboratory should be without. Do they come in different models? Yours is no doubt of the Cadillac variety. Is there something on the order of a Volkswagen available? Is there a model a poor institution could afford and still gain some of the advantages of this useful instrument? Do you have to have a different instrument for electron ionization and chemical ionization? Could you tell us a bit about the practical aspects?

H. M. Fales: The advantage of the Cadillac, or double-focusing, variety is that we can separate ions of mass 100.00 from 100.01 enabling us to calculate directly the formula of the molecule in question. This is due to the very slight difference between the integral mass of the atoms; i.e., CH_4 and O are both nominally 16 but actually differ by an easily measurable 0.0364 mass unit even at m/e 300. Such information is obviously very useful, but such measurements are either very tedious to make or require the use of a very specialized computer system. I cannot believe that this audience would often require the services of such an instrument. At the other extreme, a quadrupole mass spectrometer good to about mass 300–400 can be purchased for about $28,000. It may serve you very well in much of your work but may be unsatisfactory when you desire to obtain the molecular weight of a trimethyl silyl ether of a pentahydroxylated steroid that may be very easy to see on a GLC run. In between are the so-called medium-resolution, magnetic instruments good to about m/e 1000–1500 mass costing about $60,000. I feel that if you can afford it, this is by far the best choice for the type of application that most persons here probably have in mind.

Furthermore, if you have a sample you wish to have analyzed using high resolution (or low resolution, for that matter), several commercial organizations still perform the analysis for a fee that may amount to a very small fraction of the cost of upkeep of such an instrument. Currently, the National Institutes of Health are assisting in the analysis of compounds of biological importance; no charge is made if the sample qualifies (contact R. Foltz, Battelle Memorial Institute, Columbus, Ohio). An instrument modified for chemical ionization work can easily be used for electron ionization by just turning off the gas, although it will probably be a shade down in sensitivity. However the reverse is not true due to inadequate pumping in the chemical ionization mode.

F. G. Peron: To what extent can the techniques utilized in your laboratory be used to assess the purity of a particular compound?

H. M. Fales: One of the very strong features of the mass spectrometer is that it is extremely sensitive in disclosing the presence of an impurity, unless it happens to be a very closely related stereoisomer. On the other hand, using fractional evaporation from the probe, impurities may not seriously interfere with

one's ability to obtain a useful mass spectrum. High purity is a convenience, but definitely not a general requirement of the method.

L. L. Engel: At a Laurentian Hormone Conference many years ago [R. N. Jones, *Recent Progr. Horm. Res.* **2,** 2 (1948)] the subject of infrared spectroscopy was presented to the endocrinologists present. This physical tool had a profound impact on the development of the field. I think Dr. Fales has described for us a tool which may have an even greater impact on endocrinology.

Social Insect Pheromones: Their Chemistry and Function

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synopsis. Exocrine secretions of social insects are often characterized by extraordinarily complex mixtures of natural products. Thus, chemical communication in social insects must be interpreted in terms of signals generated by multicomponent systems, the individual constituents of which can affect the informational content of the message.

Alarm pheromones have been identified chiefly in three subfamilies of ants and their distribution appears to be chemosystematically significant. Myrmicine genera emphasize 3-alkanones as alarm releasers, whereas methyl ketones, primarily of terpenoidal origin, are widely utilized as alarm pheromones in the subfamily Dolichoderinae. Formicine species may employ formic acid as an alarm pheromone in addition to the compounds produced in the mandibular and Dufour's glands. The mandibular gland pheromones are chiefly acyclic monoterpene aldehydes (e.g., citronellal) which are relatively low boiling compounds. Higher boiling *n*-alkanes are produced in the Dufour's glands and may serve as more persistent releasers of alarm behavior. Alarm pheromones, as well as the caste-specific pheromones of male bees and ants, probably also serve as defensive products. In many cases it is likely that pheromones were originally utilized as defensive compounds and their communicative function is a secondary development.

René de Réaumur (1926), when he wrote on *The Natural History of Ants* in 1742, clearly recognized that these social insects are a rich source of volatile compounds, ". . . a great deal of volatile spirits is being continually exhaled from the bodies of ants. It is peculiar to them, or at any rate there are few other insects that are similarly able to produce an exhalation so penetrating and so abundant." This accurate description of the odoriferous qualities of ants, however, is equally applicable to a vast multitude of social insect species. Although, even now, only the major substances present in the exocrine glands of relatively few social insects have been characterized, the variety of new animal natural products which already has been identified indicates that these invertebrates possess widely varied biosynthetic capabilities.

More than 300 years have passed since

Wray (1670) isolated formic acid, the simplest of fatty acids, from a distillate of formicine ants. While subsequent investigators continued to isolate more complex substances, it is only in the last score of years that the exocrine products of the Hymenoptera and Isoptera have been established as being extraordinarily diverse. Almost ten years ago Callow et al. (1964) showed that the mandibular glands of the queen honey bee contain more than 30 compounds. Recently, in the most thorough investigation of insect-derived compounds yet reported, Bergström and Löfqvist (1971) identified 39 out of more than 50 volatile compounds present in the Dufour's gland of the formicine, *Camponotus ligniperda*. These investigations, in particular, underscore the natural products potential that these arthropods possess, and such chemical complexity of the exocrine gland secretions of social insects may not be too unusual.

It would seem, therefore, that there are no substantive grounds for the statement of Wilson (1970) that vertebrate exocrine secretions are considerably more complex

than those of insects. Wilson based his conclusion on the gas chromatographic separation of the products in the apocrine and sebaceous sudoriferous glands (plus possibly urine) of the black tailed deer (Brownlee et al., 1969) *vis-à-vis* those in the Dufour's gland of a formicine ant. As the volatile sex stimulant in the vaginal secretion of the rhesus monkey has been demonstrated to consist primarily of six simple fatty acids (Michael et al., 1971) it is probable that vertebrate exocrine products may sometimes be rather simple mixtures. Therefore, until additional investigations have been undertaken on the chemistry of vertebrate exocrine secretions, it is obviously premature to generalize about their complexity.

The exocrine glands of social insects are frequently fortified with a diversity of chemical primers and releasers of social behavior. These compounds, the pheromones, constitute many of the key elements which regulate the biology of the social insects, and the elucidation of their chemistry has provided behaviorists with an elegant tool with which to probe many of the dimensions of sociality. Although chemical analyses of social insect pheromones have outstripped the complementary behavioral studies on these exocrine compounds, the results of the former nevertheless present an opportunity to evaluate some of the broad bases which presently characterize this area of comparative exocrinology. However, this review will be restricted, for the most part, to pheromones whose chemical identities have been determined, and no serious attempt will be made to delineate the multitude of pheromones whose existence has been ascertained by behavioral investigations.

The products synthesized in the exocrine glands of arthropods generally consist of mixtures of compounds, and the glandular exudates of social insects are no exception. Although many of the constituents that have been identified in exocrine secretions are now known to possess pheromonal functions, there are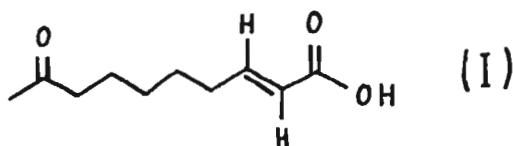 a multitude of natural products with no demonstrated behavioral activities which accompany these communicative chemicals. Since the nature of this review demands some degree of terminological exactitude, we shall endeavor to identify those compounds that appear to be the *bona fide* pheromones. In addition however, the glandular concomitants of the pheromones whose functions are currently unknown will be discussed when appropriate or necessary. In this way it is hoped that the exocrine secretions of different species can be compared both in terms of the magnitude of their biosynthetic peculiarities and by the emphasis placed on particular chemical types and functionalities.

THE LANGUAGE OF CHEMISOCIALITY

1. PRIMER PHEROMONES

Chemical primers of social behavior appear to be widespread, especially among functional female reproductives, but the difficulties in establishing a precise bioassay for these compounds have generally militated against their being isolated and identified. Although at this juncture it is impossible to ascertain whether primer pheromones are normally produced in substantial quantities by functional queens, it may not be insignificant that in the two cases in which these pheromones have been identified, they were present as major exocrine constituents.

Barbier and Lederer (1960) and Butler et al. (1961) identified (E)-9-oxo-2-decenoic acid (*trans* isomer) (I) as the queen substance of the honey bee *Apis mellifera*. This compound, which is pro-


duced in the mandibular glands, plays a role in inhibiting queen cell construction, and in addition, it is one of the pheromones which functions to inhibit ovarian development in worker bees. However, the

primer activity of (E)-9-oxo-2-decenoic acid is increased by other volatile compounds produced by the queen (Butler et al., 1961) and a mated queen is more effective in inhibiting oogenesis in workers than any combination of queen-derived pheromones which has been evaluated (Butler and Fairey, 1963).

The mandibular glands of the queen bee have also been demonstrated to be the source of a second primer pheromone which works in concert with (E)-9-oxo-2-decenoic acid. This compound, (E)-9-hydroxy-2-decenoic acid (trans isomer) (II), acts synergistically with the 9-oxo acid to inhibit queen cell construction by
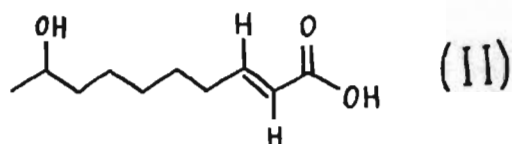
workers (Butler and Callow, 1968). As these two acids are still not quite as effective as a mated queen in inhibiting queen rearing by workers, it is obvious that additional primer pheromones are present. Whether (E)-9-hydroxy-2-decenoic acid is one of the primer pheromones which functions along with the 9-keto acid to inhibit oogenesis in workers has not been determined. However, it will not prove surprising if the mandibular glands of the queen, which are richly fortified with other ten-carbon acids (Callow et al., 1964), are also the ultimate source of the unknown primer pheromones.

(Z)-9-Oxo-2-decenoic acid (cis isomer) like its trans counterpart, has also been reported to inhibit queen rearing by workers (Pain et al., 1962). However, it has now been determined that the apparent primer activity of the cis acid results from its conversion to the trans isomer (Doolittle et al., 1970). Although the photoisomerization of the cis isomer to its trans form can be demonstrated to be initiated by sunlight, the occurrence of the cis-trans isomerization inside the beehive clearly indicates that this transformation

can occur in the absence of sunlight.

Heads of Oriental hornet queens (Vespa orientalis) have been shown to contain a chemical primer for workers of this species. This pheromone has been isolated from the acidic fraction of an extract of heads and identified as δ-n-hexadecalactone (III) (Ikan et al., 1969).
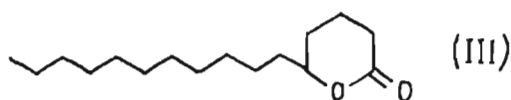
The head of each queen contains about 6 μg of this pheromone. At the end of the season, workers construct queen cells in the absence of the queen if supplied with this compound, whereas ordinarily this type of activity is expressed only in the presence of the queen (Ikan et al., 1969).

Finally, larval growth has been reported to be depressed by the acidic fraction derived from the heads of fertile queens of the ant Myrmica rubra (Brian and Blum, 1969).

2. RELEASER PHEROMONES

Although releaser pheromones with a wide variety of functions have been identified in social insects, it is nevertheless impossible to catalogue these exocrine compounds into strict behavioral categories. Many pheromones are known to subserve multiple functions depending upon the behavioral context in which they are secreted, and this pheromonal parsimony appears to be widespread among hymenopterous species (Blum, 1970). As a consequence, although most of the identified releaser pheromones have been assigned to specific behavioral classes, our classification of these compounds is intended simply to reflect the known functions with which they are currently identified. Indeed, several compounds are reported to be pheromones for some species, whereas in other species the same compound has either a different function or no demonstrable behavioral activity whatsoever.

Releasers of Trail Following

Ants. The major trail pheromone utilized by the ant *Atta texana* has been identified as methyl 4-methylpyrrole-2-carboxylate (Tumlinson et al., 1971) (IV).
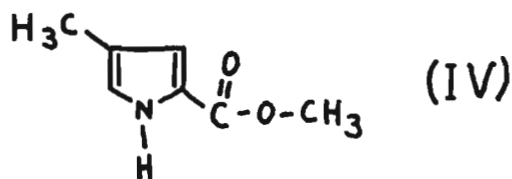
This compound, which is a trace constituent of the poison gland secretion, can be detected by workers at a concentration of about 0.8 pg/cm. In addition to this pyrrole, compounds in four other fractions were demonstrated to possess trail-following activity (Tumlinson et al., 1971). These unidentified trail pheromones are present in substantially lower amounts than the disubstituted pyrrole (IV) and some of these chemical releasers of trail following are certainly identical to the relatively non-volatile trail pheromone(s) which is produced by this species (Moser and Silverstein, 1967).

Sonnet and Moser (personal communication) undertook a thorough study of the relation of pyrrole structure to trail-following activity. Positional isomers of methyl 4-methylpyrrole-2-carboxylate (IV), in which the methyl group is shifted on the pyrrole ring, do not possess any trail-following activity. All other isomers in which the carbomethoxy and methyl groups were no longer present as 2- and 4-substituents respectively were completely inactive. Since methyl pyrrole-2-carboxylate had no demonstrable pheromonal activity, it is clear that the 4-alkyl substituent on the ring is essential. The slight activity of methyl 4-ethylpyrrole-2-carboxylate indicates that the length of the alkyl group can be slightly extended without completely eliminating trail-following activity.

Several of the pheromonal mimics evaluated by Sonnet and Moser (personal communication) possessed pronounced activity as trail pheromones. Significantly, the most active pheromonal surrogates were 2, 4-disubstituted pyrroles containing a carbomethoxy group adjacent to the pyrrolic nitrogen. On the other hand, a variety of pyrroles containing different substitutents on position 4 of the pyrrole ring possessed strong trail-following activity. Thus, methyl 4-chloropyrrole-2-carboxylate was as active as the natural trail pheromone, methyl 4-methylpyrrole-2-carboxylate (IV). The presence of 4-substituted bromine atom on the pyrrole ring also resulted in a potent trail pheromone. Indeed, moderate activity was present in pyrroles which contained a carboxaldehyde group in the 2-position and a halogen atom in the 4-position (e.g., methyl 4-chloropyrrole-2-carboxaldehyde). Conformational analysis of these data by Sonnet and Moser (personal communication) clearly indicates that this structure-activity investigation may have great value in probing the *modus operandi* of olfactory receptor sites.

Atta texana is a species in the tribe Attini, a taxon which is characterized by a series of genera whose recognized phylogentic relationship is supported by both morphological and behavioral data (Weber, 1958). Since *Atta* is regarded as the most specialized genus in the Attini, an evaluation of the sensitivity of species in the more primitive transitional genera to methyl 4-methyl pyrrole-2-carboxylate (IV) may provide an insight into the evolution of trail pheromones in this taxon. Moser and Blum (unpublished) assayed the *A. texana* pyrrole (IV) on species in the genera *Cyphomyrmex*, *Apterostigma*, *Trachymyrmex*, *Acromyrmex*, and *Atta*, a series of taxa which are believed to reflect the increasing evolutionary specialization of genera in this tribe. All species were equally sensitive to the disubstituted pyrrole (IV), and it thus appears probable that little change has occurred during the evolution of this tribe in either the trail pheromone or the olfactory receptors

SOCIAL INSECT PHEROMONES 557

which detect this exocrine compound. The same conclusion was reached by Blum et al. (1964) after demonstrating that poison gland extracts of species in these genera, which contain the trail pheromone, lack any detectable specificity at the generic level.

These results on the trail pheromone of *A. texana* lead to the obvious conclusion that it is comprised of a mixture of compounds, all of which release trail following, but not all the compounds are equally active (Tumlinson et al., 1971). It is widely recognized for many different species of ants that a newly laid trail, e.g., from a food source to the nest, produces a strong recruitment response, and Wilson (1971) states that in most cases analyzed to date, the recruitment trail substances have turned out to be strong attractants. We have noticed how workers of *Solenopsis invicta*, following a strong trail to and from a regular source of food, can be drawn off this trail by the freshly laid trail of only one worker which has found a new food source. It would appear that a freshly laid trail contains a more volatile fraction, which functions particularly for recruitment, in addition to the regular trail-following substances.

Any behavioral conclusions pertaining to trail following that are based on the responses of ants to extracts of trail substances should take into account the possibility that the true trail substance is a mixture of compounds differing in volatility (Tumlinson et al., 1971). The information content of such a mixture may vary appreciably. In numerous bioassays of an active trail-following extract of *Crematogaster peringueyi*, Fletcher and Brand (unpublished) were never able to obtain the same degree of trail following with any fractions collected from a gas chromatographic column as with the original extract. Recombination of the various collected fractions still did not give the same degree of activity as the equivalent amount of material that was injected. However, certain collected fractions of relatively low volatility which possessed weak trail-

following activity were found to contain two different heptyl benzenes and cyclohexyl benzene (Brand, Eggers, and Fletcher, unpublished). These compounds, which themselves do not cause active trail following, may act as keepers of more volatile components, thereby prolonging the perceptibility of a newly laid trail. This aspect of pheromone chemistry promises to offer an exciting field of research, and, with the technical refinements of analytical chemistry now being used and developed (Tumlinson et al., 1971), rapid progress in this area can be expected.

Termites. The odor trails of termites are generated from a secretion which originates in the sternal gland, a structure that is found universally in these insects (Stuart, 1970). The propensity of termites for following artificial trails made with a wide variety of synthetic compounds (Stuart, 1969), militates against utilizing an unnatural assay in monitoring a trail pheromone present in a termite extract. Indeed, Becker and Petrowitz (1967) reported that several species of termites readily followed the ink line made with a ball-point pen and demonstrated that the biologically active material consisted of the monoalkyl ethers of diethylene glycol. In an elegant series of experiments Stuart (1969) analyzed the mechanism of trail laying in termites and concluded, on very persuasive grounds, that unless termites on a trail display uninterrupted trail-following behavior when their natural trail is interrupted with a test compound, the candidate compounds cannot be considered identical to their trail pheromone.

Verron and Barbier (1962) isolated (Z)-3-hexenol (*cis* isomer) (V) from nymphs of

(V)
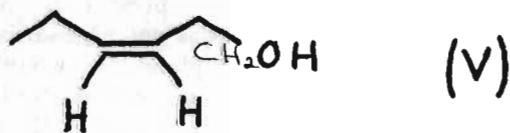
Calotermes flavicollis and from the galleries of *Microcerotermes edantatus*. This compound is attractive to both lower and

higher termites (Verron, 1963) but does not possess the potency which is identified with the trail pheromones of these species. Hummel and Karlson (1968) isolated n-hexanoic acid from *Zootermopsis nevadensis* and reported that this compound was one of the components of the trail pheromone of this species. Subsequently, Karlson et al. (1968) demonstrated that farnesol, a compound not detected in the termites, was as active as hexanoic acid when evaluated by their trail assay. The high activity of another synthetic compound, the dodecyl ester of phthalic acid, demonstrated that nymphs of *Z. nevadensis* were quite capable of exhibiting trail following in the presence of a wide variety of unrelated compounds.

A compound which is a potent attractant for *Reticulitermes flavipes* has been isolated from wood infected with the fungus *Lenzites trabea* (Matsumura et al., 1968). This compound is also present in the termites, but the fungus appears to be a much richer source of this attractant than the termites themselves. Because of the ability of this compound, n-cis-3, cis-6, trans-8-dodecatrien-1-ol (VI),

(VI)
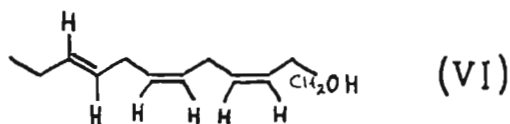
to cause trail following at very low concentrations, Matsumura et al. (1968) regard it as the trail pheromone of *R. flavipes*. However, no evidence is presented to demonstrate that the trail pheromone in the sternal gland is identical to the dodecatrienol (VI) which was isolated from the fungus. Thus, it is very possible that the minor amount of dodecatrienol which was isolated from termites was present in the gut of these insects and was not necessarily present in the sternal gland. It would seem highly desirable to present trail-following nymphs of *R. flavipes* with a choice between two branches of a trail fork, one of which is treated with the trienol (VI) and the other with a sternal

gland extract. In this way, termites should be able to distinguish easily between the fungal attractant and their trail pheromone, if they are not one and the same.

Akira et al. (1971) compared the trail-following activity of n-cis-3, cis-6, trans-8-dodecatrien-1-ol (VI) to a number of related compounds. Remarkably, the compound which lacked the trans-8 double bond, n-cis-3, cis-6-dodecadien-1-ol, was as active a trail substance as the fungally derived alcohol (VI). A completely unnatural alcohol, 4-phenyl-cis-3-buten-1-ol, was also as active as the dodecatrienol (VI) which these investigators consider to be the natural trail pheromone. Even cinnamyl alcohol, 3-phenyl-2-propen-1-ol, possessed extraordinary activity as a trail substance for *R. flavipes*.

Thus, the trail-following activity of n-cis-3, cis-6-dodecadien-1-ol, not withstanding the absence of a third double bond is not diminished in comparison to that of the trienol (VI), the presumed trail pheromone. Furthermore, if the olfactory receptors which interact with the true trail pheromone possess any degree of spatial specificity, it is difficult to reconcile this fact with the very high levels of trail-following activity of the aromatic alcohols which were evaluated. The data of Akira et al. (1971) contrast extraordinarily with those of Butenandt and Hecker (1961) who studied the relationship of sex attractant activity to geometry of the double bonds in the sex attractant of *Bombyx mori*. These investigators reported that whereas the sex attractant n-trans-10, cis-12-hexadecadien-1-ol was active at a concentration of 1×10^{-12}, activity dropped to 1×10^{-3} and lower when the geometry around the double bonds was changed.

Ritter and Coenen-Saraber (1969) isolated two fractions from wood infected with the fungus *L. trabea*, both of which released strong trail-following in *Reticulitermes lucifigus*. Bioassays were carried out by employing some isolated workers which were carefully exposed to test streaks containing the candidate fractions. One of the

fractions may contain the dodecatrienol isolated by Matsumura et al. (1968) but the compound in the other active fraction is clearly different from this trienol. Thus, for *R. lucifigus*, wood infected with *L. trabea* contains two compounds capable of releasing trail following rather than a single component as has been reported for *R. flavipes*.

The trail pheromone of *Nasutitermes exitiosus* has been characterized as an unsaturated diterpenoid hydrocarbon, $C_{20}H_{32}$, which apparently possesses a monocyclic structure with four double bonds (Moore, 1966). The same compound appears to be present in other species of *Nasutitermes* but is inactive as a trail pheromone for a species of *Coptotermes*. Since *Nasutitermes* species readily followed trails prepared with a fraction of Australian sandalwood (*Santalum spicatum*) oil (Moore, 1966), this essential oil was further characterized in order to identify the compound(s) which acted as a releaser of trail following. Birch et al. (1970) identified two sesquiterpene hydrocarbons which were shown to be responsible for the trail following activity of sandalwood oil. Two sesquiterpene hydrocarbons, 10 *cis*- and 10 *trans*-2,6,10-trimethyldodeca-2,6,10-triene, were equally identified with the trail-following activity of this oil. However, neither of these compounds was as active as the natural pheromone produced by *N. exitiosus* which was about 10^3 times more potent than the sandalwood-derived compounds.

Thus, several investigators have established that compounds produced by various plants are both potent attractants and trail pheromones for different species of termites. However, it has not been established that these compounds, notwithstanding their great trail-following activity, are identical to the natural pheromones which are present in the sternal glands of termites. If termites are dependent for their trail pheromones on the fungi which attack the wood that they ingest, then it remains to be established as to how the fungal derivatives are transported to the

sternal gland. If the termite trail pheromones which are present in the sternal gland do not have a *de novo* origin in the termite, then the sternal gland cannot be regarded as a biosynthetic tissue, at least in terms of the trail pheromone. In this case, it must be postulated that the future trail pheromone is selectively channeled to the sternal gland after its ingestion by a termite. On the other hand, if the fungally-derived compound is metabolized in the isopteran body, then it is possible that this compound is ultimately reconstructed in the sternal gland, which then would possess an important biosynthetic function. These questions, which are fundamental to our comprehension of termite biology, remain to be answered.

Bees. Many species of stingless bees lay odor trails by means of a series of droplets placed at varying intervals between the food source and the nest. The trail marking compounds, which originate in the mandibular glands, are often utilized to recruit large numbers of workers to food finds in a short period of time (Lindauer and Kerr, 1958). The presence of species-specific trail pheromones would insure that trails laid to food resources would be insulated from violations by foreign species and thus newly discovered food finds could be exploited quickly only by the bees which were recruited by the original scout.

Neral (VII) and geranial (VIII), the stereoisomers of citral,
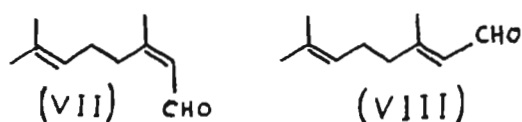
have been demonstrated to be the pheromones utilized by *Trigona subterranea* for generating trails to newly discovered food supplies (Blum et al., 1970). Workers orient to citral-impregnated objects when they discover a food source and are attracted to citral baits when objects fortified with these monoterpene aldehydes are placed on the trails along which they are flying.

Trigona species in the subgenus *Scapto-trigona* utilize a mixture of carbonyl compounds in order to lay an odor trail (Blum, Kerr, Padovani, and Doolittle, unpublished). In *T. postica* and *T. tubiba*, one of the major exocrine compounds in the mandibular glands is benzaldehyde, a powerful attractant for *T. postica* workers. In addition, the mandibular glands of *Scaptotrigona* species are fortified with methyl ketones, and workers of *T. postica* contain large quantities of 2-tridecanone and 2-pentadecanone in these exocrine structures. On the other hand, the mandibular glands of *T. tubiba* workers contain only three methyl ketones and their trail language is considerably simpler than that of *T. postica*. Since *T. tubiba* workers can follow trails laid by *T. postica* workers but not *vice versa*, it is obvious that the pheromonal composition of the mandibular glands of each species may determine the species specificity of the trails which are laid with these exocrine secretions.

Releasers of Alarm Behavior

At the present time, the chemistry of the comparative exocrinology of social insects is largely identified with the pheromones which are utilized to release alarm behavior. A wide variety of alarm pheromones have now been identified and these releasers appear to be commonly accompanied by a multitude of other exocrine compounds (Bergström and Löfqvist, 1968, 1970). With few exceptions, the characterized chemical releasers of alarm behavior have been isolated from ants, and a survey of the primary formicid alarm pheromones (Table I) is virtually equivalent to a summary of the known exocrine compounds utilized to generate alarm signals in animals. Although a few exocrine compounds have been isolated from species which belong to ant subfamilies other than the three listed in Table 1, these natural products have been excluded because they have not yet been demonstrated to release alarm behavior.

The alarm pheromones produced in the mandibular glands of species in myrmicine genera are clearly dominated by ethyl ketones (Table I). Seven 3-ketones have been identified in species of different taxa and two of these compounds, 3-octanone and 4-methyl-3-heptanone, appear to be especially characteristic releasers of alarm behavior in the Myrmicinae. The apparent absence of 3-ketones in the mandibular glands of non-myrmicine species indicates that the biogenesis of ethyl ketones in these exocrine structures is a widespread chemotaxonomic character peculiar to many genera in the large subfamily Myrmicinae. Although the myrmicine genera are united by this biosynthetic common denominator, the random distribution of 3-alkanones in these formicids clearly nullifies the phylogenetic value of these compounds as chemosystematic indicators within this subfamily.

The remarkable variation on a 3-alkanone theme which has been practiced by myrmicine species has resulted in the utilization of a variety of new natural products as releasers of alarm behavior. Thus, 4-methyl-3-hexanone is only known as a natural product because of its occurrence in *Manica* species (Fales et al., 1972). Interestingly, in an evaluation of the alarm-releasing activities of 98 ketones, Blum et al. (1971a) demonstrated that 4-methyl-3-hexanone was the only ketone which was as active as 4-methyl-3-heptanone, the natural alarm pheromone of *Pogonomyrmex badius*. 4-Methyl-3-heptanone may occur as a mandibular gland product in the apparent absence of other alkanones (e.g., *Pogonomyrmex*) or this ketone may be accompanied by 3-octanone (e.g., *Trachymyrmex seminole*) or 2-heptanone (e.g., *Atta*). The presence of 2-heptanone in *Atta* species (Moser et al., 1968) as a concomitant of 4-methyl-3-heptanone demonstrates that some myrmicines possess the ability to biosynthesize methyl ketones in their mandibular glands. On the other hand, 2-heptanone is a poor releaser of alarm for *Atta* species when compared to 4-methyl-3-heptanone, and the true func-

TABLE 1. *Typical alarm pheromones of the Formicidae.*

Compound	Subfamily	Genus	Reference
	Myrmicinae		
trans-2-hexenal		Crematogaster	Bevan et al., 1961.
4-Methyl-3-hexanone		Manica	Fales et al., 1972.
4-Methyl-3-heptanone		Pogonomyrmex	McGurk et al., 1966.
		Atta	Moser et al., 1968.
		Trachymyrmex	Crewe and Blum, 1972.
		Manica	Fales et al., 1972.
3-Octanone		Crematogaster	Crewe et al., 1970.
		Trachymyrmex	Crewe and Blum, 1972.
		Acromyrmex	Crewe and Blum, 1972.
3-Octanol		Myrmica	Crewe and Blum, 1970a.
		Crematogaster	Crewe et al., 1970.
6-Methyl-3-octanone		Myrmica	Crewe and Blum, 1970b.
3-Nonanone		Myrmica	Crewe and Blum, 1970b.
3-Decanone		Manica	Fales et al., 1972.
4,6-Dimethyl-4-octen-3-one		Manica	Fales et al., 1972.
	Dolichoderinae		
4-Methyl-2-hexanone		Dolichoderus	Cavill and Hinterberger, 1962.
2-Heptanone		Iridomyrmex	Blum et al., 1963.
		Conomyrma	Blum and Warter, 1966.
2-Methyl-4-heptanone		Tapinoma	Trave and Pavan, 1956.
6-Methyl-5-hepten-2-one		Tapinoma	Trave and Pavan, 1956.
		Conomyrma	McGurk et al., 1968.
	Formicinae		
Formic acid		Formica	Maschwitz, 1964.
		Camponotus	Ayre and Blum, 1971.
			Bergström and Löfqvist, 1971.
2,6-Dimethyl-5-hepten-1-al		Acanthomyops	Regnier and Wilson, 1968.
Citral (both isomers)		Acanthomyops	Ghent, 1961.
			Chadha et al., 1962.
Citronellal		Acanthomyops	Ghent, 1961.
			Chadha et al., 1962.
n-Decane		Camponotus	Bergström and Löfqvist, 1971.
n-Undecane		Acanthomyops	Regnier and Wilson, 1968.
		Camponotus	Bergström and Löfqvist, 1971.
2-Tridecanone		Acanthomyops	Regnier and Wilson, 1968.

tion of this 2-ketone remains to be determined with certainty.

The occurrence of 3-octanone in six myrmicine genera clearly indicates that the taxa in this subfamily also emphasize unbranched ketones as alarm pheromones. The identification of 3-nonanone in *Myrmica* species (Crewe and Blum, 1970b) and 3-decanone in *Manica* species (Fales et al., 1972) demonstrates that the capacity to produce a series of unbranched ethyl ketones, as well as 4-methyl-3-ketones, is a well-developed characteristic shared by many myrmicine species. Although the major methyl-branched 3-alkanones are substituted on the C_4 carbon atom, the ability of *Myrmica* species to synthesize alkanones which are branched on the C_6 carbon (Crewe and Blum, 1970b) further demonstrates the biosynthetic versatility possessed by species in certain myrmicine genera. The ability of species in some genera to produce dimethyl-branched 3-alkanones in which both the C_4 and C_6 carbons contain methyl substituents is exemplified by manicone, 4,6-dimethyl-4-octen-3-one (Fales et al., 1972).

Carbinols corresponding to the 3-alkanones are produced in the mandibular glands of many myrmicine species, and it is tempting to speculate that these alcohols are involved in the biosynthetic pathways for the ketones. McGurk et al., (1966) identified 4-methyl-3-heptanol as a normal concomitant of 4-methyl-3-heptanone, the alarm pheromone of *Pogonomyrmex* species. However, this carbinol has virtually

no activity as an alarm releaser for *Atta texana*, another myrmicine species which utilizes 4-methyl-3-heptanone to generate an alarm signal (Moser et al., 1968). On the other hand, in both *Myrmica* and *Crematogaster* species, 3-octanol accompanies 3-octanone in the mandibular gland secretion and this carbinol is an effective releaser of alarm behavior (Crewe et al., 1970; Crewe and Blum, 1970b). 6-Methyl-3-octanol, along with its corresponding ketone, is present in the mandibular glands of *Myrmica* species and in at least one species both the alcohol and ketone are major constituents (Crewe and Blum, 1970b). However, the apparent absence of detectable alcohols in the ketone-rich secretion of the mandibular glands of *Manica* species (Fales et al., 1972) demonstrates that carbinols do not always accompany their ketonic counterparts.

The reduction of a ketone to the corresponding alcohol with NaBH$_4$ has been used in characterizing many of these compounds. After the reduction of 4-methyl-3-heptanone or manicone we have obtained two isomeric alcohols, whereas only one peak corresponding to 4-methyl-3-heptanol was observed by McGurk et al. (1966) as a normal constituent in the mandibular secretion of *Pogonomyrmex* species. It would seem, therefore, that the various carbinols may be stereospecifically synthesized by ants, but until definitive biosynthetic studies on myrmicine alarm pheromones are undertaken, neither their metabolic role nor their function can be comprehended.

The widespread occurrence of ethyl ketones in myrmicine taxa almost guarantees that these formicids will exhibit great olfactory sensitivity to 3-alkanones. Significantly, Moser et al. (1968) reported that workers of *Atta texana* were 100,000× more sensitive to their natural alarm pheromone, 4-methyl-3-heptanone, than they were to a closely related compound, 4-methyl-2-heptanone. It is obvious that the chemoreceptors on the antennae of this myrmicine are eminently capable of resolving between closely related methyl ketones and

ethyl ketones. Furthermore, since *A. texana* workers are about 1000× more sensitive to 4-methyl-3-heptanone than they are to 2-heptanone, notwithstanding the fact that they produce both compounds in their mandibular glands, it indeed seems probable that they are selectively sensitive to 3-ketones. Blum et al. (1971a) obtained similar results with *Pogonomyrmex badius*, another myrmicine which utilizes 4-methyl-3-heptanone as an alarm releaser.

Dolichoderine alarm pheromones, which are produced in the anal glands, appear to be dominated by ketones of apparent terpenoid origin (Table 1). Three of the four known compounds employed as alarm releasers by dolichoderine species are terpenes, and one of the compounds, 2-methyl-4-heptanone, an alarm pheromone of *Tapinoma* species, is the only 4-ketone which has been identified in ants. However, the presence of 2-heptanone in some dolichoderine genera demonstrates that ketones of apparent non-terpenoid origin are also utilized as alarm releasers by dolichoderine species. 2-Heptanone is also produced by myrmicine species in the genus *Atta*, and this compound is the only ketone which is known to be shared between the subfamilies Dolichoderinae and Myrmicinae. Since methyl ketones are present in species in all dolichoderine taxa in which alarm pheromones have been isolated, the members of this subfamily are clearly identified with 2-alkanones. As a consequence, dolichoderines might be expected to be maximally responsive to methyl ketones in contrast to alkanones in which the carbonyl group is located more towards the center of the carbon chain. Indeed, a structure-activity investigation of the alarm-releasing activities of a large series of ketones demonstrated that the dolichoderine *Iridomyrmex pruinosus*, which utilizes 2-heptanone as an alarm pheromone, exhibited greatest sensitivity to 2-alkanones (Blum et al., 1966).

Species in the subfamily Formicinae have been demonstrated to generate an alarm signal with secretions from three different exocrine glands (Maschwitz, 1964), and alarm releasers have been identified in the

glandular exudates of all three structures. Formic acid, which is a typical poison gland secretion of formicine species, has also been utilized to signal alarm in species in the genera *Formica* and *Camponotus* (Table 1). This acid possesses the lowest molecular weight (46) of any of the known alarm pheromones, and its high vapor pressure results in alarm signals of rather short duration (Ayre and Blum, 1971). However, some formicine genera (e.g., *Lasius*) do not utilize formic acid to release alarm behavior (Maschwitz, 1964), although it would appear to be of obvious adaptive significance to generate an alarm signal with the copious defensive products of the poison gland.

Haskins et al. (1972) have recently described a remarkable case in which a predatory species is driven into an aggressive frenzy by formic acid, an alarm pheromone secreted by its normal prey. *Myrmecia gulosa*, an archaic myrmiciine species, is specialized for preying on *Camponotus* species, and the latter eject large quantities of formic acid during the encounters with their aggressive predators. In the presence of formic acid (which *Myrmecia* does not produce), violent attack behavior is released in workers of *M. gulosa* and the aggressive propensities of these myrmiciines is savagely directed at their formicine prey. Thus, the alarm signal generated by the formicine prey is "read" by its predator and results in lowering the attack threshold of this already aggressive myrmiciine. The alarm signal is especially disadvantageous to *Camponotus* species because it can serve to recruit more aggressive workers of *M. gulosa* to the site of the confrontation. Significantly, two other species of *Myrmecia*, *M. vindex* and *M. nigriceps*, neither of which prey selectively on *Camponotus* species, do not exhibit alarm behavior in the presence of formic acid.

The terpenes which are employed to release alarm behavior (Table 1) are produced in the mandibular glands of species in certain formicine genera (e.g., *Lasius*, *Acanthomyops*). On the other hand, volatile alarm releasers have not been detected in the mandibular glands of species in two major formicine genera, *Formica* and *Camponotus*, and, as a consequence, these glandular structures cannot always be regarded as typical sources of alarm pheromones in in the Formicinae. However, when exocrine products are biosynthesized in the mandibular gland, these compounds sometimes prove to be surprisingly novel terpenes. Bergström and Löfqvist (1970) identified both sesquiterpenes and diterpenes in the mandibular glands of *Lasius* species as well as the methyl ketone, 6-methyl-5-hepten-2-one. The presence of the latter compound, a typical dolichoderine anal gland product, demonstrates that the Formicinae share at least one ketonic product with the Dolichoderinae.

The hydrocarbons and long-chain ketone (Table 1) are alarm pheromones which originate in the secretion of the Dufour's gland. The products of this exocrine structure are secreted in admixture with the poison gland exudate (Maschwitz, 1964), and thus, an alarm signal is generated from either one (*Lasius*) or both (*Camponotus*) glandular sources which are evacuated through the venom orifice. The Dufour's gland secretion is extraordinarily complex (Bergström and Löfqvist, 1968, 1970, 1971; Schreuder and Brand, 1972) and the significance of the great chemical diversity of this glandular secretion remains to be fully established.

However, the variety of natural products identified in the Dufour's gland secretion of formicine ants has considerably extended our comprehension of the comparative exocrinology of ants. Thus, long chain methyl ketones, which usually correspond to concomitant hydrocarbons, are produced in this gland and demonstrate that 2-alkanones are not limited in their formicid distribution to the Myrmicinae and Dolichoderinae. Long-chain 3-alkanones are synthesized by some *Lasius* species (Bergström and Löfqvist, 1970) and although they probably do not function as alarm pheromones, the presence of these compounds demonstrates that the capacity to biosynthesize 3-alkanones in ants is not limited to the mandibular glands of myrmicines. Finally, bio-

genesis of terpenes in the Dufour's glands of several formicine species (Bergström and Löfqvist 1968, 1970, 1971) demonstrates that these exocrine structures share the capacity to biosynthesize this class of compounds along with the mandibular glands.

2-Heptanone, an anal gland product which signals alarm in certain dolichoderine species, is also utilized as an alarm releaser in certain bees. Stingless bees in the genus *Trigona* *(Scaptotrigona)* discharge a mandibular gland secretion which is fortified with 2-heptanone and this compound effectively releases alarm behavior in workers (Blum, Kerr, Padovani, and Doolittle, unpublished). 2-Heptanone is also a moderately effective releaser of alarm behavior for honey bee workers (Shearer and Boch, 1965), but the primary alarm signal is generated by isoamyl acetate, a compound produced by glandular tissue on the sting shaft (Boch et al., 1962, 1970).

Trigona subterranea, a stingless bee in the subgenus *Geotrigona,* utilizes both isomers of citral in order to generate an alarm signal in the vicinity of the hive (Blum et al., 1970). As mentioned previously, these two terpenes also function as trail pheromones for this species.

Releasers of Sexual Behavior

Our total knowledge of the chemistry of sex attractants in female social instects is predicated on the identification of the sex pheromone produced by the queen honey bee, *Apis mellifera.* Gary (1962) demonstrated that queen substance, (E)-9-oxo-2-decenoic acid (I) was a powerful attractant for airborne drones. This compound, which also functions as a queen substance in the milieu of the hive, thus acts both as a releaser pheromone for drones and a primer for workers. Recently, queens of the other three species of *Apis, A. indica, A. dorsata,* and *A. florea,* have been demonstrated to produce 9-oxo-2-decenoic acid in their mandibular glands (Shearer et al., 1970; Sannasi and Rajulu, 1971). Size disparities probably prevent these three sympatric

species of *Apis* from hybridizing. Indeed, even when *A. mellifera* and *A. indica,* which are not naturally sympatric, are presented with optimal mating conditions, the two species fail to hybridize (Ruttner and Kaissling, 1968).

Pain and Ruttner (1963) demonstrated that either queen honey bees or their extracts were apparently more attractive to flying drones than (E)-9-oxo-2-decenoic acid (I), whereas Butler and Fairey (1964) reported that the attractiveness of a queen bee to drones was due entirely to the 9-oxo acid (I). However, since Pain and Ruttner undertook their investigations with a level of sex attractant which was tenfold greater than that utilized by Butler and Fairey, it now appears probable that their contrasting results were due to concentrational differences. Butler and Fairey (1964) evaluated the sex attractancy of 100 μg of queen substance, a level which is quite similar to that found in a queen bee. On the other hand, Pain and Ruttner (1963) tested an inordinately high level of queen substance (>1 mg) and thus challenged the drones with a super-threshold concentration of attractant. Boch (personal communication) has observed that drones are not attracted in large numbers to lures containing 1 mg of queen substance but, rather, become visually attracted to each other at some distance from the lure where the level of attractant is sufficient to release sexual behavior.

(E)-9-Oxo-2-decenoic acid (I) appears to be more absolute in its structural specificity than any other sex attractant which has been similarly studied. An evaluation of the sex attractancies of nineteen closely related compounds demonstrated that any structural change from that of the natural pheromone resulted in a complete loss in activity (Blum et al., 1971b). The inactivity of (Z)-9-oxo-2-decenoic acid (*cis* isomer) as a sex pheromone established the critical nature of the *trans* configuration. A free carboxylic acid group is required for activity since the methyl ester of (I) possesses no sex attractancy. The requirement for an α, β-relationship to the

double bond was established by the inactivity of the β, γ-isomer. The necessity for the carbonyl group is absolute in terms of both its functionality and position. Removal of the carbonyl group or replacing it by an hydroxy group completely eliminated activity as did changing its position on the carbon chain. Either lengthening or shortening the sex attractant molecule by adding or deleting methylene groups eliminated all pheromonal activity.

A conformational analysis of (E)-9-oxo-2-decenoic acid (I) in terms of its minimal energy conformer demonstrated that any change in structure had a pronounced effect on intra-atomic distances (Blum et al., 1971b). The absolute specificity of this pheromone can best be interpreted in terms of a preferred energy conformer which derives its great specificity from its acceptability to a receptor on the antennae of the drone. Similarly, the inability of either the cis-isomer or closely related compounds to mask the activity of the natural pheromone can be most easily explained as a function of special olfactory receptors which accommodate only a preferred energy conformer, specifically that of (E)-9-oxo-2-decenoic acid (I).

The mandibular gland secretions of male bumble bees are utilized as territorial markers that attract both males and females to specific sites which the males patrol and label. Thus, these secretions, while they attract individuals of both sexes, indirectly serve as sex pheromones because they draw males and females to a common point where mating can occur. Calam (1969) studied the compounds present in the mandibular glands of males of five species of Bombus and identified long-chain alcohols, hydrocarbons, and esters as the main products. No two species contained the same qualitative mixture, and as a consequence, these secretions may be able to function admirably as species isolating devices. More recently, Kullenberg et al. (1970) analyzed the mandibular gland secretions of males of thirteen species in the genus Bombus and six species of cuckoo bumblebees in the genus Psithy-

rus. The secretions were rich in acyclic mono-, sesqui-, and diterpenes as well as an extensive series of aliphatic straight-chain compounds which included alcohols, acetates, and ethyl esters. Significantly, although a few species produced the same major compound (e.g., geranylgeranyl acetate) in their mandibular glands, no two species contained the same qualitative blend of pheromones. Thus, only B. hortorum produced nonadecene, and 2,3-dihydrofarnesol was limited in its distribution to B. terrestris. Although both Ps. silvestris and Ps. bohemicus contained hexadecen-1-ol as the major constituent in their secretions, the former species also produced tetradecan-1-ol, ethyl tetradecan-1-ol, ethyl tetradecenoate, and tetradecanal, whereas the secretion of the latter contained citronellol, tetradecanal, and hexadecanal as concomitants of the main product. The results of Kullenberg et al. (1970) provide persuasive evidence for regarding the secretory mixtures produced by male bumble bees as highly specific species attractants.

Male ants of Acanthomyops claviger, L. neoniger, and L. alienus have been shown to produce volatile compounds in their mandibular glands which do not occur in workers of the same species (Law et al., 1965). The heads of males trapped at light traps some hours after mating flights possessed little or no odor, and it was suggested that these male-derived compounds may be used in species recognition and as isolating mechanisms by attracting females during flight. In contrast to these findings, Kannowski and Johnson (1969) found that males of L. umbratus, both during a mating flight and after mating, contained the same quantity of mandibular substance as males taken from a nest before flight. Also, as Kannowski (1963) has concluded that in Lasius spp. males are attracted to females during flights, the actual function of these male-derived compounds still has to be determined.

It is interesting to note that most of the compounds obtained from the male heads of L. neoniger, L. alienus, and A. claviger

are alcohols, whereas the major com-
pounds in the heads of workers of *A.
claviger* are aldehydes (Regnier and Wil-
son, 1968). In a comparison of the volatile
products in the heads of workers and
males of *Atta sexdens* (Blum, Brand, and
Amante, unpublished), we have found
that the male heads contain a considerable
amount of 4-methyl-3-heptanol, together
with the corresponding ketone, and, rather
interestingly, nonanoic acid. This is in
contrast to the workers which contain
mainly 4-methyl-3-heptanone (Blum et
al., 1968). The fact that the alcohols have
a lower volatility than their corresponding
ketones may be particularly important in
the function of these secretions in these
male ants.

Male heads of *L. neoniger, L. alienus,*
and *A. claviger* are reported also to con-
tain an indole compound (Law et al.,
1965). We have recently identified methyl
anthranilate among the major products in
the heads of male *Camponotus nearcticus*
(Brand, Duffield, Blum, and Fales, unpub-
lished). These latter findings lead us to
make a speculation which we hope will not
turn out to be misleading. In general,
male ants have large eyes, especially when
considered in relation to the size of their
heads. A key intermediate in the synthesis
of certain eye pigments of insects is the
amino acid, tryptophan. It, therefore,
seems reasonable to assume that the heads
of male ants, with their large eyes, would
be particularly well adapted to the conver-
sion of tryptophan into these pigment sub-
stances. A number of compounds play key
roles in the pathways of tryptophan metab-
olism, among which are indole and an-
thranilic acid, the latter being an end pro-
duct of tryptophan metabolism in mam-
mals.

It is tempting to suggest that indole and
methyl anthranilate, among other com-
pounds in the heads of certain male ants,
may play some pheromonal role in the
biology of these species. If the presence of
these two nitrogen-containing compounds
is in fact related to the metabolism of tryp-
tophan, then certain products of a particu-

lar metabolic pathway may have been ex-
ploited preferentially for use in a rather
intricate behavioral system. If these specu-
lations are correct and these few com-
pounds are chemically related, then they
may be the first of a new group of formi-
cine, or even ant pheromones.

We suspect that the copious secretions
produced in the mandibular glands of
male bumble bees and male ants may have
another function which is completely un-
related to their immediate pheromonal
roles. The compounds produced in the
heads of male hymenopterans should serve
as excellent defensive substances against
predators. Free-flying males are specially
subject to predation by airborne predators,
and terrestrial arthropods undoubtedly
constitute an additional source of predato-
ry pressure when the males alight on the
ground. The mandibular gland secretions
may have a distinct value in repelling at
least some of these predators and may,
thus, possess important survival value for
the species which produce them. Indeed, it
is not improbable that these secretions
were originally employed as defensive
secretions, and their role as territorial mar-
kers and attractants may have been derived
secondarily. It is possible that the utiliza-
tion of defensive compounds to function
secondarily as volatile information-bearing
agents may be the rule among the Hy-
menoptera, rather than the social excep-
tion.

PARSIMONY:
MULTIFUNCTIONAL PHEROMONES

One of the major factors responsible for
the development of a viable chemisociality
has been the ability of social insects to
utilize single pheromones to subserve mul-
tiple functions. Evidence for widespread
pheromonal parsimony in the Hymenop-
tera is becoming increasingly common and
the development of this communicative
versatility by social insects must be regard-
ed as one of the major developments
which enabled these arthropods to expand

tremendously their social horizons. In view of the significant numer of examples of pheromonal parsimony which have already been exposed as a consequence of relatively few investigations, we believe that this phenomenon ultimately will be demonstrated as the social rule rather than the exception. Since pheromonal parsimony is usually identified with the utilization of the same pheromone in different social contexts, this phenomenon basically reflects the remarkable behavioral plasticity which characterizes the social insects.

At this juncture, queen substance, (E)-9-oxo-2-decenoic acid (I), must be regarded as a multifunctional pheromone *par excellence*. The roles of this pheromone as a sex attractant and inhibitor of both ovarian development in workers and queen cell construction have already been described, but it seems possible that the diverse functions of this oxo-acid have not been completely illuminated. Queen substance is also a powerful colonial tranquillizer which, within the context of the hive, generates a continuous signal that identifies the presence of the queen. In the absence of the queen as the source of this pheromone, the colonial cohesiveness of the workers rapidly disintegrates. Recently, Morse and Boch (1971) identified this pheromone as one of the key elements which guarantees the stability of a swarm of bees. However, other pheromones produced by the queen, which act in concert with (E)-9-oxo-2-decenoic acid (I), are necessary in order to maintain a relatively stabile swarm. These results are somewhat similar to those of Barbier and Pain (1960) who reported that the oxo-acid (I) was attractive to caged workers only when it was combined with other volatile acids which are produced in the mandibular glands of the queen bee.

Worker honey bees are almost as communicatively versatile as their queen in utilizing the products of a single exocrine gland to subserve multiple functions. An abdominal structure, the Nassanoff gland, produces a terpene-rich secretion which is dominated by geraniol, nerolic acid, and citral (Boch and Shearer, 1962, 1964;

Shearer and Boch, 1966), and this terpenoid mixture acts as a powerful attractant for both bee workers and the queen. The Nassanoff gland, which is a structure known only from species of *Apis,* is a biosynthetically unusual abdominal gland producing large amounts of terpenes, a class of natural products normally found in the mandibular glands of Hymenoptera. Worker bees scent with the Nassanoff secretion in order to (1) mark new food finds, (2) indicate the location of the nest entrance, and (3) indicate that they are separated from their queen (Renner, 1960). The Nassanoff volatiles are a vital driving force during the formation and maintenance of swarms. Scout bees signal the locations of new swarm sites by liberating Nassanoff volatiles and, thus, attract both the queen and her workers. Therefore, the formation of clusters is triggered by the Nassanoff volatiles, and after the queen has joined the cluster, additional bees scent and liberate more of the terpene-fortified secretion to attract lost bees (Morse and Boch, 1971). The vigorous scenting which results after the queen has arrived at the cluster site probably disperses queen pheromones as well, and thus produces a large air space in the vicinity of the cluster which is redolent with both queen and worker pheromones. This simultaneous utilization of pheromones from two different castes of bees as part of a phased behavioral operation (Morse and Boch, 1971) has not been previously demonstrated. However, it is not unreasonable to assume that the *modus operandi* of the queen-worker interactions of other social insects may well have pheromonal bases which are derived from individuals of both castes.

Different species of social insects frequently utilize the same compound for entirely different functions, a situation which reflects both the behavioral and biological peculiarities of each species. The isomers of citral, whose roles in trail laying, alarm release, and attraction have already been discussed, possess one critically additional function for workers of the stingless bee

Lestrimelitta limao. The mandibular gland secretion of this bee is dominated by neral and geranial (Blum, 1966) and these two terpene aldehydes appear to be the *deus ex machina* which enable *L. limao* workers to carry out facilely raids on species of stingless bees. Citral (both isomers) acts as an attractant and alarm pheromone for workers of *L. limao* when these bees are raiding colonies of either *Trigona* or *Melipona* species. Attracted by citral secreted by the initial invaders, additional workers of *L. limao* gain access to the interior of the raided colony. Copious amounts of citral soon permeate the entire nest and at the same time, organized resistance on the part of the host species disappears and the *L. limao* workers appropriate the protein-rich food in the absence of organized resistance. The colonial cohesiveness of raid-susceptible species is detroyed when their workers are exposed to the two highly stimulatory isomers of citral (Blum et al., 1970). In a sense, for the host species, neral and geranial are volatile omens of impending disaster. On the other hand, the behavior of species which are not normally raided by *L. limao* is not appreciably altered in the presence of neral and geranial, and these species would presumably be capable of defending their colonies if attacked (Blum et al., 1970).

The astonishing versatility with which the alarm pheromones are utilized by social insects strongly indicates that it is this class of releasers which will be identified most frequently with the phenomenon of pheromonal parsimony. It would seem that in view of the fact that alarm pheromones are produced in greater quantities than pheromones in other classes, they would appear to constitute the most readily available source of exocrine compounds which can be drawn upon with some frequency. As alarm pheromones, these exocrine products, which are also potent attractants, release attack behavior (4-methyl-3-heptanone in *Pogonomyrmex*) (McGurk et al., 1966), and digging behavior (2-heptanone in *Conomyrma*) (Blum and Warter, 1966), and are utilized to label an

intruder as a prelude to subsequent attack by newly recruited workers (citronellal in *Acanthomyops* (Ghent, 1961). The stingless bee *T. subterranea* labels its food find with citral (Blum et al., 1970), its alarm pheromone, and it has been suggested that ants may mark new food sources with their alarm pheromones to recruit additional workers (Ayre, 1968). The behavior of workers of the dolichoderine *Iridomyrmex pruinosus*, which are feeding for a sustained period of time at a food source, provides strong support for the suggestion of Ayre. 2-Heptanone, the alarm pheromone utilized by *I. pruinosus* workers (Blum et al., 1963), can be easily detected at feeding sites which contain large aggregations of this dolichoderine species. Holldobler (1971) has demonstrated that worker guides of the formicine *Camponotus socius* secrete one of their alarm pheromones, formic acid, along with the trail pheromone, in order to maintain a high level of excitement in workers which are being recruited to a food source. Curiously, whereas honey bees recruit workers to new food finds with their Nassanoff secretion, they mark dissipated food finds with 2-heptanone, a secondary alarm pheromone, which then serves to repel additional workers that may attempt to visit the empty food source (Núñez, 1967).

Bergström and Löfqvist (1970) identified the main components in the Dufour's glands of the slave-keeping ant *Formica sanguinea* and its slaves *F. fusca* and *F. rufibarbis*. Their results made it seem highly probable that slave raiding in this group of *Formica* was based on disarming pheromones, as it is in the case of the bee *L. limao*. The prediction that raiding in this group of ants would have a pheromonal foundation like that of stingless bees (Blum et al., 1970) was verified by Regnier and Wilson (1971) who established that slave-making species in the *Formica sanguinea* group utilized volatile pheromones to disarm the ants under attack. Since two of the three slave-making *Formica* species studied by Wilson and Regnier (1971) possess hypertrophied Dufour's glands,

these investigators believe that the presence of an over-developed gland represents a derived state. They have also suggested that the large amount of acetates present in the Dufour's gland and which play a role in subduing the raided species, also represent a derived condition. However, since relatively few species have been studied in this context and it is known that at least one slave possesses these acetates in its own Dufour's gland (Bergström and Löfqvist, 1968), it would seem premature to draw any definite conclusions about the evolutionary basis of slave making in ants at this time.

n-Undecane, which appears to be one of the characteristic components produced in the Dufours gland of formicine species, has already been described as an alarm pheromone for species in this subfamily (Table 1). However, in at least some species, this hydrocarbon functions as a potent attractant but it does not release alarm behavior. Thus, workers of *Camponotus pennsylvanicus* "home in" on emission sources of n-undecane but they do not exhibit the characteristic reactions of alarmed workers (Ayre and Blum, 1971). Furthermore, it seems likely that this alkane may be responsible for the characteristic clustering behavior of ant workers and possibly of other social insects. In *C. pennsylvanicus*, a source containing n-undecane acts as a point of aggregation for large clusters which may persist for a least twelve hours (Ayre and Blum, 1971). These alkane-induced clusters appear to be identical to those that normally form in the nests of ants. Thus, it is highly probable that an attractant which is frequently used to recruit workers in the field, also serves as a local aggregative pheromone in the milieu of the nest.

Another possible role of n-undecane in defensive secretions was suggested from electroantennagram studies on a species of *Nudaurelia,* a pine emperor moth. Bosman and Brand (unpublished) employed the usual EAG method to monitor the female sex attractant activity of various isolated fractions. An active fraction would result in the rapid depolarization of the antenna. However, if air containing n-undecane vapor was blown over the antenna prior to the sex attractant vapor, the antenna would not respond with the same rapid depolarization for a period of some minutes. This rather interesting finding leads us to speculate whether the presence of n-undecane in the formicine Dufour's gland secretion may not disrupt the olfactory acuity of an enemy during an encounter. Any mechanism that impairs the function of the antennae of a predatory insect would have an obvious advantage to a formicine, especially if the latter is not as sensitive to the hydrocarbon.

Throughout the course of evolution, mechanisms which once served one function have been readapted to fulfill another role. Ghent (1961) suggested that secretions once used for offense later have become adapted for defensive functions. Regnier and Wilson (1968, 1969) proposed the term alarm-defense system for exocrine secretions of the ants, *A. claviger* and *L. alienus.* Bergström and Löfqvist (1971) consider that more than one function is suggested for the components of the Dufour's and poison glands because of the wide difference in volatility of the constituents. These latter workers state that the volatile components of the secretion probably serve as alarm substances while the higher-boiling compounds serve the role of recognition marks. They have suggested the term *recognition*-alarm-defense system.

As many of the higher boiling compounds, which do not cause alarm, are secreted in trace amounts relative to the major components, this extension of our terminology to include the olfactory discrimination of the secretion of a particular species by the species itself, as well as by other species, seems particularly appropriate. The trace quantities of these numerous substances, which are almost all aliphatic compounds, probably cannot influence the initial primary effects of the secretion, but, by their persistence, they could easily influence the long-term behavioral response directed towards an insect

marked by them.

Bergström and Löfqvist (1971), based on their very thorough and elegant analytical work, suggest that ants of the same species can recognize one another, as well as an intruder, by these trace components. In addition, however, any enemy will have an odor peculiar to itself, and the superimposition of a species-specific odor on a foreign one may very well have a more than additive behavioral meaning. One only has to observe an attack by ants on another insect to realize that, in spite of there being ants running wildly about, they are able to distinguish between friend and foe. During such an encounter the ants themselves must possess the strong odor of their defensive secretion, but yet are seldom seriously attacked because of it. The occurrence of a foreign odor, particularly together with their own defensive secretion, may be a critical factor in the overall survival reaction of a species, whether it be one of attack or retreat.

The significance of the blend of components in the Dufour's gland secretion may not be of great importance. Regnier and Wilson (1969) have stated that the amounts of the minor components in the Dufour's gland secretion of *L. alienus* may vary as much as 50 per cent. An observation of particular importance in this connection has been made by Bergström and Löfqvist (1971). They point out that while there is no species specificity in the occurrence of the lower hydrocarbons in the nine formicines they have studied, there are species-specific differences which are apparent in those species living sympatrically. This is particularly true for related species. For example, the Dufour's gland secretion of *L. flavus* has high-boiling lactones while that of *L. carniolicus* has ketones; in *L. niger* acetates predominate, while in *L. alienus* ketones predominate. Comparisons such as these can only be made after the most careful and thorough analytical work.

It is apparent, particularly from the results of the above investigations, that the Dufour's gland secretion serves a number of functions all intricately dependent on one another. The deciphering of the evolutionary development of this and other systems which involve attraction, alarm, recognition, defense and other behavioral reactions, will depend both on elegant microanalytical work and imaginative behavioral studies.

SYNERGISTIC PHEROMONES

The complexity of the products that can be synthesized by exocrine glands should serve to emphasize the necessity of analyzing the functions of these glandular exudates in terms of multiple components rather than single compounds. The significance of the rich mixture of natural products which is found in exocrine glands cannot be determined when identified compounds are evaluated singly, as has been the general practice up to the present time. Furthermore, because a compound is present as a minor component, it should not be assumed that its contribution to the olfactory quality of the secretion is inconsequential, a fact that has not been lost on perfumerers. We would like to suggest that a simple mechanism for increasing the communicative versatility of exocrine secretions can be developed by utilizing the ability of compounds in glandular mixtures or from different glands to synergize each other. Evidence for the presence of synergistic pheromones in both social and non-social insects is already available and there are not cogent grounds for believing that these cases are exceptional.

Butler and Callow (1968) have demonstrated that two compounds produced in the mandibular glands of the queen honey bee are required to inhibit construction of queen cells by the workers. These pheromones, (E)-9-oxo-2-decenoic acid (I) and (E)-9-hydroxy-2-decenoic acid (II), both must be present in order for inhibition to be demonstrated. Since a living queen bee is a more effective inhibitor than this pair of acids, it is probable that additional synergistic compounds are required in order to suppress completely the

rearing of queen bees by workers. The same absolute requirement of two compounds for the expression of pheromonal activity in a non-social arthropod has been demonstrated by Tamaki et al. (1971). These investigators showed that the female sex attractant of the smaller tea tortrix, *Adoxophyes fasciata*, is composed of two compounds, (Z)-9-tetradecenyl acetate and (Z)-11-tetradecenyl acetate, both of which are required in order to attract male moths. Similar synergistic effects have been observed with the terpene mixture which comprises the attractant of the bark beetle *Ips confusus* (Silverstein et al., 1966).

Pheromonal synergism between compounds produced in two different exocrine glands has also been reported. The ant *Camponotus pennsylvanicus* ejects its alarm pheromone, formic acid, in admixture with the products from Dufour's gland. *n*-Undecane, the major compound produced by Dufour's gland, acts as an attractant or orienting agent for the workers which are put into a non-oriented alarm frenzy by formic acid (Ayre and Blum, 1971). *n*-Undecane also synergizes the action of formic acid while enabling the excited workers to move accurately to the site of the disturbance. Thus, *n*-undecane, notwithstanding its inactivity as an alarm pheromone, maximizes the probability that the release of alarm in workers of *C. pennsylvanicus* will be behaviorally adaptive. Brady et al. (1971) have observed a strikingly similar case of synergistic pheromones in a lepidopteran, the almond moth, *Cadra cautella*. In addition to its sex pheromone, the female produces another compound which is behaviorally inactive for males but, when combined with the active sex pheromone, results in a pronounced increase in orientation of the male moths.

In view of the potentially great communicative value which can be derived from synergistic interactions between exocrine compounds, we feel that the possible existence of this phenomenon should be determined whenever pheromones are behavior-

ally evaluated in social insects.

PHEROMONES: DERIVED DEFENSIVE COMPOUNDS

It has been frequently noted that alarm pheromones are present in inordinately large quantities relative to other classes of chemical releasers. Thus, the mandibular glands of worker honey bees contain about 40 μg of 2-heptanone (Shearer and Boch, 1965), and although this compound is moderately active as an alarm pheromone, its major function has not been considered to be fully established. However, it seems probable that the ketone, along with the other major alarm pheromones utilized by social insects, functions as a defensive secretion as well. Thus, the availability of substantial quantities of alarm pheromones in social insects may actually reflect the important role that these compounds play as defensive products. Indeed, it seems highly probable that many pheromones actually represent defensive products, which, because of their ideal properties as highly stimulatory olfactants, have been secondarily adapted to function as communicative vehicles.

The primary defensive compound which is ejected from the scent glands of the opilionid *Leiobunum formosum* is 4-methyl-3-heptanone (Blum and Edgar, 1971), a compound which is identical to the alarm pheromone utilized by several species of myrmicine ants (McGurk et al., 1966; Moser et al., 1968). There is no reason to believe that this opilionid defensive compound does not have a similar function in ants, notwithstanding the fact that this ethyl ketone is also an alarm pheromone for these formicids. Indeed, the defensive behavior of dolichoderine ants, which secrete an anal gland secretion enriched with their alarm pheromone 2-heptanone, is highly suggestive of the defensive role that this compound plays (Blum and Warter, 1966). Furthermore, the striking similarity in the natural products chemistry of opilionid defensive secretions

and ant alarm pheromones is heightened by the recent discovery that in addition to 4-methyl-3-heptanone, the defensive exudate of the harvestman *L. vittatum* contains (*E*)-4,6-dimethyl-6-octen-3-one (Meinwald et al., 1971). This ketone is an isomer of manicone, 4,6-dimethyl-4-octen-3-one, a potent alarm pheromone produced in the mandibular glands of species of ants in the genus *Manica* (Fales et al., 1972). Manicone, like its opilionid-derived isomer, should function as an excellent defensive product, especially since it is present as the major volatile constituent in the secretion of the mandibular glands.

The presence of compounds identical to hymenopterous alarm pheromones in arthropod taxa (e.g., opilionids) which are considerably more ancient than these social insects, clearly demonstrates that the ability to synthesize these compounds was present in arthropod stock before these insects arose. Thus, the structural parallel between the pheromones of social insects and the defensive compound of non-social arthropods may constitute, in a broad sense, a biochemical legacy which the former have derived from the latter. Once solitary arthropods possessed the ability to synthesize defensive compounds which were both fairly volatile and stimulatory olfactants, it only remained for the more recently derived social insects to secondarily utilize these olfactants for communicative functions. The biogenesis of these defensive substances may have only required mutations for a single enzyme in order to extend pathways which were already in existence (e.g., in order to produce formic acid). Thus, the existence of pathways for the synthesis of the same defensive compound in many unrelated non-social arthropods demonstrates that the pathway for the *de novo* biogenesis of this product has been evolved independently on many occasions. Therefore, the presence of these "defensive" pheromones in social insects should not be regarded as a surprising biochemical development.

An examination of the defensive compounds utilized by non-social arthropods

(*see* review by Weatherston and Percy, 1970) demonstrates that many of these compounds are identical to the pheromones employed by social insects. Thus, formic acid, the alarm pheromone of ant species in two formicine genera, is employed for defensive purposes by carabid beetles and some species of notodontid larvae. *trans*-2-Hexenal, one of the major products in the defensive sprays of cockroaches and many true bugs, is utilized as an alarm releaser by ant species in one subgenus of *Crematogaster* (Bevan et al., 1961; Blum et al., 1969). The defensive secretion of several species of pentatomids is dominated by the hydrocarbon *n*-tridecane, a compound which is reported to release alarm in some formicine species. Even benzaldehyde, a potent defensive product which is normally present in the cyanogenic secretions of polydesmid millipedes and in the mandibular gland of an harvester ant, is employed as one of the key pheromones with which some species of stingless bees generate trails.

Ghent (1961), in a detailed study of the defensive mechanisms employed by the ant *A. claviger*, established clearly for the first time the alarm-defensive function of pheromones. Ghent demonstrated that citronellal, the main mandibular gland product of *A. claviger*, was both an alarm pheromone and defensive product. This terpene aldehyde acted as a carrier for formic acid by disrupting the epicuticle and facilitating the penetration of the poison gland product. However, because of the variation in insect cuticles, increased penetration of formic acid should not be considered general and, in fact, could not be demonstrated for *Galleria* larvae (Ghent, 1961). In addition, citronellal acted as a topical irritant *per se*, a property which considerably extended the defensive utility of this compound. The results obtained by Ghent (1961) are probably applicable, at least to a degree, to alarm pheromones as a whole, and provide substantive grounds for regarding this class of releasers as cryptic defensive compounds. Thus, these are often no valid grounds for mak-

ing distinctions between pheromones and defensive compounds. They are frequently one and the same compound, a fact which once again underscored the parsimonious utilization of pheromones by social insects.

At least four species of ants in the genus *Solenopsis* have a venom rich in dialkyl piperidine alkaloids (MacConnell et al., 1971; Brand et al., 1972), and the defensive function of this secretion will be evident to anyone who has encountered these ants. The lipophilic character of these compounds should enable them to spread and penetrate an insect cuticle thereby giving a long-term effect, and studies on certain physiological properties of these compounds are being undertaken in order to ascertain their toxic properties. If some of the Dufour's gland secretion is released, together with the venom, during an encounter, it would act as a powerful attractant and excitant drawing nearby ants to its source. As this Dufour's gland secretion contains *n*-heptadecane (Brand et al., 1972), the occurrence of hydrocarbons in this gland may be more widespread in the Formicidae than was previously recognized.

CONCLUSIONS

The complexity of the exocrine secretions of social insects demonstrates that these arthropods possess outstanding biosynthetic capabilities. The presence in social insects of a number of pheromones which are unique animal natural products provides persuasive evidence for regarding these compounds as major factors in developing a viable sociality. A multitude of devices has been utilized to increase the communicative potential afforded by the availability of a finite number of pheromones. Qualitatively distinct blends of pheromones provide species with elegant isolating agents, especially when closely related species are sympatric.

Pheromonal secretions may contain highly volatile compounds which function to recruit workers rapidly in a highly excited state, but, in addition, these secretions may be fortified with very persistent compounds that serve to mantain a large number of individuals at the site of attraction. Synergistic interactions between compounds can considerably increase the specificity of the chemical message, thereby adding to the informational yield that may be derived from the utilization of a limited number of exocrine products. Although the exocrine secretions of relatively few species of social insects have been analyzed, it appears that the alarm-releasing signal of most species may constitute a recognition-alarm-defense system, which can be highly specific (Bergström and Löfqvist, 1971).

The compounds in some classes of pheromones are excellent defensive products, and as a consequence, no clear-cut distinction can be made for classifying these substances as either defensive products or pheromones. The identity of social insect pheromones with the defensive compounds of many non-social arthropods indicates that the ability of social insects to biosynthesize these compounds may represent the expression of a metabolic capability which had already been realized in more ancient lines of arthropods.

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THE CHEMISTRY OF DUFOUR'S GLAND AND THE POISON GLAND SECRETIONS OF *ANOPLOLEPIS CUSTODIENS* (HYMENOPTERA: FORMICIDAE)

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ABSTRACT

The chemistry of the secretions of Dufour's gland and the poison gland of *Anoplolepis custodiens* was investigated to determine the nature of the alarm-defense system of this ant. A series of aliphatic hydrocarbons and their corresponding olefins in the range C_{10} to C_{21} was shown to be present in the Dufour's gland, and formic acid was identified in the poison gland. The relative amounts of certain hydrocarbons, particularly *n*-tridecene and *n*-pentadecane, were found to vary considerably with the season. In the fall and early winter an increase in *n*-pentadecane and a decrease in the amounts of *n*-tridecane and *n*-tridecene were observed.

Key Words: *Anoplolepis custodiens,* alarm-defense system, hydrocarbons, formic acid, seasonal variation.

INTRODUCTION

A substantial part of the diet of the ant, *Anoplolepis custodiens* (Smith), consists of honeydew excreted by certain Homoptera, and it is largely for this reason that this ant is of economic importance in South Africa. A positive correlation between the incidence of red scale on citrus and the presence of *A. custodiens* in the orchard was demonstrated by Steyn (1954), and it has also been established that colonies of aphids tended by ants grow considerably faster than colonies from which ants are excluded (Way 1963). While Steyn (1954) accumulated much valuable information concerning the biology of this ant, methods for its specific control have not been forthcoming.

Both the economic value and the ready availability of this ant prompted us to investigate its alarm-defense system. This led to the isolation of a series of aliphatic hydrocarbons and their corresponding olefins from Dufour's gland and the isolation of formic acid from the poison gland. The occurrence of hydrocarbons in Dufour's gland of formicines is not new (Bergstrom and Lofqvist 1970, and references therein) and it seems that the major compounds secreted from this gland are non-polar substances, especially *n*-undecane.

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Aliphatic hydrocarbons with eleven and thirteen carbon atoms are not confined to defensive secretions of formicines but have also been obtained from these secretions of certain hemipterans (Blum *et al.* 1060; Waterhouse *et al.* 1961), beetles (Mainwald and Eisner 1964) and a myrmicine ant (Vick *et al* 1969).

MATERIALS AND METHODS

Ants were collected and kept at 4 C for four days prior to being subjected to steam distillation with continuous liquid-liquid extraction into methylene chloride. The CH_2Cl_2 solution was concentrated to give the whole ant extract.

Dufour's gland was exposed, the common glandular duct pinched shut and the gland pulled free. The intact Dufour's gland was placed in $8\mu l$ CS_2 and after several seconds was raised above the solvent, washed with an additional $8\mu l$ CS_2 and removed to give a Dufour's gland extract.

Even-numbered saturated *n*-hydrocarbons ranging from *n*-octane to *n*-eicosane were purchased from Applied Science Laboratories Inc. while the odd-numbered hydrocarbons, *n*-nonane, *n*-undecane and *n*-tridecane, were obtained from Microtek Instruments Inc.

Formic acid (90% w/w) was supplied by May and Baker Ltd.

A beckman GC-4 gas chromatograph equipped with dual flame ionization detectors and a stream splitter was used for all analyses. The columns and conditions used for all separations are indicated in the results.

The low boiling components (C_9-C_{13}) in a whole ant extract were collected by direct adsorption from the stream-split effluent on spectroscopic graphite, while the higher boiling components (C_{16}-C_{21}) were collected by passing the effluent through CH_2Cl_2 in a test tube plugged with glass wool, followed by adsorption on graphite.

All mass spectra were obtained on an AEI-MS-9 mass spectrometer by direct insertion of the fractions, absorbed on graphite, into the ion source.

Precoated silica gel plates (Merck, F254, 20 × 20 cm) were spotted both with varying amounts of formic acid and with the contents of poison glands from small, medium and large worker ants. The plates were developed with isopropanol-ammonium hydroxide-water (100: 5: 15), sprayed with 0.005 N $AgNO_3$ in 2.5 N NH_4OH and heated for 10 min. at 110 C for color development (Regnier and Wilson 1968).

RESULTS AND DISCUSSION

When either the CS_2 extract of a single Dufour's gland or an aliquot of the whole ant extract was subjected to the same temperature program on Carbowax 20 M, it was clear that both extracts essentially contained the same GC-detectable components. The retention times of the major peaks on both Carbowax 20 M and SE-30 indicated the presence of two main groups of compounds which could be broadly classified as either high boiling or low boiling. While SE-30 resolved these two groups into a number of major peaks, Carbowax 20 M resolved each of these major peaks into a major and an associated minor peak. To achieve complete resolution of the major and minor peaks, the high and low boiling groups of compounds were treated as separate entities both for preparative gas chromatography and the comparison of retention times.

The low boiling group was resolved into nine peaks (A, B, b, C, c, D, d, E, and e) by Carbowax 20 M while only peaks A, B, b and C could be demonstrated in a headspace sample on this phase. The only syringe reaction that had any effect

Table 1.   Retention times of *n*-hydrocarbons with those of both the low-boiling components (A-E) and the high-boiling components (F-L) from either the whole ant extract or a Dufour's gland extract on SE-30 and Carbowax 20 M (see below for conditions).

Compound	Retention time (min)	
	10% SE-30	10% Carbowax 20 M
n-Nonane	19.50	4.50
Peak A	19.60	8.50
n-Decane	22.80	11.50
Peak B	22.80	11.50
n-Undecane	26.50	18.60
Peak C	26.50	18.60
n-Dodecane	30.40	25.60
Peak D	30.40	25.60
n-Tridecane	34.60	32.00
Peak E	34.60	32.00
n-Tetradecane	24.20	4.60
Peak F	24.20	4.60
Peak G	26.50	6.75
n-Hexadecane	27.00	9.50
Peak H	28.75	11.80
n-Octadecane	30.50	16.00
Peak I	30.50	16.00
Peak J	33.00	20.50
n-Eicosane	36.10	24.25
Peak K	26.10	24.25
Peak L	40.75	28.50

Low boiling components: (a) Carbowax 20 M: 20 C—100 C in 30 min, hold 100 C, (b) SE30: 60 C—200 C in 30 min, hold 200 C.

High boiling components: (a) Carbowax 20 M: 120C—200 C in 30 min, hold 200 C, (b) SE30: 120C—270 C in 30 min, hold 270 C.

on a headspace sample of the whole ant extract was hydrogenation with PtO_2 and H_2 which resulted in the reduction of the size of peak b.

The high boiling fraction could be resolved, on Carbowax 20 M, into four major peaks (I, J, K and L) each of which had a corresponding minor peak (i, j, k and l). In the region between this group and the low-boiling group of compounds, using an extended temperature program, there were three small peaks (F, G and H), each of which had an associated minor peak (f, g and h) at high detector sensitivity.

Previous investigators have found the content of the Dufour's gland of formicines to consist largely of hydrocarbons (Bergstrom and Lofqvist 1970). The gas chromatographic behavior of the compounds in the Dufour's gland of *A. custodiens* suggested an homologous series and the unreactive nature of the initial major peaks in the headspace sample indicated that they might be hydrocarbons.

Table 2.   The amount and proportions of the constituents in a single Dufour's
gland of *A. custodiens*, collected in early summer.

Compound[1]	Amount (μg)	Percentage of low boiling fractions	Percentage of high boiling fractions	Percentage of total
Branched nonane	0.02	0.3	–	0.015
n-Decane	0.06	0.8	–	0.45
n-Undecane	6.60	95.6	–	48.90
n-Undecene	0.02	0.3	–	0.15
n-Dodecane	0.02	0.3	–	0.15
n-Tridecane	0.10	1.5	–	0.75
n-Tridecene	0.08	1.2	–	0.60
n-Octadecane	0.20	–	3.03	1.47
n-Nonadecane	4.00	–	60.60	29.40
n-Eicosane	0.40	–	6.06	2.94
n-Uneicosane	2.00	–	30.30	14.70

[1] Only those compounds for which mass spectra were obtained are listed. All the
other compounds were present in an amount of less than 0.02 μg of each.
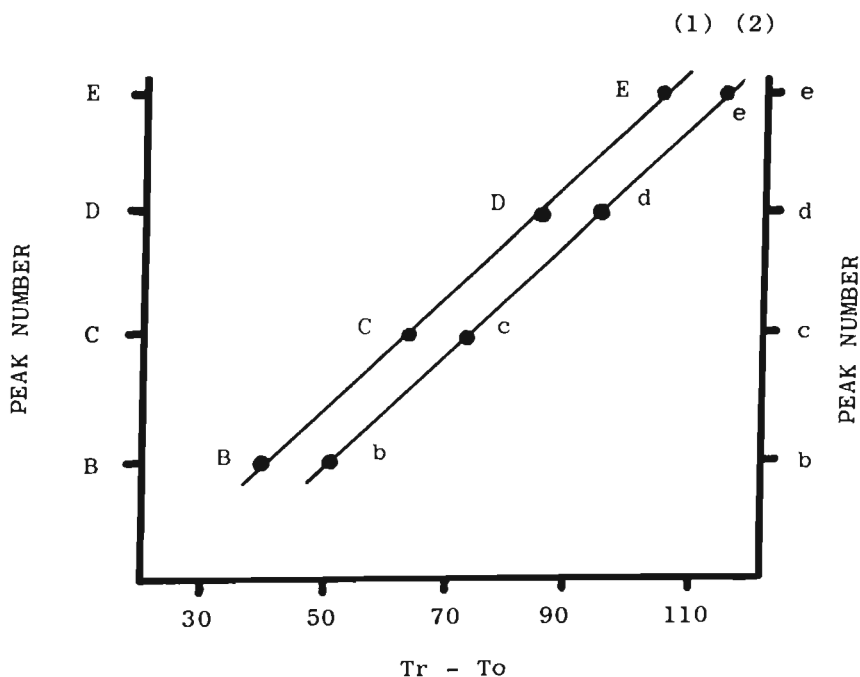
Figure 1.   Plot of retention temperature (Tr) minus initial temperature (To)
against peak number for (1) the major peaks B-E, (2) the minor
peaks b-e, on Carbowax 20 M. (See Table 1 for retention data and
GC conditions).
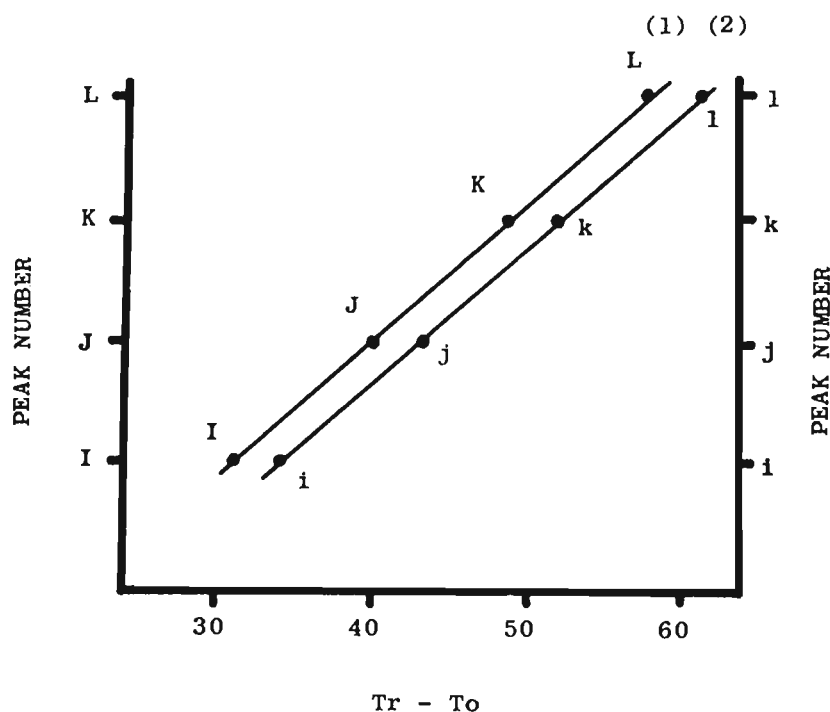
Figure 2.   Plot of retention temperature (Tr) minus initial temperature (To)
against peak number for (1) the major peaks I-L (2) the minor peaks
i-l, on Carbowax 20 M. (See Table I for retention data and GC
conditions).

This suggestion was supported by a comparison of the retention times of the
various peaks on both Carbowax 20 M and SE-30 with those of certain saturated
n-hydrocarbons (Table 1).

The retention times, and the estimated carbon number of the various
components, indicate that with the exception of peak A, all major peaks could
belong to the homologous series of saturated n-hydrocarbons extending from
C_{10} to C_{21}. To investigate the relationship between the major and minor peaks, a
plot of retention temperature minus initial temperature aginst peak number was
set up for the relevant components in both the high and low boiling groups (Figs.
1 and 2).

In temperature programmed gas chromatography, retention time increases
linearly with carbon number, and this linearity generally holds over a range of
about five carbon atoms (Harris and Habgood 1966). The plots in Figs. 1 and 2
show that two parallel lines were obtained, one for the major peaks and the other
for the minor peaks. These results substantiate the result of hydrogenation of
peak b and suggest that all the minor peaks are of a similar chemical series as are
the major peaks.

All the major peaks, B, C. D, E, I, J, K and L, yielded mass spectra with
molecular weights corresponding to saturated alkanes and had fragmentation

patterns characteristic of n-hydrocarbons, thereby confirming the gas chromatographic data. Of the minor peaks, only c and e were present in sufficient amount to be collected and for mass spectra to be recorded. Their molecular weights conformed to the formula $C_n H_{2n}$ showing that each possesses a double bond. While their fragmentation patterns were characteristic of straight chain olefins, the position of the double bond could not be determined from the spectra. It is on the basis both of these latter two mass spectra and on the homologous series plots in Figs. 1 and 2 that all the minor peaks are considered to be mono-unsaturated hydrocarbons with the same carbon number as the n-alkane series.

An exception to the n-alkane fragmentation pattern was obtained with peak A. This spectrum had a considerably more intense fragment at m/e 85 than did n-nonane and it is expected that peak A is a branched hydrocarbon. The position of branching has not been determined with certainty but the two most likely candidate compounds, as determined from the mass spectrum alone, would appear to be either 2-methyl octane or 4-methyl octane.

Quantitation of the contents of Dufour's gland was achieved by approximating the concentration of all components in the low boiling fraction from a standard curve of n-undecane. A standard curve of n-eicosane was used to approximate concentrations of the high boiling components and the amounts of the various hydrocarbons observed in a single Dufour's gland are given in Table 2. Although very little quantitative work has been done on the components involved in the alarm-defense systems of formicines, the results obtained for the hydrocarbons in *A. custodiens* are similar to those obtained by Regnier and Wilson (1968) with *Acanthomyops claviger*. In both species of ants n-undecane appears to be in the 1-$10\,\mu$g range, while the other n-alkanes are present in the 0.01 to $0.09\,\mu$g range.

It should be pointed out that these data constitute the analysis of only a few glands and are not an average of a large number of analyses. They are intended to illustrate the magnitude of the amounts of the compounds in a single gland and are representative of the amounts observed in various analyses on single glands conducted during this investigation. It is considered more important, at this stage, to have a rough assessment of the amounts of the various compounds in a single gland than to have exhaustive quantitative data. One must appreciate that these substances will vary in both ratio and absolute amount from one ant to another, one nest to another, and from season to season. Precise quantitative data will have value when presented together with a thorough elucidation of the role of the components in this glandular secretion and the manner in which they are used by the various ages and sizes of ants.

Extracts prepared from ants caught in spring and early summer (September, October, November) contained a high proportion of the unsaturated compounds while those prepared towards the start of winter (February onwards) had a low proportion of these substances. An indication of the amount of unsaturated compounds present in a whole ant extract could be obtained by examining the relative amounts of n-tridecane and n-tridecene in a glandular extract. If they were quantitatively equal then fairly substantial amounts of the other n-alkenes could be expected, but if n-tridecene was less than half of n-tridecane then the majority of the other n-alkenes would not be detectable even at high detector sensitivity.

A further quantitative variation observed to occur with the encroachment of winter was the sudden appearance of large amounts of n-pentadecane in the Dufour's gland secretion. This compound, normally present in amounts of about $0.01\,\mu$g/gland, became quantitatively important at $0.4\,\mu$g/gland, compared with 6

µg n-undecane/gland. The increase in n-pentadecane was observed to occur with a simultaneous substantial decrease in the amounts of n-tridecane and n-tridecene. It was also found that formic acid was present in amounts up to 20% of the body weight of the ant, a result similar to that obtained by Regnier and Wilson (1968) for A. claviger.

Hydrocarbons have been isolated from a number of formicine species and the available data strongly indicate that their source is the Dufour's gland. Regnier and Wilson (1968) have suggested that hydrocarbons in the C_{10} to C_{13} range will be the most efficient with respect to alarm function. This proposal is supported by the fact n-undecane is usually the most abundant compound and is claimed to be the alarm substance of certain species (Regnier and Wilson 1968; Bergstrom and Lofqvist 1970).

While the alarm function of n-undecane may be correctly assigned to some species, the function of those high-boiling hydrocarbons in A. custodiens which almost certainly are not serving as alarm pheromones, is cryptic. The high concentration of n-pentadecane (in late summer), n-octadecane, n-nonadecane, n-eicosane and n-uneicosane indicated that these compounds are of importance in fulfilling some biological function in this ant. That these high boiling compounds are not merely precursors, found only in secretory cells of Dufour's gland, was illustrated by collecting, in CS_2, the numerous droplets of "venom" left on a glass rod that had been "attacked" by several workers. After the solvent was concentrated and chromatographed, it was found that all the high boiling hydrocarbons were present in the same relative proportions as found in the glandular extracts and whole ant extracts.

The most obvious of the possible functions of the aliphatic hydrocarbons is the spreading effect that these compounds have on formic acid. Regnier and Wilson (1968) have reported that a synthetic venom (50% formic acid in a hydrocarbon mixture of about 51% undecane, 0.4% tridecane, 38% 2-tridecanone and 10% 2-pentadecanone) spreads over a ten times greater surface area on Periplenata americana cuticle than an equal volume of 50% formic acid alone. It can be seen, therefore, that in the case of n-undecane, two roles may be played by the same compound in that it might act as both a venom component and an alarm pheromone for A. claviger, and probably for a number of other formicines as well. In rough bioassays conducted in the field with n-undecane we were unable to obtain a complete and convincing alarm reaction to this hydrocarbon alone. Workers of A. custodiens would respond in a more definite pattern of alarm when confronted with a crushed gaster of another ant of the same species. Vick et al. (1969) stated that these hydrocarbons did not cause alarm in Novomessor cockerelli, and probably serve as a cuticle-pentrating agent. We wish to suggest that the hydrocarbons of the Dufour's gland serve additional, but as yet undefined functions, as well as those of a spreading-penetrating agent and of alarm. One possible function of the high-boiling hydrocarbons is that they may be of importance in the establishment and recognition of the broad extensive trails that these ants follow.

Each of the n-alkanes found in the Dufour's gland of A. custodiens has a small amount of the corresponding mono-unsaturated n-alkene. Stumpf (1965) has proposed that α-oxidation of fatty acids may be part of the route whereby epicuticular hydrocarbons are synthesized. An interesting aspect of this scheme is that the immediate precursor of the n-alkane is a corresponding n-alkene with one double bond. If the initial oxidation is performed on an even-numbered fatty acid then the resultant n-alkane will be odd numbered. As the quantitatively most important hydrocarbons in A. custodiens' defense system are odd numbered n-

alkanes and *n*-alkenes, the applicability of Stumpf's scheme to the synthesis of hydrocarbons in insects appears worthy of investigation.

Finally, it has long been realized that the contact toxicity of alkenes is greater than that of alkanes (Brown 1951). Although the absolute amount of *n*-alkenes in the Dufour's gland of a single ant is extremely small, they nevertheless may contribute significantly to the effectiveness of the role of this secretion. If this is the case then the information conveyed by these various hydrocarbons in the Dufour's gland will change with the season, possibly as a necessary adaptation for predation on seasonally-different prey. These results should emphasize the fact that the functions of various exocrine products produced by ants may be highly variable and the roles played by the same compound secreted by various species of ants may be characterized by outstanding differences.

ACKNOWLEDGMENTS

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Male Armyworm[1] Scent Brush Secretion: Identification and Electroantennogram Study of Major Components[2]

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ABSTRACT

Benzaldehyde, benzyl alcohol, and benzoic acid were identified in extracts of the abdominal scent brushes of adult male *Pseudaletia unipuncta* (Haworth). A 4th compound(s) remains unidentified. Benzaldehyde is the major component and represents 86 to 97% of the volatile compounds in 2-day-old moths.

Electroantennograms were recorded from male and fe-
male antennae in response to stimulation with the scent-brush compounds benzaldehyde and benzyl alcohol, with 2 related aromatic compounds (2-phenyl ethanol and benzyl acetate), and with a carboxylic acid iso-butyric acid. Male and female antennae responded equally to each chemical and the scent-brush compounds were no more stimulating than the non-scent-brush compounds.

Many male Lepidoptera possess modified scales which release sex pheromones to promote successful male courtship (Birch 1970a, Brower et al. 1965, Dahm et al. 1971, Pliske and Eisner 1969, Tinbergen et al. 1942). The armyworm, *Pseudaletia unipuncta* (Haworth), possesses such scales which form a pair of anterior abdominal scent brushes quite similar to those of *Phlogophora meticulosa* (L.) described by Birch (1970b). The electroantennogram (EAG) technique was used to demonstrate that the armyworm brushes release an odorous substance(s) that stimulates both male and female antennae (Grant 1971).

The compounds identified from scent scales of male moths are of a diverse nature (Aplin and Birch 1968, 1970; Dahm et al. 1971; Röller et al. 1968); those secreted by moths related to the armyworm are aromatics and a carboxylic acid. This paper reports the identification of 2 major components of the armyworm scent-brush secretion. In addition, since preliminary studies (Birch 1971, Grant 1971) have indicated a lack of antennal (EAG) specificity in response to stimulation with male scent compounds, the identified armyworm compounds and related compounds were used in EAG tests to further study this apparent nonspecificity.

MATERIALS AND METHODS

The armyworms[4] were reared for 9 generations in our laboratory on an artificial diet similar to that described by Burton (1969) except lima beans were substituted for pinto beans. Insects were sexed as pupae and placed in separate emergence jars. Adults were collected daily and maintained on a 5–10% sugar solution. Moths of various ages were dissected to compare the size of Stobbe's gland (Alplin and Birch 1968, Birch 1970b) with age and content of scent brushes.

Brushes were collected from male moths of known age by anesthetizing them with either CO_2 or cold. Neither process caused the brushes to be prematurely everted. The brushes were pulled out of their pockets with forceps, cut at their base, and quickly placed in a test tube containing either pentane or methylene chloride chilled by applying "dry ice" to the outside of the tube. All solutions were sealed and stored in a freezer until ready for gas chromatographic (GC) analysis. This analysis was carried out on a Varian Aerograph 2100 gas chromatograph equipped with flame ionization detectors and 2m × 4 mm ID glass columns. The following column packings and temperatures were used: (1) 20% butane-1, 4-diol succinate (BDS) on Chromosorb W (60/80 mesh) at 150°C, (2) 3% OV-17 on Chromosorb W (100/120 mesh) at 120°C, and (3) 3% Apiezon L on Chromosorb W (100/120 mesh) at 90°C. The carrier gas was nitrogen used at a flow rate of 50 ml/min.

Scent-brush compounds (GC peaks) were collected in the usual way by means of glass capillary tubes and analyzed on a Bell and Howell 21-490 mass spectrometer.

Electroantennogram studies were carried out as previously described (Grant 1971) except that authentic chemical samples were tested in place of the scent scales. Test chemicals were made up in ethereal solutions in 10-fold dilutions so that 10 µliters of a solution would contain from 0.1 to 1000 µg of chemical. This volume of test solution was deposited evenly on the inside of a glass tube cartridge (5 mm ID) by rotating the tube as the solvent evaporated. A 0.5- to 1.0-sec pulse of air delivered at 0.5 liter/min was allowed to pass through the cartridge and over the antenna. Successive tests with the same antenna using the same or different chemicals always proceeded in the direction of increasing concentration.

Table 1.—Comparison of retention times of armyworm scent brush components with those of authentic samples of known compounds on 3 GC columns.

Compound	Retention time (min)		
	BDS	OV-17	Apiezon-L
P. unipuncta unknown	3.5	—[a]	—[a]
P. unipuncta benzaldehyde	8.3	2.3	6.7[b]
P. unipuncta benzyl alcohol	24.7	3.6	10.7[b]
Benzaldehyde	8.4	2.3	6.3[b]
Benzyl alcohol	24.7	3.6	11.4[b]

[a] Compound was not resolved on this column.
[b] Scent brush components and authentic samples were analyzed on different days.

RESULTS

Removal of the scent brushes from the male abdomen and their use in EAG tests (Grant 1971) established the presence of an almond-like odor similar to benzaldehyde associated with the brushes. Dissection of the abdomen to reveal Stobbe's gland showed that in most moths the gland was large and swollen at eclosion but several hours later noticeable shrinkage had occurred and complete atrophy was observed at 1–2 days. The exact time course for retraction of the brushes into their pockets was not determined but the process took at least several hours following eclosion.

GC analysis of scent-brush extracts established the presence of 3 major peaks which could be attributed to the brushes themselves and a 4th peak which probably represents an oxidation product of one of the other compounds (see below). Two of the peaks were found to have retention times on all 3 GC columns identical to benzaldehyde and benzyl alcohol (Table 1). The 3rd peak with a retention time of 3.5 min on BDS could not be observed on the other 2 columns despite using a variety of temperature and flow rate conditions. We succeeded only once in collecting this material in capillary tubes as it eluted from a BDS column but the mass spectrum of this small quantity of material proved inconclusive. Mass spectral data for peaks corresponding to benzaldehyde and benzyl alcohol were congruent with spectra for authentic samples of benzaldehyde and benzyl

alcohol which had previously been collected from BDS under the same conditions as the scent-brush compounds. The 4th peak collected from BDS gave a mass spectrum identical with authentic benzoic acid. Although precise retention times were not obtained, the behavior of authentic benzoic acid on BDS was apparently the same as the compound in the scent-brush extract.

The quantities of each of these compounds extracted from the scent brushes, with the exception of benzoic acid which was not analyzed further, are listed in Table 2 according to age of moths and solvent used. Each set of data in Table 2 represents a different generation of moths. Extracts made from brushes of newly emerged moths (0–2 hr) contain none of the above compounds, while extracts of brushes from slightly older moths contained sizeable amounts of benzaldehyde and benzyl alcohol. One generation of moths was reared on a diet whose chief component was wheat germ. The scent-brush composition of these moths ($\geq$4 days) was: unknown compound 0.04 μg/brush, benzaldehyde 10.24 μg/brush, benzyl alcohol 0.04 μg/brush.

Electroantennogram responses were obtained in response to the 2 identified scent brush compounds, benzaldehyde and benzyl alcohol, and to 2 related aromatic compounds, 2-phenyl ethanol and benzyl acetate (Fig. 1). Each point in Fig. 1 represents an average of from 6 to 34 trials. Iso-butyric acid was tested to a lesser degree and, therefore, is not included in Fig. 1. Since male and female armyworm antennae responded equally the results in Fig. 1 are combined male and female tests. Over the range 0.1 to 100 μg, benzaldehyde and benzyl alcohol produced EAGs of similar amplitude. Over most of this range 2-phenyl ethanol and particularly benzyl acetate produced larger EAGs. The results with isobutyric acid paralleled those of benzaldehyde over the range 0.1 to 100 μg with only slightly smaller EAG amplitudes.

DISCUSSION

The armyworm belongs to the subfamily Hadeninae which Aplin and Birch (1970) found secreted aro-

Table 2.—Quantity of armyworm scent brush components (μg/brush) extracted by either methylene chloride or pentane from males of different ages. Number of brushes extracted for each set of data is in parenthesis.

Age (days)	Compound	Methylene chloride		Pentane		
0–2	Unknown	(22) 2.72		(58) 1.56		
	Benzaldehyde	3.40		17.08		
	Benzyl alcohol	11.31		3.11		
2	Unknown	(62) 0.99	(64) 0.20	(170) 0.06	(94) 0.13	(130) 0.24
	Benzaldehyde	13.32	7.35	7.39	18.05	22.85
	Benzyl alcohol	.73	1.04	.43	.39	1.90
4	Unknown	(10) 1.51	(16) 1.59			
	Benzaldehyde	1.84	17.59			
	Benzyl alcohol	0	.08			

matic and carboxylic acid compounds. Two species of this group produce benzaldehyde and benzyl alcohol but these compounds together represent only 12–18% of the total secretion. The major component (70–85%) is 2-phenyl ethanol. Three other species of the genus *Leucania* secrete benzaldehyde (80%) and iso-butyric acid (20%). The armyworm, formerly placed in the genus *Leucania*, secretes neither iso-butyric acid nor 2-phenyl ethanol as judged by GC retention times; however, the major component of the secretion, like the *Leucania* species, is benzaldehyde. In 2-day-old moths, which are taken as the standard because Stobbe's gland is completely atrophied and because the males are probably just reaching full sexual maturity, benzaldehyde represents 86 to 97% of the volatile components (Table 2) while the remaining components represent only 3 to 14% of the total. Brushes from newly emerged moths lacked all scent brush constituents probably because they were not contained in their abdominal pockets or had not been in them long enough. In the noctuid *P. meticulosia*, material to be deposited on the brushes when they are in the pocket originates from Stobbe's gland in the form of a precursor (Birch 1970b).

Although the proportion of armyworm benzaldehyde was relatively constant the actual amounts present in the extracts varied widely from 7.35 to 22.80 μg/brush. The cause of this variation is not known but premature eversion of brushes before analysis may be one reason. Similar wide fluctuations in hairpencil constituents were observed for the queen butterfly *Danaus gilippus berenice* (Cramer) (Meinwald et al. 1969). The presence of some benzoic acid in the armyworm extract may be due to oxidation of benzaldehyde when the brushes were pulled out and put into solution or while they were stored in solution. However, oxidation of benzaldehyde on the brushes before their removal must also be considered as a source of benzoic acid. If the oxidation occurs in solution it could cause some variation in benzaldehyde content, but based on GC analyses of several fresh extracts the amount of benzoic acid represents only a small percentage of the benzaldehyde. Variations in scent brush compounds with age of moth or solvent (Table 2) were not systematic with the exception of benzyl alcohol which was present in large amounts in young moths. Although diet was not studied, previous EAG tests (Grant 1971) indicated a difference in stimulating effectiveness between wild- and laboratory-reared male armyworms which was probably due to quantitative differences rather than qualitative ones. Table 2 would suggest that quantitative differences are common.

The EAG results (Fig. 1) confirm earlier observations on moths and butterflies (Birch 1971; Grant 1970, 1971; Schneider and Seibt 1969) that male scent brushes or the identified scent-brush compounds generally do not elicit antennal responses that are specific either with respect to sex tested or chemical compounds employed. In the case of the armyworm, there was no obvious difference between male and female responsiveness. By extrapolating
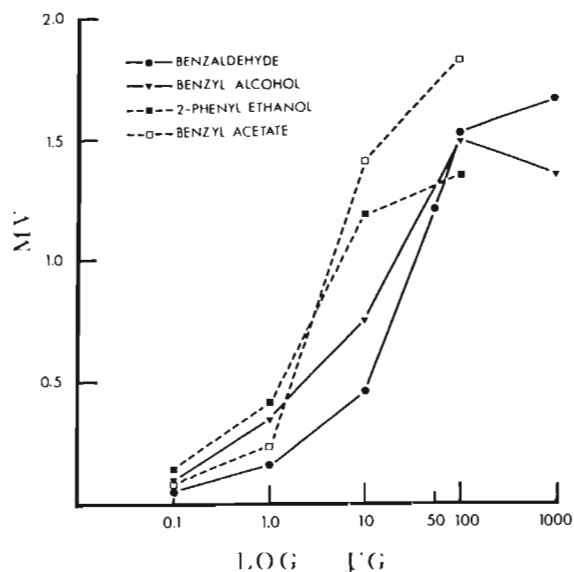
FIG. 1.—Antennal (EAG) responses to 2 scent-brush compounds (benzaldehyde and benzyl alcohol) and to 2 similar non-scent-brush compounds.

the lines in Fig. 1 it appears that the EAG threshold concentration for the 4 aromatic compounds is the same and lies between 0.01 and 0.1 μg. Iso-butyric acid has a similar threshold value. Although the non-scent-brush compounds benzyl acetate and 2-phenyl ethanol produced larger EAGs over part of the concentration range, it is not known at present whether this effect is due to differences in volatility, in stimulating effectiveness, or in the type and number of olfactory cells responding. In any event, it appears that a consistent distinction cannot be drawn between EAGs produced by scent-brush compounds and related chemicals or even between scent-brush compounds and unrelated chemicals (e.g. a carboxylic acid). By contrast, lepidopteran female sex pheromones generally elicit EAGs in male antennae only (but see Grant 1970), they have decidedly the lowest threshold values of any chemical including those closely related, and they produce the largest EAGs of any compound at all concentrations within the normal physiological range (Grant 1970,[5] Roelofs et al. 1971).

A few situations exist, however, in which moths exhibit some specificity in EAG responses to male scent-brush secretions. Schneider and Seibt (1969) found that whereas hairpencils or the synthetic sex pheromone of the queen butterfly was fully effective in eliciting EAGs in the antenna of the monarch butterfly, *Danaus plexippus* (L.), hairpencils from the monarch were ineffective in stimulating the queen antenna. Further, Birch (1971) reported that some standard laboratory compounds (not identified) related to noctuid hairpencil compounds failed to elicit EAGs in these moths. Thus it seems likely that there will be cases where moths respond to male

[5] G. G. Grant. 1970. Ph.D. Thesis, Virginia Polytechnic Institute, Blacksburg, Va.

pheromones with some degree of electrophysiological specificity perhaps similar to that seen for female pheromones. These male pheromones will most likely act as attractants as most female pheromones do rather than close-range aphrodisiacs.

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NOTE ADDED IN PROOF

Recently, Clearwater (1972, J. Insect Physiol. 18: 781–9) has identified benzaldehyde as the major constituent of the scent brushes of a New Zealand armyworm, *P. separata*. A minor 2nd constituent remains unidentified.

Correlation of Ant Alarm Pheromone Activity with Molecular Vibration

THE alarm pheromones of certain species of ant provide extraordinarily favourable tools with which to examine the relationships between molecular characteristics and biological activity.

After the identification of 2-heptanone as the alarm pheromone of *Iridomyrmex pruinosus* (Roger)[1], a number of substances, many with structures related to 2-heptanone, were tested for pheromone activity which was estimated on a 0 to 5 scale of intensity, depending on the number of ants showing a typical alarm response[2]. The tests were made both in the laboratory and in the field and were replicated many times. The pattern of response looked for included faster movement, attraction toward the stimulus, and occasionally attacks by one ant on another. In the absence of evidence to the contrary, a similar response can be regarded as evidence of similarity in the sensory input from the stimulus molecules.

J. E. Amoore believes that molecular shape plays an important and perhaps a dominant role in governing the biological activity of these quasi-alarm pheromones[3]. It is important, therefore, to see whether the biological activity of these compounds is equally compatible with the alternative hypothesis that olfactory specificity is a function of the molecular vibration pattern[4].

Through the courtesy of **Prof**essor M. S. Blum and **Dr** Amoore, compounds showing varying degrees of activity on the 0–5 scale were available for examination in the far infrared range from 500 to 100 cm^{-1}. The results were evaluated by comparing the spectra of nineteen substances having activities of 5, 4, and 3 with the spectra of twenty-six substances with activity 0, setting aside as only marginally active, those with ratings of 2 and 1. Inspection of the data suggested a possible correlation of the biological activity with the following specification:

"Favourable frequencies" (any one or more likely to confer biological activity): 210–222; 325–335; 428–438; 440–449; 479–489 cm^{-1}; "adverse frequencies" (either one likely to negate pheromone activity): 177–197; 350–360 cm^{-1}.

Of the nineteen unequivocally active compounds, seventeen met this specification, whereas of the twenty-six inactive ones, only six did so. The six inactive compounds which conformed with the specification may owe their lack of biological effect to

insufficient volatility or to steric requirements affecting the probability of a quantum exchange with the receptors. Nevertheless, the coefficient of association $(17 \times 20 - 2 \times 6)/(17 \times 20 + 2 \times 6)$, is 0.93, demonstrating the close correlation that can be found between a simple pattern of vibrational bands and the biological activity. For this association χ^2 is 16.8, indicating a highly significant correlation. The two active compounds which did not fully meet the vibrational specification were 2-nonanone and 2-butyl acetate. Each had two "favourable" frequencies, but the first had an "adverse" one at 196 and the second one at 352 cm^{-1}. In view of the limited data and the uncertainty in the precise position of absorption maxima in survey-type spectra run on liquid or dissolved samples, these apparent exceptions cannot be regarded as insuperable obstacles to the theory.

The critical test of a theory is its ability to generate verifiable predictions, and especially predictions which would not be derivable from alternative or competing theories. Accordingly, available far infrared spectra were searched for compounds which met the above tentative specification and the molecular configurations of which seemed to be quite unlike that of the natural pheromone, 2-heptanone.

Nine such compounds were selected and available for testing (Table 1). It was not possible to repeat the very comprehensive laboratory and field tests which led up to the assignment of ratings on the 0–5 scale. Instead, 0.5 μl. of the substance was drawn into a glass capillary and the tip brought to within 2 cm of quietly feeding ants and the response looked for was a general agitation, increased running about, and attacks on other ants, all manifested within 10 s of introducing the test chemical. If the agitation was especially marked and appeared within 5 s, the response was rated $++$. A distinct but less marked response appearing within 10 s was rated $+$, and little or no response was rated $-$. All the compounds were assayed on five separate laboratory colonies of ants.

Table 1 shows that seven of the nine compounds which met the vibrational specification gave a distinct response, and there is an indication that the 440–449 cm^{-1} band may not be as significant as the rest. Two compounds, isovaleric acid and triethyl amine induced additional "cleaning movements" by the ants.

These results follow other predictive successes of the vibrational hypothesis, namely, the successful prediction of wasp attractant properties[5], the anti-attractant effect of compounds carrying "adverse frequencies"[6], and the successful selection of compounds with a "green" type of odour[7]. A correlation of pheromone activity with the shape of 2-heptanone seems to be wholly incapable of accommodating, not to mention predicting, the alarm pheromone activity of such substances as

Table 1 Infrared Spectra and Bioassay of Putative Pheromones

Compound	Bioassay	Infrared bands						
		Favourable					Adverse	
		210–222	325–335	428–438	440–449	479–489	177–197	350–360
Triethylamine	+ +	—	328	438	—	483	—	—
Heptyl butyrate	+ +	217	334	428	—	479	—	—
2-Methylpentyl crotonate	+ +	—	333	432	—	480	—	—
4-Methyl-3-heptanone	+ +	215	333	438	—	—	—	—
Terpineol acetate	+	216	333	432	—	481	—	—
Isovaleric acid	+	221	328	428	—	489	—	—
d-Limonene	+	—	329	428	—	489	—	—
o-Anisil	—	—	333	—	443	483	—	—
Geranyl formate	—	—	328	438	447	—	—	—

triethyl amine, heptyl butyrate or terpineol acetate. It can, of course, be argued that the response to these compounds is not a true "alarm response", but until means are found for discriminating very subtle differences in the response pattern, similarity of response must be regarded as *prima facie* evidence of similarity of sensory input.

The ability to predict is a more stringent test of a theory than the ability to correlate existing knowledge. The success of the vibrational hypothesis in both predicting and correlating lends confidence to a belief that the relationship between alarm pheromone activity and molecular vibration is indeed a valid general principle.

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THE CHEMISTRY OF THE DEFENSIVE SECRETION OF THE BEETLE, *DRUSILLA CANALICULATA*

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Abstract—The tergal gland of the beetle, *Drusilla canaliculata*, contains defensive products which exhibit an extraordinary chemical diversity. This glandular exudate is fortified with alkanes, alkenes, saturated and unsaturated aliphatic aldehydes, 1,4-quinones, and hydroquinones. The aldehydes, *n*-dodecanal, *n*-tetradecanal, *n*-tetradec-5-enal, and *n*-tetradeca-5,8-dienal, constitute a major group of components. In addition, a new constituent in arthropod defensive secretions, 2-hydroxy-3-methylhydroquinone, has been identified as a minor component in this exocrine exudate.

INTRODUCTION

SINCE the various glands producing alarm pheromones in the social Hymenoptera are often not homologous, their function as liberators of alarm releasers must have developed polyphyletically (MASCHWITZ, 1964). It now seems clear that this statement is also applicable to the defensive glands of many beetles. For example, many species of staphylinid beetles, such as those in the genus *Stenus*, in the subfamily Steninae, secrete defensive substances from their pygidial glands located near the tip of the abdomen (SCHILDKNECHT, 1970). On the other hand, the staphylinid *Lomechusa strumosa*, a species in the large subfamily Aleocharinae, discharges its defensive exudate from the tergal gland, a dorsal structure which is located between the sixth and seventh abdominal tergites (PASTEELS, 1968). The tergal gland, which in the family is limited to the Aleocharinae, is often highly developed in both myrmecophilous and free-living species.

The tergal gland exudate of *L. strumosa*, which is reported to be an extremely effective defensive product (JORDAN, 1913), is fortified with a series of 1,4-benzoquinones and aliphatic hydrocarbons (BLUM *et al.*, 1971). We now report on the chemistry of the defensive secretion of a second aleocharine, *Drusilla* (= *Astilbus*) *canaliculata*, a free-living staphylinid that scavenges on dead ants. The secretion from its tergal gland is remarkably effective in repelling ants and was reported to react with Schiff reagent, thus presumably containing aldehydic constituents (PASTEELS, 1968). The characterization of these aldehydes as part of a defensive secretion in which 15 compounds have been identified, demonstrates that this

tergal gland exudate is one of the most complex yet analysed from any coleopterous species.

MATERIALS AND METHODS

Collection of secretion

Adult beetles were laboratory reared at the Université Libre de Bruxelles, Belgium. The tergal gland secretion was collected from beetles by inserting small filter-paper triangles, held with forceps, between the sixth and seventh abdominal tergites (counting system of BLACKWELDER, 1936). The impregnated triangles were extracted with n-hexane and the concentrated extract used for all subsequent analyses.

Gas chromatographic and mass spectrometric analysis

All gas chromatographic-mass spectrometric analyses were carried out on a LKB-9000 instrument at 70 eV, with a source temperature of 270°C, separator temperature of 260°C and 60 μA ionizing current. Either one of two gas chromatographic stationary phases, 10% SP-1000 or 1% OV-17, were used in 2 m × 2·5 mm columns. Preparative gas chromatographic separations were carried out on a Tracor MT-220 gas chromatograph with 10% Carbowax 20 M as the stationary phase in a 2 m × 5 mm column.

Ozonolysis

Certain compounds in the extract known to contain double bonds were collected after elution from a 10% Carbowax 20 M column and ozonized according to the procedure of BEROZA and BIERL (1966, 1967) or the modified procedure of BRADY et al. (1971). Gas chromatographic analyses of ozonolysis products were conducted either on 10% Carbowax 20 M (pentanal to dodecanal) or on Chromosorb 102 (acetaldehyde to butanal).

Hydrogenation

A small portion of the crude extract was hydrogenated, after the addition of PtO_2, by passing hydrogen gas through it for approximately 15 min. The reaction mixture was analysed on the 10% SP-1000 column in the LKB-9000 mass spectrometer.

Acetylation

The crude extract was acetylated with acetic anhydride in pyridine and the reaction mixture analysed on either 10% SP-1000 or 1% OV-17.

Sodium borohydride reduction

The usual procedure for reduction with $NaBH_4$, using ethanol as a solvent, was employed prior to acetylation and GC-MS analysis.

RESULTS

The defensive secretion from *D. canaliculata* is distinctly yellow suggesting the possible presence of quinones, and the positive reaction obtained to Schiff's reagent indicates that aldehydes probably are also present (PASTEELS, 1968). The temperature-programmed gas chromatographic separation of the crude extract on SP-1000 showed the presence of at least four major peaks and a number of minor peaks (see Fig. 1). On this phase, the mass spectrum of the peak eluting after the
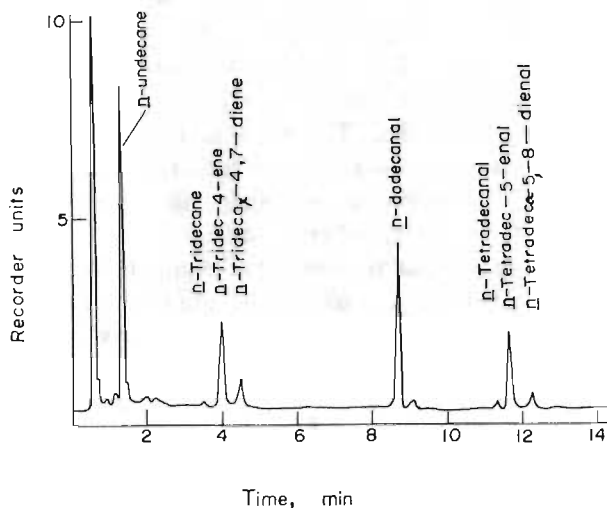
FIG. 1. Gas chromatographic separation of the hydrocarbon and aldehydic fractions in the defensive secretion of *D. canaliculata* (10% SP-1000; temperature programmed from 100 to 200°C at 8°C/min).

solvent gave a molecular ion at m/e 156 and a fragmentation pattern characteristic of a normal hydrocarbon. A scan of a very minor peak on the tail of this first peak showed a similar fragmentation pattern with a molecular ion at m/e 154, contaminated by m/e 156. A comparison of the retention time and mass spectrum of the larger peak with that of *n*-undecane confirmed its identity as this hydrocarbon. The small amount of *n*-undecene present did not permit the position of the double bond to be determined by ozonolysis.

The second major peak, together with its two associated minor peaks, gave mass spectra characteristic of normal hydrocarbons. The spectrum of the first peak gave a molecular ion at m/e 184 and a comparison of its retention time and fragmentation pattern with those of standard hydrocarbons established its identity as *n*-tridecane. The major peak of this group gave a molecular ion at m/e 182, corresponding to the formula $C_{13}H_{26}$ and its mass spectrum indicated that it was a *n*-tridecene. This peak was collected after elution from a Carbowax 20 M column and ozonized. Analysis of the reaction mixture on both Chromosorb 102 and Carbowax 20 M established that nonanal was the main product, confirming that this component in the defensive secretion was *n*-tridec-4-ene. The stereochemistry of this alkene remains

unknown. The third peak in this group gave a molecular ion at m/e 180 and a mass spectrum corresponding to a *n*-tridecadiene. Ozonolysis of the collected material produced both butyraldehyde and hexanal as major products, thereby establishing the structure of this component as a *n*-trideca-4,7-diene of unknown stereochemistry.

The third major peak eluting at approximately 8·5 min in the chromatogram in Fig. 1 gave a mass spectrum corresponding to that of *n*-dodecanal. This identification was further confirmed by a comparison of the retention time of the authentic compound on the various stationary phases. The structure of the minor peak eluting immediately after the *n*-dodecanal was not determined.

The final group of compounds eluting near 12 min in Fig. 1 gave mass spectra characteristic of aliphatic aldehydes. The minor first peak had a retention time and mass spectrum which was congruent with an authentic sample of *n*-tetradecanal. The major peak of this group gave a mass spectrum corresponding to a *n*-tetradecenal. Ozonolysis of this peak, collected after elution from Carbowax 20 M, gave nonanal as the major product thereby establishing its formula as *n*-tetradec-5-enal of unknown stereochemistry. This identification was confirmed by the congruence of the mass spectrum of the reduced and acetylated *n*-tetradec-5-enyl acetate (see Fig. 2) with that of a synthetic sample of this compound. The third
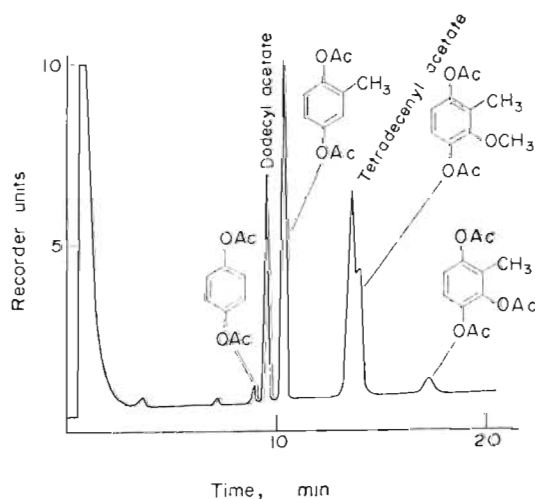
FIG. 2. Gas chromatographic separation of the defensive secretion of *D. canaliculata* after reduction and acetylation (1% OV-17; temperature programmed from 100 to 200°C at 8°C/min).

peak of this group gave a mass spectrum corresponding to a *n*-tetradecadienal, and ozonolysis of collected material showed hexanal to be the main mono-aldehydic product. It is therefore evident that one point of unsaturation in the *n*-tetradecadienal is in position 8, and, by extrapolation from the *n*-tetradec-5-enal, *n*-tridec-4-ene, and *n*-trideca-4,7-diene present in the extract, it would seem most probable that this compound is *n*-tetradeca-5,8-dienal of unknown stereochemistry.

Additional support for the Δ^5 position of the one double bond in the *n*-tetradecadienal was obtained from the following data. The methoxime of the *n*-tetradec-5-enal from the extract had a base peak at m/e 73 ($^+H_2C = CH—NH—O—CH_3$) resulting from McLafferty rearrangement of a γ-hydrogen, its intensity suggesting that the γ-hydrogen is allylic. Direct allylic cleavage, without rearrangement of a hydrogen atom, furnishes an intense peak at m/e 86 ($^+CH_2CH_2CH = N—O—CH_3$). Both of these peaks were observed in the tetradecadienal methoxime spectrum strongly suggesting that there is a double bond in the Δ^5 position in this case also. The only model compound available to us to confirm this latter point was synthetic tetradeca-3,5-dienyl acetate. Reduction and acetylation of the *D. canaliculata* extract provided a gas chromatographic peak whose mass spectrum was similar, but not identical, with that of the model dienyl acetate. Importantly, its retention time was much shorter than that of the conjugated model suggesting that the unknown was unlikely to be a conjugated diene. We therefore conclude that the last peak in the gas chromatogram in Fig. 1 is *n*-tetradeca-5,8-dienal.

The crude extract, after hydrogenation and GC–MS analysis, contained only the expected saturated aldehydes and alkanes.

The GC–MS analysis of the crude extract on OV-17 revealed the presence of two 1,4-benzoquinones which were not obtained on the more polar SP-1000 stationary phase. Their retention times and mass spectra were identical in all respects to those of authentic samples of *p*-toluquinone and 3-methoxy-*p*-toluquinone respectively.

Due to difficulties encountered with their gas chromatographic detection, the presence of hydroquinones was established by acetylation of the crude extract with acetic anhydride in pyridine followed by GC–MS analysis on OV-17. Mass spectra corresponding to hydroquinone diacetate, 2-methylhydroquinone diacetate, 2-methoxy-3-methylhydroquinone diacetate, and 2-hydroxy-3-methylhydroquinone triacetate were obtained from peaks observed in the gas chromatogram of the reaction mixture.

Reduction of the crude extract from *D. canaliculata* with NaBH$_4$, followed by direct acetylation with acetic anhydride in pyridine and GC–MS analysis, produced an increase in the relative amounts of 2-methylhydroquinone diacetate and 2-methoxy-3-methylhydroquinone triacetate obtained (see Fig. 2). Other peaks in this chromatogram corresponded to dodecyl acetate and tetradecenyl acetate formed from the corresponding aldehydes.

A standard sample of 2-hydroxy-3-methylhydroquinone triacetate was synthesized by treating 3-methoxy-*p*-toluquinone with HBr at 100°C for 10 min, evaporating the excess acid, followed by the addition of acetic anhydride and a little powdered zinc. After a few minutes the reaction mixture was cooled, diluted with water and the 2-hydroxy-3-methylhydroquinone triacetate extracted with CHCl$_3$. The mass spectrum and retention time of this synthetic compound were identical to that obtained from the last peak in the chromatogram in Fig. 2.

Thus, in addition to the hydrocarbons and aldehydes, the defensive secretion of *D. canaliculata* contains *p*-toluquinone, 3-methoxy-*p*-toluquinone, hydroquinone,

2-methylhydroquinone, 2-methoxy-3-methylhydroquinone, and 2-hydroxy-3-methylhydroquinone (I).

OH

CH$_3$ (I)

OH

OH
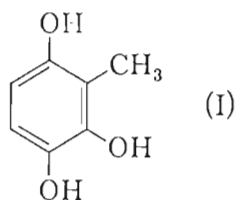
A comparison of Figs. 1 and 2, together with other chromatograms which were obtained, shows that the major components in the secretion are p-toluquinone followed closely by 2-methylhydroquinone. The two hydrocarbons, n-undecane and n-tridec-4-ene, occur in amounts approximately equivalent to the aldehydes, n-dodecanal and n-tetradec-5-enal. These six compounds, together with 3-methoxy-p-toluquinone, account for more than 80 per cent of the volatiles observed in the secretion. The two quinones, p-benzoquinone and 3-hydroxy-p-toluquinone, were not shown to be present during any of the analyses but, nevertheless, are likely to be trace constituents of the secretion.

DISCUSSION

In general, the defensive secretions of staphylinid beetles originate from the so-called pygidial glands. However, as the morphology of these glands in *Stenus* (Steninae), and, in particular, *Staphylinus* (Staphylininae), is distinctly different from that of the analogous glands in *Bledius* (Oxytelinae) (PASTEELS and ARAUJO, unpublished), the pygidial glands of genera within the same family cannot necessarily be considered homologous. Rather, 'pygidial gland' is a term which more appropriately refers to the paired glands located near the tip of the abdomen.

In contrast, the defensive substances of aleocharines are secreted by the tergal gland. This organ is not homologous with any of the known glands found in species in other staphylinid subfamilies. Although certain myrmecophilous species, e.g. *L. strumosa*, and non-myrmecophilous species, e.g. *D. canaliculata*, possess a particularly well-developed tergal gland, in termitophilous species it regresses. The degree of glandular regression appears to depend on the extent to which the species is integrated into the termite society in which it co-exists (PASTEELS, 1968, 1969).

One of us (PASTEELS, 1968) has demonstrated that the tergal gland secretion of *D. canaliculata* renders this aleocharine virtually immune to attack by ants. The defensive secretion of *Drusilla* is utilized only when this aleocharine is subjected to sustained molestation by ants, and this secretory frugality may represent an effective conservation mechanism. *D. canaliculata* does not spray the products of its tergal gland; rather, the secretion oozes from the glandular orifice and is wiped on the assailant. When molested, a beetle will rotate its abdomen so as to bring the gland-bearing segments into proximity to the point of stimulation (Fig. 3) where the copious secretion can be smeared on the source of tactile irritation.
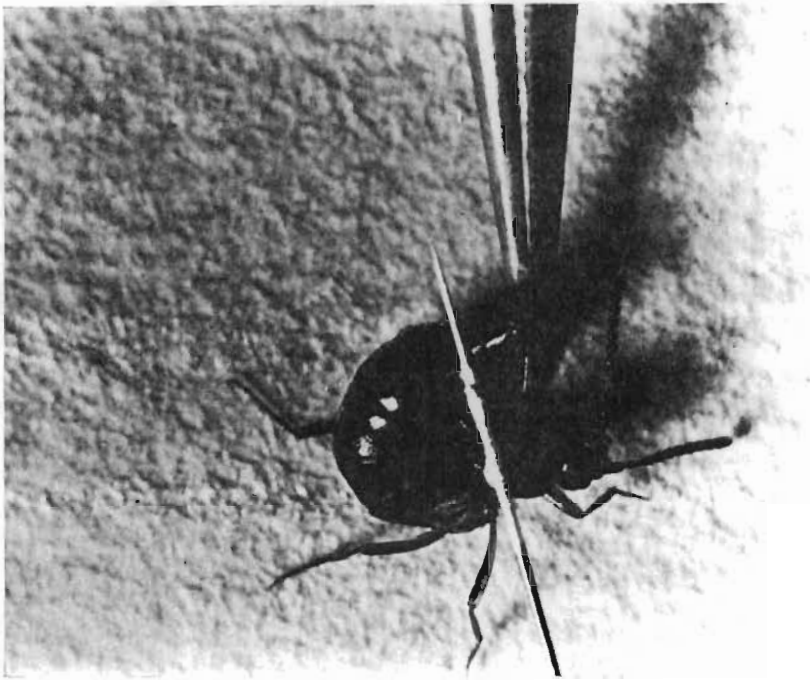
FIG. 3. *D. canaliculata* twisting the abdomen in response to pinching and applying the tergal gland secretion to the forceps.

The gas chromatographic separations of the secretion of *D. canaliculata* presented in Figs. 1 and 2 illustrate the complexity of the secretion in terms of the number of components present. In addition, the variety of chemical types identified in this product comprises a combination which previously has not been associated in any insect defensive secretion. This study on the tergal gland secretion of *D. canaliculata* has shown the presence of alkanes, alkenes, aliphatic aldehydes, 1,4-quinones, and hydroquinones. The chromatogram in Fig. 1 shows that *n*-undecane, *n*-tridecane, *n*-tridec-4-ene, and *n*-trideca-4,7-diene constitute the major hydrocarbons present. A mass spectrum obtained from the tail of the *n*-undecane peak also showed the presence of *n*-undecene. The position of the double bond in *n*-tridec-4-ene is not that expected if it was derived from a common unsaturated fatty acid.

A possible relationship between the hydrocarbons, undecane, tridecane, tridecene, and tridecadiene, and the aldehydes, dodecanal, tetradecanal, tetradecenal, and tetradecadienal offers some additional grounds for relating the biosynthetic origins of these compounds as the relative amounts of these two groups of compounds strongly indicate a quantitative relationship. For example, two of the most abundant compounds are *n*-undecane and *n*-dodecanal, each of which is present in approximately equivalent amounts. Indeed, the possible metabolic relationship between hydrocarbons and aldehydes becomes even more suggestive when one considers the C-13 hydrocarbons and C-14 aldehydes. The similarities in relative proportions of these two groups of compounds is strikingly evident from Fig. 1 and, significantly, the position of the double bonds in *n*-tridec-4-ene and *n*-tetradec-5-enal and the doubly unsaturated *n*-trideca-4,7-diene and *n*-tetradeca-5,8-dienal correspond if the aldehydic group is removed. Thus, it would appear that the hydrocarbons may be formed from the corresponding aldehydes and that both the hydrocarbons and their corresponding aldehydes are related in terms of a common precursor.

The quinoidal mixture present in the tergal gland exudate of *D. canaliculata* represents a qualitatively unique blend of quinones and hydroquinones among the characterized defensive secretions of arthropods. *p*-Toluquinone, which is the major component of this secretion, is a common quinone found in defensive secretions of insects. 3-Methoxy-*p*-toluquinone, however, is a characteristic component of millipede defensive glands (WEATHERSTON and PERCY, 1970) and has not been encountered frequently as a defensive product of insects. This latter quinone is found in one species of scaratine carabid (SCHILDKNECHT *et al.*, 1968) and has also been identified in members of the subgenus *Blaphylis* of the tenebrionid genus *Eleodes* (TSCHINKEL, personal communication). Whether its occurrence in the exocrine secretion of *D. canaliculata* is typical of aleocharines cannot yet be ascertained. The fact that it is absent from the defensive exudate of another aleocharine, *L. strumosa*, raises the possibility that it may not be widespread in members of this subfamily.

Thin-layer chromatography has been used to show that the quinone-rich secretions of some insects also contain the corresponding hydroquinones

(SCHILDKNECHT and HOLOUBEK, 1961; SCHILDKNECHT and KRAMER, 1962; IKAN *et al.*, 1970). Due to difficulties encountered in the gas chromatographic separation of hydroquinones, together with the fact that the mass spectra of a quinone and its corresponding hydroquinone are similar, we undertook their analysis as the acetylated derivatives. Acetylation of the defensive exudate of *D. canaliculata* yielded products corresponding to hydroquinone diacetate, 2-methylhydroquinone diacetate, 2-methoxy-3-methylhydroquinone diacetate, and 2-hydroxy-3-methyl-hydroquinone triacetate. It may be concluded therefore that this secretion contains these four compounds in the hydroquinone form. The fact that hydroquinones have not been reported in most of the quinone-rich secretions analysed by gas chromatography probably indicates that they were not seen due to their gas chromatographic behaviour rather than that they were not present.

Following the establishment of four hydroquinones in the secretion, we re-investigated both the crude extract and its acetylated reaction mixture for the presence of 1,4-benzoquinone and either 3-hydroxy-*p*-toluquinone or 3-acetoxy-*p*-toluquinone. However, none of these compounds could be identified from the numerous mass spectra obtained. In any mixture of quinones, the proportions of the various compounds actually present will depend on their redox potentials (assuming the absence of a strong oxidant or reductant). As a result, it is to be expected that 1,4-benzoquinone, in the presence of large amounts of 2-methyl-hydroquinone, will be reduced to hydroquinone. In addition, both the hydro-quinone and the 2-hydroxy-3-methylhydroquinone are present in the secretion as minor constituents. While we were unable to establish the presence of the two corresponding quinones, we consider it very likely that they nevertheless are present in trace amounts as the oxidized concomitants of their hydroquinones. This state-ment is supported by the fact that the analyses of all other hydroquinone-containing secretions have established the presence of the corresponding quinones.

Since the tergal gland is not homologous with any other known gland, it is apparent that the glandular source of the quinoidal defensive secretion of many beetles has evolved independently on several occasions. As a number of unrelated glands apparently have the ability to synthesize certain quinones, the genetic in-formation for the biosynthetic enzymatic systems must have been retained by various insects over a considerable period of time. Many members of the Coleop-tera synthesize a number of 1,4-benzoquinones in spite of differences in both the location and morphology of the secretory areas of the defensive glands. The differences noticed between the secretions of the related beetles, *Drusilla* and *Lomechusa*, suggest that the genetic information required to make both *p*-ethyl-quinone (*Lomechusa*) and 3-methoxy-*p*-toluquinone (*Drusilla*) may be present in the same organism, but that if one metabolic route is expressed, the other will not be operational. Therefore, the inability to correlate the chemical nature of the various characterized defensive secretions with the taxonomic classification of the species which produce these secretions may not be too surprising.

While a number of biosynthetic studies have been conducted on the formation of substituted quinones in fungi, only a few investigations have been carried out on

arthropod quinones. MEINWALD *et al.* (1966), studying *Eleodes longicollis*, concluded that 1,4-benzoquinone arises from a preformed aromatic ring whereas *p*-toluquinone is formed from acetate and *p*-ethylquinone is formed from acetate and propionate. The substituted quinones, 6-hydroxy-5-methoxy-*p*-toluquinone, 3,6-dihydroxy-5-methoxy-*p*-toluquinone, 5,6-dihydroxy-*p*-toluquinone, and 6-methoxy-5-hydroxy-*p*-toluquinone, found in *Aspergillus fumigatus* and *Penicillium spinulosum* also arise from acetate and malonate (PETTERSSON, 1963, 1964). It appears that the O-methyl group of the various quinones is derived from either the C_1-pool or the S-methyl group of L-methionine in both fungi (PETTERSSON, 1963, 1964) and arthropods (WEATHERSTON and PERCY, 1970). The occurrence of 2-hydroxy-3-methylhydroquinone in the secretion of *D. canaliculata* suggests that this compound is a likely precursor of the 3-methoxy-*p*-toluquinone and/or 2-methoxy-3-methylhydroquinone found in this and other arthropod secretions. However, the opposite could be true as O-methylquinones undergo hydrolysis fairly readily and biosynthetic demethylation is quite common.

Like the defensive exudates of many other beetles, the tergal gland secretion of *D. canaliculata* has a yellow-orange colour. In this respect it is similar to the analogous secretion of the myrmecophilous beetle *L. strumosa*, the only other aleocharine species which, so far, has been chemically characterized (BLUM *et al.*, 1971). However, while the odour of the secretion of *L. strumosa* is clearly dominated by that of quinones, the odour of the *D. canaliculata* secretion possesses a pleasantly fragrant aldehydic note. Since *Drusilla* and *Lomechusa* belong to the same tribe of the subfamily, one might anticipate definite similarities between their secretions. However, since the quinone-rich exudates of these two aleocharines are dissimilar in a very major qualitative sense, only *p*-toluquinone (and possibly 1,4-benzoquinone) being common to both, there are really no substantive grounds for concluding that the defensive secretions of members of this subfamily possess a characteristic composition. Conceivably, the complex defensive secretion of *D. canaliculata* may represent a qualitative extreme in the Aleocharinae, but until the secretions of other species in the taxon are chemically characterized, the full synthetic potential of these staphylinids cannot be ascertained.

The diversity of aldehydes which accompanies the quinones in the defensive exudate of *D. canaliculata* demonstrates that this glandular exudate is atypical of the exocrine secretions utilized by coleopterous species to repel predators. The aleocharine *L. strumosa* also produces quinones in its tergal gland but no aldehydic constituents are detectable (BLUM *et al.*, 1971). Similarly, species in the tenebrionid subfamily Tenebrioninae synthesize quinones in their pygidial glands, as do species in about five subfamilies in the Carabidae, but aldehydes are not reported to accompany the quinones in any of these defensive exudates. Indeed, the quinoidal secretions of carabids apparently do not contain additional constituents (MOORE and WALLBANK, 1968), whereas the tenebrionid exudates may be fortified with the alkenes, 1-nonene, 1-undecene, 1-tridecene (HURST *et al.*, 1964), 1-pentadecene (VON ENDT and WHEELER, 1971), or caprylic acid (MEINWALD and

EISNER, 1964). The terminally unsaturated alkenes in tenebrionid defensive secretions contrast markedly with those in the secretion of *D. canaliculata* in which the double bonds are internal.

None of the aldehydes fortifying the defensive secretion of *D. canaliculata* have been isolated previously from coleopterous exocrine secretions and the significance of this class of compounds as a concomitant of the 1,4-quinones is not readily apparent. Indeed, the only example reported of a fairly long-chain aldehyde accompanying a quinone in an arthropod defensive secretion is the case of the julid millipede, *Rhinocricus insulatus*, which produces a defensive secretion containing a mixture of *trans*-2-dodecenal and *p*-toluquinone (WHEELER *et al.*, 1964). Of the four aldehydes produced in the tergal gland of *D. canaliculata*, only *n*-tetradecanal has previously been identified in insects. This compound, along with *n*-hexadecanal, is utilized by several species of *Bombus* as one of the components in the territorial-marking pheromones which are evacuated from the mandibular glands of male bees (KULLENBERG *et al.*, 1970).

Although the chemistry of the defensive secretions of only six species of staphylinids have been examined, it is nevertheless quite obvious that the members of this family possess a remarkable repertoire of natural products. The tergal gland products of two members of the Aleocharinae are identified with five of the six 1,4-benzoquinones that do occur (or are likely to occur e.g. 3-hydroxy-*p*-toluquinone) in insect exocrine secretions (WEATHERSTON and PERCY, 1970), and, in addition, one of these species synthesizes a novel series of aliphatic aldehydes and hydrocarbons. In contrast, the pygidial gland defensive products of beetles in three other staphylinid subfamilies reflect a strong terpene emphasis. Thus, *Stenus bipunctatus* (Steninae) synthesizes 1,8-cineole, *iso*-piperitenol, and 6-methyl-5-hepten-2-one in its pygidial glands (SCHILDKNECHT, 1970), whereas *Staphylinus olens* (Staphylininae) utilizes a defensive exudate which is dominated by iridodial (ABOU-DONIA *et al.*, 1971), a monterpene aldehyde which is also produced by several species of dolichoderine ants. Monoterpene aldehydes are also present in the pygidial gland secretion of *Bledius mandibularis* and *B. spectabilis* (Oxytelinae), but these compounds do not constitute the major products in the exocrine exudate (WHEELER *et al.*, personal communication). Like the aleocharines, the *Bledius* species also produce 1,4-benzoquinone and, in addition, 1-undecene is present. However, the main defensive compound produced by these *Bledius* spp. is γ-dodecalactone, a compound not previously isolated from arthropod defensive secretions (WHEELER *et al.*, personal communication). These facts strongly indicate that the members of the Staphylinidae probably possess as versatile a natural products chemistry as any coleopterous family which has been investigated.

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Phenol and Benzaldehyde in the Defensive Secretion of a Strongylosomid Millipede[1]

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The defensive secretions of millipedes have proven to be a rich source of natural products (Weatherston and Percy 1970). Species in the order Polydesmida characteristically produce a defensive exudate which is fortified with benzaldehyde and hydrogen cyanide (Blum and Woodring 1962, Eisner et al. 1963a). We report here on the secretion of *Oxidus gracilis* (Koch), one of the 2 species in the polydesmid family Stronglysomidae which are present in North America. Although *O. gracilis* has been previously demonstrated to produce hydrogen cyanide in its defensive exudate (Eisner et al. 1963a), in our experience, the secretion of this species possesses a distinctly phenolic odor which is not typical of those of other polydesmids which we have examined.

Several hundred individuals of *O. gracilis*[4] were collected in Athens, Ga., and their secretions were absorbed on filter-paper squares which were lightly tapped on their gland-bearing diplosegments. The squares were extracted in ethyl ether and the solution was concentrated with nitrogen down to a small volume. Analysis by GLC (6 ft × ¼ in., 10% Carbowax 20 M, 170°C) demonstrated the presence of 2 components, the second of which possessed a typical phenolic odor. Comparison with authentic samples showed that the *Oxidus* volatiles had retention times identical to those of benzaldehyde and phenol. Analysis of extracts by GLC-mass spectrometry unequivocally confirmed the presence of these 2 compounds in the millipede secretions. Phenol constituted 1–5% of the mixture.

The presence of benzaldehyde in the secretion of *O. gracilis* is not surprising, especially because hydrogen cyanide has already been identified as a defensive product of this millipede (Eisner et al. 1963a). Both of these compounds are presumably generated from mandelonitrile, a precursor of these 2 constituents in other millipede secretions (Eisner et al. 1963b). On the other hand, the presence of phenol in the defensive exudate of *O. gracilis* cannot be readily reconciled with the biosynthetic origins of benzaldehyde and hydrogen cyanide, and phenol must be regarded as an anomolous constituent of this secretion. Although the quinonoid secretions of the spirobolid *Rhinocricus insulatus* (Chamberlin) and the julid *Oriulus delus* Chamberlin contain an aliphatic aldehyde (Wheeler et al. 1964) and o-cresol (Kluge and Eisner 1971), respectively, secretions of polydesmid millipedes have been reported to contain only benzaldehyde and hydrogen cyanide. The addition of a few percent of phenol to the secretion of *O. gracilis* undoubtedly increases the defensive value of this exudate, which we have observed to be very effective against workers of the fire ant *Solenopsis richteri* Forel. At this juncture, it seems certain that the exocrine exudates of millipedes will continue to yield a wealth of chemical surprises.

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20

IDENTIFICATION OF MELLEIN IN THE MANDIBULAR
GLAND SECRETIONS OF CARPENTER ANTS

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Summary

The mandibular gland secretions of males of the
ants Camponotus herculeanus, C. ligniperda, and C.
pennsylvanicus contain three major volatile sub-
stances. These compounds have been identified as
3,4-dihydro-8-hydroxy-3-methylisocoumarin (mellein
or ochracin), methyl 6-methylsalicylate, and 10-methyl-
dodecanoic acid. Mellein has not been isolated pre-
viously from animal sources.

The swarming of the sexual forms of the carpenter ant,

Camponotus herculeanus, is affected to a large extent by cli-

matic factors such as season, temperature, and time of day. On

a favorable afternoon the departing flight is initiated by the

males which are followed, at the peak of their flight, by the

females. The coordination of this synchronized swarming of both

sexes is governed by volatile substances secreted from the man-

dibular glands of the males (1). In both laboratory and field

experiments, Hölldobler and Maschwitz (1) demonstrated that fe-

males of C. herculeanus could be stimulated similarly by the

mandibular gland secretion of males of either C. herculeanus or

the closely related species, C. ligniperda.

We have analyzed the major volatiles in the mandibular gland

secretions of both of these species, as well as C. pennsylvanicus,

and have established that the secretions are dominated by two
substances, 3,4-dihydro-8-hydroxy-3-methylisocoumarin (mellein
or ochracin) and methyl 6-methylsalicylate. The former compound
is known to occur as a metabolite of certain fungi (3, 4, 5, 6,
7), while the presence of methyl 6-methylsalicylate in certain
North American Camponotus species has been reported (2). C.
herculeanus and C. pennsylvanicus also contain appreciable quan-
tities of a free acid, 10-methyldodecanoic acid.

Materials and Methods

Heads of male ants of C. herculeanus (collected near Belle-
ville, Ontario, Canada), C. ligniperda (collected at Erlangen,
Germany), and C. pennsylvanicus (collected at Jefferson City,
Tennessee) were crushed in n-pentane and the concentrated extracts
used for all subsequent analyses. Excised mandibular glands were
examined separately and determined to be the source of the vola-
tile compounds present in the heads of Camponotus males.

Gas chromatographic analyses were carried out on four sta-
tionary phases, 3% OV-1, 10% OV-17, 10% SP-1000, and 10% Carbo-
wax 20 M, and mass spectra were obtained on either a Bell and
Howell 21-490 instrument or a LKB-9000 gas chromatograph-mass
spectrometer. The NMR spectrometer used was a Varian XL-100-15
equipped with a Digilab Fourier transform system.

Results

Gas chromatographic separation of the components in the head
extracts of C. herculeanus and C. ligniperda indicated that the
same two major volatile substances, which comprise more than 80%
of the observed compounds in C. ligniperda, were present in all
extracts (peaks 1 and 3 in Fig. 1). The compound eluting first
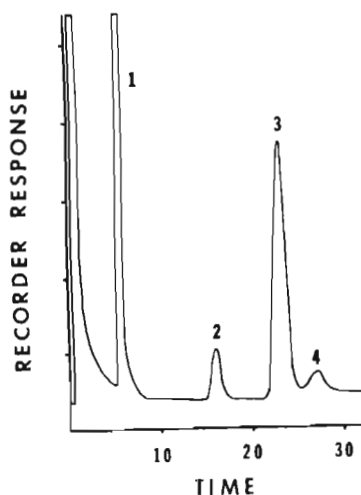
FIG. 1

Gas chromatogram of the major volatile components in the heads of
male C. herculeanus (10% Carbowax 20 M, 180°). 1. Methyl 6-
methylsalicylate; 2. unidentified compound of molecular weight
164; 3. mellein; and 4. 10-methyldodecanoic acid.

on all four phases had a retention time relative to n-heneicosane

on Carbowax 20 M at 200° of 0.75 and had a mass spectrum iden-

tical to that of methyl 6-methylsalicylate, a compound previously

identified in the mandibular gland secretion of male C. pennsyl-

vanicus (2).

The second major peak (peak 3 in Fig. 1) had a retention

time relative to n-heneicosane of 3.04 under similar conditions

and gave a mass spectrum (Fig. 2) with a molecular ion at m/e 178

at ionizing voltages of both 70 eV and 10 eV. The fragmentation

pattern showed a great similarity to that of the accompanying

methyl 6-methylsalicylate. In particular, both exhibited intense
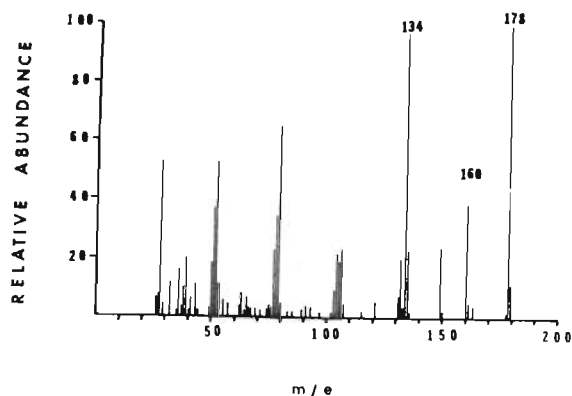
FIG. 2

Mass spectrum of mellein (3,4-dihydro-8-hydroxy-3-methyl-isocoumarin)

ions at m/e 134, assigned in the latter compound to
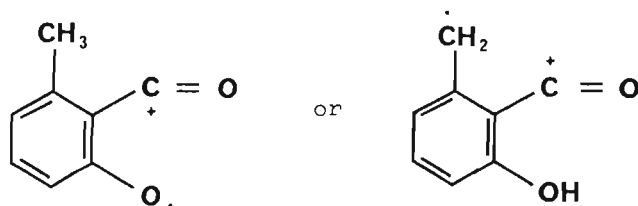
from loss of methanol (32 amu) from the molecular ion. Thus, the loss of 44 amu from the parent ion of the unknown could represent rearrangement to such an ion by loss of the elements of either acetaldehyde or carbon dioxide.

These facts suggest the presence of a carbonyl function. However, both the retention time and mass spectrum of this second major component were unchanged by treatment with sodium borohydride, 0-methylhydroxylamine, and hydrogen and PtO_2, whereas a

reaction was obtained with bis(trimethylsilyl)trifluoroacetamide. The mass spectrum of the product of this reaction exhibited a molecular ion 72 amu higher in mass, suggesting the presence of one OH group in the molecule.

Confirmatory evidence for the presence of a phenolic hydroxyl group was obtained from the ultraviolet absorption spectrum of a sample of this substance collected from GLC from approximately 600 $\underline{C}$. $\underline{pennsylvanicus}$ males. Absorption maxima, in ethanol, were found at 245 nm and 313 nm in the ratio of 1.6 : 1, and the peak at 313 nm was shifted to 346 nm by the addition of alkali. Insufficient compound was available for measurement of rotation at the D line, but this sample showed a negative ORD curve with a valley at 265 nm of -60° and a peak at 245 nm of +108°.

This same sample ($\sim$ 200 µg assuming the UV extinction coefficient of the deduced structure, see below) was examined by Fourier-transform NMR. The spectrum, in $CDCl_3$, showed peaks relative to tetramethylsilane as follows: CH_3 $C\underline{H}(O)$ CH_2 : δ 4.73 (six lines, spaced $\sim$ 6.6 Hz), Ar CH_2 CH $C\underline{H}_3$: δ 1.52 (d, J 6.2 Hz), Ar $C\underline{H}_2$ CH : δ 2.94 (d, J 7.60 Hz), three adjacent aromatic hydrogens : δ 6.67 (broad d, spaced 6.6 Hz), δ 6.87 (broad d, spaced 8.2 Hz), δ 7.39 (broad t, spaced 8.0 Hz), and a hydrogen-bonded phenolic proton, lost on exchange with D_2O : δ 11.03 (s).

This pattern, together with the other data, is in accord only with structure I, a substance identified previously as a
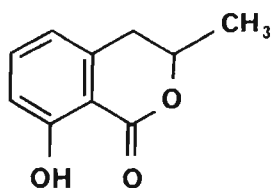
I

metabolite of the fungi, Aspergillus melleus and A. ochraceus, and assigned the trivial epithets of mellein and ochracin (3, 4, 5, 6). The NMR spectrum and the UV data obtained above are in agreement with those published by Patterson et al. (7) for the optical antipode of mellein isolated from an unidentified fungus. The retention time on SP-1000 and the mass spectrum of an authentic sample of (±)-mellein are completely congruent with our data. Since mellein contains only one chiral center, the negative ORD curve of our sample suggests that its rotation at the D line will also be (-) as in (-)-mellein rather than its antipode.

The enigmatical loss of 44 amu in the mass spectrum of I is therefore seen to result from a retro Diels-Alder reaction of the B ring with expulsion of the elements of acetaldehyde. This reaction is completely superceded in the trimethylsilyl ether of I by loss of methyl from the molecular ion, presumably because the resulting ion can be stabilized by the adjacent carbonyl oxygen atom.
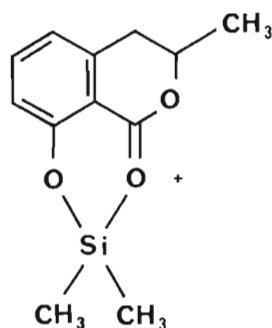
In addition to methyl 6-methylsalicylate and mellein in the extract of C. ligniperda male heads, a trace amount of a

substance with a molecular weight of 151 was also detected. The
retention time and mass spectrum of this minor component are
identical to those of methyl anthranilate, a compound isolated
previously from the mandibular gland of C. nearcticus (2).
While methyl anthranilate could not be detected in the extracts
of C. herculeanus and C. pennsylvanicus, two additional compounds
were readily observed in both. A minor component (peak 2 in Fig.
1) with a retention time relative to n-heneicosane of 2.08 on
Carbowax 20 M at 200° gave a molecular ion at m/e 164 but as yet
we have been unable to identify it.

The compound eluting immediately after mellein on both SP-
1000 and Carbowax 20 M (peak 4 in Fig. 1) gave a mass spectrum
with a molecular ion at m/e 214 and intense ions at m/e 60 and
73 typical of fatty acids. If this assignment were correct, its
formula would be $C_{13}H_{26}O_2$, i.e., a tridecanoic acid. Intense
ions were also observed at m/e 155 and 157 corresponding to loss
of CH_2COOH and C_4H_9 respectively, and this latter fragmentation
suggests the presence of a methyl branch at C_{10} and, therefore,
the structure, 10-methyldodecanoic acid. Both diazomethane and
boron trifluoride in methanol react with this acid to produce a
derivative eluting before methyl 6-methylsalicylate on both polar
phases and it is clear that the free acid is a major component of
the mixture in both of these species. The mass spectrum of the
methyl ester, formed by treatment with diazomethane, has a mole-
cular ion at m/e 228 and typical intense methyl ester peaks at
m/e 74 and 87. As expected, the peak at m/e 155 was unchanged
(loss of CH_2COOMe) while the m/e 157 ion was increased by 14 mass
units to m/e 171, thus confirming the structure, 10-methyl-
dodecanoic acid. This acid was also found to be present as a

minor component in the extract of C. ligniperda.

Discussion

Whether C. herculeanus and C. ligniperda should be regarded
as separate species, or whether C. ligniperda is a subspecies of
C. herculeanus remains to be determined (8); however, the dis-
tribution of these two forms is not in doubt. C. herculeanus
is a common species in Europe, northern Asia, and North America,
while C. ligniperda is found only in Europe and adjacent parts
of Asia. C. pennsylvanicus, which has also been considered by
certain taxonomists to be a subspecies of C. herculeanus (9),
occurs only in North America. Whatever the actual taxonomic
status of these three forms of Camponotus, it is obvious that
they are all closely related.

These studies on the major volatile substance in the man-
dibular gland secretion of their males demonstrate a general
similarity in composition. The glandular exudates of males of
both C. herculeanus and C. ligniperda contain methyl 6-methyl-
salicylate, as does that of C. pennsylvanicus males (2). In
addition, males of all three species contain equal, or slightly
greater, amounts of the related and less volatile compound,
mellein. It has been identified as a fungal metabolite (3, 4, 5,
6, 7) and synthesized as (±)-mellein methyl ether (10) or as
(±)-mellein (11) by rather complex chemical procedures. However,
to our knowledge, it has never been identified as an animal
natural product. Both 6-methylsalicylic acid and mellein appear
to be biosynthetically derived from acetate (12). All three of
these ant species also contain a free fatty acid identified from
mass spectral data as 10-methyldodecanoic acid. This acid is

known to occur in wool wax (13, 14, 15) and the lipid fraction of a bacterium (16).

This similarity of the compositions of the mandibular gland secretions of C. herculeanus and C. ligniperda is in full accord with the taxonomists' views and also with the analyses of Bergström and Löfqvist (8) on Dufour's gland contents of these two species. It may also explain why C. herculeanus females respond similarly to the secretion of either their own males or of that of C. ligniperda males (1).

The essential difference between these three extracts appears to lie in the presence of a minor component. C. ligniperda contains a trace of methyl anthranilate which is not detectable in either C. herculeanus or C. pennsylvanicus. These latter two species, however, contain a small amount of an unidentified substance with a molecular weight of 164. In this connection, we have also determined from gas chromatographic data, that males of C. noveboracensis, another species in the subgenus Camponotus, contain the same four substances as C. herculeanus and C. pennsylvanicus.

Hölldobler and Maschwitz (1) state that the mandibular gland contents of both C. herculeanus and C. ligniperda have a similar odor and we have noticed that our extracts of these two species, at room temperature, smell like methyl 6-methylsalicylate. However, at 4°, the C. ligniperda extract has a distinct grape-like odor, due to the trace of methyl anthranilate present, which is not perceptible in that of C. herculeanus. As certain behavioral characteristics of Camponotus females have been convincingly demonstrated to be due to the mandibular gland contents of the

males (1), the way is now clear to investigate the roles of specific chemicals as potential regulators of both the swarming behavior and flight coordination of at least four Camponotus species.

Acknowledgements

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Caste-Specific Compounds in Male Carpenter Ants

Abstract. *Three caste-specific substances new to arthropod glandular secretions occur in the mandibular glands of male ants of five species in the genus* Camponotus. *These volatile compounds, which are not found in alate females or workers, have been identified as methyl 6-methyl salicylate, 2,4-dimethyl-2-hexenoic acid, and methyl anthranilate. The free acid has not been described previously.*

Coordinating the pairing and mating of the two sexes of an insect species is an obvious fundamental necessity. Many species of termites and ants exhibit a synchronized swarming of male and female alates from many nests, ensuring that large populations of reproductives are airborne at the same time. This swarming behavior and flight coordination of males and females of the carpenter ant, *Camponotus herculeanus*, has been described by Hölldobler and Maschwitz (*1*), who concluded that the simultaneous swarming from a large number of nests is dependent upon season, temperature, and time of day. However, it was also convincingly demonstrated that the mandibular gland secretion of the males is a critical factor in initiating the activity of the females before swarming. This secretion is used to scent the area immediately surrounding the nest entrance and to entice the females to swarm from the nest when the male flight is at a maximum (*1*). This releaser activity of the male-derived exudate would appear to constitute a new function for a caste-specific secretion.

We have identified the major volatile components in the mandibular glands of males of *Camponotus noveboracensis*, *C. pennsylvanicus*, *C. nearcticus*, *C. rasilis*, and *C. subbarbatus* as the initial part of a program investigating factors governing the swarming behavior of certain species of ants. Three new arthropod natural products have been chemically characterized from among these species and found to be methyl 6-methyl salicylate, 2,4-dimethyl-2-hexenoic acid, and methyl anthranilate. The free acid has not been described.

Heads of male *C. nearcticus* were crushed in *n*-pentane; the resulting pentane extract was subjected to gas chromatographic–mass spectrometric analysis (*2*). Of the three peaks detected, the first and major showed a molecular ion (M) at m/e 166. This compound appeared to be aromatic (m/e 77 and 78), containing both a $COOCH_3$ group (loss of CH_3OH and $HCOOCH_3$ from m/e 166) and a phenolic hydroxyl (conversion to an *O*-acetate, M^+ 208; and a slow reaction with CH_2N_2 forming a methyl ether, M^+ 180). Both the retention time and the mass spectrum of methyl 6-methyl salicylate (*3*) correspond to those of this peak in the male heads, whereas the retention times of methyl 5-methyl salicylate and methyl 3-methyl salicylate were distinctly different.

The second peak from this extract is an unsaturated acid with a molecular ion at m/e 142 (conversion to a methyl ester, M^+ 156). Reduction of the methyl ester provided a dihydro derivative (M^+ 158) whose fragmentation pattern showed it to be an ester of an α-methyl substituted acid (intense peaks at m/e 88 and 101). Ozonolysis of the acid yielded 2-methylbutanal, suggesting the structure 2,4-dimethyl-2-hexenoic acid. This acid was synthesized by slow hypochlorite oxidation of the known 3,5-dimethyl-3-hepten-2-one (*4*) and also from hydrolysis of the product of the Wittig reaction between 2-methylbutanal and the ylid derived from triphenylphosphine and methyl 2-bromopropionate (*5*). The resulting acids had retention times and mass spectra identical to those of the natural product. The geometry of the double bond and the configuration of the asymmetric center in the natural substance are unknown. This relatively simple

Table 1. Volatile substances in the mandibular glands of males of five *Camponotus* species (+ + denotes major component, + denotes minor component, − denotes not detected).

Species	Methyl 6-methyl salicylate	2,4-Dimethyl-2-hexenoic acid	Methyl anthranilate
C. nearcticus	+ +	+	+
C. rasilis	−	+	+ +
C. subbarbatus	+ +	−	−
C. noveboracensis	+ +	−	−
C. pennsylvanicus	+ +	−	−

compound has not heretofore been described.

The third peak exhibits a molecular ion at m/e 151, suggesting that it contains one nitrogen atom. It appeared to be aromatic (doubly charged ions, intense M^+ ion, and the like) and lost CH_3OH and $COOCH_3$, suggesting it to be an aminobenzoic acid methyl ester. This structure was confirmed by the formation of an N-acetate ($M + 193$) that lost both the elements of ketene and C_2H_2O plus CH_3OH. The methyl ester of p-aminobenzoic acid gives a loss of CH_3O, rather than CH_3OH, and the retention times of both the para and the meta isomers are incorrect for this third peak. However, the ortho isomer, methyl anthranilate, has an identical retention time and mass spectrum to those of the natural product.

The extract of C. *nearcticus* male heads also contained several long chain fatty acids having α-methyl branching and unsaturation. Of the other species investigated, males of C. *rasilis* produce both the 2,4-dimethyl-2-hexenoic acid and methyl anthranilate, whereas males of C. *pennsylvanicus* and C. *noveboracensis* apparently produce only methyl 6-methyl salicylate as a major component. The mandibular glands of C. *subbarbatus* (6) males yield methyl 6-methyl salicylate in addition to several other components, one of which may be either geranic or nerolic acid. These results are summarized in Table 1. Excision of the mandibular glands from the males established that all compounds were present in these exocrine structures. None of these substances could be detected in the heads of either alate females or workers of any of these species.

As the mandibular gland secretion of males of C. *ligniperda* was shown to cause an identical response in females of C. *herculeanus* during swarming (1), this exudate cannot be regarded as species-specific or as a species isolating mechanism. Both of these European *Camponotus* species belong in the subgenus *Camponotus*, as do the two North American species, C. *pennsylvanicus* and C. *noveboracensis*, and this investigation has established that these latter two species contain the same major volatile compound, methyl 6-methyl salicylate, in their mandibular glands. The three other species studied, C. *nearcticus*, C. *rasilis*, and C. *subbarbatus*, belong in the subgenus *Myrmentoma*, and all have considerably more complex secretions. In spite of the fact that we have analyzed only five species in a genus with more than 600 species (7), it appears that some species have the same major volatile substance, whereas others may have a blend distinctive of the species.

In C. *herculeanus*, it is the male mandibular gland secretion which stimulates the females and induces them to fly off. In contrast, this same secretion of another formicine, *Lasius niger*, elicits an indifferent response from females of its species and, instead, excites the males themselves and causes them to fly off from the nest (1). This gross difference in their behavior may not be surprising as *Camponotus* and *Lasius* are not closely related formicine genera. While heads of males of *L. alienus, L. neoniger* and *Acanthomyops claviger* contain an indole, possibly skatole, which does not occur in the heads of workers, both workers and males of *L. alienus* and *A. claviger* contain ap-

preciable quantities of volatile substances (8). Our investigation of these five *Camponotus* species has shown that it is only the males that produce detectable quantities of volatile substances. In at least the five *Camponotus* species studied these compounds are therefore truly caste-specific. The identification of these novel and caste-specific compounds may provide the means for comprehending the function and significance of these exocrine products in the mandibular glands of many male *Camponotus* species.

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2. Analyses were conducted on an LKB-9000 GC-MS utilizing 10 percent SP-1000 as the stationary phase. Additional gas chromatographic analyses were carried out on a Tracor MT-220 instrument with Carbowax 20M as the stationary phase. In this case, mass spectra of collected samples were obtained on a Bell & Howell 21-490 mass spectrometer.
3. An authentic sample of the free acid was provided by Dr. S. W. Tanenbaum. Columbia University.
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Chemistry of the Venom of *Solenopsis aurea* (Hymenoptera: Formicidae)[1]

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The venom of the fire ant, *Solenopsis invicta* Buren, consists primarily of the *trans*-isomers of a series of dialkylpiperidines (MacConnell et al. 1971) which may be partly responsible for the toxic properties of this secretion. Recently, the venoms of *S. geminata* (F.), *S. xyloni* McCook, and *S. richteri* Forel have also been demonstrated to contain piperidine alkaloids and, significantly, the venom of each species possessed a characteristic alkaloidal "fingerprint" (Brand et al. 1972). There is, in addition to these 4 species of *Solenopsis* (*Solenopsis*), one additional fire ant species in North America. The present report describes the venom chemistry of *S. aurea* Wheeler.

METHODS AND MATERIALS

Workers of *S. aurea* were collected at Copper Canyon, Huachuca Mts., Cochise Co., Ariz.

Venom from worker ants was collected in capillary tubes and stored in *n*-pentane. GLC analyses were undertaken on a 10% Carbowax 20 M column (2 m $\times$ 5 mm; Chromosorb W, H.P., 60/80 mesh) operated at 180°C. Samples for mass spectrometric analysis were adsorbed on graphite after preparative gas chromatographic separations and mass spectra obtained by the direct insertion technique on a Bell and Howell 21-490 instrument (70 eV; probe temperature, ca. 150°).

RESULTS AND DISCUSSION

GLC analyses demonstrated that the venom consisted almost exclusively of 2 compounds with retentions identical to *cis*-2-methyl-6-*n*-undecylpiperidine (*cis* C_{11}) and *trans*-2-methyl-6-*n*-undecylpiperidine (*trans* C_{11}). The identity of these 2 compounds was established unequivo-

cally by the complete congruency of their mass spectra with those of the 2 standard compounds (MacConnell et al. 1971). In addition, the venom contained a trace of a compound with the same retention time as *cis*-2-methyl-6-*n*-tridecylpiperidine.

S. aurea, which was formerly considered a subspecies of *S. xyloni*, was elevated to specific status by Creighton (1950). The taxonomic treatment afforded this species is clearly supported by the chemical composition of its venom, which is very similar to that of *S. xyloni* but differs in one important feature. Both venoms are completely dominated by the *cis* C_{11} and *trans* C_{11} piperidines and in both cases the ratios of these 2 compounds are about 4:1. However, the venom of *S. xyloni* contains a characteristic minor constituent, 2-methyl-6-*n*-undecyl-$\Delta^{1,2}$-piperideine (Brand et al. 1972), a compound which could not be detected in the venom of *S. aurea*.

The venom compositions of each of the 5 North American species of *Solenopsis* (*Solenopsis*) appear to be sufficiently distinct to possess some chemotaxonomic value. The venoms of *S. invicta* and *S. richteri*, which are dominated by piperidines with C_{13} or C_{15} side chains (Brand et al. 1972), are easily distinguished from those of the other 3 species and from each other. *S. xyloni* venom possesses a piperideine which has not been identified in any other *Solenopsis* venoms. Finally, although the venoms of *S. aurea* and *S. geminata* are both dominated by the *cis* C_{11} and *trans* C_{11} piperidines, in the former the average ratio of these 2 compounds in pooled samples is 4:1 whereas in the latter it is about 1.5:1 (Brand et al. 1972). Analyses of the venoms of South American *Solenopsis* species indicate that rather characteristic venom compositions may be the rule among the species in this highly successful myrmicine taxon.

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23

Apparatus for Discontinuous Electrophoresis in Polyacrylamide Gel Slabs

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Construction of an inexpensive slab discontinuous electrophoresis apparatus is described. Using this apparatus 23 human serum proteins were resolved and the gel could be scanned with a standard densitometer to yield a trace with discrete peaks or pronounced shoulders for each protein band. The advantages of a single homogeneous slab for comparative studies are indicated.

A comprehensive account of the instrumentation available for polyacrylamide electrophoresis and its applications has been presented by Gordon (1). Original apparatus for discontinuous electrophoresis by Ornstein (2) and Davis (3) utilized cylindrical gels with concomitant difficulties in scanning. As specialized scanners were unavailable in many laboratories, Petrakis (4) designed an inexpensive scanner capable of tracing such gels.

The alternative of using polyacrylamide slabs resulted in the construction of numerous apparatus each with its own advantages and disadvantages (5–10). Slabs afford the advantage that gel homogeneity allows comparison of a number of samples on a single gel. The problem most commonly encountered with commercially available slab polyacrylamide electrophoresis apparatus is that of sample slot forming. These apparatus make use of a sample slot former which is inserted into the cell and removed after the gel has polymerized around it. In the case of gels with acid or neutral pH as well as low gel concentration the gel is damaged and sample slots unsuitable for use in a discontinuous system result (11). In this paper an alternative method of pressing a sample applicator into the preformed gel is described. The slight indentation of the spacer gel by the applicator does not seriously distort the flat gel surface although it effectively isolates the sample channels from each other. This permits

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the ready use of slab discontinuous systems for all ranges of pH and concentration. The apparatus also has a cooling coil system instead of a jacketed buffer reservoir (11) which allows the use of high voltages without excessive ohmic heating and concomitant gel distortion with consequent loss of resolution. An effective, yet inexpensive flat-slab electrophoretic apparatus is constructed and the technique, which provides a homogeneous medium ideally suited to comparative studies, affords all the advantages of discontinuous electrophoresis without some of the more obvious disadvantages.

METHODS AND MATERIALS

Apparatus[3]

The apparatus is constructed of a 6-mm Perspex sheet and Perspex tube with wall thickness 6 mm and internal diameter of 12 cm, and is made in the sections illustrated in the scale diagram in Fig. 1. The assembled apparatus is presented in Fig. 2.

Method

The method of gel preparation and sample application is essentially that of Davis (3) with the exception that "Cyanogum" 41 (B.D.H.) was used instead of the two acrylamide monomers. Care in preparation of the gel is essential for good results as many distortions are the result of poor preparation (12). After washing and thoroughly rinsing the cells they were rinsed once with a 1/200 solution of a wetting agent (Agepon, Agfa-Geveart) and allowed to air dry. This was done to ensure gentle filming of distilled water used to create a smooth interface between the spacer and running gel (3). The top of the cell was sealed with masking tape after which the cell was inverted and clamped vertically in preparation for gel polymerization. A sucrose spacer (60%) was introduced to a depth of 1 cm, followed by the spacer gel solution (3%) to a total depth of 3 cm. A finely drawn out pasteur pipette was used to layer distilled water above the spacer to ensure a smooth interface between the spacer and running gel. After photopolymerization the water was poured off and the cells thoroughly rinsed with aliquots of the running gel solution (7%). The cells were then filled with running gel solution and sealed with polythene sheet to prevent air inhibition of polymerization. Polymerization was carried out at room temperature but using gel solutions at 0–5°C. The heat of polymerization was air dissipated. After completion of polymerization the masking tape was removed and the sucrose spacer

[3] Further written details of construction may be obtained from the author upon request.
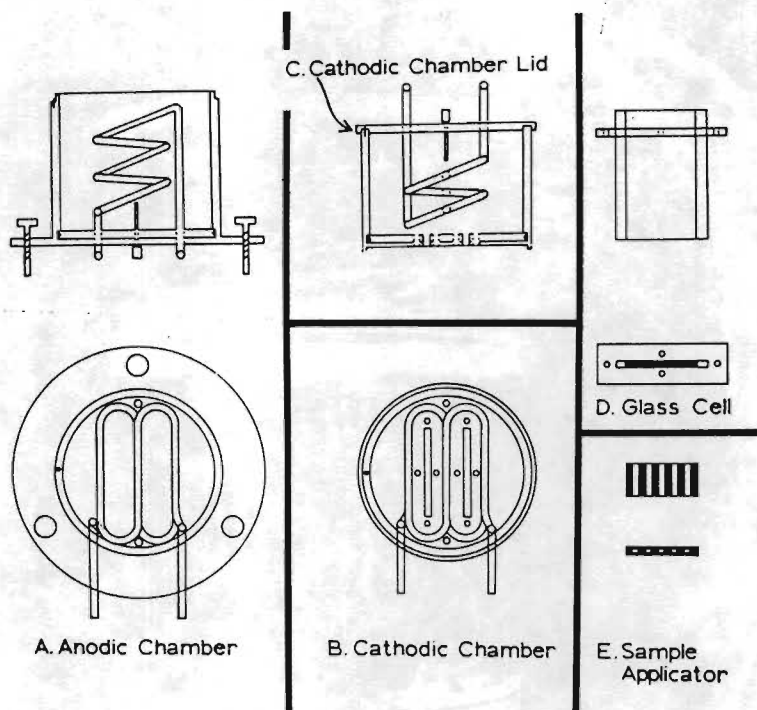
FIG. 1. Constructional plans of slab discontinuous electrophoretic apparatus. (A) The anodic chamber has a double-floored base, leveling screws, cooling coils and a platinum wire electrode centrally situated. (B) The cathodic chamber has a removable floor with centrally placed parallel cell slots and eight threaded holes for cell alignment and sealing. (C) The lid has cooling coils and a platinum wire electrode suspended from it. (D) The glass cells have perspex collars containing four holes each which align with those through the cathodic chamber floor. (E) The sample applicators are made of cellulose acetate sheet and perspex spacers to provide five equal 3-mm sample slots.

rinsed out. The cells were reinverted, screwed into place in the cathode chamber and the apparatus filled with cold Tris/glycine buffer (3). Sample applicators were freed of bubbles and pressed into the spacer gel so that the individual sample slots were isolated from one another. Samples containing 150 μg protein were applied with a Hamilton microsyringe.

Initially only 2 mA per sample slot was applied until the bromphenol blue had migrated into the running gel. The sample applicators were removed and the current increased to 5 mA per sample slot. When the marker was 1 cm from the end of the cell the gels were extracted by gently rimming around them with a syringe.

The gels were stained in an acetic acid solution (7%) of Amido Black 10B (0.1%) for 1 hr. Destaining was effected between filter paper wads between the copper plate electrodes. Complete destaining was achieved in 15 min at 500–1500 mA.
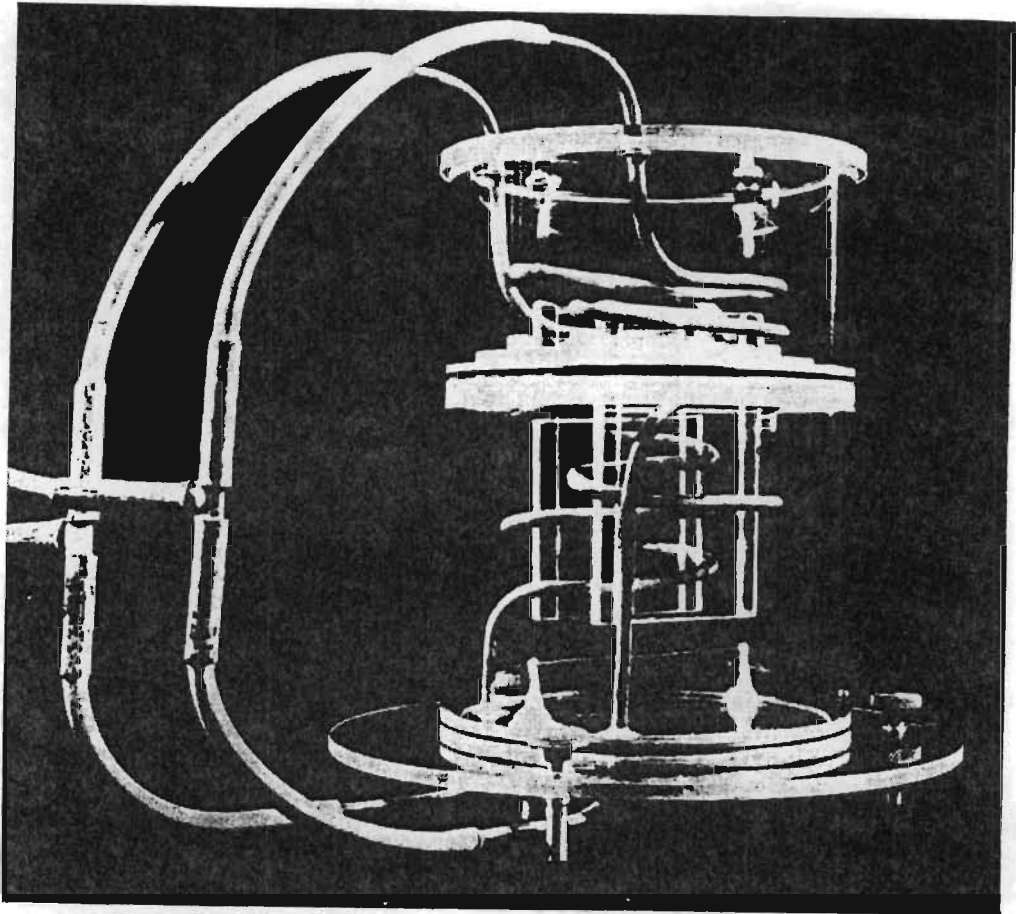
FIG. 2. Slab discontinuous electrophoresis apparatus fully assembled.

The results were recorded by scanning the gel with a Zeiss absorbance meter Model 3 equipped with a red filter.

RESULTS

Pherograms of human serum obtained by Ornstein (2) and the author, respectively, are illustrated by densitometric traces in Fig. 3.

These results serve to illustrate the comparable resolution obtained by the two techniques and scanners. This technique has also been successfully applied to studies of insect hemolymph proteins in relation to taxonomy and ontogeny (13).

DISCUSSION

The problem of obtaining an inexpensive instrumental record of protein separations by a high-resolution electrophoretic technique may be approached in two ways: (a) the use of an inexpensive scanner (4); (b) the design of a slab electrophoretic apparatus which produces gels readily scanned by standard densitometers.

The results on human serum presented in this paper substantiate the
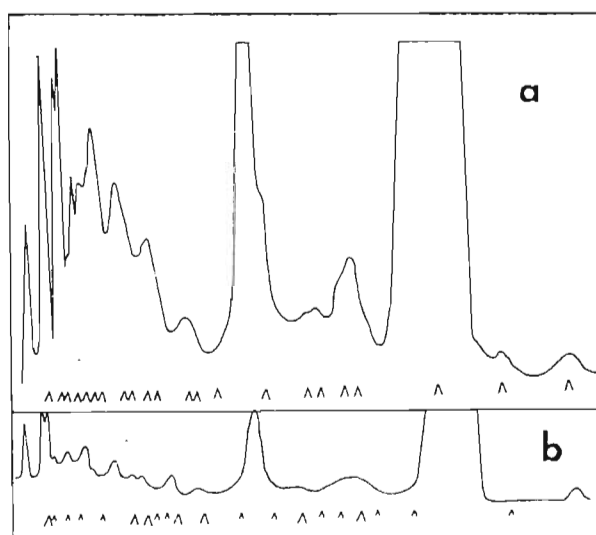
Fig. 3. Densitometric traces of protein separation of human serum from: (a) slab discontinuous electrophoresis: 7 μl on 7% gel 5 mA for 70 min at 5°C, (b) disk electrophoresis: 3 μl on 7% gel (3).

validity of the second approach. The densitometric trace shows all bands as discrete peaks or definite shoulders on major peaks. A further advantage of the slab technique is its applicability to comparative studies where the conditions of separation must be identical. Five samples can be simultaneously resolved on each slab in a homogeneous gel and under identical conditions.

Although the problem of wall adherence has been frequently encountered using gels of other pH or concentrations this problem never arose in the use of the described apparatus under the conditions detailed above. The use of a dilute solution of detergent to permit water filming with resultant flat interfaces between gels did not detrimentally affect adherence.

Gel casting in an inverted vertical cell using a sucrose spacer and distilled water layer ensured parallel surfaces between the sample, spacer gel and running gel which is a prerequisite for a discontinuous system.

The necessary use of materials with different thermal expansion coefficients in the glass cells would appear to restrict the temperature range at which the instrument could be operated. No difficulties were encountered in the range 0–25°C as the epoxy resin (Araldite) used to attach the collar to the cell compensates for expansion.

By virtue of their proximity to the glass cells the cooling coils ensure rapid heat dissipation and limit ohmic heating. Thin glass walls and a favorable surface area to volume ratio also permit the use of high voltages without heating and gel distortion. For further resolution of a multicomponent system (serum or hemolymph) a molecular sieve gradient as de-

scribed by Margolis and Kenrick (14) could be formed in the cell. A theoretical treatment of the factors controlling resolution within such a system are thoroughly discussed [Rodbard, Kapadia, and Chrambach (15)].

APPENDIX: ADDITIONAL DETAILS OF CONSTRUCTION FOR SLAB DISCONTINUOUS ELECTROPHORETIC APPARATUS

(A) *Anodic Chamber*

The anodic chamber is 11 cm deep and has a double-floored base. The inner floor, 12 cm diameter, extends into the chamber to form a seal with a 3-mm "O" ring fitting into a recessed channel in the wall of the reservoir. The lower floor is a 20 cm diameter disk with three leveling screws at the apices of a 15-cm equilateral triangle. The anode is a 8 cm long platinum wire and supported 2 cm above the floor by plugs insulated with epoxy resin. An insulated copper tube (5 mm o.d.) passes through both floors and is coiled so that it passes above the electrode but between and around the cells which project downwards from the cathodic chamber. A variation would be to use a glass coil and so avoid any possible electrical leaks through damage of the insulation. A 3 × 6 mm step cut into the upper rim permits a snug joint with the upper chamber which is orientated by a locating pin in the step.

(B) *Cathodic Chamber*

The cathodic chamber is 8 cm deep and is turned to fit snugly over the stepped rim of the anodic chamber. The removable floor is sealed by a 3 mm "O" ring fitting into a channel 1 cm from the lower edge.

Two centrally placed parallel slots 7 cm × 5 mm, 2.5 cm apart, are cut into the floor which is also provided with eight 4-mm threaded holes located as shown in Fig. 1 (B). The upper rim has a locating pin on one side to permit alignment of the lid.

(C) *Lid*

The lid of diameter 14 cm has a 6 × 3 mm channel turned to fit tightly over the walls of the cathode chamber. The platinum cathode and copper colling coils pass through the lid and are as described in (A). Alternatively glass coils may also be used.

(D) *Cells*

The cells in which the polyacrylamide gels are cast are of 1 mm thick glass. The glass used was soft standard glass obtained from discarded thin layer plates (Merck). It is possible to have one side of the cell free

and to use an O-ring seal in the cathodic chamber floor thereby facilitating removal of the gels after electrophoresis. Each measures 10 cm × 7 cm × 4 mm and has a Perspex collar 9.7 cm × 3.0 cm × 5.0 mm at right angles to the cell and 1.5 cm from one end. The collar has four 4-mm holes through it which align with those in the cathode chamber floor and can be screwed down by nylon screws to form a water-tight seal.

(E) *Sample Applicators*

Two sample applicators are constructed from cellulose acetate sheets 5.7 cm × 0.2 mm divided into five equal 3-mm slots by Perspex strips 3 cm × 5 mm × 2 mm.

The destaining apparatus consists of a circular buffer bath 20 × 7 cm containing two copper plate electrodes 10 cm × 7 cm × 0.5 mm connected to a battery charger producing a maximum current of 2.0 A at 12 V.

ACKNOWLEDGMENTS

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BIOCHEMICAL EVOLUTION IN FIRE ANT VENOMS

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ABSTRACT

The distribution of 2,6-dialkyl(and alkenyl-)piperidine alkaloids in the venom of fire ant workers of *Solenopsis xyloni, S. geminata, S. richteri,* and *S. invicta* has been compared with the occurrence of these compounds in the venom of alate queens of the same species. Whereas the venoms of workers of *S. invicta* and *S. richteri* contain piperidines with C_{13} or C_{15} side chains, the queens of these species produce venoms in which these compounds are essentially lacking. A comparison of the ratio of *cis*-2-methyl-6-*n*-undecyl-piperidine to *trans*-2-methyl-6-*n*-undecylpiperidine in all of these venoms, together with the qualitative differences of other alkaloidal components, particularly in workers of *S. richteri* and *S. invicta*, has led to the proposal that the venoms of *S. xyloni* and *S. geminata* are similar to the ancestral type, whereas those of *S. richteri* and *S. invicta* are more highly evolved.

IT is generally accepted (and certainly true in our experience) that the sting of both imported fire ant species, *Solenopsis invicta* and *S. richteri*, causes more discomfort than the sting of either *S. xyloni* or *S. geminata*, two species indigenous to the southern U.S.A. The difference in the reaction of human beings to the sting of workers of *S. invicta* and *S. xyloni* has been pointed out by Blum, Roberts, and Novak (1961). These stings are similar only during the first few hours, after which time the sting of *S. xyloni* usually causes only mild itching. Blum and others (1961) suggested that these facts strongly indicate that the venoms of these two species of *Solenopsis* differ chemically although they are both basic and contain secondary amines.

An investigation of the chemical nature of the venom of *S. invicta* established the presence of various 2,6-dialkylpiperidine alkaloids (MacConnell, Blum, and Fales, 1971). A comparative analysis of these piperidine alkaloids in the four main North American forms of *Solenopsis* (*S. invicta, S. richteri, S. geminata,* and *S. xyloni*) has shown that the venom components of each species are indeed characteristic of the species (Brand, Blum, Fales, and MacConnell, 1972).

To our knowledge almost all previous studies on hymenopterous venoms have utilized pooled samples of venom. Certainly, previous work on *Solenopsis* venom has been carried out on pooled samples collected from many worker ants. Recently we have refined our experimental techniques in order to analyse the piperidine alkaloids present in the venom of individual ants, and during these studies we have sampled both workers and alate queens of these four species of *Solenopsis*. The results obtained have shown that the venoms of workers of *S. invicta* and *S. richteri* contain alkaloidal components which are not present in their alate queens or in either the workers or alate queens of *S. xyloni* or *S. geminata*. We have used these results to propose a model for the evolutionary development of the various alkaloidal components in the venom of *Solenopsis* species.

MATERIALS AND METHODS

Worker ants and alate queens of *S. xyloni*, *S. geminata*, *S. richteri*, and *S. invicta*, and worker ants only of an unidentified species in the *S. geminata* group from South America (hereafter termed *S.* sp.) were utilized for venom analyses. Venom was collected by either one of two procedures, depending upon whether or not the ant could be milked easily with a capillary. In cases where the yield of venom was low, the poison gland was removed, placed in 10 μl. CS_2 and the extract injected into the gas chromatograph (Carbowax 20 M, 180° C.). Generally, however, ants were milked exhaustively with a 1-μl. capillary, the venom was transferred to a 1-μl. syringe and injected directly into the gas chromatograph.
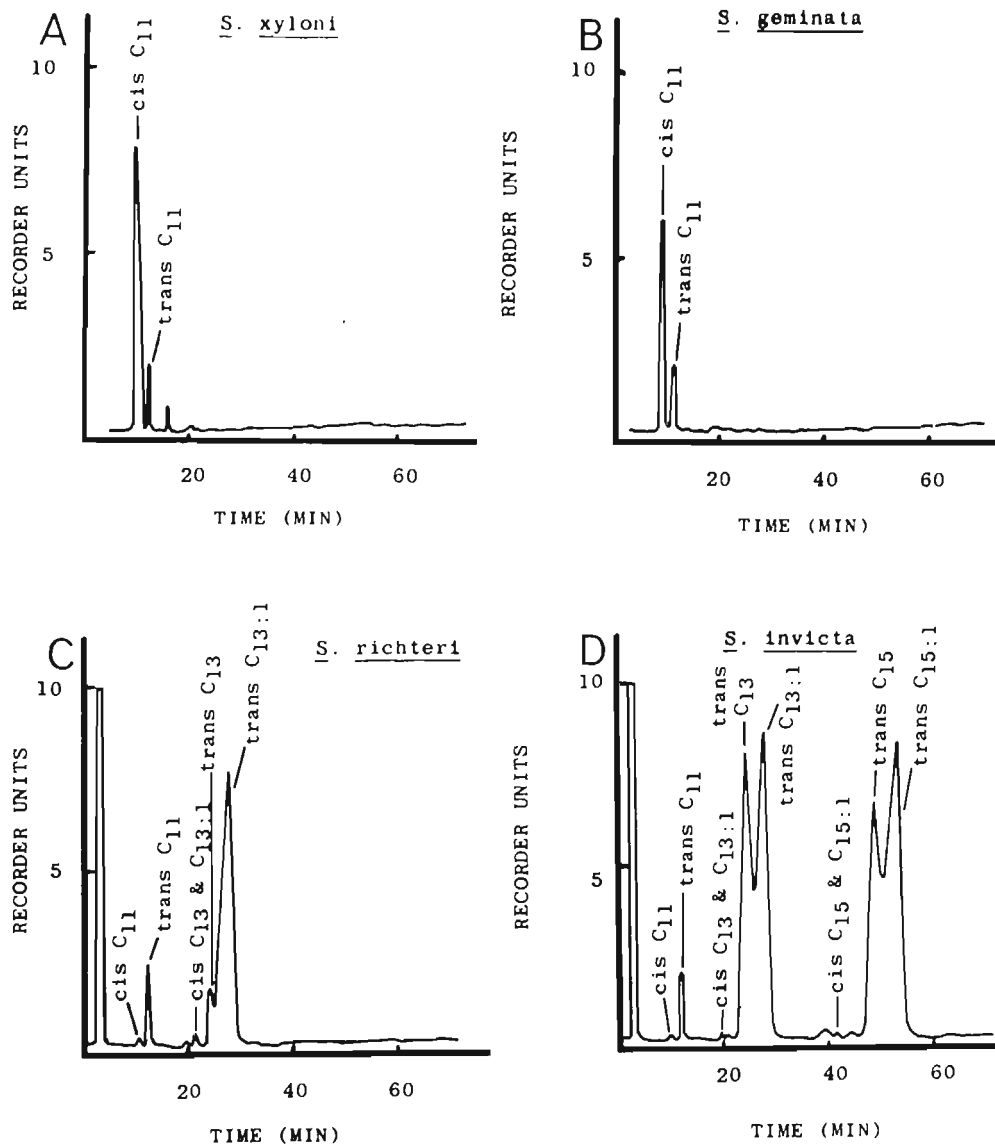
FIG. 1.—Gas chromatograms of the venom alkaloids from worker ants of four species of fire ant.

RESULTS

Typical chromatograms of the major alkaloidal components in the venom of worker ants and alate queens of four species of *Solenopsis* are presented in *Fig.* 1A, B, C, D, and the mean ratios of the two compounds, *cis-* and *trans-*2-methyl-6-*n*-undecylpiperidine, common to both castes of the four species, are presented in *Table I*. A comparison of

Table I.—RATIOS OF *cis*-C_{11} TO *trans*-C_{11} IN THE VENOM OF THE DIFFERENT CASTES OF FOUR SPECIES OF *Solenopsis*.

SPECIES	ALATE QUEENS	WORKERS	SOLDIERS
S. xyloni	17·6:1 (7)	3·97:1 (10)	—
S. geminata	3·36:1 (10)	1·50:1 (40)	4·94:1 (40)
S. richteri	1·86:1 (6)	<0·1:1 (10)	—
S. invicta	3·24:1 (27)	<0·1:1 (10)	—

Numbers in parentheses indicate the number of individuals sampled. Queens were taken from a minimum of 3 colonies per species.

the chemical nature of these piperidine alkaloids in the various venoms indicates a most interesting series of these compounds.

In the venom of workers of *S. xyloni* and *S. geminata* (*Fig.* 1A, B), and in soldiers of *S. geminata*, only C_{11} components* (i.e., *cis-* and *trans-*2-methyl-6-*n*-undecylpiperi-dine) are abundant, while C_{13} components (i.e., *cis-* and *trans-*2-methyl-6-*n*-tridecyl(and tridecenyl-$C_{13:1}$) piperidine) are present in trace amounts. We have found no indica-tions of C_{15} components (i.e., *cis-* and *trans-*2-methyl-6-*n*-pentadecyl(and pentadecenyl-$C_{15:1}$) piperidine) in these venoms. In both of these species *cis*-C_{11} is considerably more abundant than *trans*-C_{11}, and the trace amount of C_{13} present is virtually all *cis*-C_{13} and *cis*-$C_{13:1}$. In contrast to these results, the venom of workers of *S.* sp. contains *trans*-C_{11} as the major component.

Workers of *S. richteri* (*Fig.* 1C) contain both C_{11} and C_{13} components, the latter being considerably more abundant, and the *trans*-form of all components dominating. In this species the *trans*-$C_{13:1}$ is extremely abundant compared with *trans*-C_{13}. In the venom of *S. invicta* (*Fig.* 1D) the C_{11} components are quantitatively small, while both the C_{13} and $C_{13:1}$ components and the C_{15} and $C_{15:1}$ components are abundant. For all three groups of compounds the *trans*-isomers dominate and *cis*-isomers are present only as trace constituents.

The venom of the queens of these four species (*Fig.* 2A, B, C, D) contains essentially only C_{11} components and, in all cases, *cis*-C_{11} is twice or more as abundant as *trans*-C_{11}.

DISCUSSION

The venom characteristics of the workers of these five species form a phenocline suggesting one of two series of evolutionary events, proceeding either from *Fig.* 1D to

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* The terms C_{11}, C_{13}, $C_{13:1}$, C_{15}, and $C_{15:1}$ are employed to indicate the carbon length and the presence or absence of unsaturation in the 6-alkyl substituent on the piperidine ring and do not signify the total number of C-atoms in the molecule. The prefixes *cis-* and *trans-* give the orienta-tion of the 2-methyl and 6-alkyl groups on the piperidine ring.

Fig. 1A or from *Fig.* 1A to *Fig.* 1D. If *Fig.* 1D represents the ancestral condition of the alkaloidal components, then evolution proceeded first by a loss of C_{15} components, then by a drastic reduction of C_{13} components, and finally by a switch from abundant *trans*-C_{11} to abundant *cis*-C_{11}. If *Fig.* 1A represents the ancestral state, then the evolution of the venoms would have begun with a condition in which the venom contained abundant C_{11} and minute traces of C_{13}, with the *cis*-isomers dominating and the *trans*-isomers at a much lower level. The evolutionary steps in this case would have been first a switch from abundant *cis*-C_{11} to abundant *trans*-C_{11}, followed by the evolution of
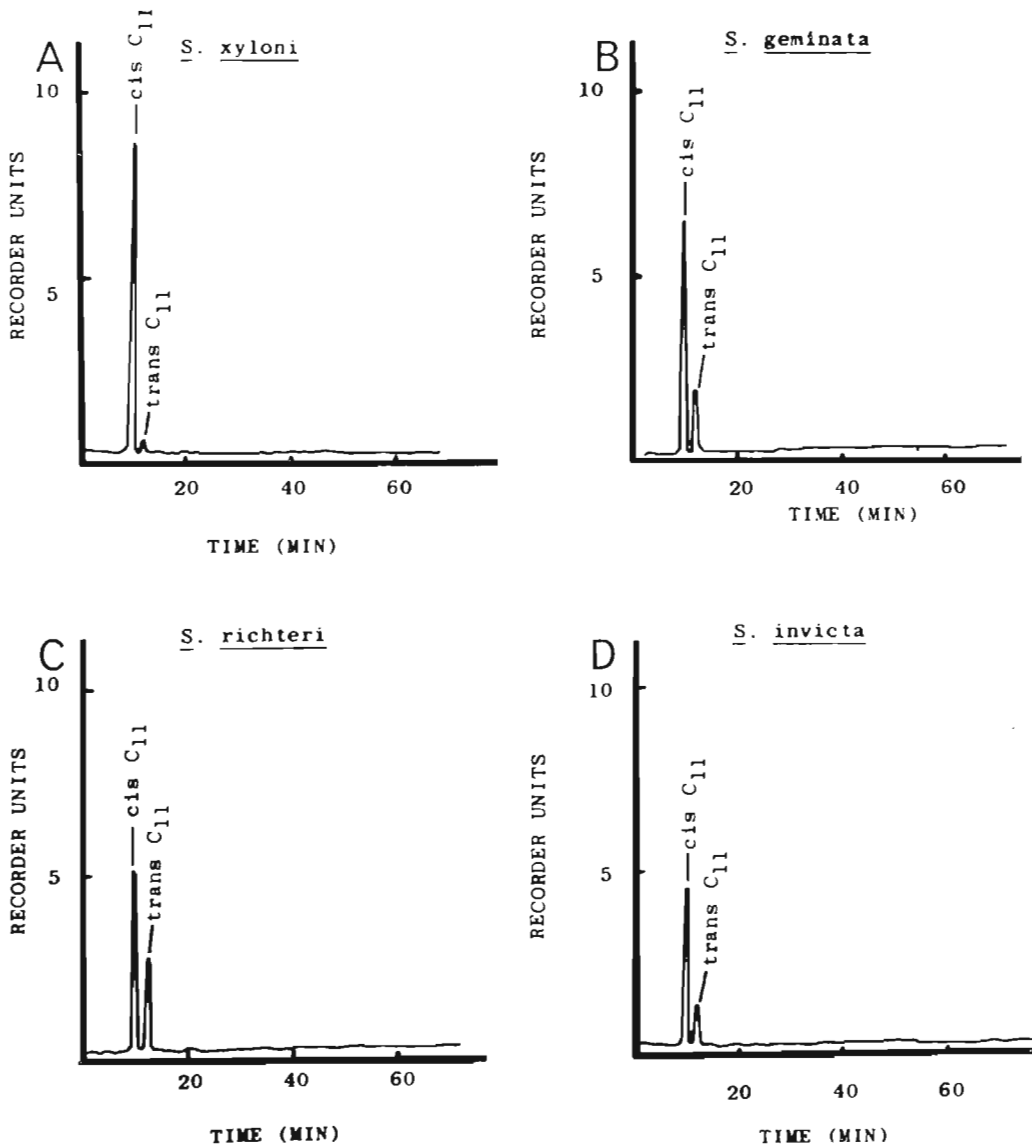
FIG. 2.—Gas chromatograms of the venom alkaloids from alate queens of four species of fire ant.

abundant $trans$-C_{13}, and then the addition of $trans$-C_{15} accompanied by a reduction in the production of $trans$-C_{11}.

If there was available a highly probable phylogeny of the genus $Solenopsis$, the above chemical data could be arranged according to the phylogenetic scheme and the direction of evolution of these alkaloidal compounds established. Unfortunately, no such phylogeny is available and the present diagnoses of the species (Creighton, 1950; Buren, 1972) are based on differences that do not lend themselves readily to deciphering phylogeny. We thought that characters of the male genitalia might shed light on the problem of evolutionary direction, but the four species for which we have males ($S.$ $xyloni$, $S.$ $geminata$, $S.$ $richteri$, and $S.$ $invicta$) have virtually identical structures. It therefore appears that the chemical characters of the venoms are the only ones so far discovered that can give any indication of evolutionary direction.

We wish to emphasize that in deciding which are the ancestral and the derived states of a character, it is most helpful to compare the states in the taxon studied with similar character states in related taxa. This is impossible in the case of the piperidine alkaloids, as they have not been identified in the venoms of ants in any other genus. The question as to whether the alkaloidal complex in either $Fig.$ 1 A or Fig 1 D represents the ancestral state of $Solenopsis$ must therefore be resolved, if possible, from information available on the species examined.

THE PROBABLE ANCESTRAL ALKALOIDAL STATE

Indications concerning the ancestral state of these alkaloids come from two sources: (1) Thermodynamic considerations of the production of cis- and $trans$-isomers; and (2) The relative proportion of cis- and $trans$-isomers in workers and queens.

1. In other stinging ants the venom is largely proteinaceous, whereas in $Solenopsis$ the amount of protein present is extremely small. It appears, therefore, that in $Solenopsis$ there has evolved a new type of venom component as yet found in no other ants, and that this has replaced a more primitive proteinaceous constituent. The evolutionary newness of the alkaloid suggests that an arrangement of the different alkaloidal states in the order of their complexity would mirror the pattern of evolution. On this basis, $S.$ $xyloni$ and $S.$ $geminata$ would appear to be the most primitive as they contain primarily cis-C_{11} and only traces of cis-C_{13}. The chemical synthesis of these 2,6-dialkylpiperidines described by MacConnell and others (1971) gives, at best, a ratio of the cis-isomer to the $trans$-isomer of 6:1. Such a situation occurs in $S.$ $geminata$ and $S.$ $xyloni$; in $S.$ sp., however, cis-C_{11} is greatly reduced and $trans$-C_{11} is the predominant form. This reversal involves a switch from a probable normal production ratio of cis- and $trans$-C_{11} to a thermodynamically highly unfavourable decrease in cis- and an increase in $trans$-isomers. Such a switch would seem to be a derived condition, undoubtedly through the evolution of a new enzymatic system and/or the restriction of an old one. After this switch had occurred, first $trans$-C_{13} then $trans$-C_{15} components were added to the venom arsenal. In this connexion it is appropriate to mention the presence of 2-methyl-6-n-undecyl-$\Delta^{1,2}$-piperidine in the venom of $S.$ $xyloni$ workers (Brand and others, 1972). This compound could be either a precursor of the cis- or $trans$-isomers or an intermediate in the conversion of the one isomer to the other.

2. A comparison of the ratio of cis-C_{11} to $trans$-C_{11} between queens and workers ($Table$ I) brings out a most surprising circumstance. Of the four species for which both workers and queens are available, we found that in the workers of $S.$ $xyloni$ and $S.$

geminata cis-C_{11} was much more abundant than *trans* C_{11}, whereas in workers of *S. richteri* and *S. invicta*, *cis*-C_{11} was at a much lower level than *trans*-C_{11}. In queens of all four species, however, *cis*-C_{11} was always more abundant than *trans*-C_{11}. A possible significance of this difference is suggested by the different behaviour patterns of queens and workers. With extremely few exceptions, when an ant nest is disturbed the workers are the aggressive defenders. The queens (certainly in *Solenopsis*) move as far as possible from the point of disturbance; even if cornered and caught they seldom attempt to sting but rather to evade and hide. With this difference in behaviour there would be a strong positive selection pressure for the production of a more effective venom in the workers, but probably little if any selection pressure for a more potent venom in the queens. It therefore seems reasonable that the more primitive condition was one in which the workers and queens had roughly the same venom composition (as in *S. geminata* and *S. xyloni*), and that increasing differences in venom chemistry between queens and workers indicate evolutionary progression. Along this line of thinking, there is a difference in the effect of the various venoms on human subjects. In our collective experience, stings by *S. invicta* queens and *S. xyloni* workers produce little pain and seldom a discernible pustule or necrotic tissue; stings of *S. invicta* workers produce considerable pain, subsequent irritation, pustule formation, and eventually well-developed necrosis.

One other item of information supports the idea that the venom composition represented in *Fig.*1 A, B is the ancestral state of the alkaloidal characters. Of the species studied, only *S. geminata* has a true soldier caste. As soldiers occur commonly throughout the subfamily Myrmicinae, to which *Solenopsis* belongs, it is highly probable that *S. geminata* represents one of the ancestral species in the genus. This indication of phylogenetic position supports the idea that the venom components of *S. geminata* are also ancestral.

On the basis of the above considerations, we would like to suggest the following model for the evolution of the alkaloidal compounds in *Solenopsis*. In the precursor of the genus, piperidine alkaloids were added to the original proteinaceous venom and later essentially supplanted these proteinaceous elements. The venom of the ancestral species of the genus as we know it contained principally C_{11} compounds and a trace of C_{13} compounds, with a predominance of *cis*-isomers to *trans*-isomers. At this stage the soldier caste was present and the worker, soldier, and queen venoms were essentially similar. The next evolutionary step was the loss of the soldier caste, with little change in worker and queen alkaloidal chemistry. The first significant change in alkaloidal chemistry occurred in the lineage leading to *S.* sp., in which *cis*-C_{11} decreased and *trans*-C_{11} became the abundant compound in the venom. In the next step *trans*-C_{13} and $C_{13:1}$ became the predominant venom components, resulting in an ancestral form much like the venom of *S. richteri*. This ancestor then gave rise to a little-changed lineage represented by *S. richteri*, and a lineage in which *trans*-C_{15} and $C_{15:1}$ were added to the venom, resulting in a species like *S. invicta*. This progression of chemical changes was accompanied by an increase in the effectiveness of the worker's stinging action as a defensive or offensive mechanism.

Although this model appears logical, it is based on only five species in a group of the genus that contains many species for which no biochemical analysis of the venom is available. We feel that the greatest value of this model is in presenting a hypothetical construct in biochemical evolution that invites further investigations and testing. Two obvious questions which remain unanswered are (1) the biosynthetic origin of the

piperidine alkaloids, and (2) the basis of their differential composition in queens and workers of the more highly evolved species.

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Key Word Index: Fire ants, *Solenopsis*, venoms, piperidine alkaloids, biochemical evolution.

FIRE ANT VENOMS: INTRASPECIFIC AND INTERSPECIFIC VARIATION AMONG CASTES AND INDIVIDUALS

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Abstract—The ratio of *cis*-2-methyl-6-*n*-undecylpiperidine (*cis* C_{11}) to *trans*-2-methyl-6-*n*-undecylpiperidine (*trans* C_{11}) in the venom of individual workers and soldiers of *Solenopsis geminata* and in individual alate queens of *S. geminata*, *S. xyloni*, *S. invicta*, and *S. richteri* was estimated. A considerable variation in this ratio was demonstrated between individuals of a particular caste within a species. In spite of considerable individual variation, the various species nevertheless exhibit a fair degree of control of the biosynthetic system for these 2,6-disubstituted piperidine alkaloids. Results obtained on one colony of *S. geminata* soldiers suggested that these individuals may have arisen from at least two fertile queens in the nest.

INTRODUCTION

INVESTIGATIONS on the chemical nature of insect exocrine secretions have been devoted primarily to studies of defensive products and sex attractants, while in social insects such as ants and bees, these investigations have focused on analyses of the products of the mandibular gland, the Dufour's gland, and the poison gland. Alarm pheromones and defensive secretions have almost always been shown to comprise more than one compound, but, due to the small amount of material that can be obtained from a single insect, most studies have used a pooled sample obtained from many individuals. Thus, variations in the relative amounts of the various components secreted by individual insects are virtually unknown.

The venom alkaloids of certain species of ants in the genus *Solenopsis* offer excellent candidate compounds for investigating the variation that exists between the secretions of individuals. The organic phase of these venoms contains 2,6-dialkyl piperidine alkaloids (MacConnell *et al.*, 1970, 1971; Brand *et al.*, 1972b), that are characteristic of the species from which they are obtained. We chose *S. geminata* for the main part of this study as it has the simplest venom of the four species investigated previously (Brand *et al.*, 1972b). The venom of workers and soldiers of this species essentially contains two main components differing only in their stereochemistry. This investigation reports on the relative proportions of *cis*-2-methyl-6-*n*-undecylpiperidine (*cis* C_{11}) to *trans*-2-methyl-6-*n*-undecylpiperidine (*trans* C_{11}) in the venom of workers and soldiers of *S. geminata* and of alate queens of *S. geminata*, *S. xyloni*, *S. invicta* and *S. richteri*. The results illustrate that the relative proportions of these two compounds in the venom may vary considerably between individual ants of the same caste and, particularly, between individuals in different castes.

MATERIALS AND METHODS

Ten medium sized workers and ten soldiers of *S. geminata* were removed from each of

325

four colonies while alate queens were obtained from each of three separate colonies collected at Gainesville, Florida. Alate queens of *S. invicta* were taken from seven colonies collected near Gainesville, Fla., alate queens of *S. richteri* were obtained from four colonies collected at Starkville, Miss., and alate queens of *S. xyloni* were taken from three colonies collected at Athens, Georgia.

All individual ants, with the exception of *S. xyloni* alate queens, were milked exhaustively with a 1 µl capillary tube. The volume of venom obtained from an individual ant is termed the sting volume and was calculated for all workers and soldiers of *S. geminata*. The venom collected from each ant was transferred to a 1 µl syringe and injected directly into the gas chromatograph (Carbowax 20 M, 180°). As the yield of venom from *S. xyloni* alate queens was very small, each poison gland was carefully removed, placed in 10 µl CS_2 and the total soluble extract injected into the gas chromatograph. The relative amount of *cis* C_{11} to *trans* C_{11} was estimated by a comparison of the two peak heights.

RESULTS

The sting volume and the ratio of *cis* C_{11} to *trans* C_{11} in worker and soldier ants from four colonies of *S. geminata* are given in Table 1. These results illustrate that the ratio of *cis* C_{11}

TABLE 1. MEAN STING VOLUME AND MEAN RATIO OF *cis* C_{11} TO *trans* C_{11} IN THE VENOM OF WORKERS AND SOLDIERS OF *S. geminata*

Colony	Caste	Mean sting vol.* (µl)	Mean ratio* (*cis* C_{11}/*trans* C_{11})
1	Worker	0·020±0·014	1·98±0·62
	Soldier	0·072±0·035	4·64±0·98
2	Worker	0·014±0·008	1·43±0·55
	Soldier	0·066±0·043	5·03±1·57
3	Worker	0·016±0·007	0·71±0·16
	Soldier	0·047±0·017	2·89±1·05
4	Worker	0·014±0·007	1·85±0·75
	Soldier	0·074±0·018	7·21±3·13

*All means are calculated from 10 determinations and presented as the mean ±1 S.D.

to *trans* C_{11} is much wider for soldier ants than it is for worker ants. It is also apparent that a considerable variation in the relative proportions of these two stereoisomers exists between members of the same caste within a nest. Soldiers, which are usually larger than the workers, generally yielded considerably more venom than did medium-sized workers.

The head length, excluding the mandibles, for many of the soldier ants in colonies 2, 3 and 4, was measured and plots of head length versus the ratio of *cis* C_{11} to *trans* C_{11} are presented in Figs. 1 and 2. A linear relationship between this ratio and the size of the ant, as estimated from the head length, is obvious for colonies 2 and 3 (see Fig. 1). However, the plot of the values for colony 4 shown in Fig. 2 suggest that, either no correlation between these factors exists, or possibly at least two separate relationships exist.

The ratio of *cis* C_{11} to *trans* C_{11} for alate queens of *S. geminata*, *S. invicta*, *S. richteri* and *S. xyloni*, presented in Table 2 illustrates that considerable variation exists between individuals of this caste. *S. geminata* alate queens give a ratio of these two compounds closer to that of soldiers, rather than that of workers of this species (cf. Tables 1 and 2). The relative proportion of *cis* C_{11} to *trans* C_{11} in workers of *S. invicta* and *S. richteri* is <0·1: 1 (BRAND *et al.*, 1972a). The results in Table 2 for the alate queens of these two
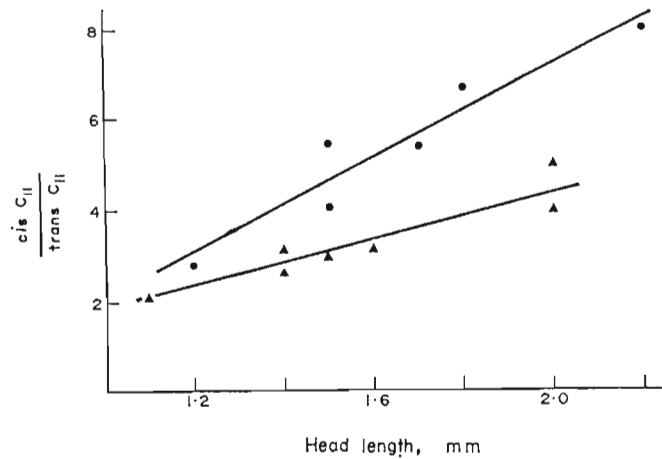
FIG. 1. PLOT OF THE HEAD LENGTH OF *S. geminata* SOLDIERS (COLONY NO. 2 AND NO. 3 FROM
TABLE 1) VERSUS THE RATIO OF *cis* C_{11} TO *trans* C_{11} IN THEIR VENOM.
●—Colony No. 2 showing the line of best fit, $r = 0.957$**;
▲—Colony No. 3 showing the line of best fit, $r = 0.927$**.
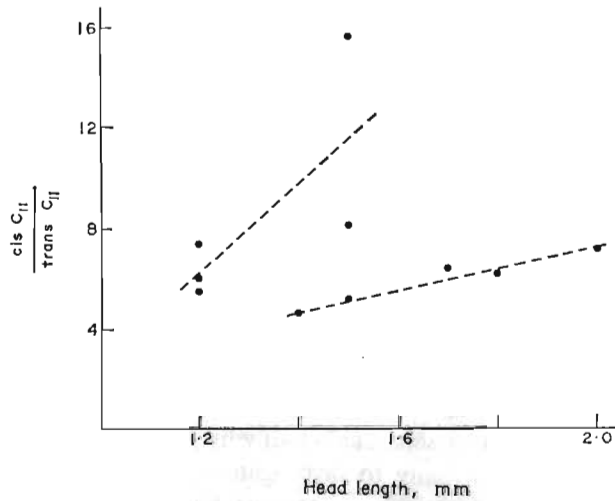
FIG. 2. PLOT OF THE HEAD LENGTH OF *S. geminata* SOLDIERS (COLONY NO. 4 FROM TABLE 1)
VERSUS THE RATIO OF *cis* C_{11} TO *trans* C_{11} IN THEIR VENOM.
Dotted lines represent the lines of best fit if the points are considered as belonging to two
separate groups.

species indicate a drastic switch in this ratio to the situation where *cis* C_{11} is the more
abundant isomer in the venom of alate queens. Results obtained with the few alate queens
of *S. xyloni* sampled indicate that individuals of this caste may have a particularly wide
ratio of *cis* C_{11} to *trans* C_{11}. Workers of this species have been shown to have a ratio of
these two compounds similar to that of workers of *S. geminata* (BRAND *et al.*, 1972a).

DISCUSSION

It seems likely that the efficacy of a secretion, in either a defensive context or its

TABLE 2. MEAN RATIO OF *cis* C_{11} TO *trans* C_{11} IN THE VENOM OF ALATE QUEENS

Species	Colony	No. sampled	Mean ratio $\pm$ 1 S.D. (*cis* C_{11}/*trans* C_{11})
S. geminata	1	5	3·34 $\pm$ 0·28
	2	3	2·53 $\pm$ 0·11
	3	2	3·90
S. invicta	1	3	2·68 $\pm$ 0·36
	2	3	2·54 $\pm$ 0·30
	3	3	3·46 $\pm$ 0·70
	4	3	3·29 $\pm$ 0·18
	5	3	3·46 $\pm$ 0·66
	6	3	4·31 $\pm$ 0·66
	7	9	3·15 $\pm$ 0·34
S. richteri	1	1	3·14
	2	1	1·10
	3	1	1·68
	4	3	1·74 $\pm$ 0·88
S. xyloni	1	3	20·75 $\pm$ 2·44
	2	3	12·37 $\pm$ 4·32
	3	1	19·67

information-bearing content, is often dependent on a mixture of substances. If this is the case, it is reasonable to assume that the relative proportion of the components may, at least in certain instances, be critical. It has been shown that the relative proportions of certain components in a pooled sample of an ant alarm pheromone are characteristic of a species within a genus (CREWE and BLUM, 1970). One intuitively assumes that the composition of a glandular secretion of one insect is either the same or very similar to that of another insect of the same species, especially in the case of a social insect in which the individuals are from the same nest. However, it has recently been pointed out by TRICOT *et al.* (1972) that individual worker ants of *Myrmica rubra*, one of the species studied by CREWE and BLUM (1970), differ appreciably in the ratio of 3-octanol to 3-octanone which is present in their mandibular glands.

Unlike other insect venoms, the secretion of the poison gland of four species in the genus *Solenopsis* is not rich in protein, but is comprised largely of certain 2,6-disubstituted piperidine alkaloids (MACCONNELL *et al.*, 1970; MACCONNELL *et al.*, 1971; BRAND *et al.*, 1972b). As these alkaloids may be separated readily by gas chromatography, these venoms afford us with a unique opportunity to investigate variation between the venom components of individual ants in a particular caste, a nest, or a species.

The species used primarily in this study is *S. geminata*, collected in Gainesville, Florida. This species, which is abundant in the coastal areas of the Southeastern U.S.A., was selected for studies on individual variation of alkaloidal components for two main reasons. Firstly, it is the only species of fire ant in North America which has an easily distinguishable soldier caste, and secondly, both workers and soldiers have only two major alkaloids in their venom that are readily separated and which differ only in their stereochemistry. The venoms of workers of *S. invicta*, *S. richteri* and *S. xyloni* are more complex as they contain other alkaloidal components in addition to the two major alkaloids of *S. geminata* venom (BRAND *et al.*, 1972b).

Analyses of venom milked from forty workers and forty soldiers of *S. geminata* showed that in all cases *cis*- and *trans*-2-methyl-6-*n*-undecylpiperidine were the major alkaloidal constituents. Estimates of the sting volume and the ratio of *cis* C_{11} to *trans* C_{11} listed in

Table 1 indicate that these two factors vary widely between individuals. The soldiers, which are much larger than the workers, yielded a consistently greater sting volume of venom when milked exhaustively. No clear correlation exists between the sting volume and the ratio of *cis* C_{11} to *trans* C_{11}. Also, it is not known how much venom remains in the poison gland after an ant has been milked exhaustively. However, it appears that since no correlation exists between the ratio of the two compounds and the sting volume, a small sample is as representative as a large one.

In the four colonies surveyed, the ratio of *cis* C_{11} to *trans* C_{11} was markedly wider for soldiers than it was for workers from the same nest. The results obtained from colony 3 suggest that the lower the value of this ratio for workers, the lower it will be for soldiers of the same nest. This may imply that the mean ratio of *cis* C_{11} to *trans* C_{11} in a particular nest may be characteristic of the genetic origin of the individuals in that nest, or that this ratio may change with developmental stages, nutritional status, or some unknown factor to which the colony responds.

Numerous analyses conducted in this laboratory on pooled samples of *S. geminata* venom have given a ratio of *cis* C_{11} to *trans* C_{11} of about 3 to 1. We now realize that this ratio was obtained because soldiers and the larger workers are generally chosen for milking as they give a greater yield of venom than smaller individuals. It must be understood therefore that any single value or chromatogram obtained from a pooled sample is a mean of values pertinent only to that sample, the variation within which is unknown.

In addition to calculating the sting volume and the ratio of *cis* C_{11} to *trans* C_{11} in 80 individuals of *S. geminata* (Table 1), the head length excluding the mandibles, was measured for many of these individuals in colonies 2, 3 and 4. A comparison of the results of some of the workers sampled indicated that, in general, the larger the worker's head, the wider the ratio of *cis* C_{11} to *trans* C_{11} in the venom. It therefore appears that the venom of the larger workers is closer in composition to that of soldiers than is the venom of smaller workers, but this result is not firmly established as the number of large workers sampled was not great. We have also noticed a variation in composition of *S. invicta* venom. In this species, small worker ants appear to contain relatively more of the *trans*-2-methyl-6-*n*-tridecenyl-piperidine than *trans*-2-methyl-6-*n*-tridecylpiperidine whereas in the venom of large workers the opposite is true.

The plots in Fig. 1 for many soldiers in colonies 2 and 3 show a high correlation between the head length and the relative proportion of *cis* C_{11} to *trans* C_{11}. This rather remarkable finding suggests that the larger a soldier ant is, the wider will be the ratio between the amounts of these two compounds. Any significance between the size of a soldier ant and the ratio of the major alkaloids in its venom is cryptic. In contrast, any correlation between similar values obtained for colony 4, presented in Fig. 2, is negligible. However, the points in this Figure may follow at least two separate lines as indicated by the dotted lines. In this case the points along the lower line show a high degree of correlation. It would appear therefore, that the individuals sampled from this nest could belong to at least two groups, each group having their own characteristic ratio of venom components in relation to their size. If this nest had two or more functional queens, each of which gave rise to soldier ants, such a situation may well be feasible.

It has recently been shown, that on occasion, *S. geminata* (BANKS *et al.*, 1972) and *S. invicta* (GLANCEY *et al.*, 1972) colonies may contain more than one functional queen. Multiple queens could give rise to a number of genetically distinct populations of worker and soldier ants, thereby explaining the presence of groups of ants with ratios of venom

components related to their size. In the other two colonies that we investigated, such a difference was not observed, suggesting possibly that each of these colonies had a single queen.

The venom of workers of *S. invicta* and *S. richteri* contains mainly *trans* 2,6-disubstituted alkaloids. In these worker ants the *trans*-2-methyl-6-*n*-undecylpiperidine is not the major component (BRAND *et al.*, 1972b) whereas their alate queens contain essentially *cis* C_{11} and *trans* C_{11} as the only alkaloidal components. Furthermore, the alate queens of these two species synthesize mainly *cis* C_{11} in their venom (see Table 2). There is, therefore, a remarkable difference between the venoms of workers and of alate queens of *S. invicta* and *S. richteri* with respect to the major alkaloidal components and to the stereochemistry of these substances. The significance of this difference between two castes of the same species is not clear. However, it is obvious that within a genus the venom composition between castes can either be very similar e.g. *S. geminata*, or widely different e.g. *S. invicta*. The possibility of a physiological difference in the proteinaceous venom of workers and alate queens of the ant, *Paraponera clavata*, has been suggested by WEBER (1939). His personal reaction to the sting of both these castes showed that the worker caused a far more severe reaction than did the alate queen.

One may question whether these differences in venom composition have some significant biological function or merely reflect the genetic origin or latitude of the biosynthetic pathway leading to the formation of *cis* and *trans* isomers. As yet insufficient data are available on the pharmacology of these two isomers, the only studies being those of BUFFKIN and RUSSELL (1972) and JOUVENAZ *et al.* (1972). Such data are needed to establish whether the *cis* and *trans* isomers differ in their toxicities and functional roles in the biology of fire ants. The variation in the relative proportions of the two isomers between individuals suggests a certain biochemical latitude but the fact that two species favor the synthesis of *trans* isomers, while the other two favor the *cis* isomers, indicates a fair degree of control of the biosynthetic system. The presence of 2-methyl-6-*n*-undecyl-$\Delta^{1,2}$-piperideine in the venom of *S. xyloni* workers presents the possibility of this compound being either a precursor of both the *cis* and *trans* C_{11} ring isomers or an intermediate in their interconversion. One can only speculate that this compound may play a key role in determining which isomer will predominate should an enzymatic system be present for converting *cis* ring isomers to *trans* ring isomers.

Variations in the composition of certain glandular secretions have been reported previously. PAIN *et al.* (1967) estimated the amount of 9-oxo-2-decenoic acid in individual heads of queen honeybees and showed that it can vary by at least a factor of ten. REGNIER and WILSON (1969) stated that the minor components of the Dufour's gland secretion of the ant *Lasius alienus* may differ as much as 50 per cent between individuals. Recently, MORGAN and WADHAMS (1972) stated that the volatile constituents of the Dufour's gland of *Myrmica rubra* showed considerable quantitative variation in the components of different samples. While the differences were most noticeable at the colony level, there were also significant differences between individuals of the same colony. In addition, the likelihood of a seasonal variation of certain components in the Dufour's gland of *Anoplolepis custodiens* has been pointed out by SCHREUDER and BRAND (1972). As our method of obtaining venom from a fire ant involves killing the ant, we have no information on whether the composition of the venom of an individual ant varies with the season or with increasing age.

It is evident that the composition of a glandular secretion from certain insects can vary considerably and this investigation has established that the ratio of the major venom

components of genetically similar worker ants from the same nest can also vary widely. Such variations do not seem particularly surprising. If the relative proportions of the components in a glandular secretion can vary from one individual to another of the same species, then the assumption that a particular blend of components may often be important for either species recognition or as an isolating mechanism should be viewed more critically. Extremely careful behavioral work is needed to evaluate whether subtle differences in response can be detected by minor changes in the proportion of the compounds to be tested. As the chemical nature of many insect glandular secretions has been well established, a more thorough understanding of the significance of blends of exocrine products may not be too far off.

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New Alkaloids in the Venoms of Fire Ants[1]

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AND MURRAY S. BLUM[2]

A recent comparative analysis of the venom components of 4 species of fire ants (*Solenopsis geminata* [F], *S. invicta* Buren, *S. richteri* Forel, and *S. xyloni* MacCook) has demonstrated that their alkaloidal compositions may be of some taxonomic utility (Brand et al. 1972). In addition, although the chromatographic patterns may vary quantitatively in detail among individuals in a nest, pooled venom samples of the same species, even from widely separated sites, are very similar. As part of our continuing studies of the alkaloidal components of the venoms of various *Solenopsis* species, we have examined venom samples from a large number of Neotropical species. We plan to compare our gas-liquid-chromatographic (GLC) data for these venom samples with the taxonomy of these same populations as reflected in morphological characters; these chemotaxonomic aspects of this work will be reported later. Generally speaking, the components of all venoms so far investigated exhibit the same molecular weight range as do those from the venom of *S. invicta* (MacConnell et al. 1971); that is, side chains of 11, 13, and 15 carbon atoms at the 6-position of a 2-methyl-substituted piperidine. In this paper, however, we report 2 piperidine alkaloids of lower molecular weight as the sole components in the venom of a *Solenopsis* population (new species to be described elsewhere) from Brazil, and as minor constituents in those of alate females of both *S. geminata* and *S. richteri* from the United States.

S. geminata was collected in Gainesville, FL; *S. richteri* was collected near Starkville, MS. The Brazilian sample came from the area of Corumbá, Mato Grosso State. Venom was collected from the ants as previously described (Blum et al., 1958) and was subjected to GLC analysis on nonpolar (OV-1) and moderately polar (Carbowax 20M or FFAP) stationary phases. For the compounds of interest, column temperatures of about 160° were appropriate on these stationary phases.

In the chromatogram of the venom from alate females of *S. geminata*, a small peak (about 0.2% of the total peak area) is observed eluting before the major *cis* and *trans* C_{11} (that is, 11 carbon atoms in the 6-alkyl side chain) venom peaks. If the *cis* and *trans* isomers of the major C_{11}, minor C_{13}, and trace C_{15} components are used as internal standards, a plot of log retention time vs. carbon number for an isothermal run on either OV-1 or Carbowax 20M indicates that the retention time of this 1st trace peak is that expected of *cis* C_9 (*cis*-2-methyl-6-*n*-nonylpiperidine). The same is true for the first 2 small peaks observed from the venom of alate females of *S. richteri;* here, however, both *cis* and *trans* C_9 are observed. The 1st peak, *cis* C_9, comprises about 1% of the total peak area, and *trans* C_9 about 0.8%. The venom sample from Brazil, designated Corumbá MT #9, has large amounts of the C_9 alkaloids only. It consists of about 15% *cis* C_9 and 85% *trans* C_9.

To provide further support for these tentative GLC identifications, mass spectra were obtained for the C_9 compounds in the venoms of *S. richteri* alate females and of the Corumbá MT #9 sample. The mass spectra of the *cis* and *trans* isomers are indistinguishable, as was anticipated (MacConnell et al. 1971). The natural materials exhibit a base peak at m/e 98, a molecular ion (M-1) at m/e 224, and significant peaks at M-15, M-29, M-43, etc., representing cleavage of alkyl fragments with retention of charge on the nitrogen-bearing moiety. In all respects, the spectra are fully in accord with our expectations.

The mixed C_9 isomers were prepared synthetically by the same route as described earlier for the higher homo-

logues (MacConnell et al. 1971). Satisfactory analytical data were obtained for both the intermediate 2-methyl-6-*n*-nonylpyridine (bp, 146–150° at 14 mm) and for the sodium-in-ethanol reduction product mixture of *cis*- and *trans*-2-methyl-6-*n*-nonylpiperidine (bp, ca. 140° at 10 mm). The mass spectral data for the synthetic compounds are congruent with those of the natural alkaloids, as are the GLC retention data on both the OV-1 and Carbowax 20M columns.

In connection with other work, we have steam-distilled whole colonies of *S. richteri* ants. Among the volatile products we have found very small traces of compounds that by GLC retention times and mass spectra (base peak at m/e 98, parent [M-1] at 196) appear to be C_7 alkaloids. Close inspection of the chromatograms of the venom from the alate females indicates that very small traces of both *cis* and *trans* C_7 alkaloids may be present in this caste's venom, accounting for its presence in the extract prepared from whole colonies. Because of the small amounts involved, this identification is not nearly as certain as that of the C_9 alkaloids.

According to Dr. W. F. Buren (personal communication), this Corumbá species is an aberrant member of the *Solenopsis saevissima* complex. The significance of a worker caste venom consisting solely of these shorter-chain alkaloids is not at all clear. The GLC patterns of the venom alkaloids of the workers and alate females of 4 *Solenopsis* species have recently been used by Brand et al. (1973) to construct a hypothetical model for the evolution of these compounds in this genus. In short, the ancestral venom was proposed to have been the simplest, consisting primarily of the C_{11} isomers, *cis* predominating over *trans*. One evolutionary possibility is that descendants of this ancestor became able to synthesize *trans* C_{11};

the descendants of this latter ant may then have diverged in 2 biosynthetic directions, one favoring *trans* piperidines of higher molecular weight (C_{13} and C_{16}), the other favoring *trans* piperidines of lower molecular weight (C_9 as in Corumbá).

Brand et al. (1973) also found that the venoms of alate females (of the 4 species studied) are remarkably similar, consisting of varying mixtures of *cis* and *trans* C_{11} with the *cis* predominating. Because of the unusual composition of the venom of these worker ants from Corumbá, it would be particularly interesting to examine the venom of alate females of this population.

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Monoterpene Hydrocarbons in the Poison Gland Secretion of the Ant *Myrmicaria natalensis* (Hymenoptera: Formicidae)[1]

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The occurrence of the monoterpene hydrocarbons, *d*-limonene and *l*-limonene, in the poison gland secretion of *Myrmicaria natalensis* F. Smith (Grünanger et al. 1960) has made this ant unique among the Formicidae. No other ant species so far investigated has been reported to contain any monoterpene hydrocarbons in an exocrine secretion. In a brief study to determine whether *M. natalensis* collected in Natal, South Africa, also contained limonene, we found that the poison gland secretion of this species contained a number of additional terpenoid constituents. This report concerns the identification of these other components as additional monoterpene hydrocarbons.

Mature workers were collected at Selsdon Park, Margate, South Africa. During collection, the ants would often discharge their poison gland contents, while, at the same time, biting any foreign object with which they came in contact. At this stage, a strong turpentine-like odor was apparent. Pentane extracts were made either from whole ants or from excised poison glands. Gas chromatograms of extracts of excised poison glands on Carbowax 20 M (80°–200° at 5° per min) established that, while limonene was the major component, a number

of other low-boiling components were also present. Pentane extracts of whole ants gave similar chromatographic patterns in the low-boiling range and were therefore used for the subsequent analyses of the substances.

A comparison of the retention times of these compounds on both Carbowax 20 M and SE-30 with authentic α-pinene and β-pinene and published retention data of other terpenes strongly indicated the presence of these two hydrocarbon terpenes as well as β-myrcene, α-phellandrene, and terpinolene. In order to identify the various minor components with certainty, the extract was chromatographed in a LKB-9000 gas chromatograph-mass spectrometer on a 12-ft SP-1000 column temperature-programmed from 70°–200° at 5° per min and the mass spectrum of each peak recorded. Both the mass spectra and retention times of the *Myrmicaria* volatiles were completely congruent with those of the standard compounds. The chromatogram and the identities of the various peaks are presented in Fig. 1.

The poison gland secretions of ants, with the exception of the formic acid-rich venoms of formicines, are generally proteinaceous (Blum 1966). However, the identification of small nitrogen-containing compounds, as well as proteins, in the venoms of the myrmicines *Atta texana* (Buckley) (Tumlinson et al. 1971) and *Monomorium pharaonis* L. (Ritter et al. 1973) has been reported. In addition, certain species of *Solenopsis* have venoms dominated by 2,6-dialkyl piperidines (MacConnell et al. 1971, Brand et al. 1972). Therefore, the monoterpene hydrocarbon-rich secretion produced by the poison gland of *M. natalensis* appears to constitute a distinctly unusual myrmicine venom.

The frontal gland secretions of soldier termites of several species in the Termitidae contain various combinations of α-pinene, β-pinene, limonene, terpinolene, α-phellandrene, and myrcene (Moore 1964, 1968). We now have evidence that these same compounds, together with
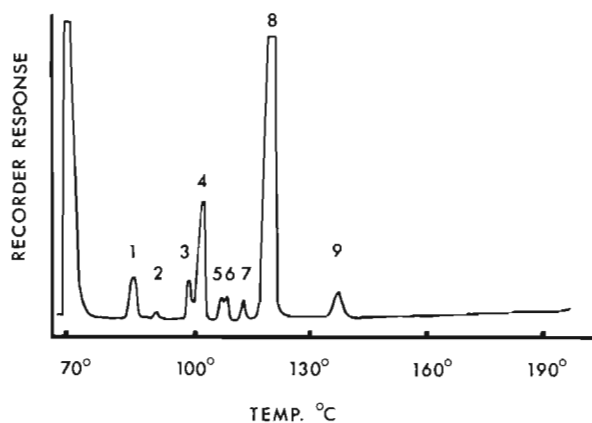
FIG. 1.—Gas chromatogram of a pentane extract of *Myrmicaria natalensis* (10% SP-1000 as stationary phase). The identities of the peaks are: 1, α-pinene; 2, camphene; 3, β-pinene; 4, sabinene; 5, β-myrcene; 6, α-phellandrene; 7, α-terpinene; 8, limonene; 9, terpinolene.

additional terpene hydrocarbons, are also used in a defensive context by *M. natalensis*. To our knowledge the terpenes, camphene, sabinene, and α-terpinene, are not known from animal sources although these compounds have been identified in the essential oils of several plant species. Thus, the chemical defense arsenal of *M. natalensis*, which is dominated by monocyclic and bicyclic monoterpenes with the exception of β-myrcene, easily rivals that of termitid soldiers in terms of its qualitative complexity.

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Guaicol in the Defensive Secretions of Polydesmid Millipedes[1,2]

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The presence of benzaldehyde and hydrogen cyanide in the secretion of the polydesmid millipede, *Oxidus gracilis* (Koch) (Family: Paradoxosomatidae), was demonstrated as early as 1882 (Guldensteeden-Egeling 1882). Since that time these 2 compounds have been

Table 1.—Phenolic concomitants of the benzaldehyde-rich defensive secretions of polydesmid millipedes.

Family	Species	Compounds	Authority
Euryuridae	*Euryurus leachii*	guaicol	This paper
	Euryurus australis	guaicol	This paper
Paradoxo-somatidae	*Orthomorpha coarctata*	guaicol, phenol	Monteiro, 1961,[a] this paper
	Oxidus gracilis	phenol	Blum et al 1973

[a] Phenols identified in total extracts.

identified in defensive secretions from a number of polydesmid millipedes (Blum and Woodring 1962, Eisner et al. 1963a, Casnati et al. 1963) and they appear to be characteristic of species in this taxon. We wish to report the presence of certain phenolic compounds as minor constituents in the benzaldehyde-rich secretions of the polydesmids *Euryurus leachii* (Gray), and *E. australis* Boll. (Euryuridae), as well as *Orthomorpha coarctata* (De Saussure) (Paradoxosomatidae).

Individuals of *E. leachii* and *E. australis* were collected in Lafollette, Tenn., and Athens, Ga., respectively, whereas specimens of *O. coarctata* were collected in Cuiaba, Brazil. During the collection of all species it was noted that their secretions possessed distinctive phenolic odors. The defensive secretions were absorbed on filter paper squares after lightly tapping the ozopores on the diplosegments with a glass rod. The paper squares obtained from each species were extracted with *n*-pentane and the concentrated extracts were used for all subsequent analyses.

Temperature-programmed GLC analyses (10% Carbowax 20 M on Chromosorb W, H.P., 60/80 mesh; 6 ft. × 0.25 in., 80–180° at 5°/min.) of *E. leachii* and *E. australis* extracts indicated the presence of a major and a minor component. These 2 substances were adsorbed directly on powered graphite during elution from the column and their mass spectra were obtained by direct insertion in the probe of a DuPont 21-490 mass spectrometer. The mass spectrum of the 1st and major component exhibited a molecular ion at m/e 106 with major peaks at m/e 105 and 77, while that of the minor component gave a molecular ion at m/e 124 with major peaks at m/e 109 and 81. Both of these mass spectra as well as the retention times of the 2 compounds, were identical to those obtained from authentic samples of benzaldehyde and guaicol (*o*-methoxyphenol), respectively. GLC analyses indicated that in both species guaicol represented 1–2% of the amount of benzaldehyde. GLC analysis of the *O. coarctata* extract indicated the presence of the same 2 compounds together with an additional minor component. The retention time of this additional peak matched that of authentic phenol.

In order to assess the efficiency of their defensive secretions, all 3 species of millipedes were placed separately into laboratory colonies of the fire ant, *Solenopsis richteri* Forel. On each encounter with an ant, the millipedes would discharge the secretion from the regions stimulated and even after intensive molestation the diplopods effectively repelled the ants for some minutes.

Our present knowledge of the various phenolic substances in polydesmid defensive secretions is summarized in Table 1.

All polydesmid secretions so far investigated contain benzaldehyde as a major component (Casnati et al. 1963) and it is presumed that this aromatic aldehyde is generated either from a stored form of mandelonitrile (Eisner et al. 1963b) or from phenylalanine (Towers et al. 1972). The phenolic compounds *p*-cresol and *o*-cresol have been obtained as major components from the defensive secretions of species in other orders, namely the chordeumoid millipede, *Abacion magnum* (Loomis) (Eisner et al 1963c), and the parajulid millipede, *Oriulus deles* Chamberlin (Kluge and Eisner 1971), respectively. The latter compound occurs together with a quinone, but benzaldehyde does not occur in either secretion.

While hypothetical metabolic pathways can be proposed to indicate a fairly close metabolic relationship between the phenols and other aromatic compounds, no biosynthetic studies have been undertaken to verify these relationships in millipedes. The presence of minor quantities of phenolic compounds in certain polydesmid secretions is obviously not fortuitous and undoubtedly adds to the potency of these defensive exudates. However, the ecological significance of polydesmid secretions which are fortified with phenols has yet to be determined.

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SOURCE OF OXYGEN IN THE CONVERSION OF 2-TRIDECANONE TO UNDECYL ACETATE BY *PSEUDOMONAS CEPACIA* AND *NOCARDIA* SP.

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Summary

Cell-free extracts from *Pseudomonas cepacia* and *Nocardia* sp. when supplied with NADPH accumulated undecyl acetate from 2-tridecanone in the presence of an esterase inhibitor, tetraethyl pyrophosphate. Mass spectra of undecyl acetate formed in an $^{16}O_2$-atmosphere exhibited characteristic oxygen-containing ions of m/e 61, 73, and 116. In experiments with $^{18}O_2$, these ions appeared at m/e 63, 75, and 118. Absence of a significant peak at m/e 45 in the ^{18}O-containing ester established that the ^{18}O was in the C—O—C linkage rather than the carbonyl oxygen. Incorporation of molecular oxygen in the presence of NADPH into 2-tridecanone to form undecyl acetate is consistent with an oxygenase-type reaction.

Introduction

Pseudomonas cepacia is capable of growing on 2-tridecanone as its sole carbon source [1] and a degradative pathway for this long chain ketone has been proposed suggesting its direct oxidation to undecyl acetate [2,3]. Hydrolysis of this ester to undecanol and acetic acid is carried out by an inducible esterase [4,5]. A species of *Arthrobacter* [6], now reclassified as *Nocardia* [7], has also been shown to produce the ester intermediates, ethyl acetate and undecyl acetate, when grown on 2-butanone and 2-tridecanone, respectively. A similar formation of an ester directly from a methyl ketone has been reported by Rahim and Sih [8] who demonstrated the fungal conversion of progesterone to testosterone acetate followed by cleavage to testosterone and acetate. This oxidation was mediated by a NADPH-dependent oxygenase. These enzymatic oxidations of methyl ketones to acetate esters may be considered analogous to the Baeyer—Villiger oxidation of ketones by peracids [9,10].

The purpose of this report was to ascertain by $^{18}O_2$ studies if an oxy-

genase reaction was involved in the bacterial conversion of the methyl ketone to an acetate ester.

Materials and Methods

Cultures of *Pseudomonas cepacia* were harvested after 60 h growth at 30°C in basal salts medium [1] supplemented with 0.4% 2-tridecanone (Chemical Samples Co.) as the sole carbon source. The species of *Nocardia* LSU 169, obtained from F.W. Forney, University of Southwestern Louisiana, Lafayette Louisiana, was grown for 48 h at 30°C in the same basal salts medium with 0.4% 2-butanone as the sole carbon source.

Cell-free extracts of *Pseudomonas* and *Nocardia* were prepared by sonication of 25% (w/v) cell suspension in 0.05 M potassium phosphate buffer, pH 7.5. Unbroken cells and debris were removed by centrifugation for 20 min at 20 000 $\times$ g.

Erlenmeyer flasks (27 ml total volume), fitted with serum stoppers, were used as reaction vessels. Each reaction mixture contained *Pseudomonas* extract (52 mg protein) or *Nocardia* extract (25 mg protein), 50 μmoles 2-tridecanone as substrate, 40 μmoles KCN, 10 μmoles NADPH (Sigma Chemical Co.), and 165 μmoles potassium phosphate in a total volume of 5 ml at pH 7.5. To allow for accumulation of undecyl acetate during incubation, an esterase inhibitor, tetraethyl pyrophosphate (TEPP), was added at a final concentration of $4 \cdot 10^{-4}$ M to some vessels. Vessels to be enriched with $^{18}O_2$ were flushed with N_2 gas to remove the majority of $^{16}O_2$. Five ml of $^{18}O_2$ (98.3%, Miles Laboratories Inc.) were injected with a Hamilton gas-tight syringe into the sealed vessels to give a concentration of approximately 18% $^{18}O_2$. Reactions were started by adding NADPH to the flasks. After incubation at 30°C with constant shaking for 45 min, 0.5 ml 20% $ZnCl_2$ was added to stop the reaction. The reaction mixtures were extracted with 6 ml pentane to recover long-chain products and unused substrate, acidified, and reextracted with 6 ml diethyl ether to recover acetic acid. All extracts were dried over sodium sulfate and reduced in volume to approximately 0.2 ml under a stream of N_2 gas. 2-Tridecanone, undecyl acetate, and 1-undecanol, 80 μl of each in separate vessels, were shaken with $H_2^{18}O$ (12.1% ^{18}O) and 0.05 M potassium phosphate buffer, pH 7.5, for 12 h and the compounds were recovered as above.

The substrate and the various reaction products were separated gas chromatographically on either 10% FFAP (14 ft $\times$ 1/8 inch; 190°C) or 10% Carbowax 20 M (15 ft $\times$ 1/4 inch; 145°C). Collection of eluting compounds was achieved by direct adsorption on powdered graphite and the mass spectrum of each compound was obtained by the direct insertion technique on a Hitachi—Perkin Elmer RMU-6 mass spectrometer. Mass spectra of some major compounds as well as the trace amount of methyl laurate formed in the TEPP-inhibited reactions were also obtained on a LKB-9000 GC-MS with 10% SP-1000 as the stationary phase. All mass spectra were obtained at an ionizing voltage of 70 eV.

Results and Discussion

Pertinent regions of the mass spectra of undecyl acetate formed from
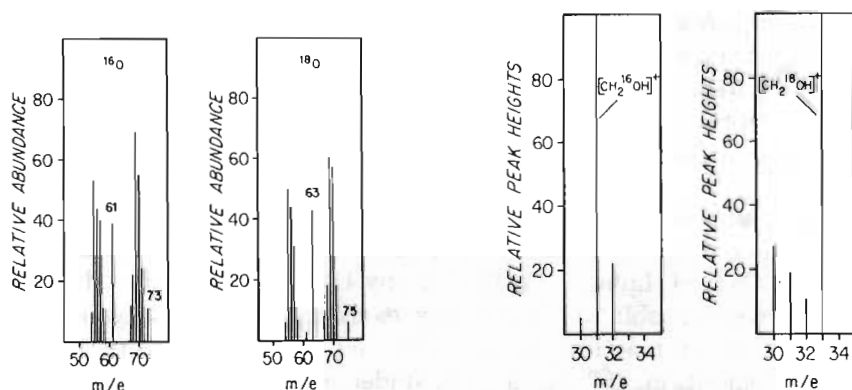
Fig. 1. Partial mass spectra of undecyl acetate formed from the enzymatic conversion of 2-tridecanone in an $^{16}O_2$ atmosphere and in an $^{18}O_2$-enriched atmosphere.

Fig. 2. Partial mass spectra of 1-undecanol recovered from reaction mixtures containing 2-tridecanone and no esterase inhibitor (TEPP) in an $^{16}O_2$ atmosphere and in an $^{18}O_2$-enriched atmosphere.

2-tridecanone by incubation with cell-free extracts of *P. cepacia* in $^{16}O_2$ and $^{18}O_2$ atmospheres are presented in Fig. 1. The oxygen-containing ion of undecyl [^{16}O] acetate at m/e 61 $[CH_3COOH + H]^+$ appears at m/e 63 in the undecyl acetate formed in an atmosphere of $^{18}O_2$, confirming the presence of ^{18}O in this ester. The ratio of these ions in the spectrum of undecyl [^{18}O] acetate indicates a 92% incorporation of ^{18}O. In addition, other oxygen-containing ions of undecyl [^{16}O] acetate at m/e 73 and 116 appeared at m/e 75 and 118 in the spectrum of undecyl [^{18}O] acetate. The absence of a peak in the ^{18}O-containing ester at m/e 45, corresponding to $[CH_3C^{18}O]^+$, established that the incorporated ^{18}O occurs only in the C—O—C oxygen. Undecyl acetate was hydrolyzed rapidly to undecanol and acetate in reactions which did not contain the esterase inhibitor, TEPP, and was not detectable by gas—liquid chromatography in these reactions.

The partial mass spectra of undecan[^{16}O]ol and undecan[^{18}O]ol in which TEPP was omitted are given in Fig. 2. The oxygen-containing ion of undecan[^{16}O]ol at m/e 31 appears at m/e 33 in the undecanol produced in an atmosphere of $^{18}O_2$ demonstrating that approximately 84% of the undecanol present contained ^{18}O. Ninety-two per cent incorporation, as observed in the undecyl [^{18}O] acetate, was not obtained since a small amount of undecan-[^{16}O]ol from prior growth on 2-tridecanone was present in the lysates. In addition, the undecanol obtained from $^{18}O_2$-enriched reaction vessels with TEPP present showed a lower, yet significant, incorporation of ^{18}O indicating that the esterase was not completely inhibited by TEPP. It may be concluded from the lower percentage of ^{18}O in undecanol, compared to undecyl [^{18}O]-acetate, that undecyl acetate was formed from the incorporation of an oxygen atom into 2-tridecanone and not from the prior formation of undecanol followed by esterification with acetate.

In unfractionated cell-free extracts supplied with $^{18}O_2$, the possibility exists that some $^{18}O_2$ may be reduced to $H_2^{18}O$ via respiration. If a monooxygenase was operating on 2-tridecanone, $H_2^{18}O$ would be formed during the

reaction. Cohn and Urey [11] demonstrated significant oxygen exchange reactions between $H_2^{18}O$ and acetic acid and acetone. The following mass spectral data were obtained to ascertain if our results were due to exchange with $H_2^{18}O$. Mass spectra of unused substrate (2-tridecanone) recovered from $^{18}O_2$-experiments indicated that no ^{18}O-exchange had occurred with the ketone. Mass spectra of acetic acid extracted from uninhibited reactions in an atmosphere of $^{18}O_2$ did not show any ions at m/e 47 and 62 as would be expected if it contained one atom of $^{18}O_2$. This finding verified that the ^{18}O in undecyl acetate was in the C—O—C linkage. Further, if any $H_2^{18}O$ was produced during the reaction we were unable to detect any exchange with acetic acid. The addition of KCN to the reaction mixture reduced the possibility of $H_2^{18}O$ formation from respiration. 2-Tridecanone, undecyl acetate, and 1-undecanol recovered after shaking with $H_2^{18}O$ for 24 h showed no ^{18}O exchange.

Chromatograms of extracts of TEPP-inhibited reactions showed the presence of a trace component eluting at the same retention time as methyl laurate. The presence of this ester was confirmed in extracts obtained after incubation in an $^{16}O_2$-atmosphere by mass spectrometry on the LKB-9000 instrument. Insufficient material was available to obtain a good mass spectrum from $^{18}O_2$ incubations but peaks at m/e 76 and 89, and not m/e 74 and 87, were apparent in spectra at the correct retention time, suggesting the possibility of ^{18}O-incorporation in this ester. An unsymmetrical ketone, such as 2-tridecanone, can theoretically give two different esters depending on whether the alkyl or the methyl group migrates to the entering oxygen atom. Tentative identification of methyl laurate indicates that some methyl migration may be occurring.

The oxidation of 2-butanone by *Nocardia* LSU 169 has been reported to occur by way of an ester intermediate, ethyl acetate, which is hydrolyzed to ethanol and acetate [6]. Cell-free extracts of *Nocardia* grown on 2-butanone are also capable of oxidizing 2-tridecanone. In this case, however, the esterase is not inhibited by TEPP or other common esterase inhibitors, and, therefore, no undecyl acetate accumulated in the reaction vessels. The mass spectrum of undecanol formed from the oxidation of 2-tridecanone by *Nocardia* lysates in $^{18}O_2$-atmospheres revealed 87% incorporation of ^{18}O.

This study demonstrates that molecular oxygen is the source of oxygen used for the oxygenation reaction in the enzymatic conversion of 2-tridecanone to undecyl acetate:
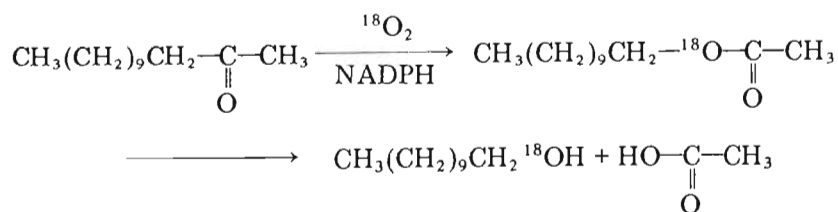
$$CH_3(CH_2)_9CH_2-\underset{\underset{O}{\|}}{C}-CH_3 \xrightarrow[\text{NADPH}]{^{18}O_2} CH_3(CH_2)_9CH_2-^{18}O-\underset{\underset{O}{\|}}{C}-CH_3$$

$$\longrightarrow CH_3(CH_2)_9CH_2\,^{18}OH + HO-\underset{\underset{O}{\|}}{C}-CH_3$$

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Production of verbenol pheromone by a bacterium isolated from bark beetles

THE aggregation pheromones of the bark beetle, *Ips paraconfusus* Lanier[1] are expelled in the faecal pellets of male beetles feeding on the phloem of *Pinus ponderosa*[2,3]. These substances, *cis*-verbenol, ipsenol, and ipsdienol[1], seem to originate in the hindgut[5] but the precise site of their biosynthesis has not been determined. In various species of *Ips* a connection has been convincingly demonstrated between the production of these pheromones in the hindgut and either ingestion of phloem or exposure of the beetles to host plant oleoresin[6]. More specifically, exposure of individuals of certain species of *Ips* to myrcene can result in an increased production of ipsenol and ipsdienol[7] and exposure of certain species of *Dendroctonus* to α-pinene results in an increased production of *cis*- and *trans*-verbenol[8-10] in the hindgut. It seems therefore that a precursor-product relationship exists between certain host plant substances such as α-pinene and myrcene and the three aggregation pheromones mentioned above.

A symbiotic relationship between an insect and a species of bacterium with respect to the production of the insect's sex pheromone, has been found in the New Zealand grass grub beetle[11] which carries a phenol-producing bacterium in the colleterial glands of the female. To our knowledge, however, the presence of microorganisms within the gut of an insect capable of transforming certain precursors to its pheromones has not been suggested.

Certain microorganisms can transform α-pinene into various substances including *cis*-verbenol and verbenone[12-15]. We therefore isolated a number of different microorganisms from the gut of adult male and female *I. paraconfusus* and determined their ability to transform α-pinene into *cis*- and *trans*-verbenol. All isolated species were grown aerobically in 100 ml of 3% yeast extract in a mineral salts medium[16] and shaken continuously. Good growth occurred in all cases within 24 h at which time 0.5 ml α-pinene was added and the flasks shaken for a further 24 h. Controls without either the microorganisms or α-pinene were run in the same manner. All flasks were extracted with ether, and, based on retention data of temperature-programmed gas chromatographic runs of the extracts (10% SP-1000, 60 C to 200 C at 10 C min⁻¹), one organism in particular seemed to be producing the verbenols and this was identified as *Bacillus cereus*. This was isolated from both male and female beetles. Additional cultures of this organism were grown in 10 ml of the same medium, shaken for 24 h, and 0.1 ml α-pinene, purified by preparative gas chromatography, added for a further 24 h. Ether extracts of the cultures, and of

α-Pinene   *trans*-Verbenol   *cis*-Verbenol

appropriate controls, were prepared and an aliquot of each extract analysed by gas chromatography on 10% EGS (140 C). Peaks corresponding to both *cis*- and *trans*-verbenol were found in the culture extracts while none was detectable, even at high sensitivity, in the controls.

The verbenols were purified from the remaining culture extracts by the consecutive separation, collection, and rechrom-

atography of the appropriate peaks on four different stationary phases. These were, in order of use, 10% Apiezon L (140 C), 10% SP-1000 (140 C), 10% EGS (140 C), and 3% OV-225 (100 C). Mass spectra of the purified substances eluting from the OV-225 column, at the same retention time as *cis*- and *trans*-verbenol, were recorded and the congruence of these spectra with those of standard spectra, also purified by gas chromatography, confirmed that both verbenol isomers had been synthesised from α-pinene by *B. cereus*. The yields of *cis*- and *trans*-verbenol were approximately 0.1% and 1% respectively of the added α-pinene. Another major product of this transformation has been tentatively identified as myrtenol.

In spite of the numerous studies on the aggregation pheromones of *Ips*, the site of their synthesis has not been clearly defined. Hughes[10] has argued that detection of the verbenols and other oxidation products in the haemolymph of the bark beetles, *D. ponderosae* and *D. valens*, after exposure to α- or β-pinene, established that their production occurs outside the alimentary canal. It is assumed that these oxidation products are secreted into the hindgut and concentrated there by the reabsorption of water. In contrast to this hypothesis, we consider that exposure of an individual beetle to the vapour of a particular monoterpene will saturate both the haemolymph and the gut contents with the monoterpene. The oxidation of α- or β-pinene by microorganisms within the gut would then account for the greatly increased amounts of their oxidation products in this region and the detectable amounts in the haemolymph could occur either by diffusion out of the gut or by production of small amounts in the haemolymph, or by both processes.

We have found that α-pinene, the most likely precursor of both the verbenols and myrtenol, is a major component of the monoterpenes present in the phloem of ponderosa pine. Therefore, by either feeding or exposure, or both, this monoterpene would be present in the gut of male *I. paraconfusus* during nuptial chamber excavation. The occurrence of α-pinene throughout the gut of feeding *I. calligraphus*[6] has been shown, and a number of *Dendroctonus* species produce increased amounts of the verbenols in their hindgut after exposure of individuals to α-pinene[8-10]. These and other observations indicate that either ingestion of α-pinene, or intimate contact with α-pinene, or a combination of both of these conditions, is a prerequisite for the production of the verbenols by the various bark beetles. It is obvious that under natural conditions of a beetle excavating a gallery both mechanisms would be operative. *B. cereus* present in the gut must come into contact with this monoterpene and presumably could bring about the oxidation of α-pinene to the verbenols. Significantly, myrtenol is also known to be present in the gut of certain bark beetles after exposure to α-pinene[6,10].

A *Bacillus* sp. capable of converting α-pinene to the verbenols has also been isolated from both males and females of *I. grandicollis* and three species of *Dendroctonus*. While our preliminary results do not prove conclusively that *B. cereus* actually synthesises the verbenols from α-pinene in the hindgut, our data clearly indicate that this is a distinct possibility and substantiate the hypothesis that microorganisms may play a significant role in the synthesis of certain pheromones occurring in the frass of these bark beetles. The known differences in the quantitative production of the aggregation pheromones between males and females in these two genera remains enigmatic and is the subject of continuing investigations.

SUNY. Syracuse, New York, for a sample of *cis*-verbenol.

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FIRE ANT TRAIL PHEROMONES: ANALYSIS OF SPECIES SPECIFICITY AFTER GAS CHROMATOGRAPHIC FRACTIONATION

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Abstract—The specificity of the trail pheromones of four *Solenopsis* species was determined using natural trails, Dufour's gland extracts, and purified fractions from Dufour's gland extracts collected after gas-chromatographic separation. *S. richteri* and *S. invicta* possess species-specific major trail pheromones, while *S. geminata* and *S. xyloni* appear to have a common trail pheromone. Preliminary chemical characterization of the main trail pheromone of *S. richteri* indicates a M.W. of 218 and empirical formula of $C_{16}H_{26}$. The trail pheromone system of *S. richteri* consists of a blend of compounds and this phenomenon may also occur in the other species. The lowest concentration of their trail pheromone that workers of *S. richteri* could detect was about 10 fg per cm. The significance of blends of pheromones being utilized to generate chemical trails is discussed.

INTRODUCTION

Trail pheromones of ants constitute a vital aspect of their complex social system. In the Formicidae the glandular source of these pheromones is varied, and species in the large subfamily Myrmicinae use three different glands for the production of trail pheromones (Moser and Blum, 1963; Wilson, 1959; Fletcher and Brand, 1968). Within this subfamily there appears to be a paucity of intra- or intergeneric specificity of those trail pheromones originating in the poison gland (Blum, 1974). Chemical studies on the trail pheromones of myrmicine ants have resulted in the identification of only two compounds, methyl 4-methylpyrrole-2-carboxylate in the poison gland of *Atta texana* (Tumlinson *et al.*, 1971) and 5-methyl-3-butyl-octahydroindolizine in the poison gland of *Monomorium pharaonis* (Ritter *et al.*, 1973), as the major releasers of trail-following behaviour.

Wilson (1959) established that the source of the trail pheromone of the fire ant *Solenopsis saevissima* (=*invicta*) is the Dufour's gland. Species-specificity studies showed that workers of *S. saevissima* and *S. geminata* would not follow each other's trails; that *S. geminata* followed the trails of *S. xyloni*, but not *vice versa*; and that *S. xyloni* would follow the trails of *S. saevissima* (Wilson, 1962).

All previous studies of species specificity of trail substances, including these of Wilson (1962), have used crude extracts of the glandular source. The objectives of the present investigation were to exa-

mine the specificity of the trail pheromones of four *Solenopsis* species by using in addition to crude glandular extracts, components of the Dufour's gland that had been purified, or partially purified, by preparative gas chromatography. The species specificity of the trail pheromones of each of the four *Solenopsis* spp. studied might be more clearly determined from results obtained in this way as they would be based on both behavioural data as well as a chemical criterion. Our results have established that *S. richteri* and *S. invicta* possess species-specific major trail pheromones and that *S. geminata* and *S. xyloni* employ a common major trail pheromone.

MATERIALS AND METHODS

Four species of fire ants were used, the two introduced species, *S. invicta* (obtained near Gainesville, Florida, and Gulfport, Mississippi) and *S. richteri* (obtained in Starkville, Mississippi); and two indigenous species, *S. geminata* (obtained near Gainesville, Florida) and *S. xyloni* (obtained in Athens, Georgia). All colonies were maintained in soil and were provided with dead insects, a sucrose solution, and water.

Species-specificity studies

The bioassay technique employed was a modification of that used by Fletcher and Brand (1968). A colony of ants was connected by an inverted V-shaped wooden stick to a 2 m foraging table. A sucrose solution and a dead insect under an inverted petri dish were placed at the end of the foraging table, and the trail was established on pages of paper placed down the centre of the table. The petri dish had a

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small opening so that the ants could get to the food, but could not carry the entire insect back to the nest. In the middle of the table one sheet of paper, termed the 'test sheet', was slightly elevated in the centre and was easily removable. A pencil line was drawn from the nest over the test sheet to the food, and a trail was laid with a Dufour's gland extract along this line, using a fine, drawn out, glass capillary. The ants established a natural trail along this artificial trail and the colony could be used for bioassay about 30 min later. Bioassays were performed by carefully removing the centre sheet and replacing it with a test sheet. This sheet had a curved pencil line about 37 cm in length along which a solution of the test substance had been streaked with a fine glass capillary as described above. The artificial trail on the test sheet and the trail of the ants on the foraging table were lined up and the trail-following activity of the ants monitored by assignment of '+', '++', '+++', or '−'. The original paper was replaced in the test sheet position between bioassays and as many as ten bioassays could be performed every 30 min for 4 to 5 hr.

The species specificity of the trail pheromones was first determined by transposing test sheets which had had naturally-laid trails established on them. Subsequently, these results were verified by laying artificial trails with extracts of Dufour's glands. In this case an excised gland was crushed on 0.5 ml n-pentane and approximately 10 μl was streaked along the line on a test sheet and presented to the ants after the solvent had evaporated. Finally the species specificity of the trail pheromones was examined by the gas-chromatographic separation of the components in extracts of Dufour's glands. This was done in two ways. Firstly, 50 Dufour's glands of each species were collected and the extracts fractionated on 10% Carbowax 20 M at 150°C. The retention indices of the peaks were calcu-

lated relative to normal hydrocarbons. Secondly, the fractionation and collection was repeated using extracts of only three Dufour's glands of each species and regions corresponding to single peaks seen in the chromatograms of the fifty gland extracts and to certain elution intervals between peaks, were collected in glass capillaries. The collected material was eluted with 0.5 ml n-pentane and approximately 10 μl of each collected fraction was used for bioassay. n-Tridecane was incorporated into each extract as a check on the constancy of retention times.

Low concentration detection studies

One Dufour's gland was crushed in 5 ml n-pentane, and five two-fold dilutions were made thereby providing six solutions with concentrations ranging from one to 1/32 of a Dufour's gland. An aliquot (30 μl) of each solution was bioassayed. This experiment was performed twice with ants which had not been given food for 3 days, and twice with ants which had recently had access to food.

Mass spectrometry

Mass spectra of the main trail pheromone of workers of S. richteri were obtained both from an extract of five hundred Dufour's glands separated on 3% OV-1 in an LKB-9000 GC-MS, and from a gas-chromatographically purified active component obtained from steam distillates of whole ants on a Bell and Howell 21 490 instrument.

RESULTS

The results of the first two series of bioassays in which natural trails were transposed between species and Dufour's gland extracts were tested are presented in Table 1. They indicate that the trail pheromones of the two introduced species are more closely related to each other than they are to those of the two indigenous species, and vice versa.

Gas chromatograms obtained from extracts of fifty Dufour's glands of each of the four species are presented in Figs. 1 to 4. The results of the bioassays on collected fractions from the extracts of three Dufour's glands and their correlation with the peaks in the chromatograms of the extracts of the fifty Dufour's glands are given below.

Compounds eluting at the same time as n-heptadecane and n-nonadecane were present in all species.

S. richteri (Fig. 1)

The chromatogram of the Dufour's gland extract of S. richteri shows a peak with a retention index of 18.17. This compound is the main trail pheromone for this species but does not elicit strong trail-following behaviour in the other species. However, S. xyloni workers were sometimes able to follow it in a hesitant manner. The fraction eluting immediately after this peak, containing the n-nonadecane, was inactive for all species. However, a later fraction at 25 to 30 min

Table 1. Species specificity of naturally-laid trails and Dufour's gland extracts of the four fire ant species showing their relative response

Source species	Test species			
	S. richteri	S. invicta	S. geminata	S. xyloni
Natural trail				
S. richteri	+++	+++	−	−
S. invicta	+	+++	−	−
S. geminata	+	+	+++	+++
S. xyloni	−	−	+++	+++
Dufour's gland extract				
S. richteri	+++	+++	−	+
S. invicta	+++	+++	−	+
S. geminata	+	−	+++	+++
S. xyloni	+++	−	+++	+++

was active for *S. richteri* workers. When this later fraction was collected and re-chromatographed, the activity remained in this region and none appeared in the region of the major trail peak. The fraction in the *S. richteri* extract eluting at the same time as the trail pheromone of *S. invicta* (see Fig. 2) was active for the latter species.

S. invicta (*Fig. 2*)

Trail-following activity in *S. invicta* is associated with a compound having a retention index of 16.98. The regions immediately before and after this peak also showed some activity. None of these fractions showed any significant activity when tested on the other three species.

S. geminata *and* S. xyloni (*Figs. 3 and 4, respectively*)

The gas chromatograms of the extracts from glands of *S. geminata* and *S. xyloni* both demonstrate compounds having a retention index of 19.39. This peak corresponds to the trail pheromone of both these species, and when collected from either species, is active for the other species. In addition, workers of *S. geminata* showed some positive activity to fractions eluting after the trail pheromone in both their own Dufour's gland extract as well as that of *S. xyloni*. The workers of *S. xyloni* however showed no such activity to any other fraction of either species' glands. When the fractions were bioassayed for trail-following activity on the two other species, the fraction eluting immediately before the trail peak in the extract of Dufour's glands of *S. geminata* showed slight activity for workers of both *S. richteri* and *S. invicta*; and of the fractions collected from the extract of Dufour's
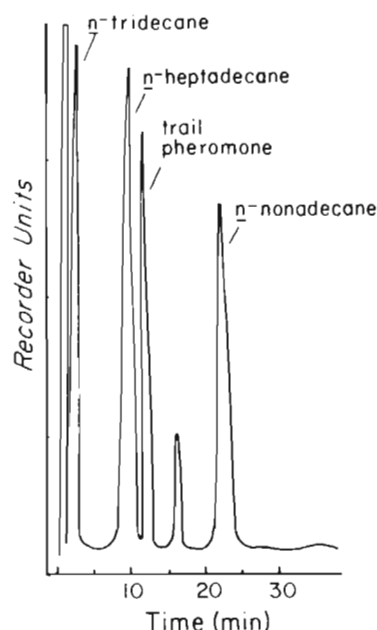
Fig. 2. Gas chromatogram of extracts of 50 Dufour's glands of *S. invicta* on 10"„ Carbowax 20 M (150°).

glands of *S. xyloni*, the region eluting at the same time as the trail pheromone of *S. richteri* showed slight trail-following activity in the latter species.

It is estimated from the various gas chromatograms obtained that a Dufour's gland from one worker of *S. richteri* contains about 1 ng of trail pheromone. Bioassays of appropriate dilutions of extracts indicated that workers of *S. richteri* which had not been
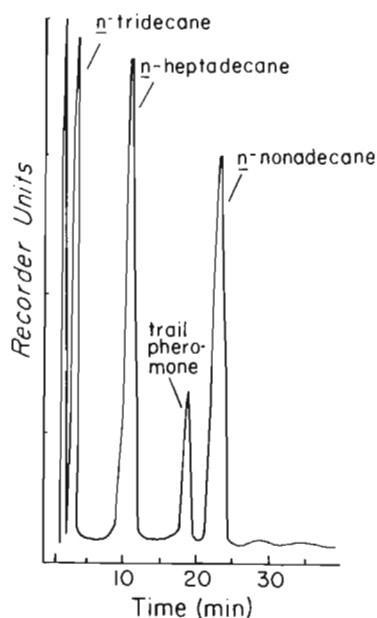
Fig. 1. Gas chromatogram of extracts of 50 Dufour's glands of *S. richteri* on 10"„ Carbowax 20 M (150°).
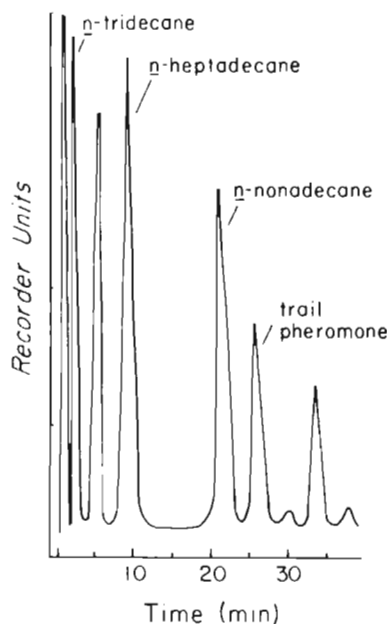
Fig. 3. Gas chromatogram of extracts of 50 Dufour's glands of *S. geminata* on 10"„ Carbowax 20 M (150°).
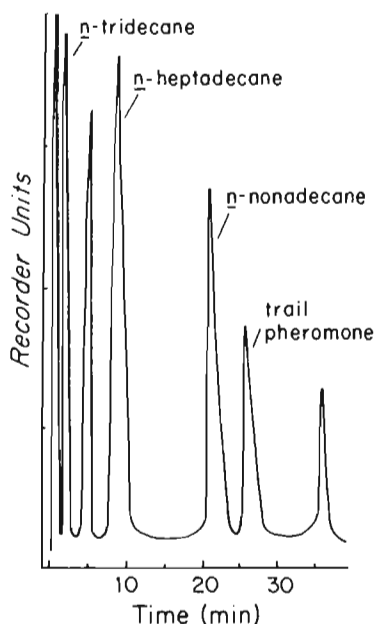
Fig. 4. Gas chromatogram of extracts of 50 Dufour's glands of *X. xyloni* on 10% Carbowax 20 M (150).

fed for at least 3 days can detect about 10 fg per cm of their trail pheromone. When this experiment was conducted with ants that had continuous access to food, their threshold level was in the order of 80 fg per cm.

The mass spectrum of the main trail pheromone of *S. richteri* showed an intense molecular ion at m/e 218 (60% relative abundance) which readily lost a methyl group (m/e 203; 70% abundance). The fragmentation pattern does not indicate the presence of oxygen and suggests the empirical formula $C_{16}H_{26}$.

DISCUSSION

As it was evident throughout this study that these species of fire ants could detect small quantities of a large number of odours, it was essential that the method of bioassay be both sensitive and discriminating. A major criterion of a successful bioassay technique is that the natural conditions be simulated as closely as possible. We therefore used the basic method of FLETCHER and BRAND (1968) since it takes advantage of ants following a natural trail between their nest and a food source about 2 m away.

The substance to be bioassayed was applied along a curved pencil line on the test sheet which was raised slightly from the table surface. The raising of the test sheet is necessary as the ants can detect an old trail on a surface even when the surface is covered by a clean sheet of paper. The curved trail also precluded the likelihood of the ants orienting to the light since *Solenopsis* spp. have been observed to orient visually (WILSON, 1962). Furthermore, whenever an ant was

observed to reinforce the substance on the test sheet with a protruded sting, that bioassay was repeated.

The concentration of the trail pheromone in an extract was also important since too high or too low a concentration of trail pheromone elicited either a negative response or repellency. As far as possible the active test substances offered to the various colonies for bioassays were in a concentration similar to the concentration of the trail pheromone of the test species that would have elicited natural trail following. The lowest detectable quantity of trail pheromone of *S. richteri* was about 10 fg per cm, an amount similar to that obtained for the workers of *Atta texana* which can detect 80 fg per cm of their trail pheromone (TUMLINSON *et al.*, 1972).

Due to the many variables encountered during bioassays the only practical method of evaluating trail-following activity was to assign four degrees of activity to the response of the ants. The ants differed in behaviour not only between species and between colonies, but also from day to day. Furthermore, the ants were easily disturbed when the papers were manipulated and in this respect *S. richteri* workers were much less so than those of the other three species. It was not practical to count the number of ants on the trail or the number reaching the end of a trail in a fixed time because the number was correlated with both size of the colony and the degree of starvation of the workers. The assignment of '+ + +' indicates natural trail following across the test sheet. A rating of '+ +' indicates that the test substance elicited trail following from most ants, with hesitation by a few workers. The assignment of '+' indicates detection of the test substance by only one or two ants which followed the trail in an unnatural manner. No trail following at all was indicated by '−'.

In the initial endeavours to establish the species specificity of the trail pheromones of *Solenopsis*, natural trails were transposed and the results in Table 1 indicate that *S. geminata* and *S. xyloni* possibly employ the same trail pheromone. The only other occurrence of lack of specificity among the four species is the ability of workers of *S. invicta* to follow trails laid by *S. richteri*; however, workers of *S. richteri* would not follow the trail of *S. invicta* in a convincing manner. From this evidence one may speculate that the Dufour's gland of *S. richteri* contains the trail pheromone of *S. invicta*, but that the reverse is not true. The ability to detect the trail of another species by a few ants, as indicated by '+' in Table 1, was not regarded as active trail following, but rather as evidence that these ants would follow substances other than their own trail pheromone. However, it is possible that the source species may contain a trace amount of the trail pheromone of the test species which is detected by workers of the latter.

The results of the response to extracts of Dufour's glands in Table 1 support the transposition studies of natural trails. *S. geminata* and *S. xyloni* readily

followed Dufour's gland extracts of each other's Dufour's glands and workers of *S. invicta* followed Dufour's gland extracts prepared from workers of *S. richteri*. However, these results demonstrate that a response is also obtained from *S. richteri* workers to the Dufour's gland extracts from workers of *S. invicta* and *S. xyloni*. As with the natural trail transposition studies, the ants may be following compounds other than their trail pheromones or the source species may contain the trail pheromone of the test species. WILSON (1962) found that workers of *S. xyloni* followed Dufour's gland extracts of *S. invicta* and would not follow Dufour's gland extracts of *S. geminata*. Our results differ from those of WILSON (1962) in these two incidents.

The species-specificity studies carried out on the fractions collected from the gas-chromatographic separations permit a more conclusive interpretation than was obtained with the previous methods, and moreover, they introduce an additional criterion to determining species specificity of the trail pheromones of ants. It has been shown by WALSH *et al.* (1965) that the trail pheromone of *S. invicta* can be recovered after gas-chromatographic separation, but activity of the partially purified compound was rapidly lost. During this investigation we have collected the purified trail pheromone of *S. richteri* and have been able to retain it in pentane at −10°C for several months without apparent loss of activity or chemical changes. All of the bioassays on partially purified trail pheromones were conducted immediately after collection of fractions from the gas chromatograph.

These results obtained with purified, or partially purified, fractions established that *S. richteri* and *S. invicta* possess species-specific major trail pheromones. Both *S. geminata* and *S. xyloni*, however, appear to employ a common major trail pheromone and neither species will follow the major trail pheromone utilized by *S. richteri* and *S. invicta* workers. From these data the extraneous activity indicated in Table 1 was interpreted in the following manner. The major trail pheromone of *S. richteri* is perhaps present in extremely small quantities in the Dufour's gland of *S. xyloni* and in the latter species it may have a minor synergistic role in the main trail pheromone complex. A compound with the same molecular weight as the suspected molecular weight of the trail pheromone of workers of *S. invicta* (mol. wt 204) is present in *S. richteri* workers (unpublished data) and this region from the Dufour's gland extract of *S. richteri* workers elicited positive trail following with *S. invicta* workers. Workers of *S. richteri* therefore may possess the trail pheromone of *S. invicta* workers, but not *vice versa*. It was firmly established that Dufour's glands of *S. richteri* workers contain at least two components that elicit trail following, one being active at a much lower concentration that the other component. No peak was ever observed for this trace compound eluting after the main trail pheromone and rechromatography of this minor component showed

that the activity was not due to contamination by the major compound. It is suspected that the trail pheromone of *S. invicta* workers is multicomponent in nature as activity was often evident in fractions eluting both before and after the trail pheromone peak. This could, however, be due to spreading of the main trail component.

Other compounds, in addition to the major trail pheromone of *S. geminata* and *S. xyloni* workers, are evident in Figs. 3 and 4. *S. geminata* workers will follow fractions eluting after the trail peak, but these additional regions of activity have not been separated unequivocally from the main trail pheromone and until this can be done, as it has for *S. richteri* extracts, there again remains the possibility of observed activity being due to tailing of the main trail pheromone.

The retention index of the *S. richteri* trail pheromone with a molecular weight of 218 is 18.17, while that of *S. invicta* in 16.98, a difference of 1.19; the retention indices of the pheromones of *S. geminata* and *S. xyloni* are both 19.39, a difference of 1.22, from 18.17. This difference is almost equal to that between *S. richteri* and *S. invicta*. If we assume that these trail pheromones are of a similar chemical type, it is tempting to suggest that as the empirical formula of the trail pheromone of *S. richteri* is $C_{16}H_{26}$, that of *S. invicta* may be $C_{15}H_{24}$, and that of *S. geminata* and *S. xyloni* may be $C_{17}H_{28}$.

It has been emphasized that pheromones are often not single components but rather a number of compounds which function synergistically (BLUM and BRAND, 1972). In addition, it may be the blend of these components that determines the specificity of the pheromone. For example, compounds other than the main trail pheromone in *Atta texana* were also found to elicit trail following (TUMLINSON *et al.*, 1972). Similarly, all four species of fire ants each have one main trail pheromone, but most of them also appear to have additional components that show activity. However, until the existence of these compounds is more clearly established, their true significance cannot be fully evaluated.

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BARK BEETLE PHEROMONES: PRODUCTION OF VERBENONE BY A MYCANGIAL FUNGUS OF *Dendroctonus frontalis*[1,2]

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Abstract—A mycangial symbiotic fungus of *Dendroctonus frontalis* is capable of oxidizing *trans*-verbenol to verbenone. Both of these compounds are known to be important behavioral chemicals for this species, and it is suggested that development of the fungus in the plant host may play a role in influencing the behavior of the beetle to a successfully colonized tree.

Key Words—pheromones, *Dendroctonus frontalis*, bark beetles, *trans*-verbenol, verbenone, microorganisms.

INTRODUCTION

The possibility that organisms within the gut of an insect might be capable of transforming dietary substrates into substances that may serve as pheromones was demonstrated by isolating a bacterium from the gut of *Ips paraconfusus* capable of oxidizing α-pinene to *cis*- and *trans*-verbenol and myrtenol (Brand et al., 1975). All three of these oxidation products occur in substantial amounts in the hindgut of various bark beetle species after exposure of individuals to α-pinene vapors (Hughes, 1973a; Hughes, 1973b;

Renwick et al., 1973; Hughes, 1975). Hughes (1973b) suggested that these oxidation products might be formed in a region of the beetle other than the alimentary canal. In contrast, Brand et al. (1975) suggested that they may be formed where they occur in the alimentary canal by microbial action on α-pinene. It must be emphasized that neither the results of Hughes (1973b) nor those of Brand et al. (1975) are based on conclusive evidence, and it is quite possible that both the beetle's enzymatic repertoire and that of the gut microorganisms are capable of these transformations. One of our main goals in this general study is to ascertain to what extent both the insect and the microorganisms associated with it are capable of synthesizing and metabolizing certain pheromones.

SJB-133 is one of two filamentous fungi usually present as yeast phases in the mycangium of female *D. frontalis* (Barras and Perry, 1972; Barras and Taylor, 1973). It occurs as a *Sporothrix* imperfect form in the beetle gallery with the perfect form being produced only rarely in vitro. In addition, two true yeasts are often associated with the mycangium. The microorganisms of the mycangium are introduced into host pines upon attack by the insect and result in rapid invasion of the gallery system and surrounding phloem. Barras (1973) has shown that growth of the total mycangial complex in the gallery system is important in insect development as the absence of the complex causes greater than 50% reduction in the number of insect progeny and greatly extends the larval generation time. In addition to the mycangial complex, other microorganisms present on the exoskeleton will also colonize the gallery system (Howe et al., 1971; Barras and Marler, unpublished data). Therefore it is clear that many organisms have ample opportunity to transform and metabolize an array of host plant substances during their development in the phloem.

In transformation experiments with liquid cultures of the fungus SJB-133, similar to those previously described (Brand et al., 1975) but using a rather impure sample of DL-α-pinene, *trans*-verbenol was consumed and verbenone accumulated. This finding led us to suspect that this fungus could transform *trans*-verbenol into verbenone. In addition, as *cis*- and *trans*-verbenol may be produced from α-pinene by other microorganisms (Prema and Bhattacharyya, 1962; Brand et al., 1975) it also suggests that a more complex system may be operative in the natural environment of bark beetles in which the products accumulated by one microorganism may be the substrates of another, thereby leading to sequential transformations.

In order to investigate the possible production of verbenone from *trans*-verbenol we did the following experiment. The fungus, SJB-133, isolated by one of us (S.J.B.), was grown in stationary 1 1 cultures of Sabouraud's dextrose for 7 days. The mycelial growth was then filtered on a Buchner funnel. Twenty-five milligrams of the damp mycelial mat were added to 2.5 ml

of phosphate buffer (0.05 M, pH 7.5) containing 10 μmoles of *trans*-verbenol (10% solution in ethanol). Either *trans*-verbenol or the fungus was omitted from the control flasks. After incubation of all flasks for 4 h at 30°C on a Dubnoff metabolic shaker, each solution was extracted with 6 ml *n*-pentane containing 1-octadecene as an internal standard. The pentane extracts were concentrated to 0.2 ml and 1-μl aliquots were injected into the gas chromatograph (10% SP-1000, 1.85 m × 3.25 mm stainless steel, 160°C).

The various chromatograms obtained showed that SJB-133 will readily convert *trans*-verbenol to verbenone under these experimental conditions. It appears that SJB-133 cannot oxidize verbenone further as the yield of verbenone was equal to the amount of *trans*-verbenol added. Similar experiments showed that SJB-133 will convert *cis*-verbenol quantitatively to verbenone and, also, that 3-methyl-2-cyclohexen-1-ol will be converted to the corresponding ketone in a yield of approximately 75%. The identity of the ketones was confirmed by mass spectrometry.

The various pheromones and chemicals identified from the Scolytidae have been summarized by Borden (1974). If one may be permitted to generalize from the table presented by Borden, it seems that the two ketones, verbenone and 3-methyl-2-cyclohexen-1-one, may both be involved in nullifying the attractive properties of various substances to certain *Dendroctonus* species. Verbenone is present as a major volatile component of the hindgut of emergent *D. frontalis* (Renwick, 1967) and is known to be an important substance in rivalry behavior between males of this species (Rudinsky and Michael, 1974). An increase in the concentration of verbenone released from a successfully colonized tree has been proposed as an important factor in inhibiting further attacks on the tree by both *D. frontalis* and *D. brevicomis* (Renwick and Vité, 1970). In addition, 3-methyl-2-cyclohexen-1-one has been termed an antiaggregative pheromone of *D. pseudotsugae* (Rudinsky et al., 1973; Rudinsky et al., 1974).

Our results show that both verbenone and 3-methyl-2-cyclohexen-1-one can be produced from their respective alcohols by SJB-133. With these results in mind, Fonken and Johnson (1972, pp. 89–93) give a number of examples illustrating that fungi and bacteria are able to oxidize monoterpene hydrocarbons and, in most cases, if the alcohol is formed then the corresponding ketone also appears. It is interesting to speculate that the termination of attack by *D. frontalis* because of an increase in verbenone concentration (Renwick and Vité, 1970) is related to the development of microorganisms that can produce it.

Our initial presentation of the possibility that microorganisms associated with the gut of bark beetles may be producing at least some of their pheromones (Brand et al., 1975) must now be extended. It is likely that SJB-133 growing in the phloem could produce verbenone from verbenol in situ, and,

if this is the case, then a microorganism external to the beetle would be responsible for part of the production of at least one of its behavioral chemicals. The proof of this hypothesis constitutes a part of our continuing investigations.

These transformation experiments have added additional evidence to biological studies that have shown a complex symbiotic relationship between the Southern Pine Beetle, associated microorganisms, and host tree components. The elaborate glandular mycangium in the female (Happ et al., 1971) indicates that such a high degree of symbiosis is not left to chance. Although the conclusion is somewhat speculative at this point, it appears that at least one fungus (SJB-133) of the mycangial complex, which is important in the beetle's nutritional regime, could play a significant role in regulating response to the plant host.

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33

162   J. Georgia Entomol. Soc. Vol. 11, No. 2

SPECIES-SPECIFICITY STUDIES ON THE
TRAIL PHEROMONE OF THE CARPENTER ANT,
CAMPONOTUS PENNSYLVANICUS
(HYMENOPTERA: FORMICIDAE)

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ABSTRACT

The source of the trail pheromone of *Camponotus pennsylvanicus* is the contents of the hind gut. Workers of *C. pennsylvanicus* follow trails prepared from hind gut extracts of two other *Camponotus* spp. The trail pheromone was isolated by gas chromatographic fractionation and demonstrated to be identified with a single compound.

Key Words: *Camponotus*, trail pheromone, species specificity, recruitment

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Chemical trails comprise an integral part of the communication system of many ants. In the Formicinae the source of the trail pheromones is the hind gut (Blum and Wilson, 1964), while the poison gland and the Dufour's gland play an important role in attraction and alarm (Ayre and Blum, 1971). The substances in the hind gut of the formicine *Lasius fuliginosus* (Latreille), responsible for trail following, are hexanoic acid, heptanoic acid, octanoic acid, nonanoic acid, decanoic acid, and dodecanoic acid (Huwyler *et al.*, 1973). In addition to chemical trails, *Camponotus (Tanaemyrmex) socius* Roger has been shown to use a waggle movement to recruit workers to a food source (Hölldobler, 1971).

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We have found that the trail pheromone of *C. (Camponotus) pennsylvanicus* (DeGeer) can be eluted from a gas chromatographic column as a single peak and species specificity studies on hind gut extracts show that *C. pennsylvanicus* is able to follow the trail of at least two other *Camponotus* species.

MATERIALS AND METHODS

Colonies of *C. pennsylvanicus* and *C. (Camponotus) americanus* Mayr were collected near Athens, Georgia. *C. pennsylvanicus* workers were kept in pieces of their original log nests, while *C. americanus* workers were maintained in Wilson nests. All ants were fed honey, cockroaches, and water. The bioassay technique was identical to that used by Barlin *et al.* (1975) for *Solenopsis* spp. Hind gut extracts were made by removing one hind gut and crushing it in 0.5 ml *n*-pentane and 20 μl of this solution was streaked over a distance of 40 cm, i.e. 10^{-3} hind guts per cm, for each bioassay. The hind gut extracts of *C. (Camponotus) americanus, C. (Tanaemyrmex) castaneus* (Latreille), *C. (Colobopsis) impressus* (Roger), *C. (Myrmothrix) floridanus* (Buckley), and *C. (Myrmentoma) nearcticus* Emery were all tested for trail-following activity with *C. pennsylvanicus* workers. The hind gut extract of *C. pennsylvanicus* workers was also tested \for activity on workers of *C. americanus*. All experiments were replicated six times.

Gas chromatographic studies were carried out on pentane extracts of fifty hind guts of *C. pennsylvanicus* workers. The components in the concentrated extract were separated on Carbowax 20M at 150 C. Fractions were collected in capillary tubes, eluted with *n*-pentane, and diluted so that approximately 10^{-3} hind guts per cm would be bioassayed for trail-following activity.

RESULTS AND DISCUSSION

The various bioassays conducted indicate that the trail pheromone is not entirely species specific. The hind gut extracts of *C. americanus* and *C. socius* workers both elicit trail following in workers of *C. pennsylvanicus*, but this latter species does not follow hind gut extracts of *C. rasilis, C. castaneus, C. impressus, C. floridanus,* or *C. nearcticus* workers. In the only trail-following experiment undertaken with workers of another species, it was found that workers of *C. americanus* will ·follow the hind gut extract of *C. pennsylvanicus* workers.

Gas chromatographic separation of the hind gut extract of *C. pennsylvanicus* workers revealed a peak with a retention index of 17.8 relative to normal hydrocarbons. When this compound was collected and bioassayed it elicited positive trail-following behavior with workers of *C. pennsylvanicus*, whereas none of the fractions collected before or after this peak were active. We have not characterized the nature of the compound further.

C. socius workers mark trails with the contents of their hind guts by dragging their abdominal tips over the substrate (Hölldobler, 1971). This is in contrast to workers of the carpenter ant, *C. pennsylvanicus*, which dot the tip of their gasters on the substrate as they move between the food source and the nest. Since attempts to elicit trail following with extracts of the whole

mid gut and extracts of the hind gut wall proved negative, we conclude that the trail pheromone of *C. pennsylvanicus* workers originates in the contents of the hind gut.

Recruitment behavior similar to that described for *C. socius* workers by Hölldobler (1971) was also observed with workers of *C. pennsylvanicus*. However, the *C. socius* recruiter, after stimulating workers to forage by its waggle movement, leads the recruited ants back to the food source. The hind gut trail pheromone does not have a recruitment effect on nestmates. On the other hand, the *C. pennsylvanicus* recruiter, after alerting workers within the nest, returns to the food source but does not necessarily function as a guide ant for the new recruits. Furthermore, in contrast to *C. socius*, the hind gut trail pheromone of *C. pennsylvanicus* had a mild recruiting function. This was apparent when the bioassay table was being prepared with a hind gut extract laid by the investigator between the nest and food source, as ants readily followed this trail. The quantity of pheromone used in our studies corresponded to 10^{-3} hind guts per cm since we found that this species would not follow a trail laid with an amount of material one or two orders of magnitude greater.

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6-Methyl-5-hepten-2-one in *Formica* Species: Identification and Function as an Alarm Pheromone (Hymenoptera: Formicidae)[1]

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ABSTRACT

6-Methy-5-hepten-2-one comprises at least 95% of the volatiles detectable in the mandibular gland secretions of workers of 8 *Formica* species in the subgenera *Neoformica* and *Proformica*. In certain of these species 6-methyl-5-hepten-2-ol is present as a trace component. The ketone functions as a releaser of alarm behavior for worker ants.

The genus *Formica* is by far the largest in the Nearctic region north of Mexico, with its members consituting approximately one-sixth of the entire ant fauna (Creighton 1950). However, relatively few studies of their exocrinology have been undertaken. The studies of Bergström and Löfqvist (1968, 1973) on the Dufour's gland constituents of certain European species of *Formica,* and the accounts of Wilson and Regnier (1971) and Regnier and Wilson (1971) on the exocrine chemistry of 9 Nearctic species of slave-making and nonslave-making *Formica* spp., provide most of our information on the chemistry of this genus.

While aspirating ants from a colony of *F. periplosa,* it was observed that they produced a strong odor which was demonstrated to originate in cephalic glands. We subsequently investigated the mandibular gland chemistry of a number of species of *Formica* representing different subgenera, and report the identification of 6-methyl-5-hepten-2-one as the major constituent in 8 species in the subgenera *Neoformica* and *Proformica*.

METHODS AND MATERIALS

The *Formica* species investigated were collected at the following localities: *F. (Proformica) lasioides* Emery, Emmet Co., IA; *F. (Proformica) neogagates* Emery, *F. (Neoformica) nitidiventris* Emery, *F. (Neoformica)* species A, Johnson Co., IA; *F. (Proformica) peripilosa* Wheeler, Big Bend National Park, TX; *F. (Neoformica) schaufussi* Mayr, Clarke Co., GA; and two *F. (Neoformica)* species, B and C, Lumpkin Co., GA.

After being excavated and the ants separated from the dirt, each colony was maintained in the laboratory on honey and mealworms. Extracts of worker heads were prepared in methylene chloride, and volatile compounds were analyzed gas chromatographically on a 10% SP-1000 column programmed from 60–200° at 5° per min. Mass spectral analyses were conducted on a Finnegan (Model 3600) gas chromatograph- mass spectrometer utilizing a 10% SP-1000 column. The ionizing potential was held at 70 eV.

RESULTS AND DISCUSSION

One major compound was evident in all species. In addition, *F. peripilosa* and those species belonging to the subgenus *Neoformica* exhibit a 2nd minor peak on the trailing edge of the 1st peak in the gas chromatograms. The mass spectrum of the major compound was identified as that of 6-methyl-5-hepten-2-one (Bergström and Löfqvist 1970), and that of the minor compound as the corresponding alcohol, 6-methyl-5-hepten-2-ol. Both the mass spectra and GLC retention times of these compounds were completely congruent with those of standard compounds. GLC retention times of the volatile substances in excised mandibular glands demonstrated that these glands were the source of these 2 compounds. Cephalic extracts of *F. schaufussi* indicated that 6-methyl-5-hepten-2-one was present at an amount of < 10 ng/head.

In order to determine the behavioral properties of this ketone, filter paper discs elevated 1 cm on pins were coated with 5 ug of synthetic 6-methyl-5-hepten-2-one and placed at the nest entrance. The behavior of worker ants drawn to the source was characterized by frenzied erratic movements, raised heads and thoraces, and outstretched antennae. These reactions are typical of the alarm behavior exhibited by these species.

The identification of 6-methyl-5-hepten-2-one in these *Formica* species represents the 1st report of a volatile mandibular gland constituent in this genus. It is not surprising to find the corresponding alcohol as a trace constituent since ant species which produce ketonic alarm pheromones often produce a trace of the corresponding alcohol (Blum and Brand 1972).

Analyses of head extracts of several other species in the subgenera *Formica* and *Raptiformica* did not reveal the presence of 6-methyl-5-hepten-2-one. These results indicate that this ketone may be restricted to mandibular gland secretions of *Formica* species in the subgenera *Proformica* and *Neoformica*. The systematics of the majority of the North American *Formica* species are generally confusing; our data indicate that the presence of 6-methyl-5-hepten-2-one in the mandibular gland secretions may represent a character state for species in the subgenera *Neoformica* and *Proformica*.

Although 6-methyl-5-hepten-2-one is a characteristic anal gland product in dolichoderine genera such

as *Iridomyrmex* (Cavill et al. 1956), *Conomyrma* (McGurk et al. 1968), and *Tapinoma* (Trave and Pavan 1956), it has only been identified as a mandibular gland product of 2 species in the formicine genus *Lasius* (Bernardi et al. 1967, Bergström and Löfqvist 1970). In the case of *Lasius,* this ketone is a trace constituent in a large blend of components whereas in these *Formica* secretions it comprises at least 95% of the detectable volatiles. Its occurrence in the mandibular gland exudates of formicine ants is considered atypical by Bergström and Löfqvist (1970), but in light of our findings, we feel that 6-methyl-5-hepten-2-one may be one of the characteristic terpenoid constituents synthesized in the mandibular glands of many species of formicine ants.

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The Major Volatile Constituents of a Basidiomycete Associated with the Southern Pine Beetle

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The female of the southern pine beetle, *Dendroctonus frontalis* Zimm., has a prothoracic mycangium which usually contains two fungi and two yeasts (1). One of the fungi, designated SJB–122, is a Basidiomycete (1) and both liquid and solid cultures of this fungus have a distinct fruity odor. This odor is also prominent in phloem of pine trees infested with the insect. As we were investigating the possibility that odorous compounds of microorganisms associated with the southern pine beetle may have some behavioral activity for this beetle, we isolated and identified the major volatile compounds produced by this fungus.

MATERIALS AND METHODS

SJB–122 was isolated from the mycangium of female *D. frontalis* and maintained on slants of Sabouraud's dextrose agar. In order to obtain material for analytical work, twenty flasks, each containing 100 ml of Sabouraud's dextrose broth, were inoculated and allowed to stand at room temperature for 2 months. Both the fungal mat and culture fluid of the 20 flasks were transferred to a 5 liter round bottom flask and the volatiles steam distilled and continuously liquid-liquid extracted into *n*-pentane. The apparatus for this procedure is described by Vogel (2). The pentane extract was concentrated to 2 ml and the volatile extractives separated gas chromatographically on Carbowax 20 M temperature programmed from 60°–220° at 10° per minute. Three major compounds were observed. The mass spectra of these compounds, eluting from a SP–1000 column, were recorded on a Finnigan 3200 gc-ms. No volatile products were obtained from the culture medium alone.

RESULTS AND DISCUSSION

Our goal at this time concerns the identification of the major volatile substances produced by microorgan-isms associated with the southern pine beetle and their possible influence on the aggregation and/or dispersal behavior of these beetles. SJB–122 grows slowly in the various media that we have used, but after good growth has occurred, its fruity odor is always apparent. The two-month growth period was arbitrarily chosen for the isolation of volatiles and one must assume that the ratio of volatiles will vary both with time and growth conditions.

Gas chromatograms of the extract established the presence of three major volatile substances. The first and major component exhibited prominent ions at m/e 29, 31, 41, 42, 43, 55, 57 and 70. Comparison of both the mass spectrum and retention times of this compound with those of authentic isoamyl alcohol confirmed its identity as this alcohol. The second component, and quantitatively least important of the three, showed a molecular ion at m/e 126 and other prominent ions at m/e 39, 41, 43, 55, 67, 69, 108 and 111. The retention times and mass spectrum of this compound are congruent with those given by 6-methyl-5-hepten-2-one. The third compound gave a molecular ion at m/e 128 with other prominent ions at m/e 39, 41, 43, 45, 55, 67, 69, 95 and 110. The chromatographic and spectral data of this compound were congruent with those of 6-methyl-5-hepten-2-ol. Other very minor peaks were evident in the gas chromatograms but have not been identified.

Many wood-rotting fungi are known to produce odors (3, 4) and

many Basidiomycetes are wood-rotting fungi. However, although SJB–122 is a Basidiomycete and grows in the phloem of pine trees after introduction by the southern pine beetle, we do not wish to suggest that it must be considered a wood-rotting fungus. Extensive culturing techniques and consultations with specialists have been undertaken over the last 10 years to identify the fungus and a review of its possible taxonomic position and a study of its fine structure has been presented by Happ *et al.* (5). At this stage we know very little about how rapidly and to what extent this fungus actually develops in the phloem or xylem and its effect on the host plant tissues. The extent of our knowledge on the development of SJB–122 in the phloem is summarized by Barras and Perry (1).

The southern pine beetle is also closely associated with two forms of *Ceratocystis.* Trees attacked by these beetles will usually end up with blue regions in the wood caused by the blue stain fungus, *C. minor* (Hedgcock) Hunt, carried on the exoskeleton of the beetle. The pronotal mycangium of the female beetle often contains specialized asexual spores of *C. minor* var. *barrasii* Taylor and the development of this fungus is considered important for the nutrition of larvae (6, 7).

6-Methyl-5-hepten-2-one and 6-methyl-5-hepten-2-ol have been identified previously from cultures of *Endoconidiophora coerulescens* (8) and *Ceratocystis virescens* (9) and methyl heptenyl ketone has been obtained from *Ceratocystis coerulescens* (10). *Endoconidiophora* is now synonymized with *Ceratocystis* (11). Neither *C. minor* nor *C. minor* var. *barrasii* associated with the southern pine beetle produce either of these two compounds on Sabouraud's dextrose broth. SJB–122, which is a Basidiomycete, produces both 6-methyl-5-hepten-2-one and its corresponding alcohol and the fruity odor of cultures of this mycangial fungus is due mainly to these

two compounds. Both the Ascomycetes, *C. variospora* and *C. virescens,* and the Basidiomycete, *Trametes odorata,* have been found to produce a number of monoterpenes (4, 9, 12) and, in the case of *C. virescens,* 6-methyl-5-hepten-2-one and its corresponding alcohol are also produced (9) It has been suggested that the monoterpene, citral, is the likely biosynthetic precursor of 6-methyl-5-hepten-2-one (13). However, *C. virescens,* which produces both 6-methyl-5-hepten-2-one and the corresponding alcohol does not produce neral and geranial, the isomers of citral, while *C. variospora* produces the isomeric aldehydes but not the ketone and alcohol (9).

Isoamyl alcohol is a common product of fermentation of glucose by yeasts (14) and we have obtained results which indicate that this alcohol can enhance the attractive quality of the pheromone blend (frontalin: *trans*-verbenol:turpentine; 1:1:12) of the southern pine beetle in laboratory bioassays (15). We plan to bioassay the effect of both 6-methyl-5-hepten-2-one and 6-methyl-5-hepten-2-ol on the southern pine beetle.

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BARK-BEETLE PHEROMONES

Enhancement of *Dendroctonus frontalis* (Coleoptera: Scolytidae) Aggregation Pheromone by Yeast Metabolites in Laboratory Bioassays[1]

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Abstract—The two major yeasts isolated from the southern pine beetle, *Dendroctonus frontalis* Zimmerman, are *Hansenula holstii* Wickerham and *Pichia pinus* (Holst) Phaff; a third yeast, *P. bovis* van Uden et do Carmo-Sousa, has been isolated far less frequently. The main volatile metabolites produced by these yeasts are isoamyl alcohol, isoamyl acetate, 2-phenylethanol, and 2-phenylethyl acetate. We have found that certain of these compounds, particularly the esters, can greatly, enhance the attractiveness of a mixture of frontalin:*trans*-verbenol:turpentine (1:1:12), at certain limiting concentrations, to walking beetles in a laboratory bioassay.

Key Words—Pheromones, bark beetles, *Dendroctonus frontalis*, southern pine beetle, bioassays, microbial metabolites, isoamyl alcohol, isoamyl acetate, 2-phenylethanol, 2-phenylethyl acetate.

INTRODUCTION

We have proposed that microorganisms associated with certain bark beetles may be responsible in part for the ultimate blend of behavioral chemicals that these insects encounter and respond to in their natural habitat (Brand et al., 1975, 1976). This idea concerning bark beetles was by no means new, as Person wrote in 1931 "... fermenting inner bark was more attractive than any of the other substances tested. This suggested the possibility that the attractiveness of attacked trees might be due to some fermentation organism, such as a yeast, associated with the beetle" (Person, 1931). After the successful initial attack by a few beetles Person concluded "... a second, stronger attraction is started by the yeast introduced by the attacking beetles, finding the inner bark a favorable medium for its growth." Person's reference was to a closely related species, *D. brevicomis* Le Conte, attacking ponderosa pine.

In principle we agree with the statements made by Person almost 50 years ago. However, our conviction is not universally shared as it had been either concluded or suggested that the role of yeasts in the secondary attraction of bark beetles is questionable (Anderson, 1948; Wood and Vité, 1961; Vité and Gara, 1962; Francke-Grosmann, 1963; Graham, 1967). It should also be pointed out that we have not yet been able to unequivocally demonstrate the involvement of microorganisms in the production of compounds utilized by bark beetles as attractants during their orientation to attacked trees. A good review of selection and colonization of ponderosa pine by bark beetles has been written by Wood (1972).

In this paper we present evidence that certain metabolites of yeasts associated with the southern pine beetle, *Dendroctonus frontalis*, can enhance the response of walking beetles to a mixture of frontalin, *trans*-verbenol, and loblolly turpentine, in laboratory bioassays.

METHODS AND MATERIALS

Isolation and Culturing of Yeasts

Yeasts were isolated on malt agar plates (pH 3.7) and peptone:yeast extract:glucose [1%:0.4%:1%; PYG; in Dworkin–Foster salts medium (Dworkin and Foster, 1958)] agar plates from whole beetles, excised whole guts, and various locations in galleries in loblolly pine. All cultures were grown at 22° and identified by the criteria of Lodder (1970).

The yeasts were grown in 100 ml PYG (1%:0.4%:2%; in Dworkin–Foster salts medium) shake cultures at 22° prior to extraction and assay of volatiles. A phloem medium for assaying production of volatiles was made by homogenizing fresh phloem in water and filtering the homogenate through

cheese cloth. Approximately 10 g of fresh phloem yielded 100 ml of filtrate, and this filtrate was used as a culture broth after autoclaving.

Identification of Volatile Metabolites

The yeast cultures were extracted with ether at various time intervals, the extracts dried over Na_2SO_4, and concentrated under a stream of nitrogen. The major volatile components in the concentrated extract were separated gas chromatographically on Carbowax 20 M temperature programmed from 60° to 200° at 10° per minute. Mass spectra of the major volatile metabolites were obtained either by the direct insertion technique of each compound adsorbed on powdered graphite, or by GC–MS (SP-1000 column) in a Finnigan 3600 instrument. Compounds were adsorbed directly on graphite as they eluted from the effluent-split gas chromatographic column. Extracts of sterile PYG medium did not contain detectable amounts of volatile substances.

Laboratory Bioassays

The bioassay procedure, which is described in detail by Payne et al. (1976), was employed for all bioassays. In this assay a group of beetles of one sex were placed on an arena. Test compounds in *n*-pentane were delivered by means of a 10-μl syringe driven by a timer motor into an air stream directed across the bioassay arena. Positively responding beetles walked upwind to the release portal.

The standard attractant mixture was frontalin: *trans*-verbenol: turpentine (1:1:12) diluted with *n*-pentane (Payne et al., 1976). This mixture will be referred to as the triplicate standard. *n*-Pentane was used as a control. The attractancy of the triplicate standard was assayed both on its own and together with each of the following compounds or mixtures respectively: isoamyl alcohol (1), 2-phenylethanol (2), isoamyl acetate (3), 2-phenylethyl acetate (4), isoamyl alcohol and 2-phenylethanol (5), isoamyl acetate and 2-phenylethyl acetate (6), isoamyl alcohol, 2-phenylethanol, isoamyl acetate, and 2-phenylethyl acetate (7).

The triplicate standard was assayed at the following rates: 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} μg μl^{-1} min^{-1}. The various compounds and mixtures were assayed together with the triplicate standard, each compound or mixture being at a rate 5 times greater than the triplicate standard, i.e., triplicate standard (10^{-5} μg^{-1} min^{-1}) and (1) (5×10^{-5} μg /l^{-1} min^{-1})

RESULTS

Identity of Yeasts

The vast majority of isolated yeast colonies were either *Hansenula*

holstii Wickerham or *Pichia pinus* (Holst) Phaff in agreement with the results of Shifrine and Phaff (1956), but occasionally we obtained isolates of *Pichia bovis* van Uden et do Carmo-Sousa.

Identification of Compounds

When ether extracts of the three yeast cultures were analyzed gas chromatographically, a number of volatile compounds were found to be present. The mass spectrum of each major compound, trapped on powdered graphite, was obtained by the direct insertion technique. In addition, mass spectra of both the major and minor components were obtained by coupled GC–MS. From these data, and by comparison with data obtained from authentic standards, the four major compounds were identified as isoamyl alcohol, 2-phenylethanol, isoamyl acetate, and 2-phenylethyl acetate.

P. bovis, in liquid culture, produced isoamyl alcohol and 2-phenylethanol after approximately 30 hr, and the amount of these two alcohols reached a maximum at about 100 hr, thereafter decreasing in amount. At approximately 70 hr significant amounts of isoamyl acetate and 2-phenylethyl acetate were produced and the amounts of these two esters equaled the amounts of the two alcohols at about 100 hr. When cultures were extracted at a time greater than 150 hr, these compounds disappeared and valeric acid was found to be the predominant compound. Both *H. holstii* and *P. pinus* produced isoamyl alcohol and 2-phenylethanol after approximately 30 hr in liquid cultures, but they did not produce the acetates under these conditions. In addition, cultures extracted up to 250 hr continued to contain 2-phenylethanol as the major volatile component, together with valeric acid.

All three yeast species also produced methanol (with the exception of *H. holstii*), ethanol, *n*-propanol, and isobutanol in their earlier stages of growth. Extracts of cultures of each of these three species on the sterile phloem medium indicated that *H. holstii* and *P. bovis* produced only 2-phenylethanol as a major component under these conditions. The other alcohols and acetates, however, were detected if the phloem medium was supplemented with glucose.

Bioassays

The major volatile compounds produced by the three yeasts were bio-assayed in a laboratory bioassay against walking beetles, and the results are presented in Tables 1 to 4. Table 1 shows the response of males and females to the triplicate standard at a rate of 10^{-3} μg μl^{-1} min^{-1} as well as their response to the triplicate standard plus each of the various compounds or combinations of compounds. While minor differences exist, it is obvious that

TABLE 1. RESPONSE OF *D. frontalis* MALES AND FEMALES IN A WALKING BIOASSAY TO FRONTALIN: *trans*-VERBENOL:TURPENTINE (TS; 1:1:12) DELIVERED AT A RATE OF 10^{-3} μg ul^{-1} min^{-1} AND TO TS PLUS CERTAIN YEAST METABOLITES, EACH METABOLITE OR MIXTURE AT A RATE OF 5×10^{-3} μg μl^{-1} min^{-1}

Compound[a]	% Response[b]			
	♂	$SE_{\bar{x}}$	♀	$SE_{\bar{x}}$
Control	17	±4	13	±4
TS	90	±4	62	±9
TS+(1)	87	±7	80	±7
TS+(2)	80	±7	60	±7
TS+(3)	90	±5	77	±9
TS+(4)	93	±4	57	±7
TS+(5)	93	±4	63	±8
TS+(6)	100		67	±9
TS+(7)	97	±3	67	±9

[a] Control = *n*-pentane delivered at a rate of 1 μl min^{-1}; (1) isoamyl alcohol, (2) 2-phenylethanol, (3) isoamyl acetate, (4) 2-phenylethyl acetate, (5) isoamyl alcohol + 2-phenylethanol, (6) isoamyl acetate + 2-phenylethyl acetate, (7) isoamyl alcohol + 2-phenylethanol + isoamyl acetate + 2-phenylethyl acetate; each compound or mixture at a rate of 5×10^{-3} μg μl^{-1} min^{-1}.

[b] 30 Beetles/value except the control which was 60 beetles/value.

no real inhibition of the high response to the triplicate standard was caused by any of the compounds or mixtures. The low response of females to the triplicate standard plus 2-phenylethanol [Table 2, test TS+(2)] is noteworthy.

The results in Table 3, however, are the most interesting. At a rate of 10^{-5} μg μl^{-1} min^{-1} the response of both males and females to the triplicate standard was not much greater than the control. The addition of either 2-phenylethyl acetate [TS+(4)] or isoamyl acetate [TS+(3)] at 5×10^{-5} μg μl^{-1} min^{-1} increases the response of male beetles to 87%. The combination of isoamyl alcohol and 2-phenylethanol, or their acetates, or all four compounds, also produced a greatly increased response from the males. The response of females, while lower overall, is of a similar pattern, 2-phenylethanol [TS+(2)] again appearing as a possible exception. None of the yeast metabolites elicited significant responses from the beetles when assayed alone at a rate of 10^{-4} μg μl^{-1} min^{-1}.

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TABLE 2. RESPONSE OF *D. frontalis* MALES AND FEMALES IN A WALKING BIOASSAY TO FRONTALIN: *trans*-VERBENOL:TURPENTINE (TS; 1:1:12) DELIVERED AT A RATE OF 10^{-4} μg μl^{-1} min^{-1} AND TO TS PLUS CERTAIN YEAST METABOLITES, EACH METABOLITE OR MIXTURE AT A RATE OF 5×10^{-4} μg μl^{-1} min^{-1}

Compound[a]	% Response[b]			
	♂	$SE_{\bar{x}}$	♀	$SE_{\bar{x}}$
Control	3	±2	5	±3
TS	73	±8	53	±5
TS+(1)	83	±6	73	±8
TS+(2)	60	±10	20	±7
TS+(3)	90	±5	80	±7
TS+(4)	83	±7	63	±8
TS+(5)	83	±9	50	±9
TS+(6)	83	±7	80	±7
TS±(7)	90	±5	47	±7

[a,b] See Table 1. 10^{-4} μg μl^{-1} min^{-1} delivery rate for TS and 5×10^{-4} μg μl^{-1} min^{-1} for other compounds or mixtures.

TABLE 3. RESPONSE OF *D. frontalis* MALES AND FEMALES IN A WALKING BIOASSAY TO FRONTALIN: *trans*-VERBENOL:TURPENTINE (TS; 1:1:12) DELIVERED AT A RATE OF 10^{-5} μg μl^{-1} min^{-1} AND TO TS PLUS CERTAIN YEAST METABOLITES, EACH METABOLITE OR MIXTURE AT A RATE OF 5×10^{-5} μg μl^{-1} min^{-1}

Compound[a]	% Response[b]			
	♂	$SE_{\bar{x}}$	♀	$SE_{\bar{x}}$
Control	7	±3	10	±4
TS	27	±8	23	±7
TS+(1)	50	±12	50	±7
TS+(2)	47	±9	27	±10
TS+(3)	87	±5	70	±9
TS+(4)	87	±7	63	±3
TS+(5)	73	±8	43	±9
TS+(6)	67	±10	47	±9
TS+(7)	57	±9	43	±9

[a,b] See Table 1. 10^{-5} μg μl^{-1} min^{-1} delivery rate for TS and 5×10^{-5} μg μl^{-1} min^{-1} for other compounds or mixtures.

TABLE 4. RESPONSE OF *D. frontalis* MALES AND FEMALES IN A WALKING BIOASSAY TO FRONTALIN: *trans*-VERBENOL:TURPENTINE (TS; 1:1:12) DELIVERED AT A RATE OF 10^{-6} μg μl^{-1} min^{-1} AND TO TS PLUS CERTAIN YEAST METABOLITES, EACH METABOLITE OR MIXTURE AT A RATE OF 5×10^{-6} μg μl^{-1} min^{-1}

Compound[a]	% Response[b]			
	♂	SE$_x$	♀	SE$_x$
Control	18	± 5	10	±4
TS	23	±11	17	±6
TS+(1)	23	±11	23	±9
TS+(2)	20	±10	10	±5
TS+(3)	43	±9	20	±10
TS+(4)	43	±10	17	±7
TS+(5)	40	±8	17	±7
TS+(6)	30	±12	23	±5
TS+(7)	23	±10	13	±5

[a,b] See Table 1. 10^{-6} μg μl^{-1} min^{-1} delivery rate for TS and 5×10^{-6} μg μl^{-1} min^{-1} for other compounds or mixtures.

DISCUSSION

Our isolation of *H. holstii* and *P. pinus* is not surprising as they were shown by Shifrine and Phaff (1956) to be the two main yeasts associated with the bark beetles *Ips* and *Dendroctonus*. Shifrine and Phaff used the synonym *Candida silvicola* for *H. holstii* (Lodder, 1970, p. 279) and *Saccharomyces pini* for *P. pinus* (Lodder, 1970, p. 515). We isolated *P. bovis* far less frequently than the above two. The association of yeasts with bark beetles has also been described by Holst (1936), Whitney (1971), and Gouger (1972). We should also point out that both *H. holstii* and *P. pinus* are found in the mycangium of female *D. frontalis* (Barras and Perry, 1972) and that their introduction in the galleries is not dependent on chance transport on the exoskeleton. This adds to our conviction that our results indicate these yeasts have an important function in the bark beetle–host ecosystem.

As yeasts produce numerous volatile products during growth, we analyzed these compounds from extracts of cultures of these three yeasts. Both *H. holstii* and *P. pinus* produced three main compounds, namely ethanol, isoamyl alcohol, and 2-phenylethanol. *H. holstii* usually grew more rapidly than *P. pinus* and produced larger quantities of these three alcohols. In

addition to these compounds, *P. bovis* also produced appreciable quantities of isoamyl acetate and 2-phenylethyl acetate.

All of these identified compounds are known to be produced by certain yeasts during the fermentation of glucose (Suomalainen, 1969), so their production in this case was not unexpected. As we have found that 2-phenylethanol was the only one of these substances (with the possible exception of ethanol) produced by *H. holstii* and *P. bovis* in detectable quantities on a phloem medium, the growth medium influences the quantity and ratios of these metabolites. Their production may be dependent on the amount of glucose or carbohydrate available, as the addition of 2% glucose to the phloem medium resulted in their production. In the laboratory bioassays that we conducted, we did not find any behavioural response to these yeast metabolites (ethanol was not tested). However, ethanol is a common end product of fermentation by yeasts, and the ambrosia beetles *Trypodendron lineatum* Olivier (Moeck, 1970, 1971) and *Gnathotrichus sulcatus* Le Conte (Cade et al., 1970; Moeck, 1971) are attracted to it. Its production by anaerobic metabolism in potential hosts has been suggested (Graham, 1968).

We consider that these various yeasts growing in the galleries during the early stages of attack by *D. frontalis*, and during the initial mass attack phase, would have an ample supply of carbohydrate available and would therefore produce these various compounds. However, at this stage in these investigations we do not know whether these yeasts growing in situ produce the various volatile compounds identified above or whether these compounds are produced in time to actually interact with other known attractants, e.g., frontalin, and thereby influence the behavior of beetles toward trees under attack. These problems are complex and are being investigated further.

If we assume that these volatile substances are produced in the galleries of a tree under active attack, we consider it very likely that they would be perceived by attacking bettles and could influence their behavior. The presence of 2-phenylethanol in the hind guts of emergent *D. brevicomis* males and feeding *Ips paraconfusus* males has recently been reported (Renwick et al., 1976). In field bioassays, the response of *D. brevicomis* was not affected by the addition of 2-phenylethanol to its known attractant, whereas the response of *I. paraconfusus* to male-infested log sections was greatly enhanced by the addition of 2-phenylethanol (Renwick et al., 1976). No effect of this compound on the behavior of *D. frontalis* has been reported.

It was for these reasons that we bioassayed the various yeast metabolites together with the triplicate standard against *D. frontalis* males and females in the laboratory. We chose a concentration range of the triplicate standard such that the attractiveness of the mixture ranged from very high to very low. With this range of response either inhibition or enhancement of attraction by any of the yeast metabolites should be evident. The results obtained indicate that

the slight response to low concentrations of the triplicate standard can be greatly improved by the addition of low concentrations of certain yeast metabolites, e.g. isoamyl acetate and 2-phenylethyl acetate. It appears, therefore, that certain yeast metabolites enhance the attractive qualities of the triplicate standard to walking beetles in a laboratory bioassay and that the delivery concentration may be an important variable in the response elicited. In addition, inhibitory substances may be produced, and in this context the results obtained from the addition of 2-phenylethanol [TS+(2)] to the triplicate standard seem particularly worthy of further investigation. We appreciate the possibility that the results of walking bioassays in the laboratory may not be directly applicable to the field (Vité et al., 1963), and we are attempting to obtain data on the effect of these metabolites under field conditions. This study is continuing, and we predict that in order to describe fully the complex of behavioral chemicals to which the southern pine beetle displays its attraction, aggregation, and dispersal behavior, volatile microbial products will have to be considered as part of the system.

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Chemistry of Mandibular Gland Secretions of Small Carpenter Bees (*Ceratina* Spp.)[1,2]

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ABSTRACT

The mandibular gland secretion of *Ceratina strenua* F. Smith contains neryl acetate, geranyl acetate, and farnesyl acetate; whereas nerolic acid and geranic acid are present in that of *C. calcarata* Robertson, and 1-hexadecyl acetate in the secretion of males of *C. cucurbi-tina* Rossi. *n*-Pentadecane is a major glandular constituent of all species and is accompanied by C_{16}-C_{23} alkanes and alkenes. The mandibular gland products appear to be effective defensive secretion against ants.

When disturbed, several species of small carpenter bees in the genus *Ceratina* secrete from cephalic glands a yellowish fluid which often possesses a lemonlike odor (Daly 1966). We have also noted that when females of *C. strenua* F. Smith or *C. calcarata* Robertson are persistently agitated with a grass stem while they are in their nests, the bees will smear the exudate over their bodies and onto the intruding object. The presumed utilization of these secretions in defensive contexts prompted us to analyze these exudates.

METHODS AND MATERIALS

Males and females of *C. strenua* and *C. calcarata* were collected in Athens, Ga., whereas those of *C. cucurbitina* Rossi were obtained in Aïn Draham, Tunisia and Draguignan, France. Heads were placed in either *n*-pentane or methylene chloride and these extracts plus additional methylene chloride extracts were employed for subsequent analyses.

Analyses of the cephalic extracts were undertaken on a combined gas chromatograph-mass spectrometer (LKB 9000 and Finnigan 3200 E) utilizing columns of 1% OV-17 temperature programmed from 70°C and 10% SP-1000 temperature programmed from 40°C at 8°/min. Supelcoport 80–100 mesh was used as a solid support for both stationary phases. Cephalic glands were dissected from both *C. strenua* and *C. calcarata* and extracts of the glands were similarly analyzed in order to determine the anatomical source of the observed volatiles.

The repellent effects of identified glandular constituents were studied by treating freshly-killed crickets with one μl of each test compound and observing the reactions of fire ant workers, *Solenopsis invicta* Buren, when the treated insects were placed on their foraging platform.

RESULTS

Mass spectral analyses of the volatiles demonstrated that the cephalic extracts of males and females of all 3 species were rich in normal alkanes and alkenes.

n-Pentadecane was a major constituent in all species and was accompanied by a series of minor or trace hydrocarbons in the range C_{16}-C_{23} (Table 1). Only *C. calcarata* produced tricosane and tricosene, whereas *C. strenua* extracts were distinctive because nonadecene and heneicosene were major constituents. *C. cucurbitina* was distinguished from the other 2 species because heptadecene was a major component (Table 1).

Extracts of *C. strenua* also contained 3 terpenes, which possessed a base peak at m/e 69 and exhibited molecular ions at m/e 196, 196, and 264, respectively. Comparison of the retention times and fragmentation patterns of synthetic neryl acetate, geranyl acetate, and farnesyl acetate with those of the unknowns established their identities. All of these were present in trace amounts compared to the alkanes and the alkenes. The ratio of neryl/geranyl acetate was ~1:2.

Similar examination of male and female heads of *C. calcarata* indicated the presence of 2 different monoterpenes. These compounds showed a base peak at m/e 69 with a molecular ion at m/e 168. The molecular ion exhibited a prominent loss of 68 mass units to m/e 100. Comparison with authentic samples established their identities as nerolic and geranic acid (~1:2). One sample of *C. strenua* female heads also exhibited nerolic and geranic acids as minor components. This was the only extract examined which contained all 5 terpenes.

Extracts of *C. cucurbitina* heads contained no detectable terpenoid constituents. However, male extracts were distinguished by the presence of a compound exhibiting a small molecular ion at m/e 284 and this was subsequently identified as 1-hexadecyl acetate by comparison with an authentic standard. Analyses of extracts of dissected cephalic glands established that all detected compounds originated in the capacious mandibular glands that are characteristic of these *Ceratina* spp.

When freshly-killed crickets were treated with either neryl/geranyl acetate, farnesyl acetate, 1-hexadecyl acetate, or *n*-pentadecane, fire ant workers rapidly approached the dead insects but immediately withdrew, often while cleansing their antennae. The retreating ants frequently stopped to perform stereotyped antennal cleaning behavior, and then moved erratically over the foraging platform. However, within 30 min, the crickets were being fed upon by large numbers of ant workers.

Table 1.—Components of the mandibular gland secretions of *Ceratina* spp.

	mw	*C. strenua*	*C. calcarata*	*C. cucurbitina*
Pentadecane	(212)	major	major	major
Hexadecane	(226)	trace	—	trace
Heptadecane	(240)	major	major	minor
Heptadecene	(238)	trace	trace	major
Nonadecane	(268)	—	trace	minor
Nonadecene	(266)	major	trace	minor
C21 ane	(296)	—	trace	minor
C21 ene	(294)	major	minor	minor
C23 ane	(324)	—	minor	—
C23 ene	(322)	—	minor	—
Neryl acetate	(196)	minor	—	—
Geranyl acetate	(196)	minor	—	—
Farnesyl acetate	(264)	minor	—	—
Nerolic acid	(168)	—	minor	—
Geranic acid	(168)	—	minor	—
1-Hexadecyl acetate	(284)	—	—	minor

DISCUSSION

The presence of a large number of hydrocarbons in the mandibular glands of these 3 species of small carpenter bees distinguishes their secretions from those of any other hymenopterous species analyzed to date. Hydrocarbons are characteristic products of the Dufour's glands of ants (Blum and Brand 1972) but these compounds have never been previously encountered in the mandibular gland secretions of hymenopterans. Indeed, except for the identification of nonadecene as the main constituent produced in the labial glands of males of *Bombus hortorum* L. (Kullenberg et al. 1970), hydrocarbons have not been reported to be present in the cephalic glandular secretions of any hymenopterans.

The cephalic glands of bees appear to be an especially rich source of terpenes, and both farnesyl acetate and geranyl acetate have been previously identified as minor labial gland products of males of *Bombus* and *Psithyrus* spp. (Kullenberg et al. 1970). Since 1-hexadecyl acetate has also been detected as a cephalic product of males of *Bombus sporadicus* Nyl. (Kullenberg et al. 1970), it is ob-vious that *Ceratina* spp. share at least some exocrine products with these male apids.

Nerolic and geranic acids, the major terpenes produced by *C. calcarata,* have been identified as exocrine products of the honey bee *Apis mellifera* L. (Boch and Shearer 1964). However, these terpenoids, as well as several others, are not produced in the mandibular glands but rather, in the Nassanoff gland, a dorsal organ located on the 7th abdominal tergite. It appears that, in the honey bee, the synthesis of monoterpenes has shifted from cephalic glands to the Nassanoff gland, and the latter structure has become an important social organ. On the other hand, the mandibular glands of honey bees, in common with those of *Ceratina* spp., still possess a deterrent function and their secretion is dominated by a well-known defensive compound, 2-heptanone (Shearer and Boch 1965). These facts serve to emphasize the varied origins of exocrine compounds in bees while at the same time indicating the natural product potential that these arthropods possess.

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DIMORPHISM IN CERATOCYSTIS MINOR VAR. BARRASII

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Abstract

Ceratocystis minor var. *barrasii*, a fungus associated with the southern pine beetle, *Dendroctonus frontalis*, exhibits dimorphism which is influenced by the concentration of both carbon dioxide and phosphate. The mycelium produces blastospores from the tips and sides of its hyphae when the concentration of carbon dioxide is high, and these blastospores germinate if the concentration of phosphate is above about 10^{-2} mM, whereas below this concentration they bud.

Introduction

Dimorphism in fungi has been divided into three classes by Romano (9). *Blastomyces dermatitidis*, in which phase conversion is solely dependent on the incubation temperature, is an example of the first class. Conversion from the mycelial to the yeast phase occurs only at temperatures above 37°C (6). In the second class, the two phases have differing nutritional requirements. For example, in *Mucor*, a high level of carbon dioxide will induce the arthrospores to bud to form yeast-like cells (2). In the third class of dimorphism the two phases have different optimal temperatures and different nutritional requirements. Conversion from the mycelial to the yeast phase in *Histoplasma capsulatum* requires temperatures of 37°C (8) as well as the presence of sulfydryl groups in small organic molecules (10). The factors influencing dimorphism in many pathogenic fungi have been ably reviewed by Gilardi (5).

Dimorphism in *Ceratocystis* species has been well established. Francke-Grosmann (4) reported that many *Ceratocystis* species associated with bark beetles are capable of growing in a yeast-like manner. Tyler & Parker (11) observed budding spores in *C. ulmi* at tem-

peratures below 18°C. Similarly, Batra & Michie (3) were able to obtain yeast-like growth of *C. bicolor* on a number of different media at temperatures up to 33°C and Mariat (7) has shown that *C. stenoceras* will grow as a yeast if the culture is continuously agitated.

The prothoracic mycangium of the female southern pine beetle, *Dendroctonus frontalis* Zimm., harbors two fungi (1) one of which produces spores that reproduce by budding. This paper deals with the dimorphism of this fungus, *Ceratocystis minor* (Hedgcock) Hunt var. *barrasii* J. Taylor var. nov. and some factors which influence it.

Materials and methods

Organism

C. minor (Hedgcock) Hunt var. *barrassii* J. Taylor var. nov. was isolated from the prothoracic mycangium of female *D. frontalis* and kindly supplied by Dr. S.J. Barras.

Media

All studies used either Sabouraud dextrose broth (SAB) or a defined medium consisting of: glucose, 40g; $(NH_4)_2SO_4$, 2g; KH_2PO_4, 3.4g; Na_2HPO_4, 6.6g; H_3BO_4, 10μg; $MgSO_4 \cdot 7H_2O$, 0.2g; $CaCl_2 \cdot 2H_2O$, 1 mg; Fe-Cl₃.6H₂O, 1.3mg; thiamine HCl 50μg; pyridoxal HCl, 25μg; per liter distilled water.

biotin, 5μg;
$MnSO_4$, 10μg; $ZnSO_4$, 10μg; $CuSO_4$, 50μg; MoO_3, 10μg;

Cultural conditions

All cultures were grown at 25°C. Shaken cultures were grown in a gyrorotary shaker at 200 rpm.

Inocula

The suspensions of yeast-like cells used as inocula were obtained by filtering 100 ml of a culture which had 10^7–10^8 yeast-like cells per ml through Whatman #4 filter paper to remove the mycelium.

Carbon dioxide determination

A 0.1 ml sample of the culture was acidified with 1.0 ml 5% sulfuric acid. Compressed air, passed through a flask of 20% KOH and a flask of water, was sparged through the reaction mixture for 5 minutes. The gas from the reaction mixture was then sparged through 100 ml of 10^{-3} M Ba (OH)$_2$. The CO_2 combined with the Ba (OH)$_2$ to form $BaCO_3$ with precipitated out. The remaining Ba(OH)$_2$ was titrated with 0.01 M HCl and the amount of $BaCO_3$ formed was determined. This equals the amount of CO_2 in the sample.

Results and discussion

Life cycle

C. minor var. *barrasii* grown on a rotary shaker exhibits the dimorphic life cycle illustrated in Fig. 1. The fungus
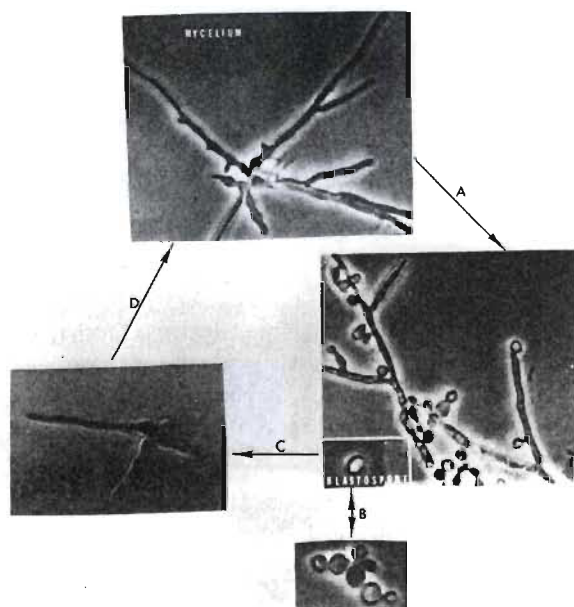
Fig. 1. Life cycle of *C. minor* var. barrassii. (A) depicts sporulation, (B) budding, (C) germination and (D) mycelial formation.

produces blastospores from the tips and sides of its hyphae (A) and these blastospores may either bud (B) or they may germinate (C). Blasospores are defined as spores that arise from the hyphae by budding. Germination of the blastospores consists of the formation of a germ tube which elongates and branches, forming a septate mycelium (D). As the blastospores have the ability to reproduce by budding, they are also called yeast-like cells.

Blastospore formation

A shaking culture grown in either SAB or the defined medium will form blastospores in 2–3 days. The number of these yeastlike cells in a shaking culture of the defined medium can be substantially reduced by the addition of 0.5 ml of 20% KOH to the center well of a Warburg flask. The KOH reduces the amount of CO_2 in the system. No yeast-like cells are formed in standing cultures of SAB which have a pH of 5.6 and have no measurable CO_2. In contrast, cultures in the defined medium with a pH of 7.2 and approximately 7 mM CO_2 show considerable sporulation. The higher pH of the defined medium allows more CO_2 to be dissolved.

If a shaking culture growing in the defined medium is sparged continuously with compressed air the number of yeast-like cells will be greatly reduced. Balls of mycelium form which contain yeast-like cells but no yeast-like cells occur on the outside of the balls or in the surrounding medium. Presumably the continuous sparging removes the dissolved metabolic CO_2 from the medium but not from within the balls. These three observations indicate that sporulation will only occur above a certain CO_2 concentration.

Budding

A shaking culture was grown in SAB until it contained 10^7–10^8 yeast-like cells per ml. This culture was filtered through Whatman #4 filter paper to remove any mycelium and the filtrate shaken again. The number of yeast-like cells per ml was determined in a Petroff-Hausser chamber. The fact that blastospores do reproduce themselves by budding is illustrated in Fig. 2. The budding occurred with a doubling time of 9 hours.

Germination

The above results which show that blastospores can reproduce by budding suggest that the medium from
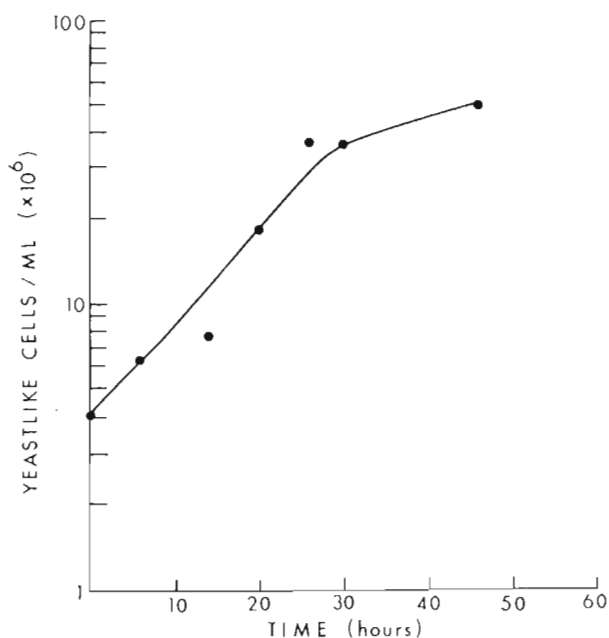
Fig. 2. Reproduction by budding of a culture of blastospores in Sabouraud's dextrose broth.

which the blastospores were taken somehow inhibited germination. There are two possible ways in which this could happen. Either the fungus had excreted some substance into the medium which inhibited germination, or the medium was depleted of some nutrient necessary for germination.

To test the first possibility, a shaking culture was grown in SAB with 1% yeast extract (SABYE) until there were more than 10^8 yeast-like cells per ml. The culture was filter sterilized and 4g glucose, 1g neopeptone and 1g yeast extract were added to 100 ml of media. The culture medium was re-inoculated with yeast-like cells and placed back on the shaker. The yeast-like cells subsequently germinated indicating that the original culture medium did not produce an inhibitor of germination.

To test the second possibility, a shaking culture of SABYE was grown and filtered through Whatman #4 filter paper. The medium was divided into 10 ml aliquots and to each flask all but one of the components of the defined medium were added, one flask had all the components present and a blank flask had none added. In each case the additional components increased the volume of the culture by no more than 1 ml. The results are presented in Table 1. The yeast-like cells in flask 1 germinated indicating that the addition of all the components of the defined medium would bring about germina-

tion. Flasks 2, 3, 4, 5 and 6 also germinated indicating that the components omitted from these cultures were not necessary for germination. Flasks 7, 8, 9, 10 and 11 did not germinate suggesting that both the buffer solution and the vitamin solution were needed for germination to occur. Neither germination nor budding occur in the complete absence of the buffer or the vitamin solutions. However, the five cultures which did not germinate did reproduce by budding showing that there were enough nutrients in the original SABYE to allow the culture to grow.

In an attempt to determine the concentration of vitamins necessary to cause germination, yeast-like cells were inoculated into the defined medium with the concentration of vitamins ranging from 10^{-7} M, the normal concentration, to 10^{-13} M. Although the amount of growth was greatly reduced at concentrations below 10^{-9} M, germination occurred even at vitamin concentrations of 10^{-13} M, suggesting that the amount of biotin, thiamine and pyridoxal available to the fungus plays a role in its growth but not directly in germination of the blastospores.

By inoculating yeast-like cells into the defined medium with different dilutions of buffer, we determined the level of buffer necessary to cause germination. Germination occurred in a culture containing at least 2.5×10^{-2} mM KH_2PO_4 and 4.6×10^{-2} mM Na_2HPO_4. If the buffer concentration was lower, budding occurred.

181

Table 2. Effect of components of buffer on germination

Flask No.	Buffer component added*	Germination
1	Complete buffer	+
2	25 mM KCl	−
3	93 mM NaCl	−
4	71 mM KH_2PO_4	+
5	None	−

*Blastospores were inoculated into defined medium with a 10^4 dilution of buffer. Different buffer components were added to the cultures, which were subsequently examined for germination.

Since the blastospores in flasks 9 and 10 reproduced by budding, neither potassium nor sodium alone can cause germination. This suggested that phosphate was the component of the buffer that caused germination. To test this, blastospores were inoculated into the defined medium with a 10^4 dilution of the buffer solution. Individual components of the buffer were added to the cultures. The results, shown in Table 2, indicate that phosphate probably influences germination directly suggesting that C. minor var. barrasii falls into Romano's second class of dimorphic fungi, namely those in which the form of the fungus is determined by the composition of the medium (9).

Barras & Perry (1) reported that sporulation of C. minor var. barrasii occurs in the galleries made by bark beetles in the phloem of pine trees. One may speculate that the carbon dioxide produced by the growing fungi and bacteria in the galleries, as well as that produced by the adult beetles and beetle larvae, could cause the observed sporulation. Blastospores seem likely to be the easiest form of the fungus to be picked up by callow adults. Barras & Perry (1) also stated that yeast-like cells reproduced in the prothoracic mycangium of the female beetle. If the level of phosphate in the mycangium was low, the blastospores would germinate by budding and when the beetle made a gallery it would distribute these yeast-like cells throughout the gallery where they would germinate. The speculative nature of these statements must be emphasized.

Conclusion

Our results suggest the following life cycle for C. minor var. barrasii. Blastospores, upon inoculation into fresh medium, germinate and form a mycelium. As the fungus grows it produces CO_2 and when the concentration of dissolved CO_2 reaches a certain level, sporulation occurs. At this stage the blastospores may either germinate or bud. If the concentration of phosphate is above about 10^{-2} mM the blastospores germinate, but if it is below this value they bud. Our results suggest that the type of growth that the blastospores undergo, whether by germination and mycelial growth or by budding and yeast-like growth, is determined by the concentration of phosphate and is independent of the CO_2 concentration.

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CHRYSOMELIDIAL IN THE DEFENSIVE SECRETION OF THE LEAF BEETLE
Gastrophysa cyanea MELSHEIMER

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Abstract—Larvae of the chrysomelid beetle *Gastrophysa cyanea* produce a defensive secretion in their eversible thoracic and abdominal glands that is an effective repellent for small predators such as fire ants. This secretion is composed primarily of chrysomelidial, 2-(2-formyl-3-methyl-2-cyclopentenyl)propanal, and a compound tentatively identified as its enol lactone. Adaptations that optimize the effectiveness of the larval defensive exudate are discussed.

Key Words—chrysomelidae, chrysomelidial, *Gastrophysa cyanea*, defensive secretion.

INTRODUCTION

The eversible glands of larval leaf beetles (Chrysomelidae) have proven to be a rich source of insect defensive products. A number of diverse compounds, including salicylaldehyde (Wain, 1943, Pavan, 1953; Wallace and Blum, 1969), benzaldehyde (Moore, 1967), and β-phenylethyl esters (Blum et al., 1972), have been identified in the secretions of the relatively few species of chrysomelid larvae that have been so far investigated. We have investigated the defensive secretion of *Gastrophysa cyanea* and have identified as its major constituent chrysomelidial, a novel cyclopentenoid monoterpene aldehyde.

47

METHODS AND MATERIALS

The secretion was collected in 0.5-μl microcapillaries from the everted glands of tactually stimulated larvae, and stored in n-hexane or methylene chloride at $-10°$. These extracts were used directly for all chemical analyses.

The secretion was analyzed on the following gas-liquid chromatography columns: 10% Carbowax 20 M, 3% SP-1000, and 3% ECNSS-M. Combined gas chromatographic–mass spectrometric (GC–MS) analyses were carried out on an LKB 9000 instrument. Nuclear magnetic spectra were obtained on a Varian XL-100-15 spectrometer equipped with a Digilab Fourier transform system.

The deterrent value of the defensive secretion was examined by placing larvae on the foraging platform of a fire ant (*Solenopsis invicta*) colony and observing the subsequent confrontations. The repellency of chrysomelidial, collected by preparative gas chromatography, was examined by treating *Tenebrio* larvae with 10 μg of this compound and noting the reactions of fire ant workers that encountered these treated larvae placed on their foraging platform.

RESULTS

Identification of Chrysomelidial

Upon GC–MS, the extract showed two peaks of nearly equal height eluting at 10.6 and 15.5. min at 160° on a 2-m 10% Carbowax 20 M column. The first peak gave a molecular ion at m/e 164 (65), and important ions at m/e 136 (60), 121 (40), 107 (100), 93 (45), 91 (76), and 80 (82), whereas the second peak gave a molecular ion at m/e 166 (6) and important ions at m/e 151 (4), 148 (21), 138 (15), 137 (4), 133 (6), 123 (10), 109 (53), 108 (26), 81 (100), and 79 (37).

After isobutane chemical ionization, mass measurement of the two protonated molecular ions led to the formulas $C_{10}H_{12}O_2.H^+$ (found 165. 0925) and $C_{10}H_{14}O_2.H^+$ (found 167.1093), respectively. Neither compound reacted with silanizing agents, but the peak from the second compound disappeared after treatment with $NaBH_4$. Furthermore, the second compound reacts with 2,4-dinitrophenylhydrazine and Purpald (Dickinson and Jacobson 1970) (after collection from the gas chromatograph) and forms a dimethoxime exhibiting a molecular ion at m/e 224 (4) and other ions at m/e 193 (7), 178 (9), 138 (100), 107 (38), 106 (40), 87 (15), and 79 (23). The base peak at m/e 138 (M-86) and the rearrangement ion at m/e 87 in the mass spectrum of the methoxime, taken with the intense peak at m/e 109 (M-57, confirmed by a

metastable ion at 71.7) in the original compound, point to the structural feature $-CHCH_3CHO$ and indicate the presence of a γ-hydrogen atom.

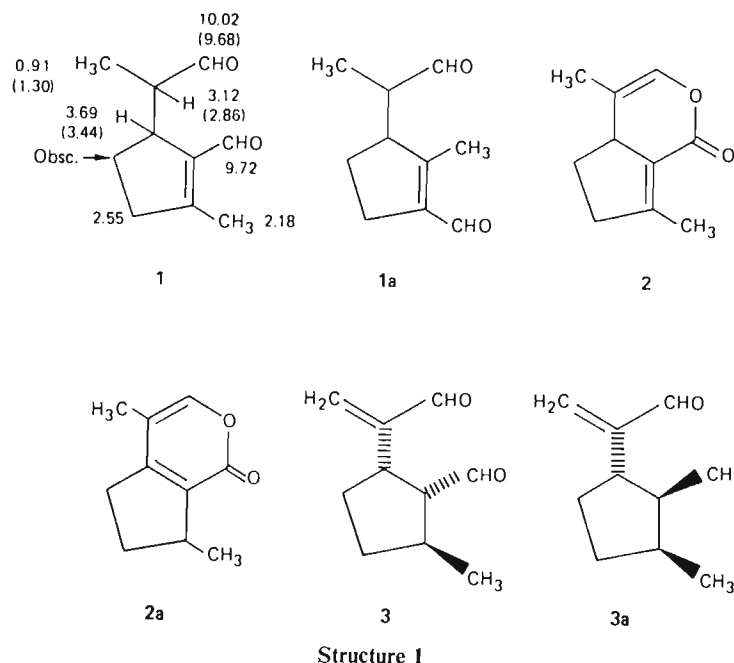Preparative gas chromatography of this second peak provided enough material (~ 50 μg) for a Fourier-transform PMR (100 mHz, $CDCl_3$, 35,000 16K free induction decays). Two aldehydic protons at $\delta 9.72$ and 10.02, were clearly visible along with an olefin-bound methyl ($\delta 2.18$) weakly coupled to allylic protons ($v_{1/2}$ 6 Hz). In confirmation of the mass spectral results, a doublet was observed at $\delta 0.91$ ($J \sim 7$ Hz) for the methyl protons of the $-CHCH_3CHO$ feature. The corresponding α proton ($\delta 3.12$) was additionally coupled to a downfield, presumably allylic hydrogen at $\delta 3.69$ ($J \sim 4$ Hz). Finally, an allylic methylene triplet was observed at $\delta 2.55$, further broadened by long-range coupling. The remaining methylene group probably appeared in the $\delta 1.5-1.7$ region, but was obscured by impurities in the solvent. The lack of olefinic protons observed indicates a ring.

The presence of a tetrasubstituted, α,β-unsaturated aldehyde was suggested by the ultraviolet spectrum (λ_{max}^{Etoh} 256 nm). This absorption disappeared on treatment with $NaBH_4$ and shifted to 277 nm (285 nm infl.) on treatment with semicarbazide. There appear to be few models for a 2-substituted 1-cyclopentene carbaldehyde,[4] but the values for 2-methyl-1-cyclohexene carbaldehyde (λ_{max}^{Etoh} 242 nm, ε 11,000) (Braude and Timmons, 1955), 1-cyclohexene carbaldehyde (λ_{max}^{Etoh} 229 nm, ε 12,000) (Heilbron et al., 1949), and 1-cyclopentene carbaldehyde (λ_{max}^{Etoh} 237 nm, ε 12,000, semicarbazone 267 nm, ε 37,500, 277 nm infl., ε 25,500) (Heilbron et al., 1949) suggest that a value near 250 nm is reasonable.

These facts can be accommodated by structures 1 or 1a for chrysomelidial; the existence of the closely related dolichodial (3 and 3a) (Cavill and Hinterberger, 1961; Cavill and Whitfield, 1962; 1964; Cavill, 1969) and anisomorphal (3 or 3a) (Meinwald et al., 1962) leads us to favor the former, and we have assigned the resonances accordingly.[5] The lack of observable coupling in structure 1 between the aliphatic aldehyde and its adjacent H indicates that the methyl and carbonyl are essentially eclipsed, as expected for an α-substituted aldehyde (Karabatsos and Hsi, 1965). The downfield shift of the adjacent allylic hydrogen is unusual, and models suggest that it may be caused by the influence of the nearby unsaturated aldehyde. Its mass

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[4] Carbaldehyde replaces carboxaldehyde according to IUPAC, nomenclature rules; c.f. J.H. Fletcher, O.C. Dermer, and R.B. Fox, *Nomenclature of organic compounds*, Advances in Chemistry Series #126, American Chemical Society, Washington, D.C., 1974, p. 161.

[5] After this work was completed, we learned that J. Meinwald had identified chrysomelidial in the secretion of another chrysomelid larva, a *Plagiodera* species. He has kindly compared a sample of chrysomelidial isolated from *G. cyanea* to an unambiguously synthesized sample of this compound and found them to be identical; cf. J. Meinwald, T.H. Jones, T. Eisner, and K. Hicks, *Proc. Natl. Acad. Sci.* 74:2189–2193 (1977).

D

10.02
(9.68)
0.91   H₃C   CHO
(1.30)
              H   3.12
3.69 H   (2.86)
(3.44)
Obsc.→   CHO
                 9.72
   2.55   CH₃ 2.18

1

H₃C   CHO

              CH₃

              CHO

1a

H₃C   O

              O

              CH₃

2

H₃C   O

              O

              CH₃

2a

H₂C   CHO

              CHO

              CH₃

3

H₂C   CHO

              CHO

              CH₃

3a

Structure 1

spectrum, involving loss of the sidechain, is, of course, markedly different from that of dolichodial. By analogy with dolichodial (Cavill and Hinterberger, 1961), hydrogenation of this compound (PtO₂–ethanol) might be expected to produce the known dihydro derivative, iridodial, or one of its isomers. Unfortunately, however, extensive hydrogenolysis occurs, giving at least seven compounds, none of which show a mass spectrum similar to that of irododial.

A second sample of chrysomelidial collected from a 3% SP-1000 GC liquid phase showed the presence of a second, very similar compound via a doubling of several major PMR peaks. When the sample was reexamined with a 3% ECNSS-M column, two GC peaks of similar intensity appeared at 14.2 and 15 min, giving nearly identical mass spectra. It is clear that the carbon alpha to the aliphatic aldehyde epimerized during GC in the presence of the somewhat acidic SP-1000 liquid phase. We have assigned to this epimer the NMR peaks shown in parentheses on structure 1.

With the later-eluting peak of the extract established as resulting from structure 1, the first peak, whose mass spectrum lacks two hydrogens compared with 1, may represent the corresponding enol lactone, 2. Its mass spectrum, characterized by loss of CO followed by loss of either methyl or CHO, may be explained as follows:

Structure 2
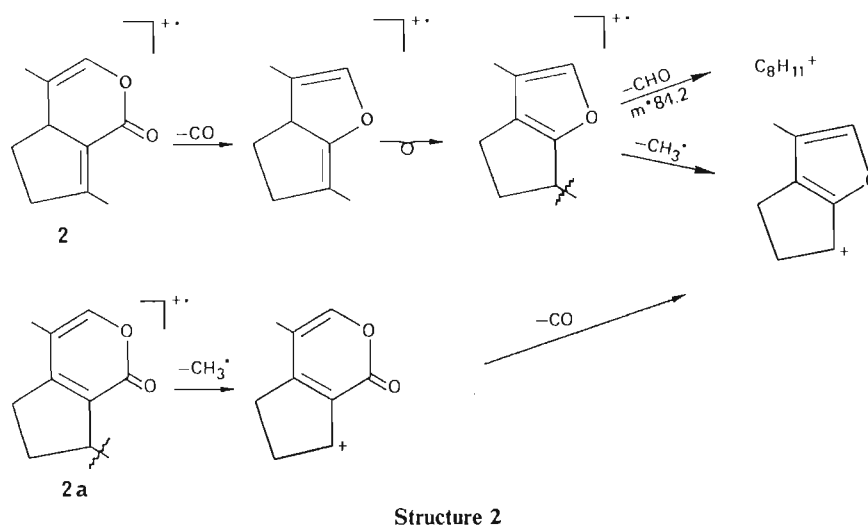
An old sample of the extract exhibited a trace of an isomer of 2 eluting just before 1 on 3% ECNSS-M and having an intense molecular ion at m/e 164 (100) and important ions at m/e 149 (64), 135 (45), 121 (53); i.e., loss of the same fragments occurred in the reverse order. We suggest this is the conjugated pyrone 2a, which would not require rearrangement prior to methyl loss as would 2.

Defensive Value of the G. cyanea Secretion

Mature larvae of *G. cyanea* that had been placed on the foraging platform of a fire ant colony were generally avoided by these normally aggressive ants. However, the occasional ant workers that attacked the larval beetles were totally disarmed by the chrysomelid secretion. Larvae that were tactually stimulated by their ant antagonists immediately evaginated their eversible glands, smearing their aggressors with the defensive secretion. Typically, a contaminated ant worker immediately withdrew from the scene of the encounter, dragging its head and antennae, and moving in a completely disoriented manner. The secretion-labeled ant was avoided by its sister workers. Furthermore, when a secretion-moistened larva fresh from an encounter moved near a group of ant workers feeding on a cockroach, the ants immediately abandoned their repast and moved rapidly from the area.

Tenebrio larvae treated with chrysomelidial were avoided by ant workers for 10 min or longer. On the other hand, untreated larvae were immediately overrun by ant workers.

DISCUSSION

The identification of chrysomelidial in the glandular exudate of *G. cyanea* further emphasizes the diversity of defensive compounds produced by larval Chrysomelinae. Salicylaldehyde dominates the secretions of species of *Phyllodecta* (Wain, 1943), *Melasoma* (Pavan, 1953), and some species of *Chrysomela* (Wallace and Blum, 1969). On the other hand, the defensive secretion of *Chrysomela interrupta* is comprised primarily of β-phenylethyl isobutyrate and β-phenylethyl 2-methylbutyrate (Blum et al., 1972). It would not prove surprising if the defensive exudates of species in other chrysomelid genera proved to be sources of interesting new insect natural products.

We consider the secretion of *G. cyanea* to be the most potent fire ant deterrent produced by any species of chrysomelid that we have examined. Several behavioral adaptations appear to optimize the effectiveness of the chrysomelidial-rich defensive exudate of *G. cyanea*. Early-instar larvae characteristically aggregate on the undersides of dock (*Rumex* sp.) leaves, where they are very inconspicuous. In a sense, the clumped larvae "pool" their limited defensive secretion. Disturbance of a larval aggregation results in the virtual simultaneous eversion of the glands of many larvae. In young larvae, only the thoracic glands are functional, whereas in older larvae, which are often solitary, the defensive secretion issues from glands through pairs of tubercles on the last two thoracic and first seven abdominal segments as has been reported for other species of Chrysomelinae (Garb, 1915).

Significantly, the pupae of *Gastrophysa*, unlike those of *Chrysomela* species, shed their pupal skin entirely, thus losing their defensive glands. *Chrysomela* pupae, which are exposed on the host plant, retain the larval integument that contains the salicylaldehyde-rich defensive glands. The glands can be discharged when the freehanging pupa is stimulated (Hinton, 1951; Wallace and Blum, 1969). Indeed, the freshly emerged adult, which is especially vulnerable to predators, is bathed in the salicylaldehyde derived from the retained larval defensive glands, and this aromatic bath renders the beetle highly repellent to invertebrate predators at a time when it is very inactive (Wallace and Blum, 1969). *G. cyanea*, on the other hand, discards its chrysomelidial-fortified glands when it pupates, and would seem to be especially susceptible to predation if exposed like the pupa of *Chrysomela*. However, the *Gastrophysa* larvae pupate in the ground litter near the host plant, and are very unobtrusive. These results illustrate the variety of defensive mechanisms evolved in the Chrysomelidae and emphasize the chemical and behavioral diversity manifested by the species in this large family.

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Fire Ant Venom Alkaloids: Their Contribution to Chemosystematics and Biochemical Evolution

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Abstract—Studies on the chemistry of the 2,6-dialkylpiperidines in the venom of various fire ant species have shown that these alkaloids have practical value as chemotaxonomic characters. In addition, the finding that variations exist in the distribution of the geometric isomers between species and castes has led to a hypothetical construct for the biochemical evolution of these compounds. These various results, obtained previously, are integrated here as a coherent whole.

Progress in chemosystematics and biochemical evolution is dependent upon the identification of new (usually) secondary substances from established taxa and the identification of already known compounds from additional taxa. Nevertheless, all available evidence morphological, physiological, anatomical as well as chemical, should be used for the description of taxa. However, as a rule, chemical characters may be a little closer to the gene than morphological ones. For example, a whole biosynthetic pathway may be affected by a change in the activity of a single enzyme and therefore by a single gene, whereas morphological characters would not be changed so easily. Taxonomically useful chemicals often are those that accumulate before a rate limiting step or at the end of a biosynthetic chain.

A problem in the application of chemical data to taxonomy is the fact that the acquisition of such data usually requires the use of specialized and expensive equipment and may thus be relatively hard to obtain. Moreover, pattern recognition using chemical data is best carried out by biochemists familiar with the ramifications of metabolic pathways. Studies on chemosystematics and biochemical evolution often would be served best by the detailed elucidation of the enzymology of the biochemical pathways involved. Knowledge of a specific biochemical pathway should be considered more important than the mere knowledge of the structure of the end product. It is not the substances produced but the homology of their biosynthetic routes that is the more

plausible indication of phylogeny. One may anticipate that closely related species will be more likely to synthesize the same compound by the same route using closely related enzymes, than will widely separated taxa. At present, however, the details of biochemical pathways to various secondary compounds within numerous taxa are generally not known in sufficient detail.

This paper synthesizes much that previously has been published about the piperidine alkaloids occurring in the poison glands of the main species of fire ants found in the southeastern United States. Two important sets of biochemical data are missing from this example, namely, the biosynthetic origin and the absolute stereochemistry of these alkaloids. These data, once obtained, will supplement and may modify what is presented here.

Ants in the large genus *Solenopsis* are distributed worldwide and in general are rather unobtrusive. Two closely related species were imported into the southern United States from South America about 40–50 years ago and one of these, *S. invicta*, has become dominant in the region. It is a particularly aggressive species and anyone who has been stung by its workers understands why it is commonly called the 'fire' ant. It is primarily the fact that its stings are so painful that has led to the spending of vast sums of money both for its control and for a better understanding of its biology.

In the southeastern United States the four main species of fire ant are *S. invicta, S. richteri, S. geminata* and *S. xyloni,* all of which

possess venoms that contain various 2-methyl-6-n-alkyl (or alkenyl) piperidines. The paucity of proteins in these venoms makes these ants unique among the stinging hymenopterans. The basic chemistry of these alkaloids has been described [1, 2] as well as their distribution among the various species [3–5] and between individuals of the same species [6, 7].

The two introduced species, *S. invicta* and *S. richteri*, belong to the large group of species termed the *saevissima* complex, while *S. xyloni* and *S. geminata* are assigned to separate species complexes [5]. Brand *et al.* [3] conducted a comparative study on the presence of piperidine alkaloids in workers of all four of these species and found chemical features that distinguish the venom of each. *S. geminata* has the simplest venom, containing both *cis-* and *trans*-2-methyl-6-n-undecylpiperidine (*cis* C_{11} and *trans* C_{11}) as the major components. The same two piperidines are found in *S. xyloni* venom together with a small amount of 2-methyl-6-n-undecyl-$\triangle^{1,2}$-piperideine. The venom alkaloids of the two imported species differ first in that the ring substituents are mainly *trans-*, and second in that the alkyl substituent in the 6-position contains 11, 13, or 15 carbon atoms. *S. richteri* venom contains mainly *trans* C_{11}, *trans*-2-methyl-6-n-tridecyl (and tridecenyl) piperidine (*trans* C_{13} and *trans* $C_{13:1}$) while *S. invicta* contains the same components together with *trans*-2-methyl-6-n-pentadecyl (and pentadecenyl) piperidine (*trans* C_{15} and *trans* $C_{15:1}$). It is apparent therefore that the alkaloidal constituents of the venom are useful characters for a chemotaxonomic study.

It was partly as a result of such observations that MacConnell *et al.* [5] investigated the distribution of the piperidine alkaloids in 29 fire ant populations, many of which were collected in South America. Their results confirmed that those species in the *saevissima* complex have piperidines with the 2,6-substituents *trans* on the ring whereas the *geminata* and *xyloni* complexes have these substituents both *cis* and *trans* on the ring. They concluded that the venom chemistry of these ants correlates reasonably well with species populations and may have practical value as a chemotaxonomic tool. Information on the enantiomeric ratios of the various piperidine alkaloids would be particularly helpful at this point.

It should be noted that the presence of a series of 2,5-dialkyl-pyrrolidines and -pyrrolines [8] has been established in the venom of *S. punctaticeps*, a South African species. MacConnell *et al.* [5] placed this small polymorphic species in a section separate from the true fire ant species mentioned above.

Studies on insect pheromones and defensive substances generally use pooled samples due to the small amount of material that usually can be obtained. Therefore, variations in the relative amounts of any components secreted by an individual are usually unknown. Fire ants, however, contain sufficient amounts of these piperidine alkaloids in their poison sacs to permit gas chromatographic determination of individual variation.

Such studies were done on *S. geminata*, a polymorphic species with the largest individuals often being called soldiers [6]. The venom contains only two major components, namely *cis* C_{11} and *trans* C_{11}. Analyses of these components established that the ratio of *cis* C_{11} to *trans* C_{11} was wider in soldiers than in workers and also that variation existed from colony to colony. If head length was taken as a measure of the size of an ant, it was found that a linear relationship may exist between this measurement and the ratio of venom components. Any significance between the size of an ant and the ratio of the major alkaloids in its venom is cryptic. However, it does suggest that if the ratio of *cis* to *trans* isomers is enzymatically controlled, then the expression of one part of the genome is correlated with size.

One colony of *S. geminata* gave results which indicated that more than one linear relationship may exist between head size and *cis* to *trans* ratio [6]. Individuals of this colony may therefore belong to more than one genetical group. It was speculated that if the nest had more than one functional queen, each of which gave rise to soldier ants, such a situation may well be feasible. It is known that *S. geminata* colonies may contain more than one functional queen [9] and each queen could give rise to a number of genetically distinct populations of workers and soldiers within the same nest.

As mentioned above, the venom of workers of the two imported species, *S. invicta* and *S. richteri*, contains mainly *trans*-2,6-disubstituted piperidines. However, the venom alkaloids of alate queens of these two species consist of only *cis* C_{11} and *trans* C_{11} in a ratio favoring the *cis* isomer [6]. In fact, the venom of alate queens of all four species is remarkably similar. Therefore, within a genus, the venom composition between castes can either be very

similar, e.g. *S. geminata,* or widely different, e.g. *S. invicta.* It is clear that the synthesis of the various alkaloids is the result of the coactivity of a series of enzymatic steps present in a single species. Therefore, there is a direct relationship of the quantitative variation of these substances and differences in the genome. Also, the genetic material in the different castes specifying the component enzymes of the biosynthetic pathways is regulated very differently. If the chemical components in a glandular secretion are to be useful in chemotaxonomy the variation among individuals may be important. Either this variation must be established and taken into account, or pooled samples from many individuals and colonies must be employed. It seems likely that studies on individual variation may prove extremely useful if a biosynthetic pathway and its genetic control is to be investigated. Here are a number of areas that invite further investigation.

Finally, the information presented on fire ant venoms can be taken one step further. The venom alkaloids of the four main species in the United States varies between the workers but does not vary much between the alate females. This information has been used to propose a model for the evolutionary development of the various piperidine alkaloids in these *Solenopsis* species [7].

The venom characteristics of the workers of the four species form a phenocline. This suggests that one of two series of evolutionary events occurred with the venom alkaloids. The simple venom components of *S. geminata* workers with their abundance of the *cis* isomer may be closest to the ancestral form. This condition was followed by a switch to an abundance of *trans* C_{11}, the addition of *trans* C_{13} and *trans* $C_{13:1}$ (*S. richteri*), and then the addition of *trans* C_{15} and *trans* $C_{15:1}$ (*S. invicta*). Or evolution proceeded by the reverse of this sequence. Because there is no probable phylogeny of the genus *Solenopsis* available, we must rely on the chemical data alone in order to present a hypothetical construct in biochemical evolution.

Since the amount of protein in the venom of these *Solenopsis* species is low, one may assume that this genus has evolved a new venom component not yet found in other ants. If the alkaloids are evolutionarily new, then an arrangement of the alkaloidal states in order of their complexity should mirror the pattern of evolution. *S. geminata* and *S. xyloni* would therefore have venom more similar to the

ancestral state than the two imported species. Chemical synthesis of these alkaloids gives a ratio of the *cis* isomer to the *trans* isomer of about 6:1 [2] again fitting the two indigenous species best. *S. eduardi,* a South American species in the *geminata* complex has a venom in which *cis* C_{11} is greatly reduced and *trans* C_{11} is the predominant form [5]. Such a switch would seem to be derived condition brought about by a change in the biosynthetic enzymes. 2-Methyl-6-*n*-undecyl-$\triangle^{1,2}$-piperideine in the venom of *S. xyloni* [3] may be the intermediate for this switch.

However, since enzymes do not always follow the easy way out thermodynamically speaking, the choice of *S. geminata* venom as being the closest to the ancestral type is decided primarily by the finding that alate females of all four species have a venom similar to *S. geminata.* Increasing differences in the venom chemistry between queens and their workers is taken to indicate evolutionary progression.

On the basis of these considerations, a model has been suggested for the evolution of the alkaloidal components in *Solenopsis* [7]. A relatively simple venom dominated by the *cis* isomers, similar to *S. geminata* and *S. xyloni,* was followed by a switch to dominance by the *trans* isomers (*S. eduardi*). The addition of further *trans* isomer homologues led to the venoms of *S. richteri* and *S. invicta.* This model is based on only five species and its value lies in inviting further investigation at the chemical, biochemical and genetical levels.

It must be emphasized that this model deals only with the evolutionary development of the venom alkaloids and in no way suggests that one species of *Solenopsis* is in any other way more primitive than another. Also, it must be considered quite possible that a species of *Solenopsis* that is primitive from a morphological and behavioral point of view could have venom alkaloids that are more advanced in the terms of this model.

These aspects of fire ant venoms illustrate chemotaxonomy and biochemical evolution with one continuous example and present what has been written previously as a coherent whole. It is evident that the elucidation of the biosynthetic pathways leading to these piperidine alkaloids and their absolute stereochemistry would add confidence to one's speculations.

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SIGNIFICANCE OF THE PYRROLIC NITROGEN ATOM IN RECEPTOR RECOGNITION OF *Atta texana* (BUCKLEY) (HYMENOPTERA:FORMICIDAE) TRAIL PHEROMONE AND PARAPHEROMONES[1]

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Abstract - Calculations of the charge of the pyrrolic nitrogen atom in the trail pheromone of *Atta texana*, methyl 4-methylpyrrole-2-carboxylate, as well as 17 analogs, showed that the most active compounds all had the same charge of -0.51 electrons on the nitrogen atom. It is suggested that the close value for this charge may be important in recognition by the receptor.

Key Words - *Atta texana*, trail pheromone, quantum calculations.

INTRODUCTION

The chemorecognition of a small molecule by a specific region of a large macromolecule is one of the most basic phenomena of living systems. This property has been demonstrated many times in both enzymology and the mechanism of drug action, but many examples are also available from studies of chemical communication in insects. Two of the main properties of both the small molecule and the receptor site are probably that their shapes and their electron densities must be complementary.

A major trail pheromone of the ant, *Atta texana* (Buckley), has been identified as the 2,4-disubstituted pyrrole, methyl 4-methylpyrrole-2-carboxylate (I) (Tumlinson et al., 1971), and trail-following activity studies of synthetic analogs of this compound have been conducted by Sonnet and Moser (1972, 1973). Their main conclusions concerning the stereochemical requirements for behavioral activity may be summarized as follows: (1) the pyrrole ring of the trail pheromone is planar; (2) the carbonyl carbon, both oxy-

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273

I
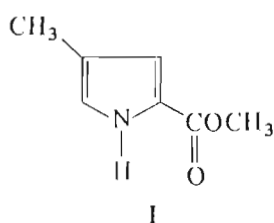
gen atoms, and the 4-methyl group, are coplanar with the pyrrole ring; (3) the pyrrole ring and the carbonyl group are conjugated; (4) the carbomethoxy group can have two conformations; (5) for activity to be retained, the substituent on position 2 cannot be enlarged but can be smaller with similar properties and the substituent on position 4 cannot be removed but can change its size and polarity; and (6) the receptor can distinguish the pyrrolic N from the ring carbons.

It was the last point mentioned above that attracted our attention. It seems likely that the pyrrolic N is important in the process of chemorecognition by the receptor site. We wondered whether the charge on this nitrogen in the most active compounds was the same, and distinct from that of the inactive compounds. By means of the extended Hückel molecular orbital procedure we calculated the charge on the nitrogen of many of the substituted pyrroles synthesized and assayed by Sonnet and Moser (1972, 1973) in the hope that this may add to the stereochemical conclusions they arrived at as requirements for activity. Our results show that the three most active compounds all have an identical charge on the pyrrolic nitrogen. It therefore seems possible that this parameter is important as an additional criterion of activity.

METHODS AND MATERIALS

The extended Hückel calculations on compounds I–XVIII were carried out according to standard methods (Hoffmann, 1963, 1964a,b,c) on an IBM 360 computer. The geometry of the pyrroles was generated from standard bond length and bond angle data, and the coulomb integrals (see Table I) were obtained from valence orbital ionization potentials (Basch et al., 1965). The basis atomic orbitals were Slater-type atomic orbitals with the orbital exponents taken from optimization of the appropriate SCF calculations (Clementi and Raimondi, 1967).

The reasonance integrals, which correspond to the off-diagonal elements of the Hamiltonian matrix, were computed from the Wolfsberg-Helmholz arithmetic mean formula (Wolfsberg et al., 1952)
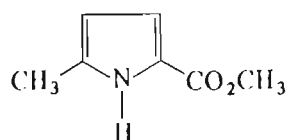
$$H_{ij} = KS_{ij}(H_{ii} + H_{jj})/2$$

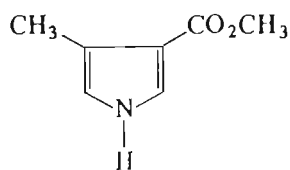where K is 1.75 and H_{ii} and H_{jj} correspond to the coulomb integrals and S_{ij} is
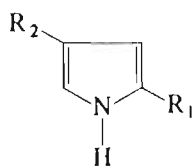
I $R_1 = CO_2CH_3$; $R_2 = CH_3$
II $R_1 = CO_2CH_3$; $R_2 = Cl$
III $R_1 = CO_2CH_3$; $R_2 = C_2H_5$
IV $R_1 = CHO$; $R_2 = CH_3$
V $R_1 = CHO$; $R_2 = Cl$
VI $R_1 = CO_2CH_3$; $R_2 = CHO$
VII $R_1 = COCH_3$; $R_2 = CH_3$

VIII $R_1 = CHO$; $R_2 = CHO$
IX $R_1 = CO_2CH_3$; $R_2 = CO_2CH_3$
X $R_1 = CHO$; $R_2 = CO_2CH_3$
XI $R_1 = CO_2CH_3$; $R_2 = COCH_3$
XII $R_1 = CO_2C_2H_5$; $R_2 = CH_3$
XIII $R_1 = CH_3$; $R_2 = CO_2CH_3$

XIV

XV

XVI

XVII $R_1 = CO_2CH_3$; $R_2 = H$
XVIII $R_1 = CHO$; $R_2 = H$

TABLE 1.   VALUES OF COULOMB INTEGRALS FOR ATOMIC ORBITALS

Atomic orbital	Coulomb integral H_u (eV)
Carbon $2p$	-11.4
Carbon $2s$	-21.4
Oxygen $2p$	-17.9
Oxygen $2s$	-35.30
Nitrogen $2p$	-14.32
Nitrogen $2s$	-27.50
Chlorine $2p$	-15.8
Chlorine $2s$	-24.00
Hydrogen $1s$	-13.6

the overlap integral between orbitals *i* and *j*. With this format, both the total energy for each molecule and the charge on each atom were calculated. Charges were computed using a Mulliken population analysis (Mulliken, 1955, 1962).

The method of calculation does not permit us to calculate the nitrogen charge of the bromo derivative tested by Sonnet and Moser (1972, 1973).

RESULTS AND DISCUSSION

Payne (1974) has suggested that the more the structure of a paraphero-mone varies from the structure of the true pheromone, the greater the amount needed to elicit a significant electroantennogram. Furthermore, the EAG response is usually considered to reflect the behavioral response. Sonnet and Moser (1972) bioassayed the various pyrroles at three different concentrations. At the highest concentration some of the compounds, including the

TABLE 2. RELATIONSHIP BETWEEN CALCULATED CHARGE ON PYRROLE NITROGEN ATOM OF VARIOUS SUBSTITUTED PYRROLES AND THEIR OBSERVED BIOLOGICAL ACTIVITY AS TRAIL-FOLLOWING SUBSTANCES

Compound	Nitrogen charge (electrons)[a]	2,4-Disubstituted	Activity (0.4 ng/μl)[b]
I	−0.51	Yes	+++
II	−0.51	Yes	+++
III	−0.51	Yes	++
IV	−0.52	Yes	−
V	−0.52	Yes	−
VI	−0.48	Yes	+
VII	−0.52	Yes	+
VIII	−0.48	Yes	−
IX	−0.49	Yes	−
X	−0.49	Yes	−
XI	−0.49	Yes	−
XII	−0.51	Yes	−
XIII	−0.53	Yes	−
XIV	−0.57	No	−
XV	−0.59	No	−
XVI	−0.49	No	−
XVII	−0.51	No	−
XVIII	−0.51	No	−

[a] The relative precision of these values is ±0.001. They are rounded to the nearest 0.01 for convenience.
[b] Data from Sonnet and Moser (1972).

natural trail pheromone, were found to cause repulsion. It should be pointed out that the published bioassay results are only roughly quantitative and no corrections are possible for effects such as differences in volatility.

Our interest was concerned primarily with determining whether any calculable parameters of those compounds which mimicked the natural trail-following pheromone were unique. We therefore decided to consider only those compounds that showed high activity at the same concentration as the natural pheromone, rather than consider all the compounds that showed some activity, no matter what the concentration.

With this limitation imposed, only three compounds are considered highly active at 0.4 ng/μl (Sonnet and Moser, 1972; compounds I, II, and III in Table 2). Each of these three compounds has a charge of -0.51 electrons on the pyrrolic nitrogen atom. None of the compounds which showed good activity at 40 ng/μl (IV–VI) has this value. Of the other three compounds with a nitrogen charge of -0.51 electrons, compound XII has an ethyl group in position 2 which is apparently too large for activity (Sonnet and Moser, 1972) and neither XVII or XVIII has the necessary 2,4-disubstitution pattern.

We therefore suggest, although not without caution, that for optimal trail-following activity to be demonstrated, the receptor requires the charge on the pyrrolic nitrogen atom to be a very precise value in a compound with the correct steric properties. If good quantitative data on a series of compounds can be obtained with a bioassay, quantum calculations on the compounds may provide some insight into their mode of action and also have predictive value for compounds as yet untested.

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DECYL ACETATE SYNTHESIS IN THE ANT, *FORMICA SCHAUFUSSI* (HYMENOPTERA: FORMICIDAE)

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Abstract—Decyl acetate occurs in high amounts in the Dufour's gland of *Formica schaufussi*. When worker ants were fed [^{14}C]-acetate the acetate moiety of decyl acetate was labelled preferentially indicating that decyl acetate is synthesized via an esterification reaction.

Key Word Index: Formica schaufussi, ant, biosynthesis, Dufour's gland, decylacetate

INTRODUCTION

THE VOLATILE compounds present in the Dufour's gland of many formicine ants probably comprise the most studied glandular secretion of any insect group (BLUM and BRAND, 1972; BERGSTRÖM and LOFQVIST, 1973; LOFQVIST, 1976). The quantitatively most important compounds occuring in this gland are the hydrocarbons (undecane and tridecane), the 2-ketones (2-tridecanone and 2-pentadecanone), and the acetates (decyl acetate, dodecyl acetate and tetradecyl acetate). However, while no one species contains large amounts of all three classes of compound, many contain appreciable quantities of two of the classes.

In the genus *Formica*, certain species contain one or more of the straight chain acetates (BERGSTRÖM and LOFQVIST, 1968; WILSON and REGNIER, 1971). In slave making species the esters are reported to be important in causing confusion among workers of a slave species during a raid by the slave maker species (REGNIER and WILSON, 1971). Individual worker ants of the slave species, *F. schaufussi*, may contain several hundred micrograms of decyl acetate in the Dufour's gland (BRAND, unpublished data) and this indication of a particularly active biosynthetic mechanism led us to choose this ant which is locally available, to study the biosynthesis of an acetate ester.

Various studies in this laboratory on the microbial degradation of hydrocarbons and 2-ketones have elucidated a pathway establishing that 2-ketones can be oxidized by the introduction of an oxygen atom between carbon atoms 2 and 3 thereby forming acetate esters (BRITTON *et al.*, 1974; BRITTON and MARKOVETZ, 1977). This metabolic relationship between 2-ketones and acetate esters made us interested in the mechanism for the synthesis of acetate esters in the Dufour's gland. We wished to establish whether the acetates in a formicine Dufour's gland arose either by the introduction of molecular oxygen into a 2-ketone or by the esterification of acetic acid with an alcohol. Our results indicate the latter mechanism to be functional.

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MATERIALS AND METHODS

Chemicals

Unless otherwise stated chemicals were of analytical reagent grade. Water was glass distilled and diethyl ether was redistilled.

Collection of ants and preparation of whole ant and Dufour's gland extracts

Formica schaufussi workers were collected in open fields bordering forested areas near Iowa City. The ants were kept in soil in plastic dishpans and fed honey once a week and cockroaches or crickets once a month. Water was always available.

Ether extracts of whole ants were made by macerating ants in ether in 15 × 125 mm test tubes and centrifuging at 1000 *g* for 10 min to remove the solid material. The ether layer was dried over anhydrous sodium sulphate and concentrated under a stream of nitrogen prior to gas chromatography (GC).

Ants were also extracted with chloroform–methanol (2:1, v/v). The chloroform–methanol extract was chilled at −20°C for 1 hr and centrifuged at 1000 *g* for 10 min to remove particulate material. The supernate was concentrated under a stream of nitrogen prior to thin-layer chromatography (TLC).

Dufour's glands were exposed, the common glandular ducts pinched shut, and the glands pulled free. Intact glands were placed in 1 ml of ether and macerated with a glass rod. The ether layer was removed and concentrated prior to GC.

Thin layer chromatography

Thin-layer chromatography was carried out on silica gel plates (EM Labs Inc., F-254) using either hexane–diethyl ether–acetic acid (90:10:1, by vol; solvent 1) or hexane as developing solvents. Radioactive spots were detected with a Packard model 7201 radiochromatogram scanner.

Gas–liquid chromatography (GLC)

Ether extracts of whole ants were fractionated using a 10"₀ Carbowax 20 M on Gas Chrom Q 80–100 (w/w) column (2 m × 0.5 cm i.d.) in a gas chromatograph equipped with a stream splitter. The temperature programmed conditions were 80°C for 10 min followed by 15 min temperature rise to 160°C and held isothermally.

^{18}O Incorporation studies

Fifteen worker ants were exposed to an atmosphere of ^{18}O$_2$ in air (50:50, v,v) in a 100-ml round bottom flask. After 72 hr the ants were extracted with ether and the dried ether extracts analyzed by gas chromatography–mass spectrometry (GC–MS) using a Varian model 1400 gas

chromatograph and a Finnigan 1015 mass spectrometer equipped with a system 150 computer (System Industries). A 1.5 m × 0.5 cm i.d. O.D. glass column packed with 3% OV-17 on Gas Chrom Q 100-120 (w/w) at 130 C was employed. Selected ion monitoring of ions at m/e 43 ($CH_3C^{16}O$)', 45, 61 ($CH_3C^{16}O^{16}OH + H$)', 63, 73 ($CH_3C^{16}O^{16}OCH_2$)' and 75 with integration of ion intensities was used.

Incorporation procedure

Fifteen *F. schaufussi* workers were fed 30 μl honey water (1:4 dilution of unfiltered honey) containing [1-^{14}C]-acetate (56 mCi/m-mol) (Amersham Searle Corp.) having 2.5 × 10⁶ dis/min/μl. After 24 hr whole ants were homogenized in ether in 12 × 100 mm glass screw-capped test tubes and extracted three times with 4 ml ether. The combined ether extracts were dried over anhydrous sodium sulphate and the volume reduced to *ca.* 50 μl under a stream of nitrogen. Extracts were fractionated by GLC and the steam-split eluate collected at 2-min intervals on activated charcoal powder. The charcoal, with adsorbed column fractions, was mixed with 15 ml LCS cocktail (Baker Chem. Co., Phillipsburg, N.J.), filtered through Whatman No. 1 filter paper and counted in a Packard model 2420 tri-carb liquid scintillation spectrometer at 95% efficiency with quench correction.

Purification and hydrolysis of labelled decyl acetate

Whole ants were extracted with ether containing 5 μl non-radioactive decyl acetate as carrier. The ether was extracted with 0.1 M Na_2PO_4 buffer (pH 11.0) to remove any free [^{14}C]-acetate present. All extracts were dried over anhydrous sodium sulphate and reduced to *ca.* 50 μl under a stream of nitrogen. The volatile components in the extract were separated by GLC and the decyl acetate from the stream-split eluate trapped in a 30-cm glass capillary cooled with dry ice. The trapped ester was eluted from the capillary with 1.0 ml 2 M methanolic KOH into a 12 × 100 mm screw-capped test tube and hydrolyzed at 22 C for 18 hr. Water (1.0 ml), concentrated HCl (0.3 ml) and sodium acetate (1 mg) to improve recovery of the liberated [^{14}C]-acetate, were then added to the tube cooled at 0 C. The mixture was extracted four times with 3 ml ether, the extract dried over anhydrous sodium sulphate and reduced to *ca.* 50 μl under a stream of nitrogen. This solution was chromatographed by GLC and two minute fractions trapped on charcoal. Adsorbed material was eluted with the LCS cocktail and ths solutions counted in a scintillation counter.

RESULTS AND DISCUSSION

Some micro-organisms possess mono-oxygenases that catalyze the oxidation of 2-ketones to acetate esters by a reaction involving the direct insertion of an oxygen atom from molecular oxygen into a carbon–carbon bond (BRITTON *et al.*, 1974). The possibility existed that this reaction may be the mechanism of decyl acetate synthesis in *F. schaufussi*. Ants were exposed to $^{18}O_2$ for 72 hr and decyl acetate in the ether extracts of these ants was separated gas chromatographically and its mass spectrum obtained by CG–MS. Selected ion monitoring of oxygen containing ions at m/e 43, 45, 61, 63, 73 and 75 showed that no significant difference in the ratio of these ions among the $^{18}O_2$ exposed groups compared to ^{16}O exposed controls was observed. Therefore, oxidation of a 2-ketone (2-dodecanone) to yield decyl acetate does not occur in *F. schaufussi*.

Radiochromatogram scans of the chloroform–methanol (2:1) extracts of whole ants fed [^{14}C]-acetate showed that most of the activity migrated with the

solvent front when developed with hexane. This incorporation of [1-^{14}C]-acetate into the nonpolar hydrocarbons of *F. schaufussi* workers was detectable after 2 hr and peaked at 22 hr. Nonpolar hydrocarbons are present in both the cuticle and the Dufour's glands. However, when Dufour's glands from [^{14}C]-acetate fed ants were extracted with chloroform–methanol, the radioactivity did not migrate with the solvent front with either solvent 1 or hexane. Therefore, the labelled acetate is rapidly incorporated into cuticular hydrocarbons but is not detectable in the undecane present in the Dufour's gland. Detection of radioactivity in these experiments employed the rather insensitive Geiger counter in the radiochromatogram scanner.

Decyl acetate, purified by preparative gas chromatography from ether extracts of whole ants fed labelled acetate, was found to be radioactive when counted in a scintillation counter. The region of the chromatogram associated with this ester contained the major portion of the radioactivity. Alkaline hydrolysis of the collected [^{14}C]-decyl acetate followed by preparative gas chromatographic separation and scintillation counting of the trapped hydrolysis products established that approximately 85% of the radioactivity was associated with acetic acid (Fig. 1). This finding suggests that an esterification of acetic acid with decanol is likely to be the major biosynthetic mechanism. We were unable to detect decanol in ether extracts of Dufour's glands by high sensitivity gas chromatography suggesting that if it is present, it comprises less than 0.1% of the amount of decyl acetate present.
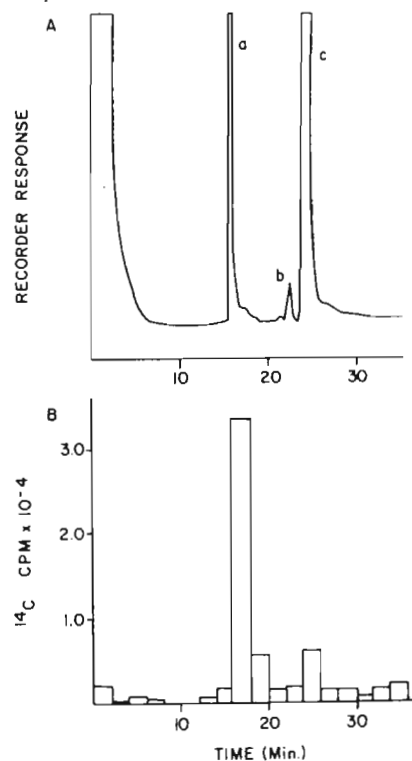

Fig. 1. Gas chromatogram of alkaline hydrolysis products of decyl acetate obtained from worker *F. schaufussi* fed [^{14}C]-acetate. (A) Peaks a, b and c are acetic acid, decyl acetate and decanol respectively. (B) Counts/min of fractions in the gas chromatogram trapped at 2-min intervals.

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Insect Pheromones: A Critical Review of Recent Advances in Their Chemistry, Biology, and Application

By

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1979

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Their Chemistry, Biology, and Application

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Contents

I. Introduction

The chemical basis of insect behavior is firmly established and forms an integral part of regulatory biology. The many and varied studies on this topic constitute part of an overall attempt to understand behavior at the molecular level. A better understanding of this subject will only come about by interdisciplinary collaboration between chemists and biologists.

We have elected to review certain aspects of recent developments in pheromone chemistry. Scientific objectivity is difficult to achieve when summarizing the many, and sometimes conflicting, reports and opinions of investigators. We confess to personal biases based on our experiences, offer no apology but ask a rational tolerance.

A number of terms have been coined to designate the various kinds of chemical interactions between individuals and some are defined below. As a particular substance may be defined to act in more than one specific manner, depending on the context, these terms should not be considered as mutually exclusive.

The chemicals delivering the message are called *semiochemicals* (1). *Allelochemics* are those semiochemicals used for interspecific communication (2). These in turn are subdivided into *allomones*, which give adaptive advantage to the emitter (e.g. the defensive secretions of many

arthropods or the spray of a skunk) or *kairomones,* which give adaptive advantage to the receiver (e. g. substances that enable predators to locate their prey) (*3*). Semiochemicals used for intraspecific communication are called *pheromones* (*4*).

Pheromones are classified according to the response they elicit. Chemical stimuli that trigger an immediate and reversible change in the behavior of the recipient are called *releasers*, whereas those inducing delayed, lasting responses are referred to as *primers* (*5*). These responses may be due to an individual chemical or, as is often the case, a mixture of chemicals. In the latter instance, the total mixture is the pheromone and the individual chemicals that make up the pheromone are termed *pheromone components.* In some semiochemical mixtures, all the components must be present to elicit a maximum response. If the total effect is greater than the sum of the effects of the individual components, the phenomenon is termed *synergism.* In some instances, synergism may occur from a combination of both insect and host produced chemicals (e. g. *6*). The major categories of semiochemicals are not mutually exclusive; the sex pheromones of some insects also serve as kairomones in that they attract predators (e. g. *7*).

The literature in the field of insect chemistry is vast; at the time of this writing, in excess of 10,000 scientific papers have been published. To make this review more manageable and meaningful and to avoid an encyclopedic approach, we have attempted to emphasize only the recent leading studies in one area, namely pheromones.

The last review of insect pheromone chemistry in this series was by EITER in 1970 (*8*). Since then, a number of other reviews have been published in the form of books (*9—16*) or review articles (*17—21*).

Sex pheromones are secreted by one sex to attract the other as an initial part of the mating process. A variety of chemicals have been identified by screening as attractive to one sex, but until these compounds have been isolated and identified from the opposite sex, they should be termed *sex attractants* or *parapheromones.* In some species, particularly among beetles (Coleoptera), the pheromones may attract both sexes and therefore serve more than one function. Such compounds are called *aggregation* or *recruitment* pheromones. Many social insects use *alarm pheromones* to alert other members of their species, or *trail pheromones* for foraging.

The importance of geometrical and optical isomers in the list of behavior-modifying chemicals is apparent, and we have taken this fact as a point of emphasis. We begin with a discussion of structure elucidation and chemical synthesis. This is followed by a section entitled "stereobiology" which includes recent reports on insect pheromones dealing with the occurrence of various isomers in insect species. Subsequently,

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studies on the biosynthesis of pheromones are covered in some detail; this section is followed by a brief mention of the application of pheromones to chemosystematics and speciation. Finally, we present a section on the present status of the practical applications of pheromones and offer some thoughts for the future.

A typical pheromone study involves intimate collaboration between a chemist and a biologist and follows a pattern (22):

Biologist

1. Selects an insect pest (often one of economic importance) and describes the behavior under pheromone control.

2. Develops a bioassay.

3. Collects source material containing the pheromone.

Chemist

1. Fractionates source material; each step must be monitored by the bioassay (biologist).

2. Isolates and identifies the compound(s) responsible for the behavior. In most cases, the insect uses a multicomponent pheromone system and a precise reproduction of the mixture may be necessary to attract or confuse; in many cases however, a single component of the blend (or a single parapheromone) may be sufficient to affect field behavior.

3. Synthesizes to confirm proposed structures and to furnish material for field studies.

4. Devises release systems for field studies.

Biologist

1. Confirms equivalent activity of isolated and synthesized compounds in the laboratory and in the field.

2. Develops protocol for survey and control.

At this point, large scale synthesis is turned over to the chemical industry and specialized industrial help is sought to develop efficient, slow-release formulation and dispersal systems. Only a handful of companies has been involved in these activities, and it is safe to say that returns have been marginal at best—in most cases developmental costs have exceeded returns.

II. Structure Elucidation

A. Isolation

1. Collection

The amount of semiochemical produced by an insect is highly variable and depends upon species and type of pheromone. Sex pheromones are usually produced in extremely small amounts [e. g. *ca.* 1 ng per female

cabbage looper *(Trichoplusia ni) (23)*] whereas up to 20% of the body weight of the ant *Formica rufa* is formic acid *(24)* which functions mainly as a defensive allomone and partly as an alarm pheromone.

In choosing the method of isolation, one must consider how the insect stores and releases the pheromone. If it is stored in relatively large quantities ready for release, one can extract the whole insect or, preferably, the gland producing the chemical. If stored in small quantities, in precursor form, and/or released slowly over an extended period, it can be collected by passing air over the insects and through a conventional cold or cryogenic (liquid nitrogen) trap *(25)*, or through an absorbent such as Porapak Q *(26)*. Alternatively one can extract paper used to line the cages. Methods of collection have been reviewed *(15, 27, 28)*.

2. Bioassay

The collected material must now be fractionated and the pheromone components isolated in pure form. Each step in this process must be monitored by the bioassay which is a test for the presence or amount of a biologically active substance with the detector being an insect. The development, problems encountered, and methods of bioassay have been extensively reviewed by YOUNG and SILVERSTEIN *(27)*. One bioassay technique deserves special mention: the electroantennogram (EAG) developed by SCHNEIDER *(29)*. In this technique, electrodes are attached to the base and sensory hairs of an insect's antenna and one then measures the change in voltage potential in response to olfactory stimuli. The EAG procedure has been especially useful in studies on lepidopteran pheromones. In addition to identification of active fractions, EAGs have been useful for investigating physiological and environmental factors, molecular specificity, and other variables on antennal olfactory responsiveness. Responses have been obtained with as little as 0.1 pg of pheromone. For reviews on EAGs see *(28, 30, 31)*.

3. Fractionation

The choice of a fractionation method will depend upon the physical state, stability, and amount of pheromone available. To avoid decomposition or rearrangement, the mildest possible conditions should be used.

Traditional purification techniques such as distillation, sublimation, and recrystallization have been used only occasionally, due in part to the small amount of material available. Extraction and derivative formation has proved useful in many instances.

The technique of choice is chromatography. Column chromatography, including gel permeation, is often used in the first stages of purification. Since many pheromones are olefinic, silver nitrate impregnated adsorbants and ion exchange resins (*32*) have proved useful in the separation of *E*- and *Z*-isomers. The relatively new technique of high-performance liquid chromatography (HPLC) is finding increased use (e.g. *33—36*). Thin-layer chromatography (TLC) can be a versatile tool for preparative separations of relatively complex mixtures, for separation of *E*- and *Z*-olefins, and through the use of chromogenic reagents for demonstrating the presence or absence of certain functional groups.

Gas chromatography (GC) is almost universally used in pheromone studies because of its speed, resolution, sensitivity, precision of analysis, and simplicity, and because most pheromones are volatile and thus ideally suited for this technique. Among the major uses of GC in pheromone studies are: to fractionate mixtures and collect the various components, to determine the purity and homogeneity of a sample, to dertermine the amounts of various components in a mixture, to detect the presence of a specific compound, and to compare synthetic with natural material. Preparative GC involves diverting much of the effluent from the end of a column and trapping the various fractions (e.g. *37*). Purity is best determined by using several GC columns of high resolving power and differing polarity. Many of the lepidopteran sex pheromones contain mixtures of *E*- and *Z*-isomers with one predominating. In early studies, however, the minor component was not well resolved from the other, and its presence was often overlooked.

Fractionation of semiochemicals has been reviewed in detail (*15, 27*).

B. Identification

As natural products go, insect pheromones have relatively simple structures. This is due in part to the requirement for high volatility and rapid diffusion in air. Identification of these compounds is no easy matter, however, since the amounts produced by an individual are frequently at the nanogram level (or less). Only a decade ago, TUMLINSON *et al.* (*38*) required 4.5 million boll weevils *(Anthonomus grandis)* to get enough material for identification. With the improvement in analytical instrumentation and microtechniques, rapid progress in the identification of pheromones has been made in the past few years. Since procedures for identification of pheromones have been reviewed at length (*15, 27, 34, 39*) and lists of identified pheromones have been adequately tabulated elsewhere (*15, 21, 40—43*), much of the emphasis here will be placed on examples to illustrate the useful techniques.

1. Spectrometric Methods

Structure determination rests heavily on information derived from mass (MS), nuclear magnetic resonance (NMR), infrared (IR), and ultraviolet (UV) spectra. Experienced chemists can frequently identify structures with no other information, using only 5—10 µg of material.

Modern mass spectrometers are capable of giving spectra from 10 ng or less of sample. In pheromone studies, the mass spectrometer is usually interfaced with a gas chromatograph (GC-MS), and, when the output is coupled to a computer, complex mixtures can be readily analyzed. The electron-impact MS sometimes gives the molecular weight, and, from the fragmentation pattern, one can often deduce the presence of functional groups and branching in molecules. High resolution mass spectrometers can give the elemental composition of the parent compound and its fragments. Chemical ionization mass spectrometry, from which the molecular weight can usually be determined, may use a reagent gas as the carrier gas with an interfaced GC. Computer searches of MS data banks may lead to identification.
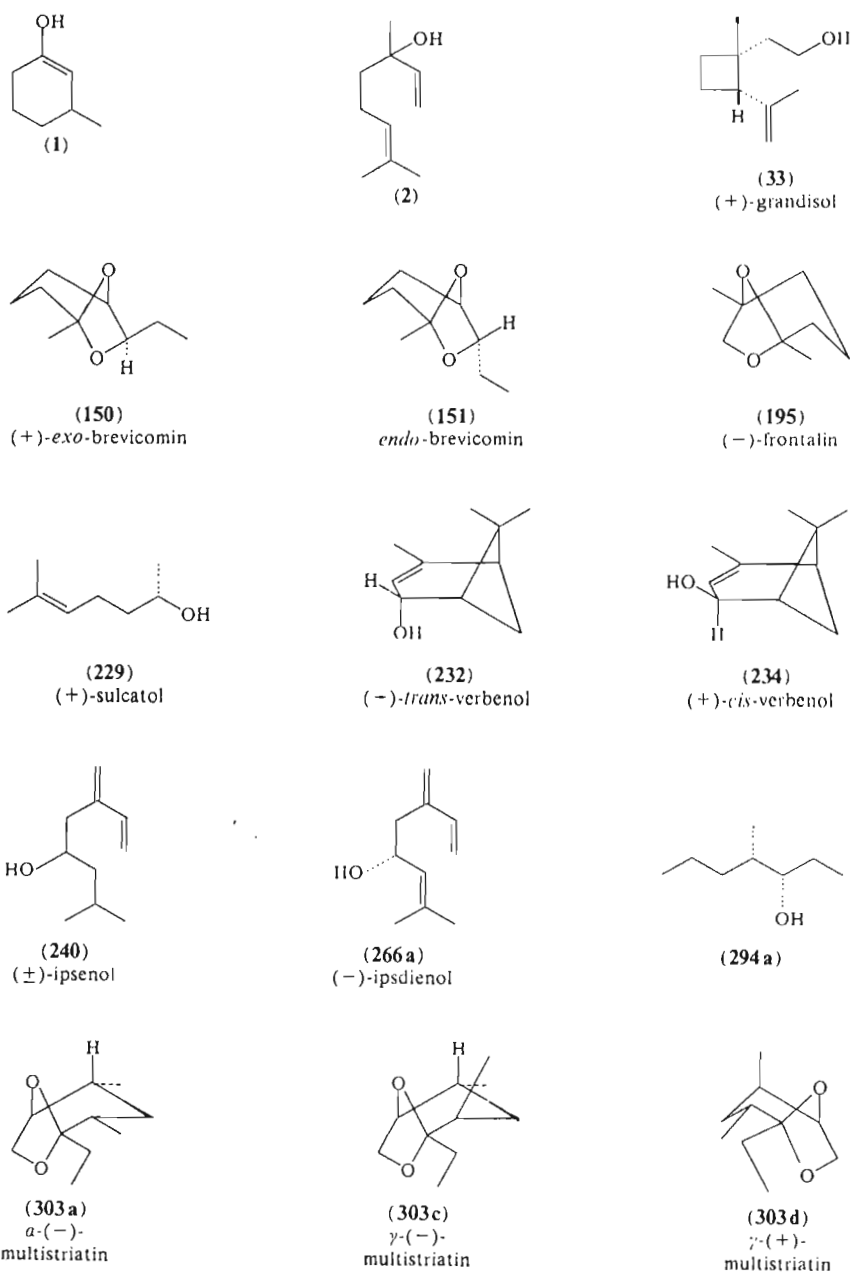
With beam condensers and Fourier transform IR spectrometers or GC-IR combinations, spectra can be obtained from less than 1 ng (e. g. 44). IR spectra are useful in identifying certain functional groups such as carbonyl or hydroxyl; the band at about 970 cm^{-1} is especially useful for confirming the E-configuration of the carbon-carbon double bonds.

UV spectrometry shows the absence or presence of conjugated systems and gives some indication of the type of conjugation. A number of pheromones have conjugated systems.

Applications of NMR spectrometry to pheromone identification have been restricted thus far to proton magnetic resonance. Until recently, the requirement for a relatively large sample size (100 µg) was a limiting factor in spectral identifications. TUMLINSON and HEATH (34) have recently reported good spectra from only 2 µg of grandisol (33, Chart 1), a pheromone component of the boll weevil. Complex molecules often produce spectra with overlapping peaks, thus making interpretation difficult. In some cases, this problem can be alleviated by use of lanthanide shift reagents such as Eu(fod)₃ (45). Chiral shift reagents have been used to determine the enantiomeric composition of several pheromone bicyclic ketals (150, 195, 303 a) (46) and alcohols (1, 229, 232, 266 a, 294 a) (45) (see Chart 1). The α-methoxy-α-trifluoromethylphenylacetyl derivatives of these alcohols were also used to determine enantiomeric composition (45, 47, 48). It is interesting to note that (266 a) from *Ips pini* and (294 a) from *Scolytus multistriatus* are 100%, (−)-enantiomers, whereas, (229) from *Gnathotrichus sulcatus* is a 65 : 35 mixture of (+)/(−), (232) from *Dendroctonus frontalis* is 60 : 40 (+)/(−), and (1) from *Dendroctonus*

Chart 1

(1)

(2)

(33)
(+)-grandisol

(150)
(+)-*exo*-brevicomin

(151)
endo-brevicomin

(195)
(−)-frontalin

(229)
(+)-sulcatol

(232)
(−)-*trans*-verbenol

(234)
(+)-*cis*-verbenol

(240)
(±)-ipsenol

(266a)
(−)-ipsdienol

(294a)

(303a)
a-(−)-
multistriatin

(303c)
γ-(−)-
multistriatin

(303d)
γ-(+)-
multistriatin

pseudotsugae is 50:50 (+)/(−). These determinations were done on 5—200 µg of substrate. Carbon-13-NMR has become a powerful tool for structure identification. At present, large sample sizes (*ca.* 1 mg) are required, which limits its use in pheromone identification. However, it has proved useful in determination of optical purity of synthetic α-multistriatin (**303a,** Chart 1), an aggregation pheromone component of *Scolytus multistriatus* (*49*), and stereochemistry of the bicyclic ketals (**150**), (**151**), (**195**), (**303a**), (**303c**), and (**303d**) (*50*).

Optical rotatory dispersion (ORD) measurements have been made on some enantiomeric pheromones (*49*) and classical optical rotations are routinely determined. It should be noted that the sign of rotation may depend upon the solvent used: (1*S*,4*S*,5*S*)-*cis*-verbenol (**234**) is dextrorotatory in methanol and acetone (*48, 51*) and levorotatory in chloroform (*52*).

2. Chromatographic Methods

Use of the Kovats system of GC retention indices (*53, 54*) can often provide considerable information on the functional groups and molecular size of an unknown compound. The retention index indicates where a compound will appear on a chromatogram with respect to straight-chain alkanes, using adjusted retention times. This method has been especially helpful with lepidopteran pheromones, which tend to be long, straight-chain unsaturated compounds with a single terminal functional group (e.g. *55*). From this and spectral evidence one can often choose model compounds for comparison. If two substances are resolved by GC, they can unambiguously be said to be different; however, identical retention times on a given column do not prove identity. Young *et al.* (*56*) observed that the terpene alcohols (**2**) and (**240**) (Chart 1) had identical retention times on three of five columns investigated.

Similarly, comparison of known and unknown compounds by TLC and HPLC can be used to establish identity. Silver salt impregnated TLC is used to distinguish between *E*- and *Z*-isomers of closely related structures (e.g. *57*).

Reaction gas chromatography (*58—61*) has become one of the most convenient methods for performing chemical reactions at the microgram level. In this technique, the unknown compound is injected into the GC system and is retained or transformed, frequently at the injection port on a precolumn. The products that elute can be collected and analyzed.

One commonly employed technique is to use hydrogen as a carrier gas and to put an appropriate catalyst on a precolumn. On injection a compound may undergo a) hydrogenation to saturated analogs; b) dehydrogenation; or c) hydrogenolysis. Carbon-skeleton chromatography utilizes

hydrogenolytic conditions to strip off all functional groups to give the parent hydrocarbon or a lower homolog. Determination of the structure of brevicomin (**150**) by Silverstein *et al.* (*62*) was facilitated when carbon-skeleton chromatography of (**150**) afforded nonane (**3**).

carbon-skeleton-
chromatography

(150)   (3)
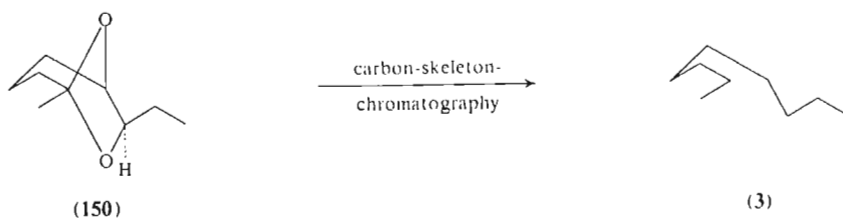
Compounds containing certain functional groups can be "subtracted" (retained) by a chemical placed in the GC pathway (*61, 63, 64*). The structure of the gypsy moth *(Lymantria dispar)* sex pheromone (**536**) was provided in part when it was subtracted by phosphoric acid, which is known to remove epoxides (*57*).

Functional group analyses can be performed by chemical reactions on TLC plates (*15*).

(535)

(536)
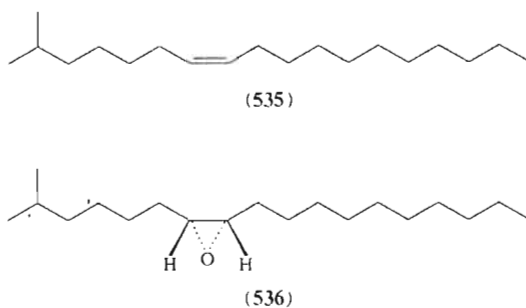
3. Microchemical Methods

When spectral and chromatographic data for an unknown compound are insufficient for elucidation of the total structure, it becomes necessary to perform chemical manipulations.

Hydrogenation by GC or in solution is frequently used to determine the number of olefinic bonds. The position of these bonds is usually determined by microozonolysis (followed by reduction) and examination of the aldehydic and/or ketonic fragments produced (*65—67*). The identification of fragments commonly encountered from ozonolysis of

References, pp. 157—190

pheromones has been studied by MOORE and BROWN (68). Epoxidation of olefins has proved useful when there are two or more olefinic bonds and the products of ozonolysis are very volatile and difficult to analyze (34). When compounds containing two olefinic bonds are epoxidized, the reaction is monitored by GC and the reaction stopped at the mono-epoxide stage. Chemical ionization mass spectrometry helps to locate the position of the epoxide (69) and the configuration of the double bond is determined from the IR spectrum.

The nature of functional groups in a pheromone can be determined by chemical modification coupled with a sensitive bioassay of the converted material. For example, if a pheromone loses activity after saponification and regains it upon acetylation it likely contains an acetate group. Loss of activity after hydrogenation indicates unsaturation. INSCOE and BEROZA (15) tabulated many of the typical functional group tests that can be used in pheromone structure elucidation. These tests need not be restricted to purified material; they can be employed on crude extracts as well and the results may suggest purification methods to be used (or avoided).

Once the functionality has been identified, a search for precursors in the extracts may yield valuable information. When the sex pheromone of the gypsy moth was shown to be an epoxide, BIERL et al. (57) treated the monoolefin fraction of the extract and obtained biological activity. Identification of olefin (535) as the precursor led directly to (536) as the sex pheromone (see p. 10).

Use of solvents in the extraction of very volatile alarm pheromones can introduce impurities or mask short retention time components in GC analysis. These problems can be avoided by drawing glandular liquid into fine glass tubes and then introducing the pheromone into the GC by the solid sampling technique (70). Chemical pretreatment, reaction GC, or selective subtraction can also be conducted to obtain useful information.

4. Electroantennogram Methods

Difficulties with traditional bioassay techniques include development of olfactometers, maintenance of sufficient numbers of insects, synergism, replication, and time taken to get significant results. The EAG technique has none of these. This method has been widely used to define chain length, functional group, and the position and configuration of double bonds in lepidopteran pheromones. After determining the first two parameters, one screens a series of standards differing only in the olefin portion. Subject to field testing, that compound showing the greatest response is assumed to be the pheromone or a pheromone component. On the as-

sumption that the response from a doubly unsaturated compound will be the summation of the responses from two monounsaturated molecules, Roelofs et al. (71) correctly predicted that (E, Z)-7,9-dodecadien-1-yl acetate (490) was the sex pheromone of the European vine moth (*Lobesia botrana*) after obtaining significant responses to the *E*-7 and *Z*-9 isomers of dodecen-1-yl acetate. Some caution in interpretation of results should be exercised since chemicals other than the pheromone may elicit an EAG response.

(490)
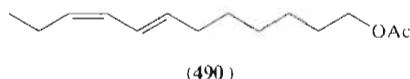
5. Screening Methods

As the number of identified pheromones increases, we are getting a clearer picture of the types of chemicals that are used by each order and family of insect. Structural variations of known lepidopteran pheromones have been tested in field traps and have attracted a wide variety of insects. It should be noted that attraction to a particular synthetic chemical or mixture does not constitute rigorous proof that the compound(s) is (are) a pheromone for that species; independent isolation and identification is required.

6. Examples

Three examples of how some of the above methods have been successfully combined to elucidate pheromone structures are given below.

a) Wild silkmoth (*Antheraea polyphemus*)

Kochansky et al. (72) purified extracts of the abdominal tips from about 1800 females by column chromatography on Florisil and 25% AgNO$_3$-silicagel. All bioassays were conducted by the EAG technique. GC analysis on polar and nonpolar columns suggested a major and minor component.

Comparison of retention times of the major component with that of hexadecyl acetate suggested that it might be an unsaturated C_{16} acetate. A sample purified by preparative GC was examined by TLC; comparison of R_f values of the sample and its mercuric acetate adduct with those of suitable standards confirmed the presence of two double bonds. Saponification and LiAlH$_4$ reduction destroyed EAG activity, which was regained on reacetylation. Acetylation had no effect and bromination destroyed activity. Hydrogenation also destroyed activity and gave a product that cochromatographed with hexadecyl acetate. MS analysis

confirmed a diunsaturated C_{16} acetate. EAG analyses of a series mono-unsaturated C_{12}, C_{14}, C_{16}, and C_{18} acetates, alcohols, and aldehydes revealed the greatest responses to be from E-6 and Z-11 C_{16} acetates, which suggested (E,Z)-6,11-hexadecadienyl acetate as the major phero-mone component. Microozonolysis on 250 ng of material confirmed the positions of the double bonds.

GC data suggested that the minor component was a C_{16} aldehyde, and (E,Z)-6,11-hexadecadienal seemed a reasonable first guess. This guess was confirmed when the purified minor component was reduced with $LiAlH_4$, the resultant alcohol preparatively collected at the C_{16} alcohol GC retention time, and the material then acetylated to give a substance having chromatographic, chemical, and EAG properties identical to those of the major component.

Field bioassay with authentic synthetic material confirmed that a 9:1 mixture of acetate and aldehyde was highly attractive and thus constituted the sex pheromone.

b) Japanese beetle *(Popillia japonica)*

TUMLINSON *et al.* (*73*) purified the rinses of glass vessels used to hold virgin females by gel permeation liquid chromatography and sequential preparative GC on five columns of differing polarity. Bioassays were conducted by pouring fractions into petri dishes, which were placed on a golf-course fairway. The number of males responding in five minutes was counted and compared with the number responding to three virgin females in a small cage placed near by during the same time.

MS, IR, and NMR data suggested a γ-lactone of a 14-carbon hydroxy acid with one double bond of possibly Z configuration. Microozonolysis yielded a major compound having MS and GC properties identical to those of nonanal. The racemic Z- and E-isomers plus the saturated analog were synthesized and their spectrometric, chromatographic, and chemical properties were compared with those of material obtained from the insects. All three compounds were present in the extracts, but only the Z-isomer (**144**) corresponded to the active compound.
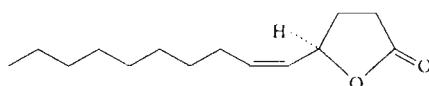

(**144**)

Since the racemic synthetic material was inactive in the field bioassay, the optically active enantiomers were synthesized. The R,Z enantiomer was attractive and as little as 1% of the S,Z enantiomer significantly reduced the number of males captured by traps baited with pure R,Z enantiomer.

c. California red scale *(Aonidiella aurantii)*

ROELOFS *et al.* (*74, 75*) obtained the crude sex pheromone by passing air over scale-infested potatoes and through a Porapak Q trap, and then extracting the absorbent with pentane. Two active compounds were isolated by Florisil column chromatography followed by HPLC on two different columns and preparative GC.

Both compounds were shown to be acetates when activity was lost on reduction with $LiAlH_4$ and regained after treatment of the products with acetyl chloride. Hydrogenation and further reduction to a hydrocarbon skeleton of both compounds afforded identical products. MS spectra were indicative of doubly and triply unsaturated C_{14} acetates and only the latter component gave a product, 3-ketobutan-1-yl acetate, on ozonolysis. Finally, NMR spectra were obtained, and the information was used to derive structures (**332a**) and (**333**) for the sex pheromones. These structures were confirmed by synthesis.


(**332a**)

(**333**)
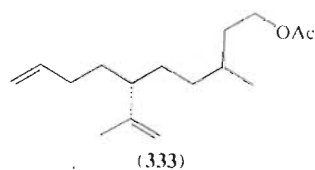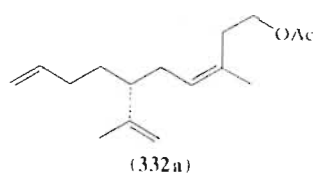
7. Problems

The ultimate proof of structure is unambiguous synthesis followed by demonstration of equivalent biological activity of synthetic material in the field. However, despite all precautions, errors still may occur. The sex pheromone of the gypsy moth was first postulated as (**4**) (*76*) and some synthetic material was reported to be active in the field (*77*). Later studies (*57*) showed that although (**4**) was present in extracts, the active material was (**536**) (see p. 10). Similarly, the sex pheromone of the pink boll worm moth *(Pectinophora gossypiella)* was first reported as (**5**) with synthetic material active in the laboratory (*78*) and later (*79*) shown to be a mixture of Z,Z- and Z,E-isomers of 7,11-hexadecadienyl acetate (**529a** and **529b**, respectively). A number of other sex pheromones have been misidentified, including those for the American cockroach

(Periplaneta americana) *(80)*, the codling moth *(Laspeyresia pomonella)* *(81)*, and the oak leaf roller moth *(Archips semiferanus)* *(82)*. EITER *(83)* offers critical comments on some pheromone identifications.

(4)

(5)
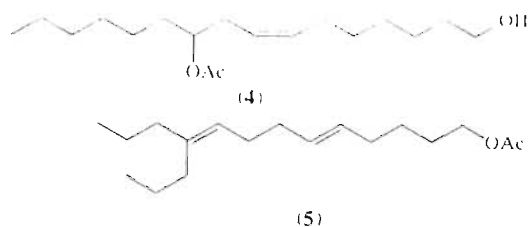
III. Synthesis

In addition to confirmation of an assigned structure, synthesis of enantiomers or *E/Z*-isomers can provide material to establish which of the compounds is actually produced by an insect or to determine the absolute stereochemistry of a chiral molecule.
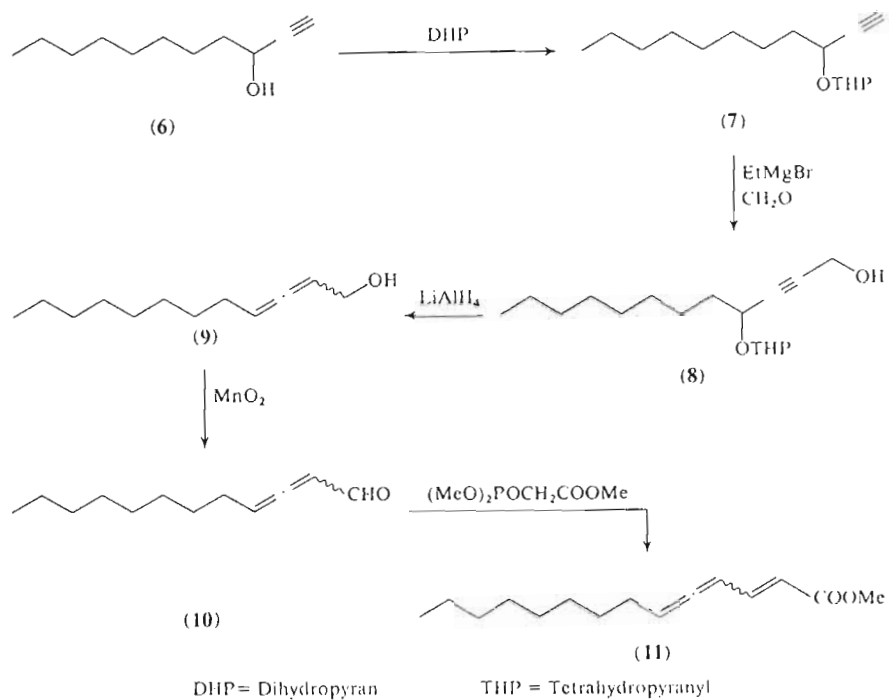
As noted above, insect pheromones have relatively simple structures. However, many of the compounds contain double bonds or possess chiral centers. Furthermore, some insects can distinguish between isomers and small amounts of the "wrong" isomer in a synthetic preparation may inhibit responses to the "correct" one. Thus the requirement for stereospecificity renders many syntheses non-trivial. Synthetic chemists have responded to the challenge and many new reactions of widespread utility have been developed; notable among these are reagents for stereospecific generation of *E* and *Z* double bonds.

The remainder of this section is devoted to a survey of novel syntheses and is organized alphabetically by Order and Family. Certain aspects of insect pheromone synthesis have been recently reviewed *(83a, 89, 90)*.

A. Coleoptera

1. Bruchidae

The sex pheromone (11) of the dried bean beetle *(Acanthoscelides obtectus)* contains the conjugated carbomethoxyeneallene system. LANDOR *et al.* *(84)* generated the allene by lithium aluminium hydride reduction of the α-hydroxy-α'-tetrahydropyranyloxy acetylene (8). Subsequent oxidation and coupling with the modified Wittig reagent, trimethyl phosphenoacetate, afforded the racemic ester (Scheme 1). DESCOINS *et al.* *(85)* prepared the masked functional groups (14) and by addition of lithium dioctyl cuprate, in one step produced the conjugated allene and completed the carbon skeleton (Scheme 2). Formation of the conjugated

(6)

DHP

(7)

EtMgBr
CH₂O

(9)

LiAlH₄

(8)

MnO₂

(10)

(MeO)₂POCH₂COOMe

(11)

DHP = Dihydropyran   THP = Tetrahydropyranyl

Scheme 1

(12)

HC≡CMgCl

(13)

Ac₂O

(14)

(n-C₈H₁₇)₂CuLi

H⁺

(15)

(16)

MeOH
MnO₂
NaCN

(11)
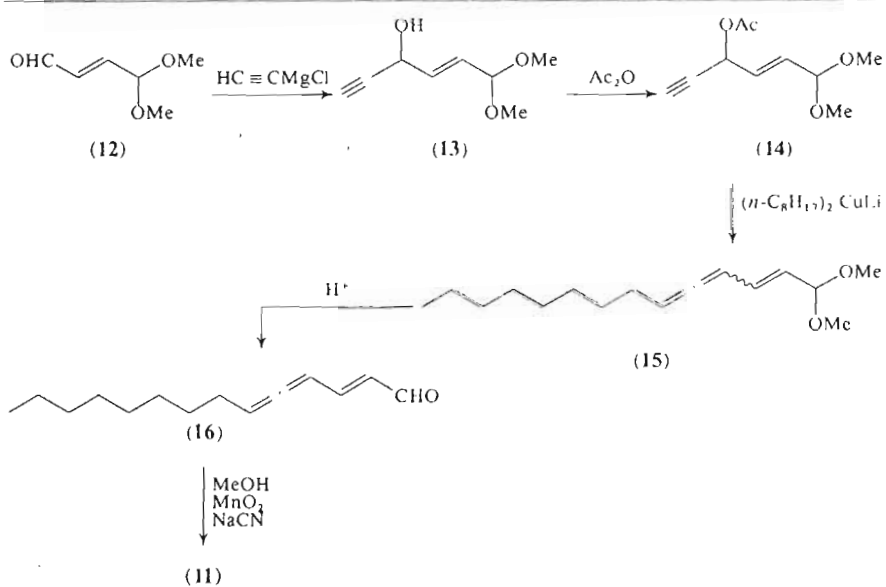
Scheme 2

allene was accomplished by BAUDOUY and GORE (*86*) in a single step by addition of 2-hydroxypropyne to the α-mesyloxy acetylene (17) (Scheme 3). Reduction of the conjugated acetylene with methoxy lithium aluminium hydride was followed by standard methods to give (11).

A novel synthesis by MICHELOT and LINSTRUMELLE (*87*) is outlined in Scheme 4. Treatment of the lithium dialkyl cuprate (22) of the substituted allene (21) with methylpropynoate afforded (11) in 94% overall yield.
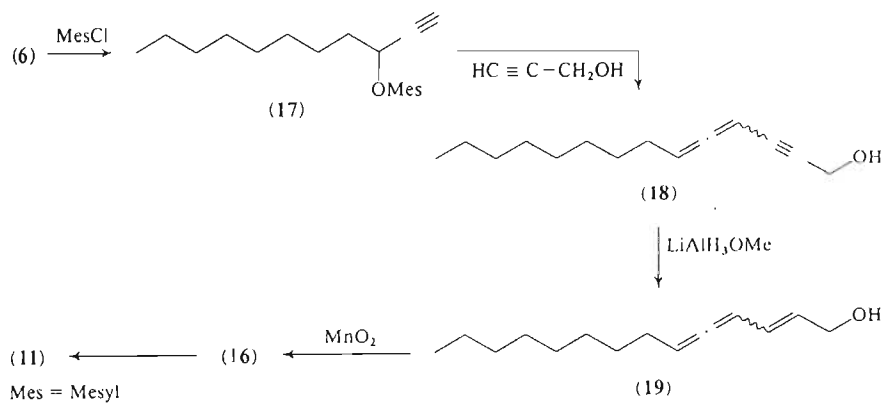
Scheme 3
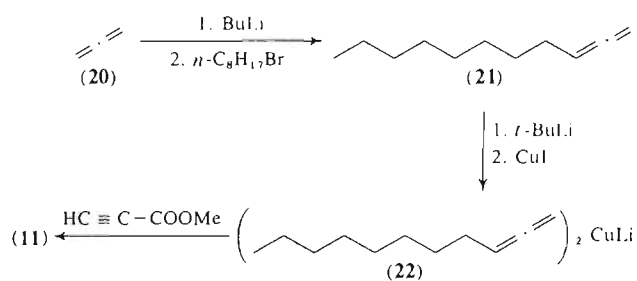
Scheme 4

Scheme 5

KOCIENSKI *et al.* (*88*) generated the β-allenic ester (23) by a modified Claisen rearrangement of ynol (6) (Scheme 5). After chain extension to a γ-allenic ester, the *E* α,β-unsaturation was introduced by selenoxide elimination.

2. Curculionidae

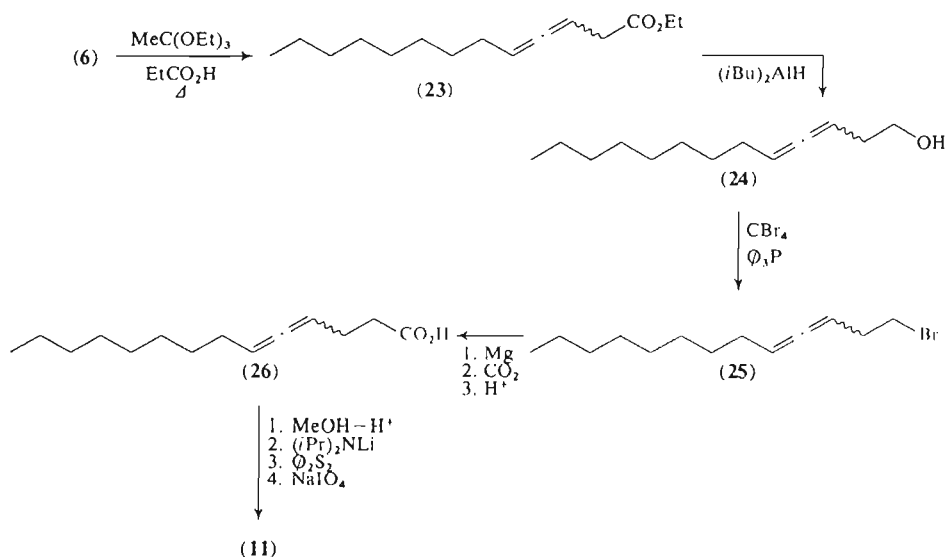Male boll weevils *(Anthonomus grandis)* produce a mixture of alicyclic terpenes (33, 110b, 111a, and 111b) which synergistically serves as an aggregation and sex pheromone. The unsaturated cyclobutane alcohol (33) (grandisol) has generated much interest and activity among synthetic chemists. Since many of the syntheses have been reviewed in detail by KATZENELLENBOGEN (*89*) and HENDRICK (*90*) the treatment here will be cursory.

A common approach to the cyclobutane ring system has been to generate it by photochemical means and then elaborate the functional groups. In the initial synthesis (Scheme 6), TUMLINSON *et al.* (*38, 91*) irradiated a mixture of isoprene (27) and methylvinyl ketone (28) and obtained the keto cyclobutane derivative (29) as a mixture of diastereomers. Alcohols (30a) and (30b), obtained by treatment of (29) with methyl Grignard, were separated and the desired *cis*-isomer (30a) was converted to grandisol by hydroboration-oxidation followed by dehydration and hydrolysis.
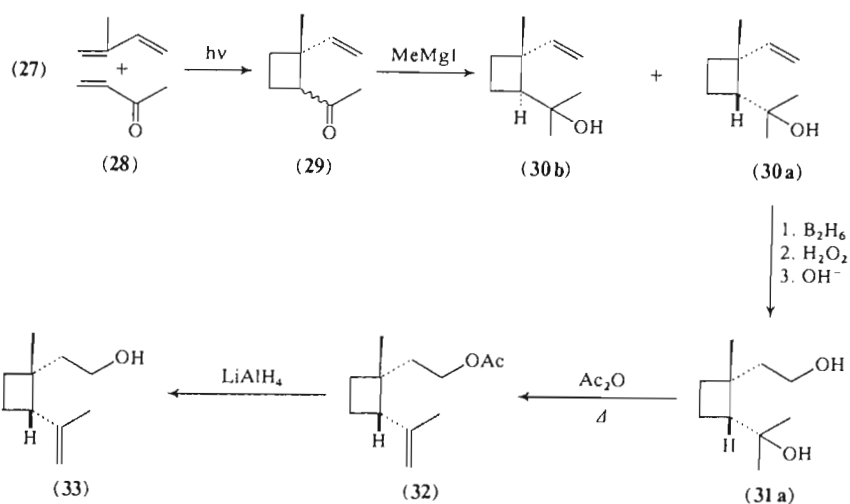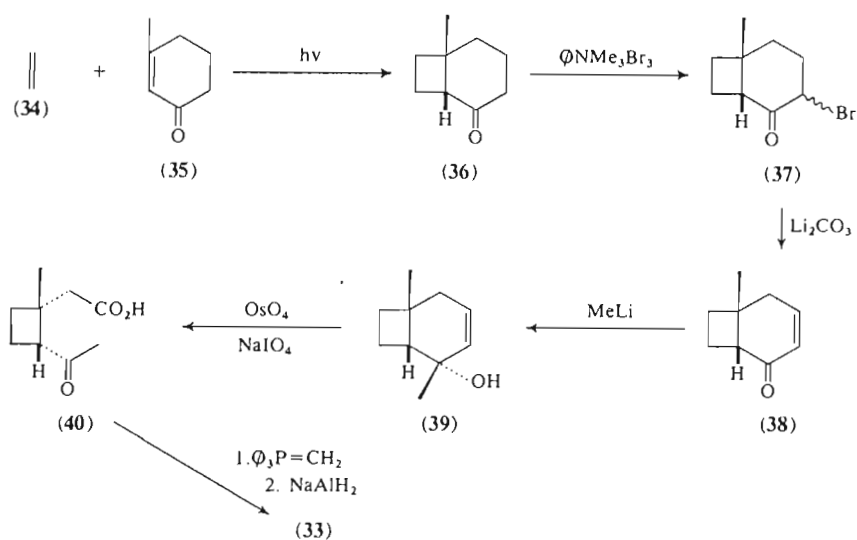
Scheme 6

Scheme 7

By generating a fused bicyclic ring system, Zurflüh et al. (92) were able to preform the required *cis*-configuration at the cyclobutane ring (Scheme 7). Cyclohexanone (36) was transformed to a cyclohexenone (38). Allylic alcohol (39) was cleaved with osmium tetroxide-periodate to keto

acid (40), and subsequent Wittig methylenation and hydride reduction gave grandisol.

Keto acid (40) was obtained by CARGILL and WRIGHT (93) via cyclopentanone (42) and the subsequently formed ozonide (45) (Scheme 8). After preparation of bicyclic lactone (47), GUELDNER et al. (94) stereoselectively generated diol (31a), which was converted to grandisol contaminated with isomeric (44) (Scheme 9). KOSUGI et al. (95) treated lactone (47) with the sodium salt of dimethyl sulfoxide (Scheme 10). Subsequent aluminium amalgam reduction, acetylation, Wittig methylenation, and hydrolysis afforded grandisol.
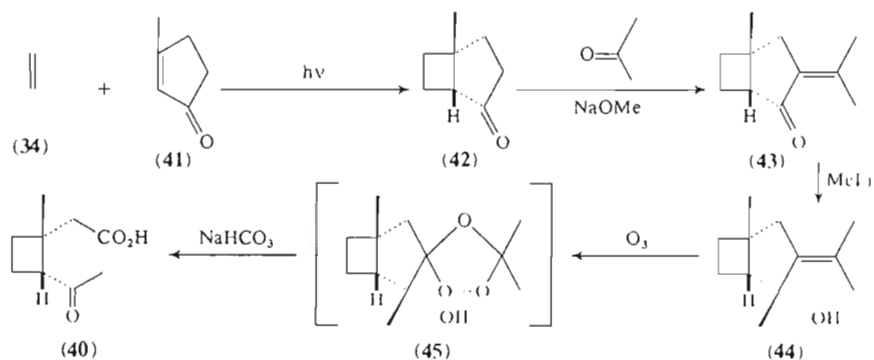
Scheme 8
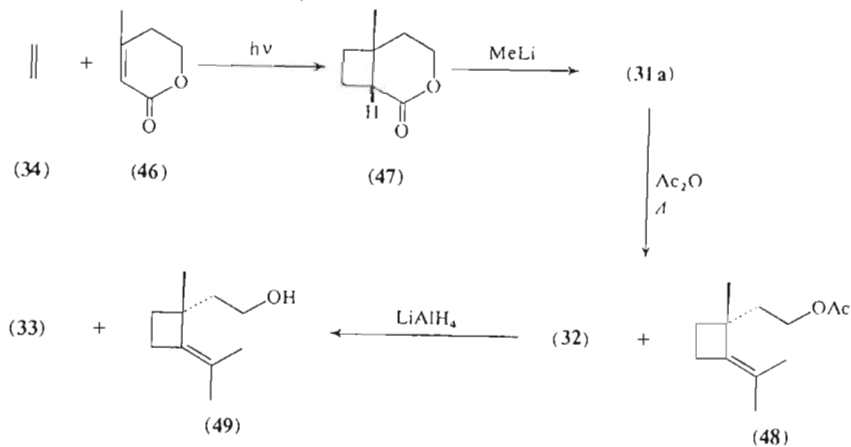
Scheme 9
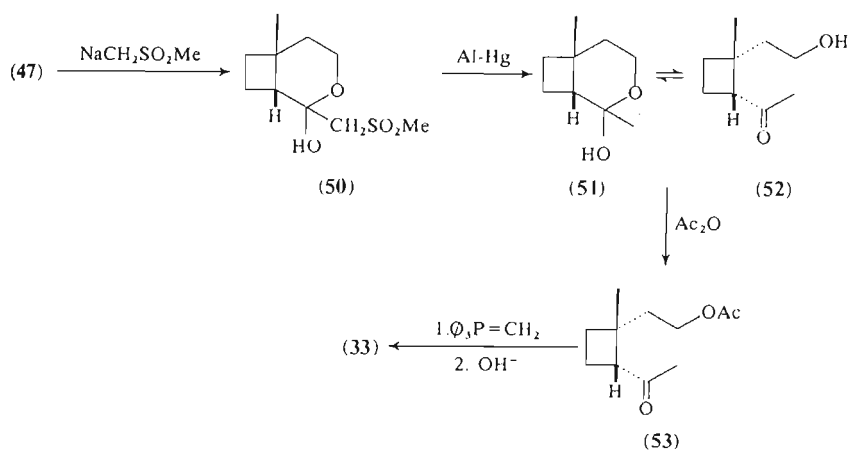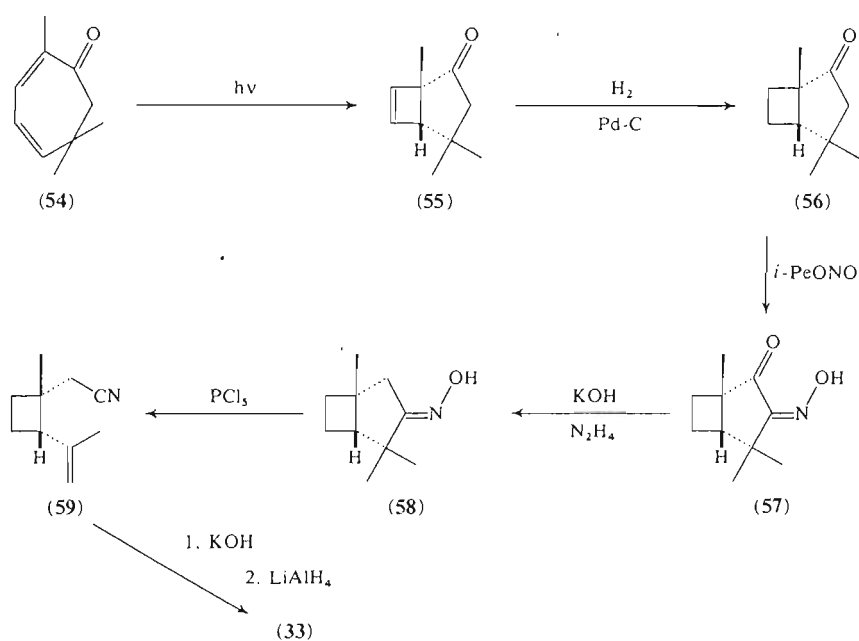
Scheme 10

Scheme 11

An intramolecular photocyclization of eucarvone (54), which contains all of the required carbon atoms, was used by AYER and BROWNE (96) to give cyclopentanone (55) (Scheme 11). Transposition of the keto group, Beckman fragmentation of the resultant oxime (58), hydrolysis and reduction completed the synthesis.

A variety of different routes to cyclobutane systems involving non-photochemical means has also been devised. Acid catalyzed rearrangement of fused cyclopropyl ether (61) by WENKERT *et al.* (97, 98) afforded the bicyclic dione (62), which through a thioketal-desulfurization process and treatment with hydroxylamine yielded oxime (58) (Scheme 12). The shortest synthesis of grandisol is that reported by BILLUPS *et al.* (Scheme 13) (99). Dimerization of isoprene in the presence of a zero-valent bis-cyclooctadienyl-nickel-phosphite complex gave the *cis*-cyclobutane di-olefin (65), which could be separated in 12 – 15% yield from the complex product mixture by low-temperature distillation. Selective hydroboration and oxidation afforded grandisol.

Scheme 12

COD = 1,5-cyclooctadiene
Sia₂ = disiamyl

Scheme 13

References, pp. 157– 190

(TMS)₂NLi = Lithium hexamethyldisilazane
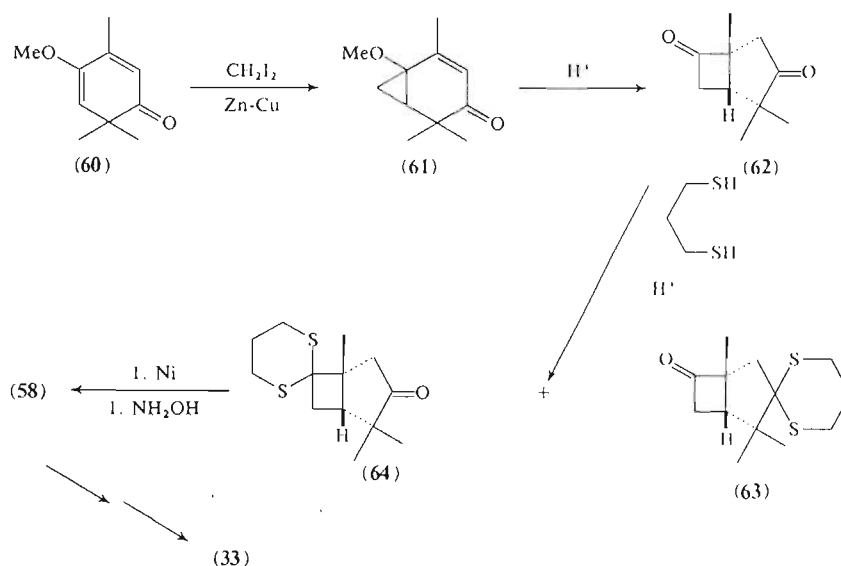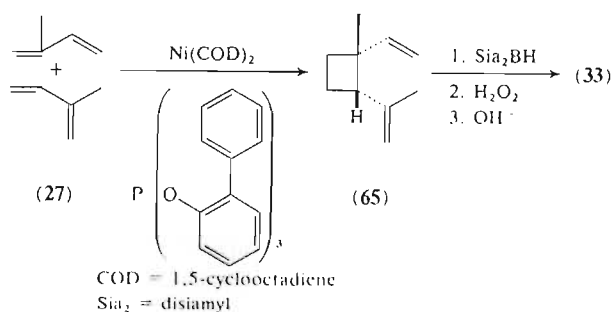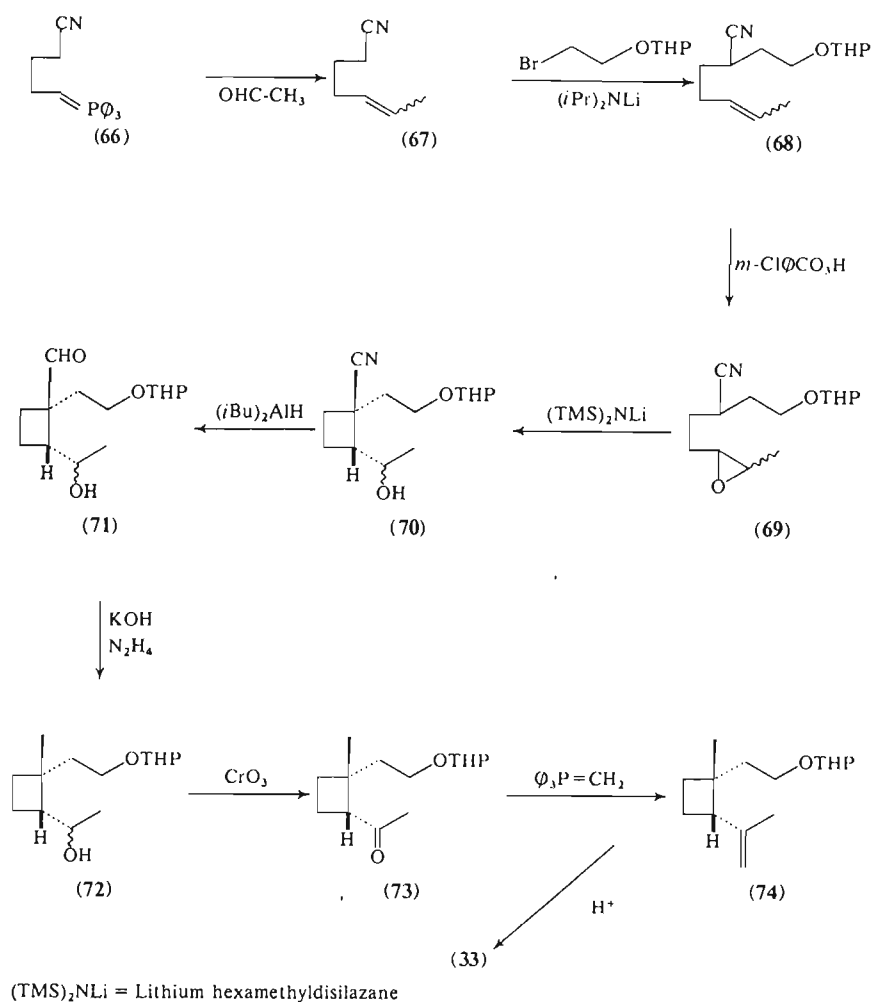
Scheme 14

STORK and COHEN (*100*) gained entry into the *cis*-cyclobutane system by stereoselective cyclization of epoxynitrile (**69**) (Scheme 14). Reduction of the nitrile to a methyl group and elaboration of the isopropenyl group completed this synthesis. Cyclization of δ-chloroester (**80**) by BABLER (*101*) gave a 65 : 35 mixture of *cis*- and *trans*-cyclobutane olefin esters (**81 a** and **81 b,** respectively), which were carried through to a mixture of grandisol (**33**) and fragrantol (**82**), respectively (Scheme 15).

Scheme 15

1. PyBr₃ – HOAc
2. NaOMe – MeOH
3. AgNO₃ – MeOH

Scheme 16

Py = Pyridinium
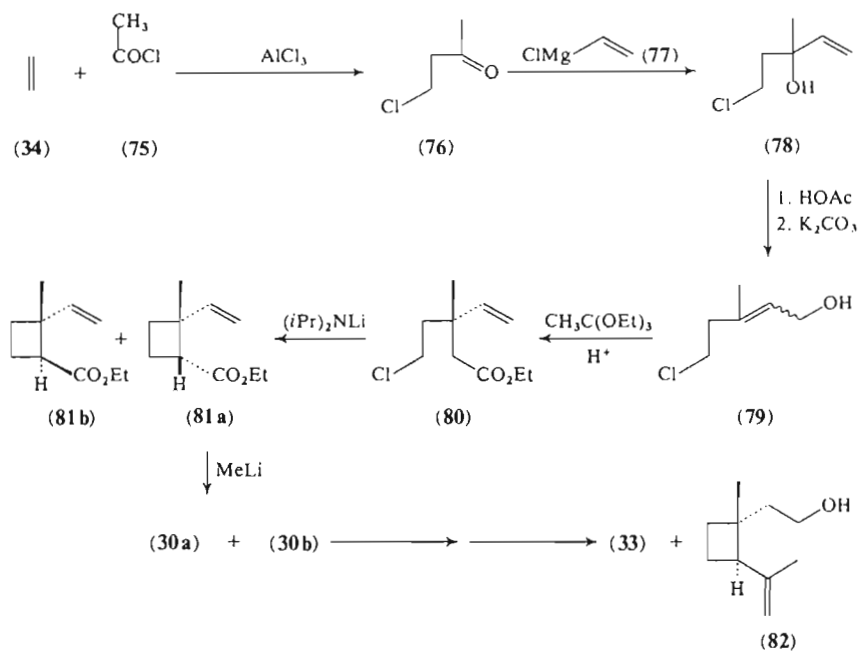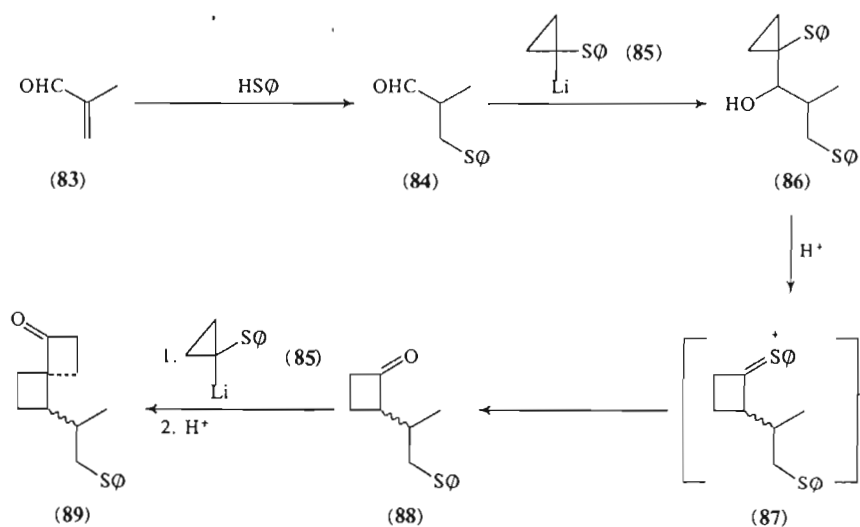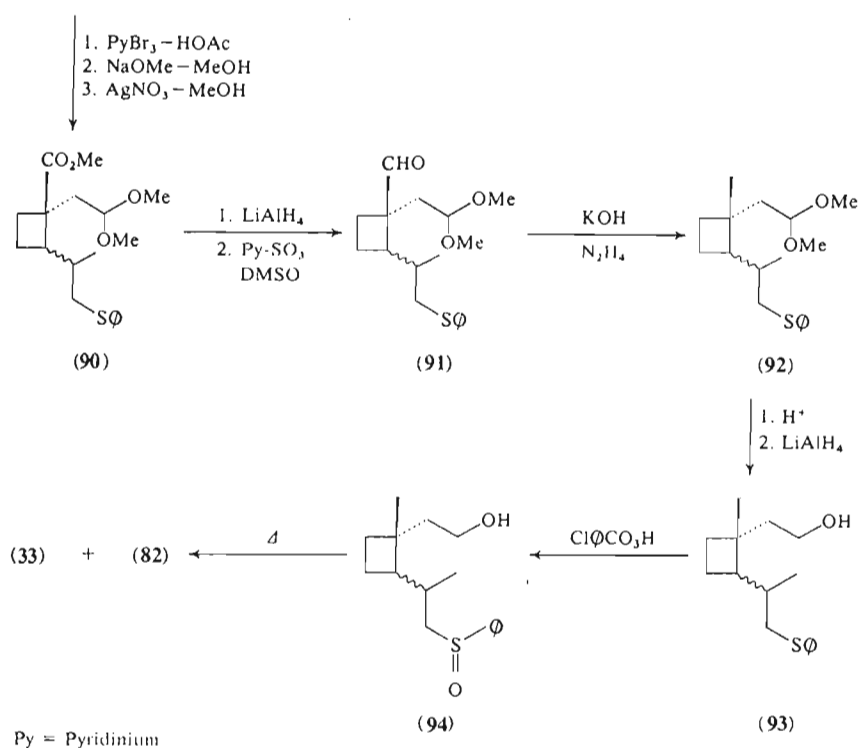
In a novel approach, TROST and KEELEY (*102*) used the annelation reagent lithiocyclopropylphenyl sulfide (**85**) to prepare first cyclobutanone (**88**) and then the fused biscyclobutane spiro system (**89**) as a mixture of diastereomers with the desired conformer in four fold excess (Scheme 16). Haloform-type cleavage afforded cyclobutane derivative (**90**), which upon transformation of the various functionalities yielded grandisol.

All of the above syntheses gave racemic grandisol. By starting with optically active (−)-β-pinene (**95**), which has a suitably substituted cyclobutane ring, HOBBS and MAGNUS (*103, 104*) were able to obtain (+)-(1R,2S)-grandisol (**33**) (Scheme 17). The ethanol side chain (in **100**) was elaborated by oxidation of the *endo*-methyl group, Wittig elongation and selective hydroboration-oxidation. Allylic oxidation of (**102**) and subsequent reduction yielded ketone (**104**). Upon irradiation this ketone underwent a Norrish type I cleavage to afford aldehyde (**105**), which contained the desired isopropenyl group. Rhodium catalyzed decarbonylation and hydrolysis afforded enantiomerically pure (+)-grandisol.
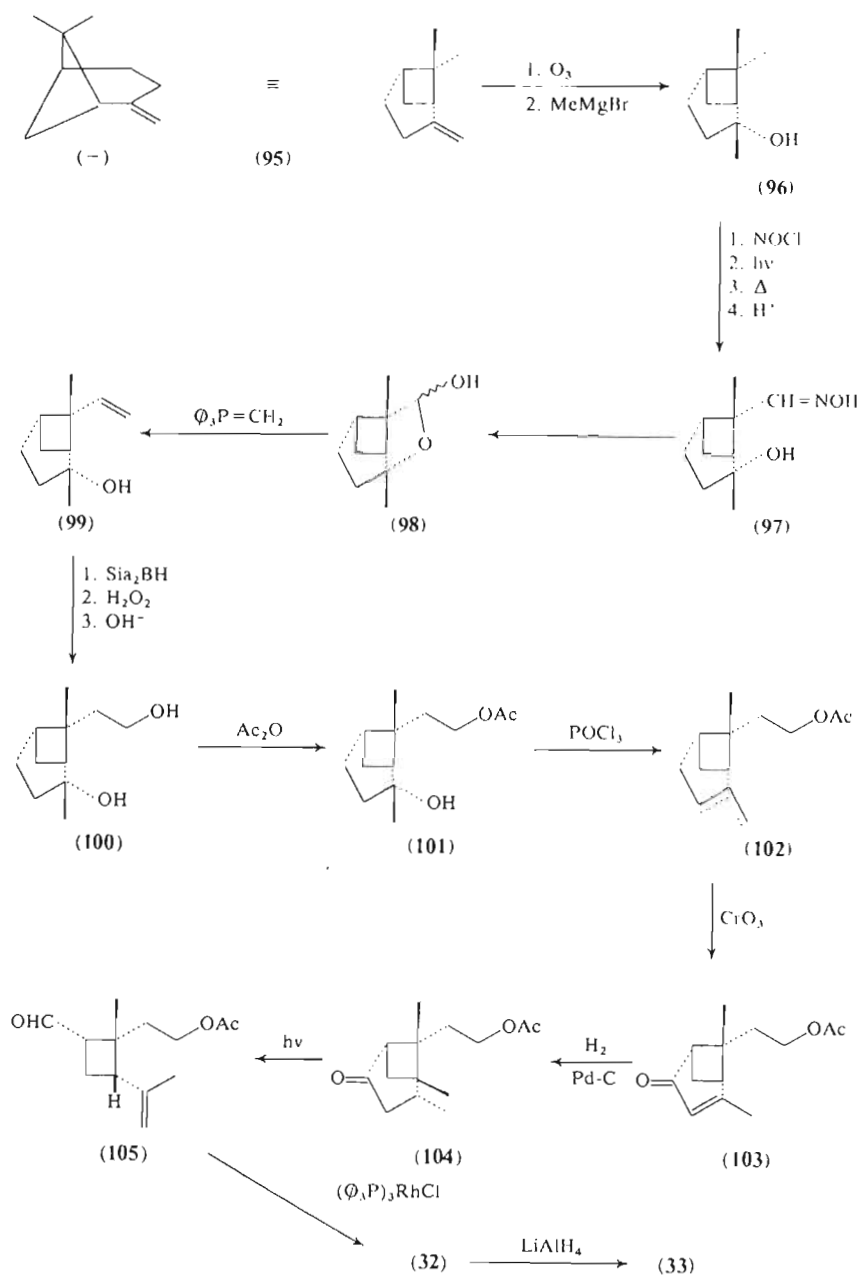
Scheme 17

Syntheses of the olefinic dimethylcyclohexane derivatives (**110b, 111a** and **111b**) have been relatively straightforward. 3,3-Dimethyl cyclohexanone (**106**) (Schemes 18 (*38, 91*), 19 (*105*), 20 (*106*), and 21 (*107*)), isophorone (**116**) (Schemes 22 (*108*) and 23 (*109*)) and geranic acid (**120**) (Scheme 24 (*110*)) have served as starting materials.
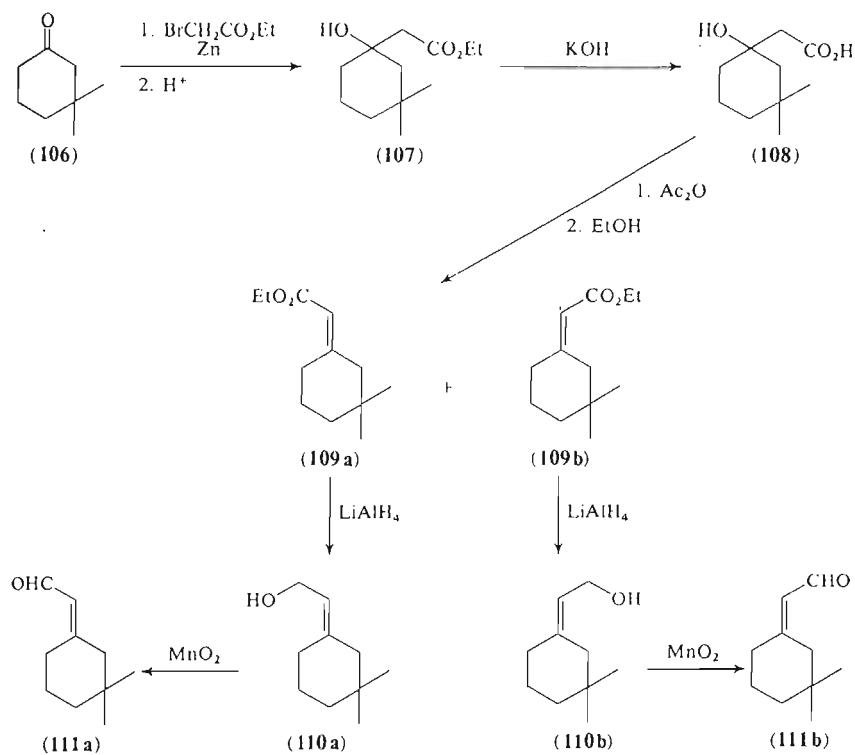
Scheme 18
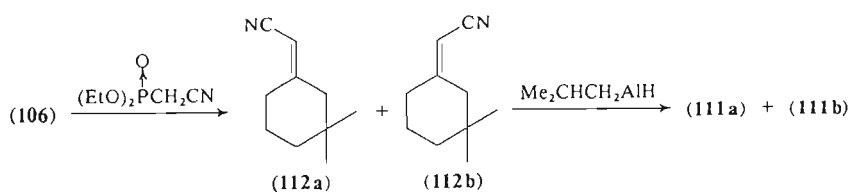
Scheme 19
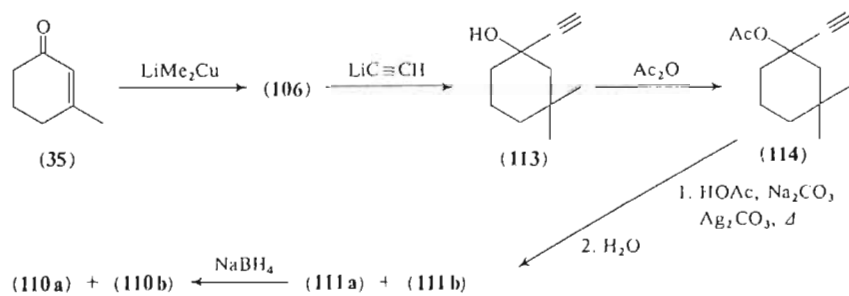
Scheme 20
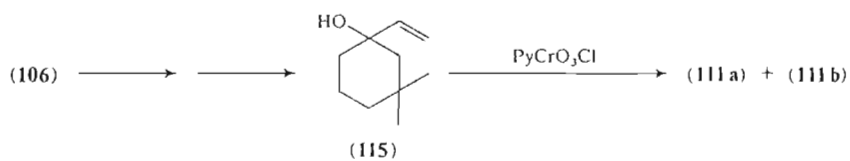
Scheme 21
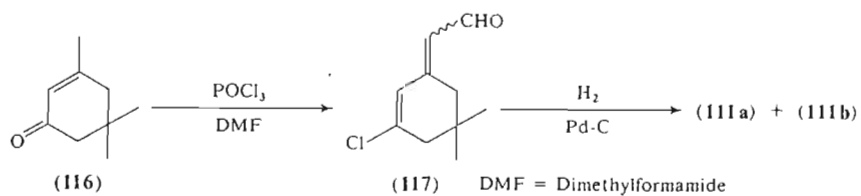
Scheme 22
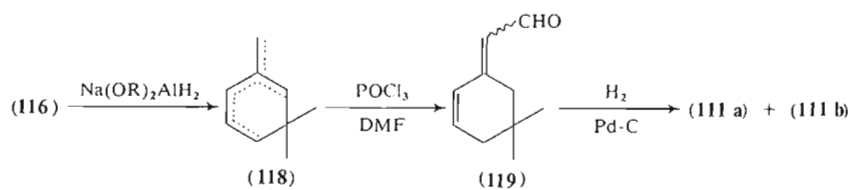
Scheme 23

References, pp. 157—190
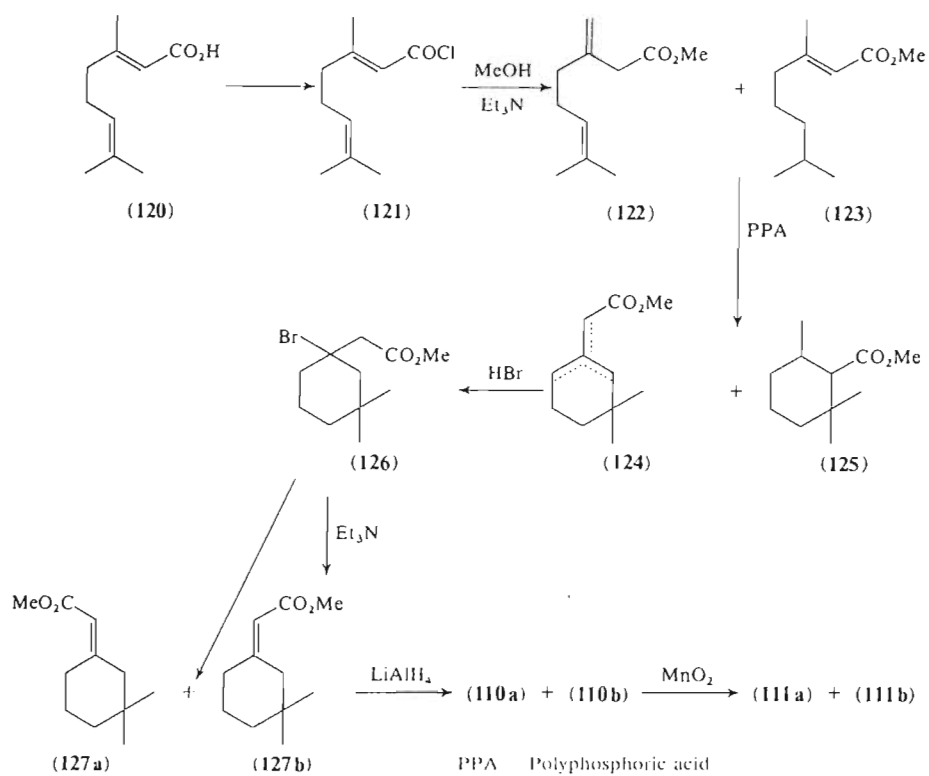
Scheme 24

3. Dermestidae

As shown in Scheme 25, SILVERSTEIN *et al.* (*111, 112*) began ela-
boration of the conjugated *E,Z* configuration of the black carpet beetle
(Attagenus megatoma) sex pheromone (**133**) by simultaneous allylic

1. CuCN
2. MeOH–H⁺

(131)

H₂
Lindlar

(132)

KOH

(133)

Scheme 25

(134)   (135)

(136)

(137)

1. H⁺
2. H₂-Lindlar

R (−)   (138)

1. CrO₃
2. CH₂N₂

R (−)   (139)
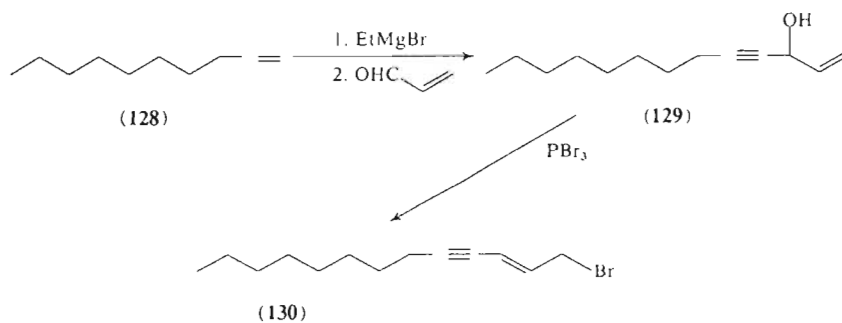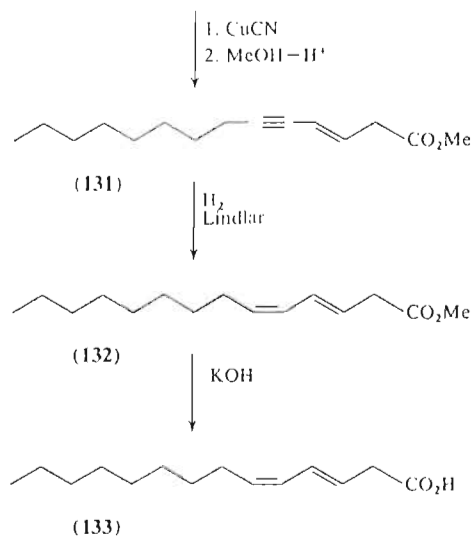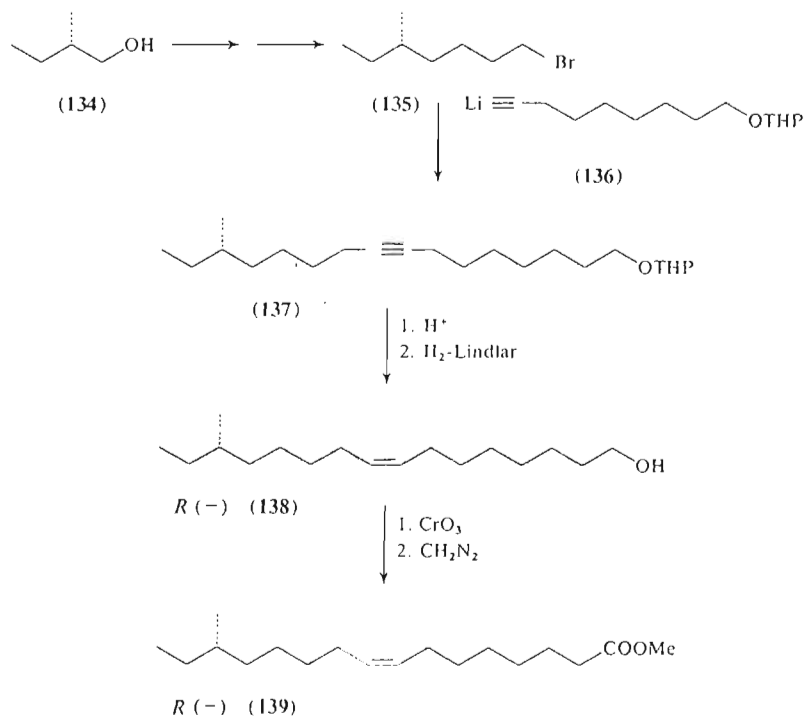
Scheme 26

rearrangement and bromination of the diunsaturated alcohol (129) to the bromide (130), which contained the desired E-isomer in two fold excess. Subsequent catalytic reduction of the triple bond afforded the diene.

The sex pheromone of *Trogoderma inclusum* is a mixture of the branched unsaturated ester (139) and its corresponding alcohol (138) and aldehyde (Scheme 26). Two syntheses giving racemic material (*113, 114*) used the Wittig reaction to form the Z double bond. Optically active products were obtained by MORI (*115*) *via* the alkyl bromide-lithium alkyne coupling reaction followed by catalytic reduction. In a similar manner, ROSSI and CARPITA (*116*) prepared optically active S (138), its E-isomer and the corresponding Z- and E-aldehydes.

4. Scarabidae

The Japanese beetle *(Popillia japonica)* sex pheromone (144) is a lactone with an unsaturated side chain (Scheme 27). Optically active (R)-(−)-glutamic acid (140a) served as the starting material in the synthesis by TUMLINSON *et al.* (*73*) shown in Scheme 27. After ring closure with retention of configuration and formation of the aldehyde, inverse addition of the Wittig reagent afforded the desired R,Z configuration.
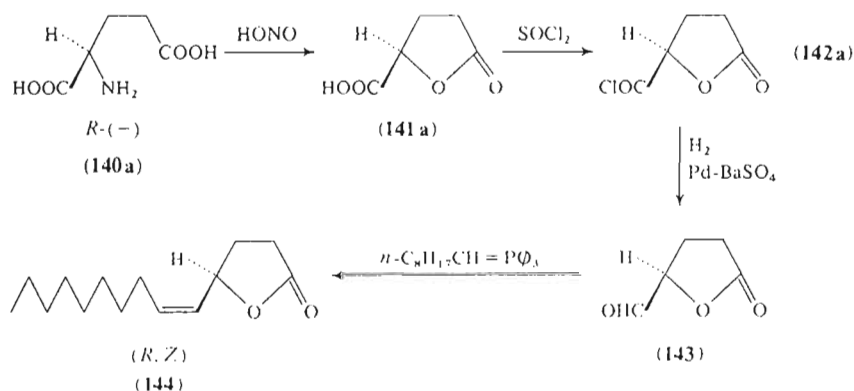
Scheme 27

5. Scolytidae

Beetles of the genus *Dendroctonus* use the bicyclic ketals *exo*-brevicomin (150, formulas in Chart 1) and frontalin (195) and the terpene *trans*-verbenol (232) as part of their pheromonal communication systems.
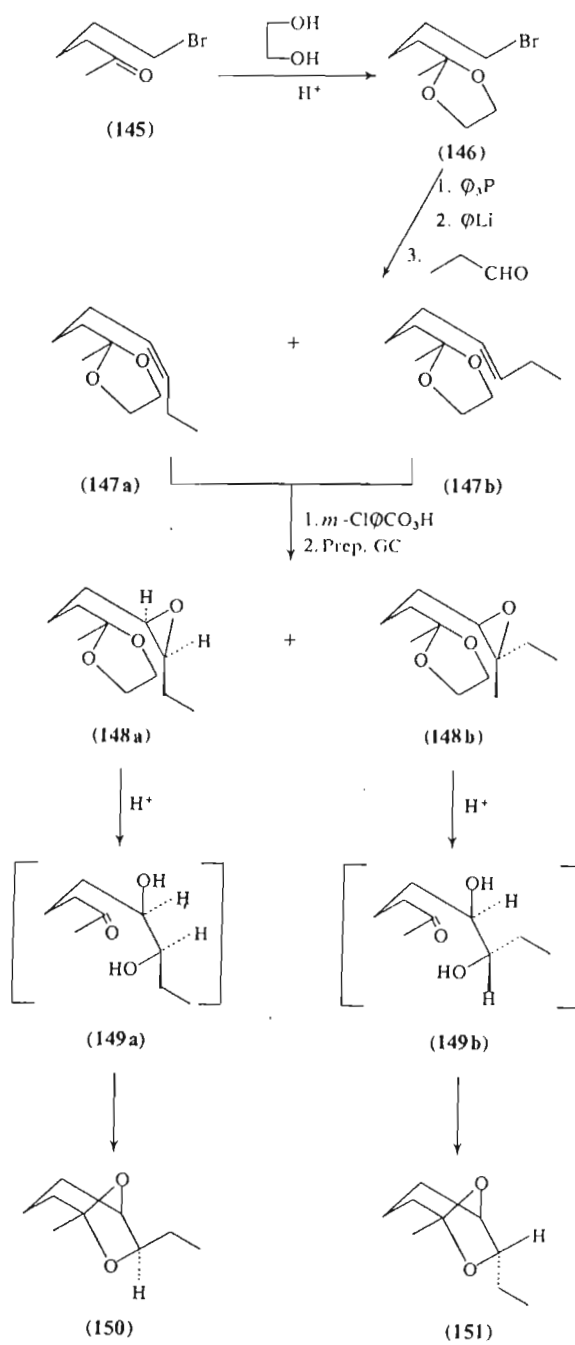
Scheme 28

Syntheses of *exo*-brevicomin and its *endo*-epimer (**151**) have followed two general approaches. The first involves generation of the ketodiol intermediates (**149a** and **149b**) of Scheme 28, which on treatment with acid give *exo*- and *endo*-brevicomin respectively. or the ketoepoxy intermediates (**184a** and **184b**) of Scheme 35, which are thermally cyclized to give (**150**) and (**151**), respectively. The second approach begins with Diels-Alder cyclizations. Syntheses of frontalin have followed the same approaches. For a discussion of *trans*-verbenol, see *Ips* spp. below.

A mixture of epimeric epoxyketals (**148a** and **148b**), separable by preparative GC, was obtained by SILVERSTEIN *et al.* (*62, 117*) upon treatment of the isomeric mixture of (**147a** and **147b**) with *m*-chloroperbenzoic acid (Scheme 28). Acid hydrolysis of (**148a**) gave the intermediate ketodiol (**149a**), which spontaneously cyclized to racemic *exo*-brevicomin (**150**). Similarly, (**148b**) afforded racemic *endo*-brevicomin (**151**). Stereoselective syntheses of (**147a**) and/or (**147b**) have been achieved by BELLAS *et al.* (*117*), KOCIENSKI and OSTROW (*118*), and MORI (*119*) (Schemes 29—31, respectively). KNOLLE and SCHAEFER (*120*) prepared the carbon skeleton (**162a**) in one step *via* Kolbe electrolysis (Scheme 32).

Scheme 29

Ts = *p*-Toluenesulfonyl
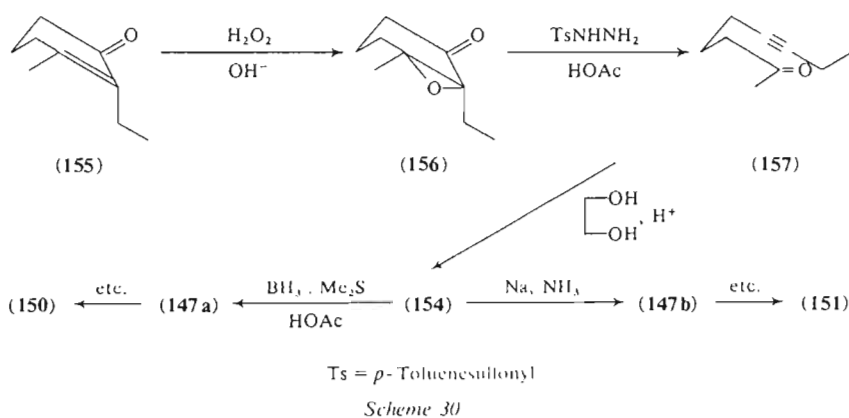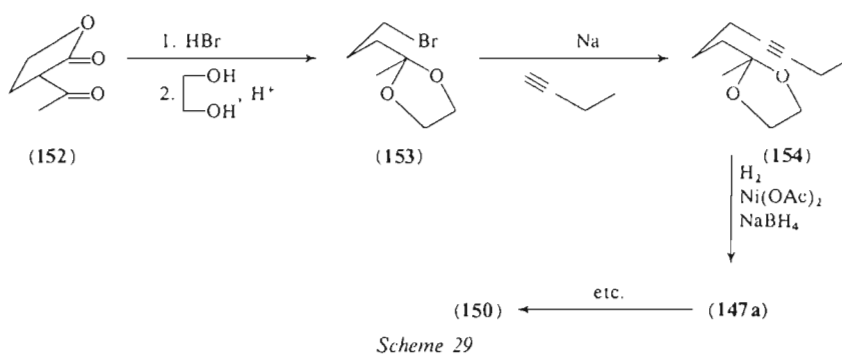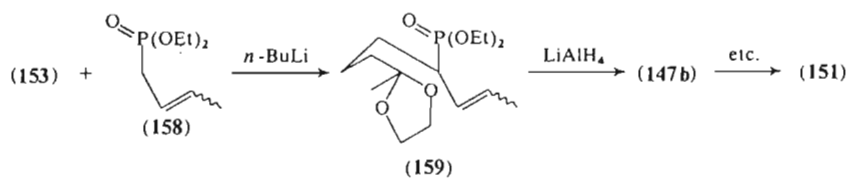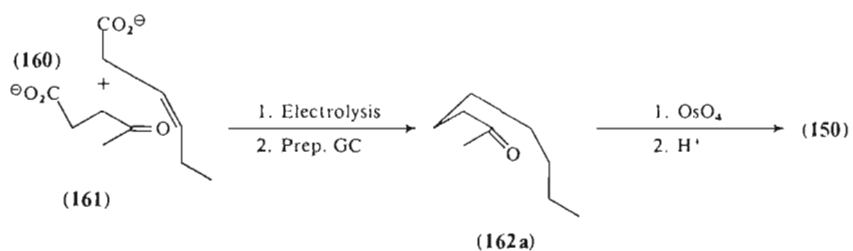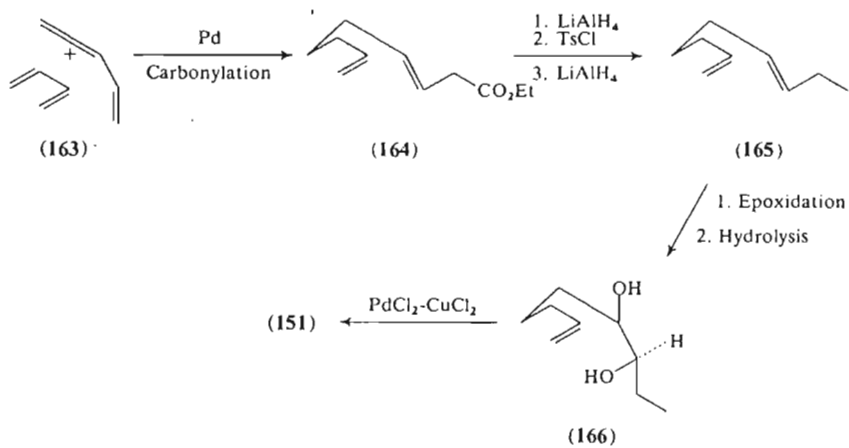
Scheme 30

Scheme 31

Scheme 32

Scheme 33
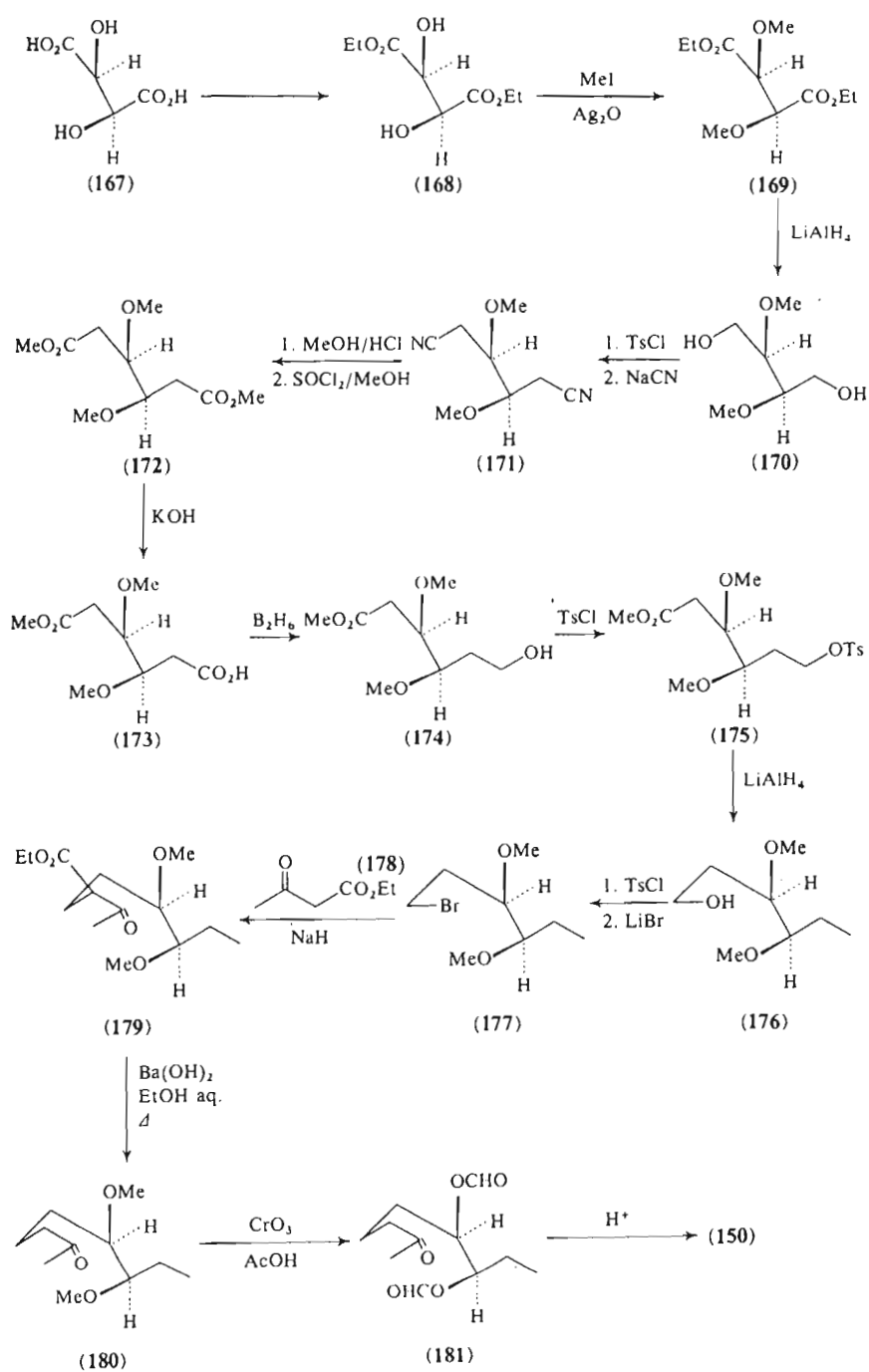
Scheme 34

After separation by preparative GC, (162a) was treated with osmium tetroxide and acid to afford brevicomin. Catalytic dimerization and carbonylation of butadiene by BYROM *et al.* (*121*), followed by reduction, epoxidation, and hydrolysis gave the alkenediol (166), which was cyclized catalytically to *endo*-brevicomin (Scheme 33). A stereoselective synthesis of optically active (1*R*,7*R*)-(+)-*exo*-brevicomin (150) from (2*S*,3*S*)-D-(−)-tartaric acid (167) has been achieved by MORI (*122*) (Scheme 34).

WASSERMAN and BARBER (*123*) observed that heating the ketoepoxides (184a and 184b) in a sealed tube stereospecifically gave *exo*- and *endo*-brevicomin, respectively (Scheme 35). A similar synthesis of the *endo*-epimer has been reported by LOOK (*124*). Acetylenic ketone (157) was prepared by COKE *et al.* (*125*) after treatment of chloride (186) with methyl lithium and thermal cleavage of the resultant intermediate (Scheme 36). Catalytic reduction to the *E* olefinic ketone (162a) was followed by epoxidation and thermolysis to give *exo*-brevicomin. RODIN *et al.* (*126*) obtained *exo*-brevicomin from acid catalyzed cyclization of ketoepoxide (184a).
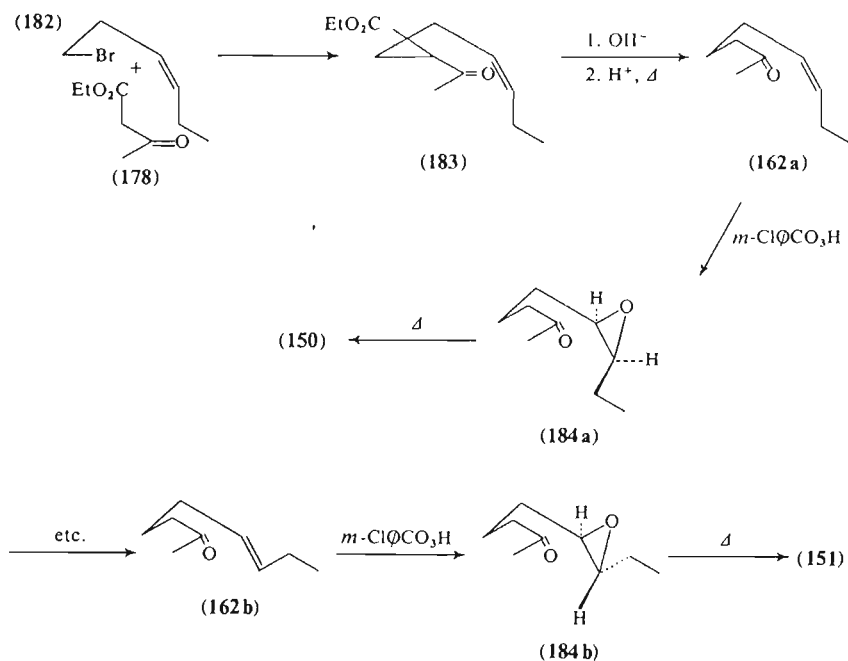
Scheme 35
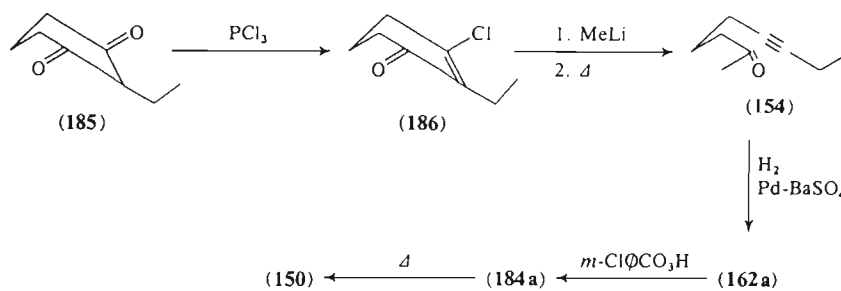
Scheme 36

As shown in Scheme 37, MUNDY *et al.* (*127*) treated the Diels-Alder adduct (**188**) of acrolein (**187**) and methylvinyl ketone (**28**) with ethyl-magnesium bromide to obtain an epimeric hydroxydihydropyran (**189**), which cyclized in the presence of mercuric acetate to a mixture of *exo*- and *endo*-brevicomin. Difficulties in purifying (**188**) precludes large-scale

Scheme 37

Scheme 38

(190) $\xrightarrow[\text{2. EtMgBr}]{\text{1.} \quad \text{—NH}_2, \; -H_2O}$ (191) $\xrightarrow{hv}$ (192) $\xrightarrow[\text{Pd-C}]{\text{H}_2}$ (150)
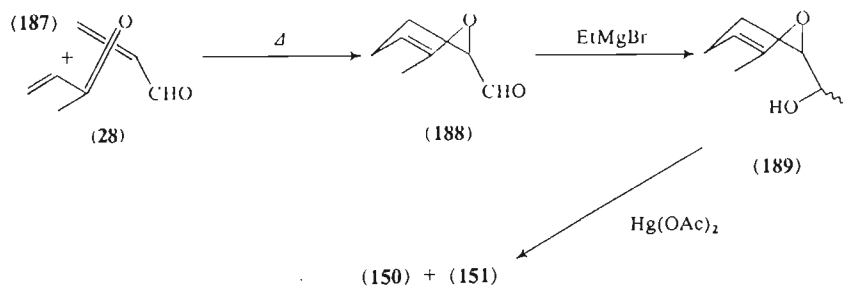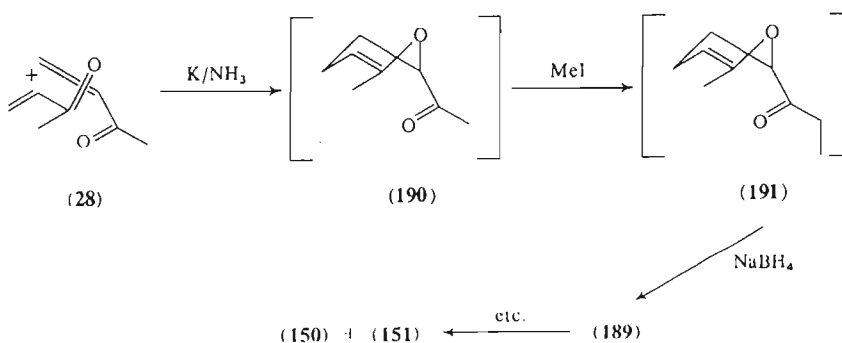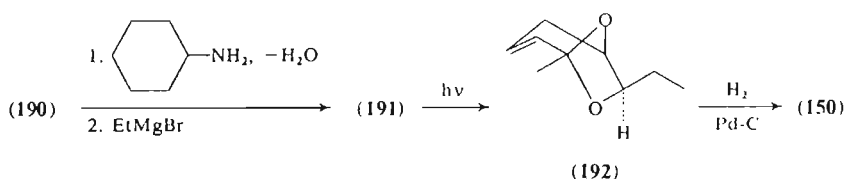
Scheme 39

preparation of brevicomin by this route.. In a later synthesis, LIPKO-
WITZ *et al.* (*128*) prepared (189) from the methylvinyl ketone dimer (190)
(Scheme 38). CHAQUIN *et al.* (*129*) recently reported the stereospecific
photolytic cyclization of keto dihydropyran (191) to an *exo*-ethylenic
ketal (192), which readily yielded *exo*-brevicomin on catalytic reduction
(Scheme 39).

The first synthesis of racemic frontalin (195) was accomplished in
one step by KINZER *et al.* (*130*) with a Diels-Alder reaction postulated
to proceed *via* the hydroxy-dihydropyran intermediate (194) (Scheme 40).
D'SILVA and PECK (*131*) improved the synthesis by substituting formal-
dehyde and acetone for methylvinyl ketone. By use of a different
dienophile, MUNDY *et al.* (*127*) were able to isolate (194), and in the
presence of mercuric acetate cyclize it to frontalin (Scheme 41). Thermo-
lysis of the ketoepoxide intermediate (205) was used by MORI *et al.* (*132*)
to obtain racemic frontalin (Scheme 42). Resolution of acid (206) with
cinchonine by MORI (*133*) afforded starting material for the synthesis of
optically active (*S*)-(−)-frontalin *via* a ketodiol intermediate (211)
(Scheme 43). The same intermediate was formed by OHRUI and EMOTO
(*134*) from *D*-glucose (Scheme 44).

(193)   (28)   (194)   (195)

Scheme 40

(196)   (28)   (197)   (194)   (195)
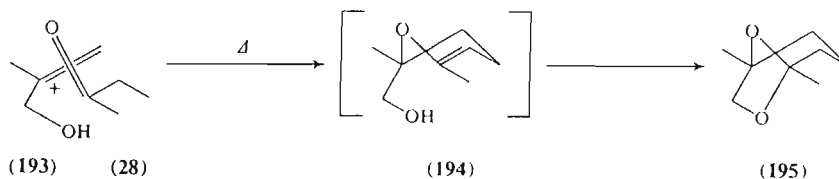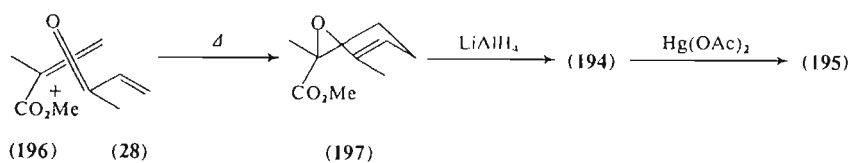
Scheme 41

Scheme 42

Scheme 43

(212)
D-glucose

(213)

(214)

1. HIO₄
2. NaBH₄

(217)

(216)

(215)

Me₂CO

(218)

(219)

(219)

HIO₄

(222)

(221)

(220)

(211) ⟶ (195)
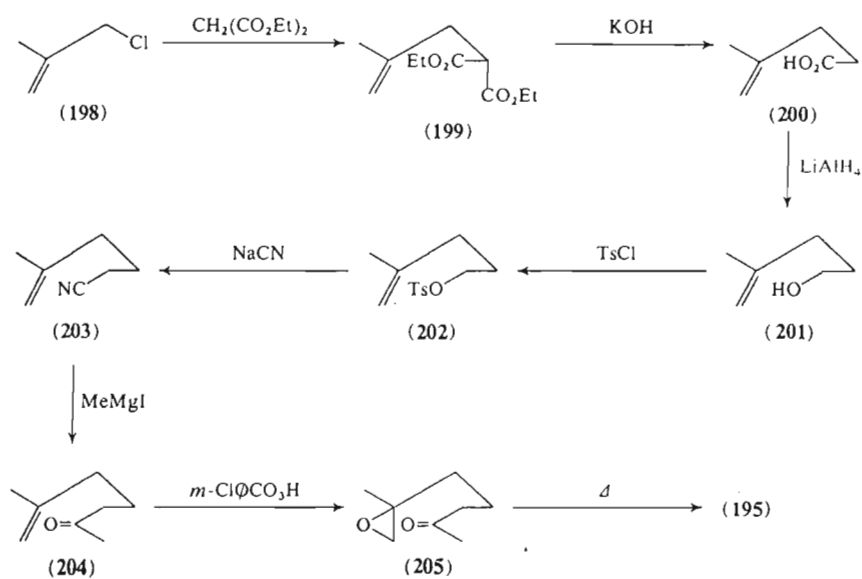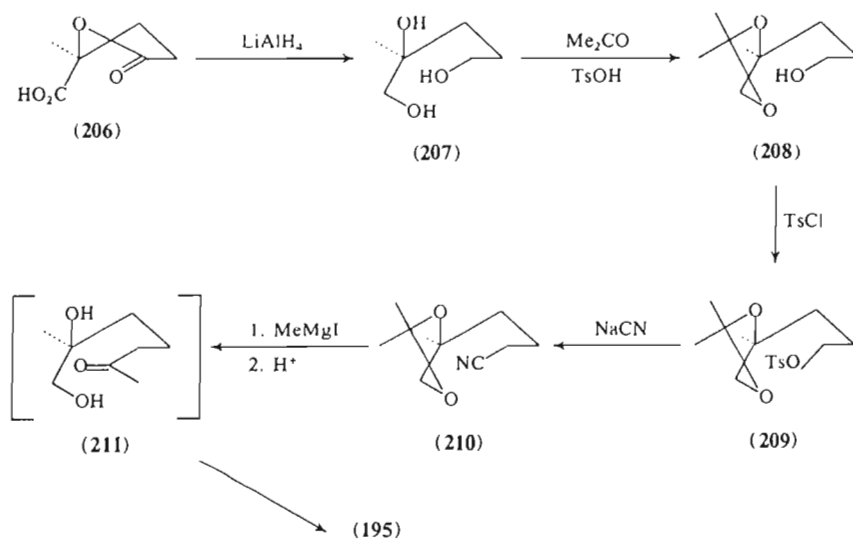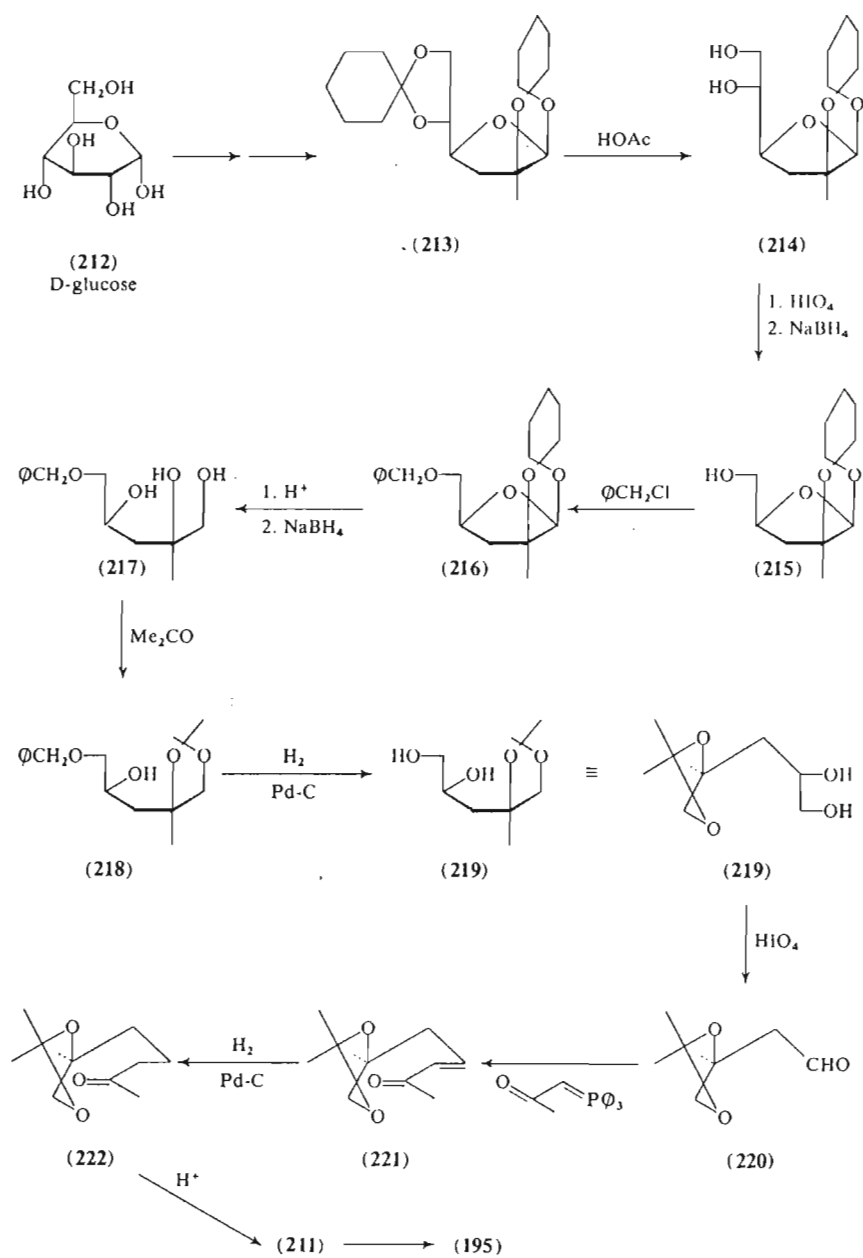
Scheme 44

References, pp. 157--190
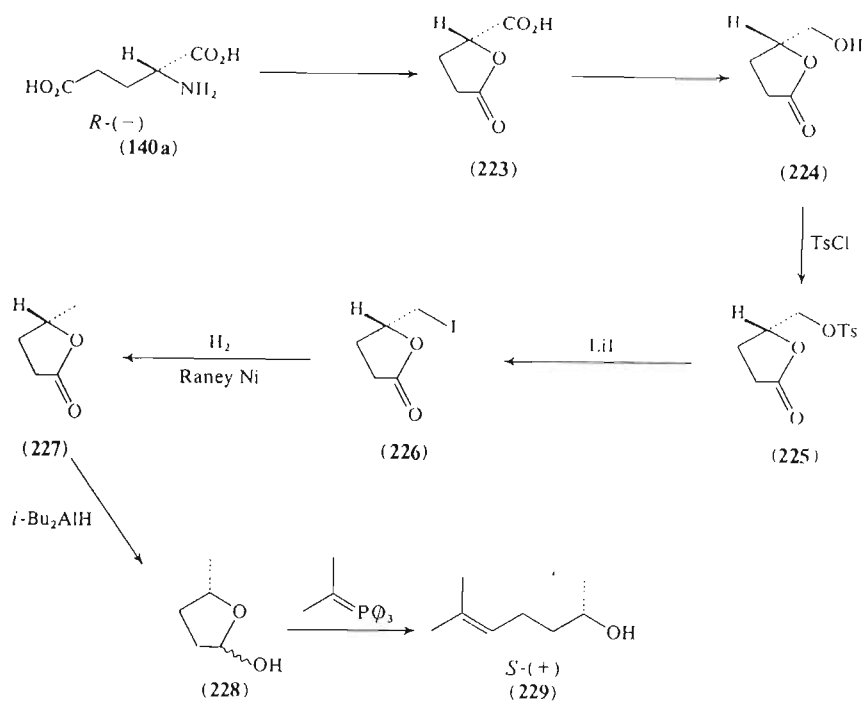
Scheme 45

Sulcatol (**229**), the sex pheromone of *Gnathotrichus sulcatus*, has been synthesized in its optically active (*S*)-(+)- and (*R*)-(−)-forms from (*R*)-(−)- and (*S*)-(+)-glutamic acid (**140**), respectively, by MORI (*135*) (Scheme 45).

Ips spp. bark beetles use a variety of terpene alcohols for communication. Among these are *cis*-verbenol (**234**, Scheme 46), ipsenol (**240a**, Scheme 50), and ipsdienol (**266a**, Scheme 52). *cis*-Verbenol was first synthesized by SILVERSTEIN *et al.* (*51, 136*) by stereospecific reduction of verbenone (**233**) with sodium borohydride. MORI (*47*) synthesized *trans*-verbenol (**232**), a *Dendroctonus* spp. attractant, by lead tetraacetate oxidation of α-pinene (**230**) followed by hydrolysis of the resultant acetate (Scheme 46). Repeated recrystallization of the 3β-acetoxyetienic acid ester afforded optically pure *trans*-verbenol. Oxidation (*48*) of the optically pure *trans*-epimer to verbenone and reduction with lithium aluminium hydride yielded optically pure *cis*-verbenol.
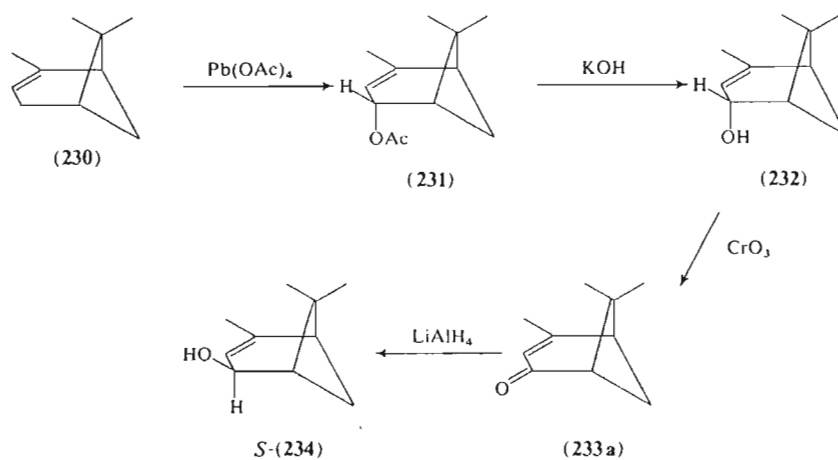
Scheme 46
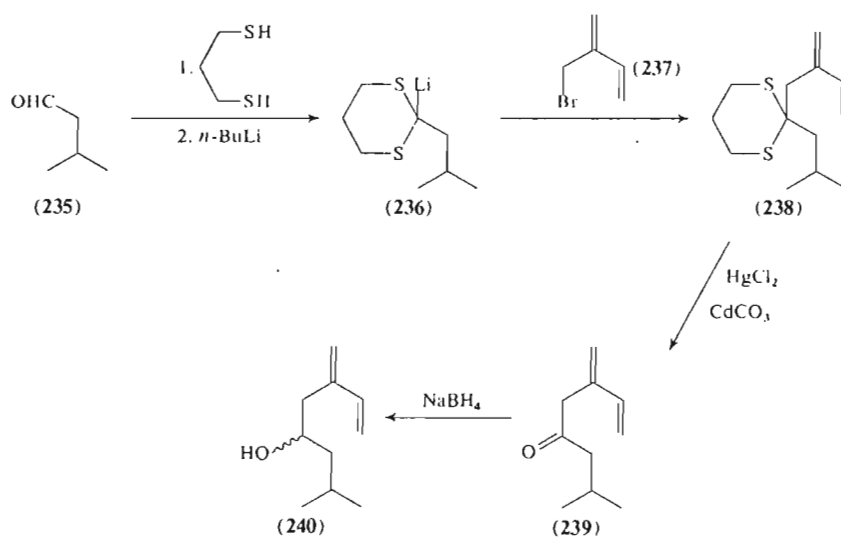
Scheme 47

SILVERSTEIN *et al.* (*51, 136*) began their synthesis of racemic ipsenol by coupling 2-bromomethyl butadiene (**237**) with the anion of the dithiane derivative (**236**) of 3-methylbutanal (**235**) (Scheme 47). Desulfurization of (**238**) and reduction of ketodiene (**239**) gave the desired alcohol (**240**).

KATZENELLENBOGEN and LENOX (*137*) were able to obtain ipsenol in one step by coupling (235) with either (237) in the presence of zinc or 2-mesyloxymethylbutadiene in the presence of lithium.

A thermal sigmatropic rearrangement of the allenic vinyl ether (243) by KARLSEN *et al.* (*138*) afforded 3-methylene-4-pentenal (244), which was treated with *iso*-butyl magnesium bromide (245) to give ipsenol (Scheme 48). CLINET and LINSTRUMELLE (*139*) prepared ketone (239) by treating the conjugated allenic ketone (247) with vinyl cuprate (Scheme 49). (*S*)-(+)-Leucine (248) served as the starting material for MORI's stereoselective synthesis (*140, 141*) of (*S*)-(−)-ipsenol (240 a) (Scheme 50). Epoxide (255) was condensed with diethylmalonate, cyclized, and dehydrated to give the unsaturated lactone (257). After protection of the methylene group as a phenylseleno derivative and reduction to a hemiacetal (*259*), the second methylene was added by treatment with a Wittig reagent.
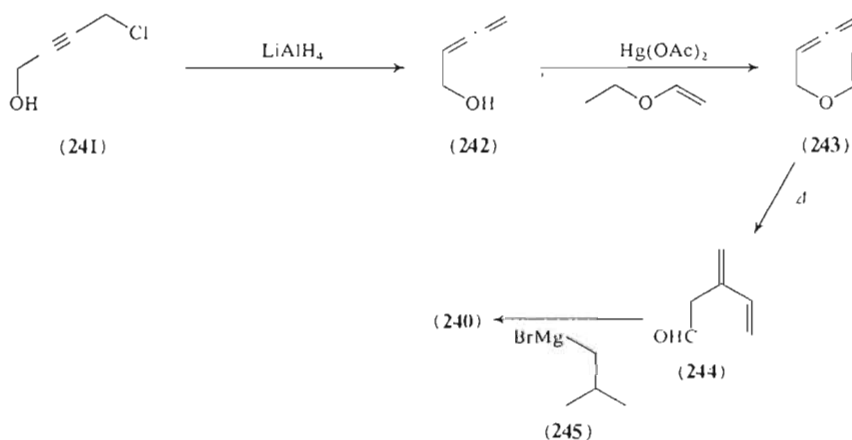
Scheme 48
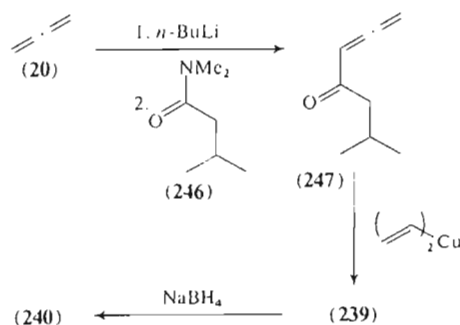
Scheme 49
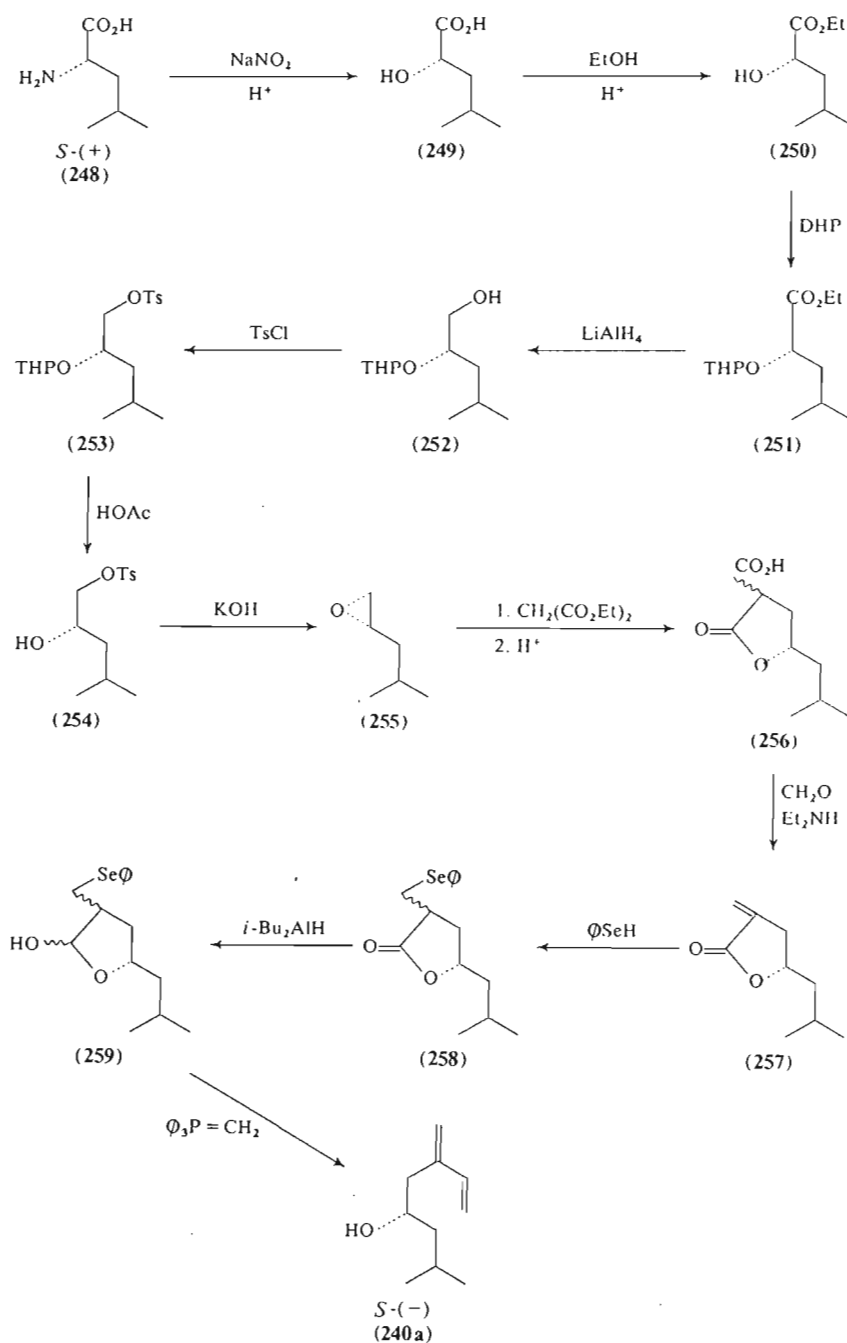
Scheme 50
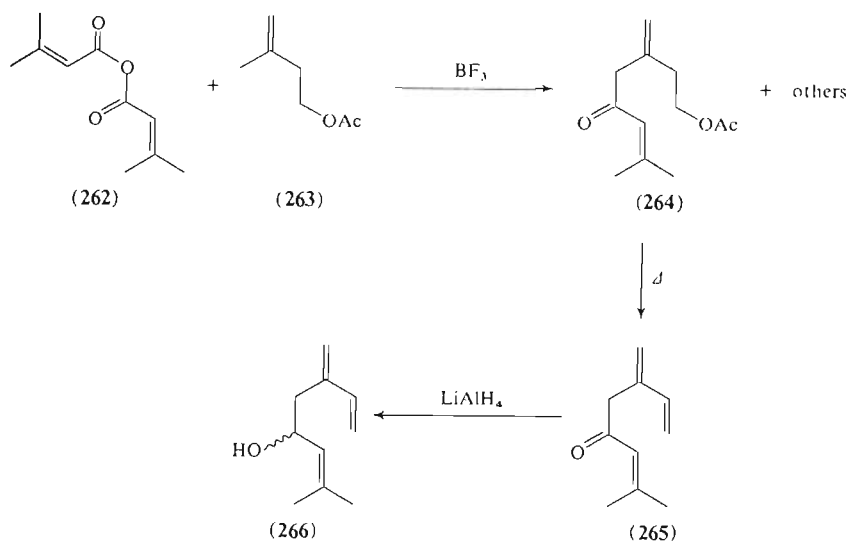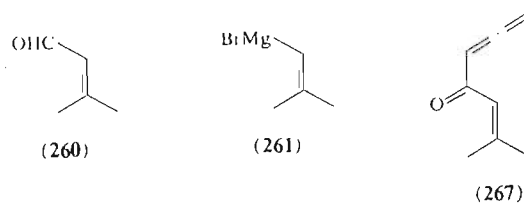
Scheme 51

Syntheses of racemic ipsdienol (**266**, Scheme 51) have tended to follow the approaches used for ipsenol. SILVERSTEIN *et al.* (*51, 136*) began with the dithiane derivative of 3-methyl-2-butenal (**260**) and proceeded as in Scheme 47. RILEY *et al.* (*142*) coupled (**260**) with (**237**) in the presence of zinc, and KARLSEN *et al.* (*138*) coupled (**244**) with the Grignard reagent (**261**). Condensation of senecioic anhydride (**262**) with acetate (**263**) by GARBERS and SCOTT (*143*) gave a mixture of ketoacetates, with (**264**) predominating (Scheme 51). Pyrolytic elimination of acetic acid followed by hydride reduction of ketone (**265**) afforded racemic ipsdienol. By using the cross conjugated ketone (**267**) and vinyl cuprate, CLINET and LINSTRUMELLE (*139*) prepared ketone (**265**). MORI's synthesis (*144*) of (*R*)-(−)-ipsdienol (**266a**) began with *D*-mannitol (**268**) (Scheme 52) and followed essentially the same path as that of (*S*)-(−)-ipsenol (Scheme 50).
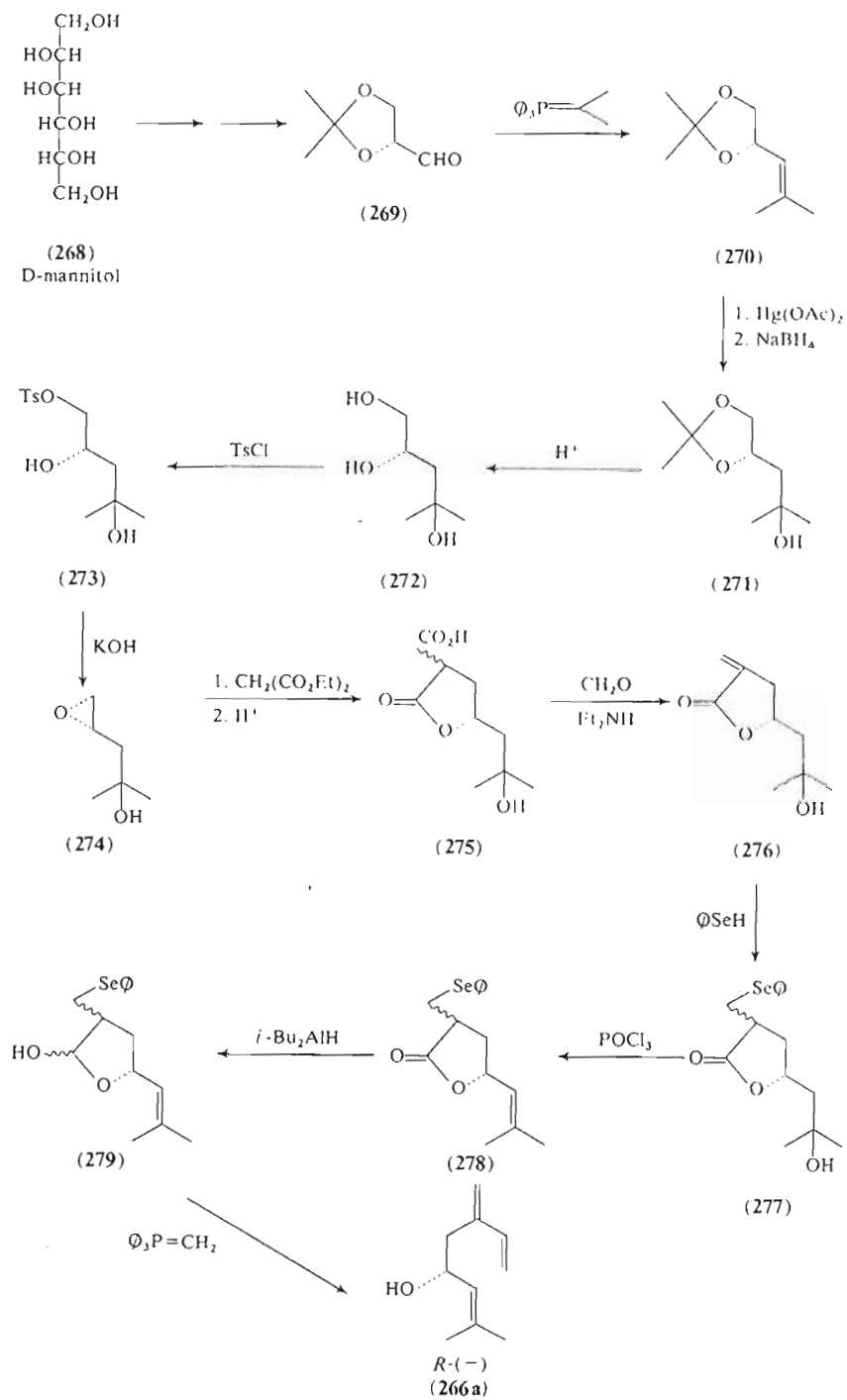
Scheme 52
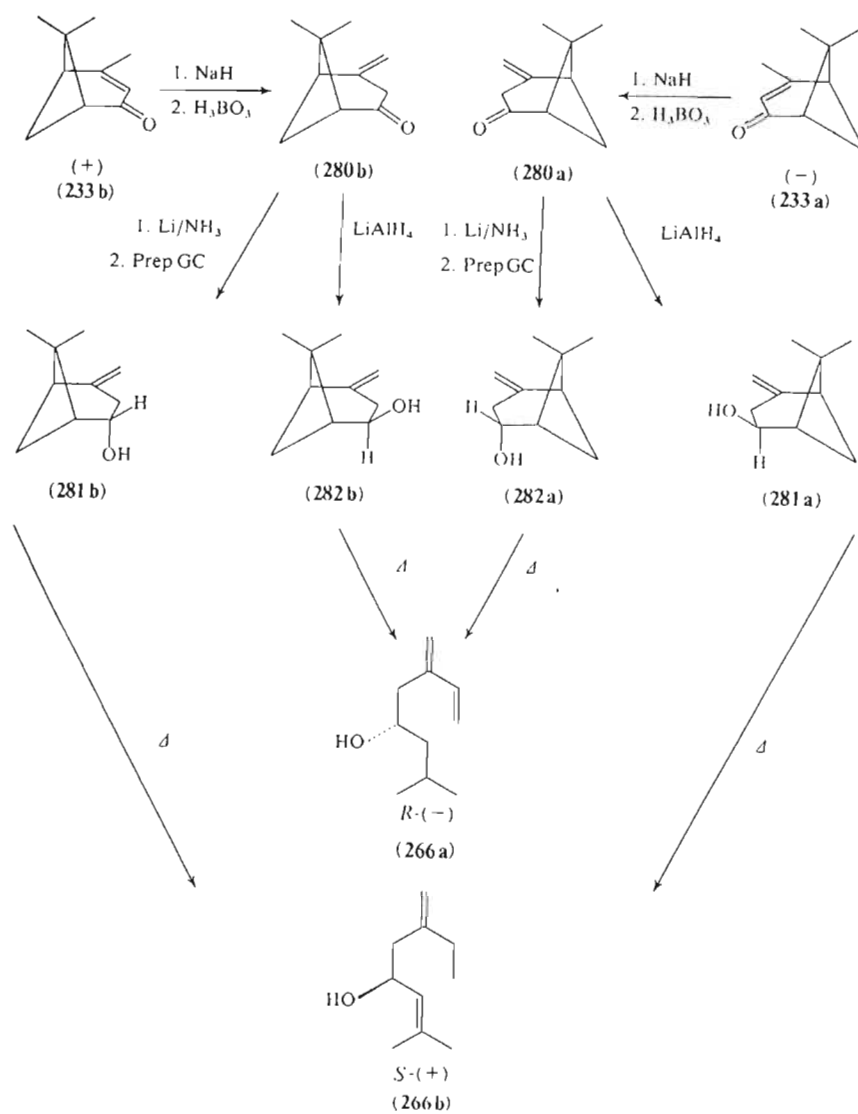
Scheme 53

The best route to (R)- or (S)-ipsdienol is that devised by OHLOFF and GIERSCH (145), which commences with the optically active verbenones (233a and 233b) (Scheme 53). The α,β-unsaturated ketones were deconjugated by sequential treatment with sodium hydride and boric acid to give (280a) and (280b). Reduction with lithium aluminium hydride

Scheme 54

(283)

(296)   (297)   (297a)

LiAlH₄

(300)   (299)   (298)

(301)   (302)

m-ClOCO₃H

SnCl₄
or
H⁺

α-(−)-(1S,2R,4S,5R)   γ-(−)-(1S,2R,4R,5R)
(303a)   (303c)

β-(+)-(1R,2R,4S,5S)   δ-(+)-(1R,2R,4R,5S)
(303b)   (303d)
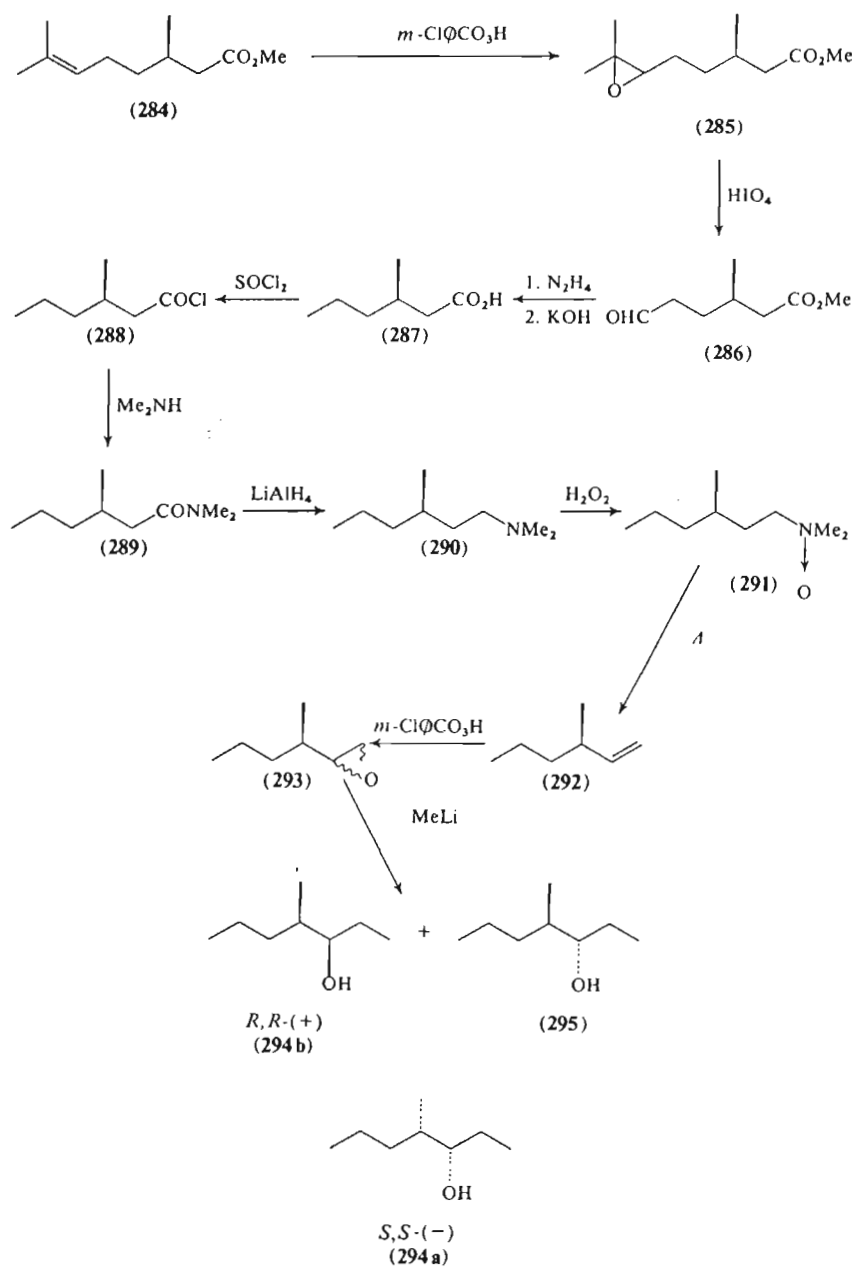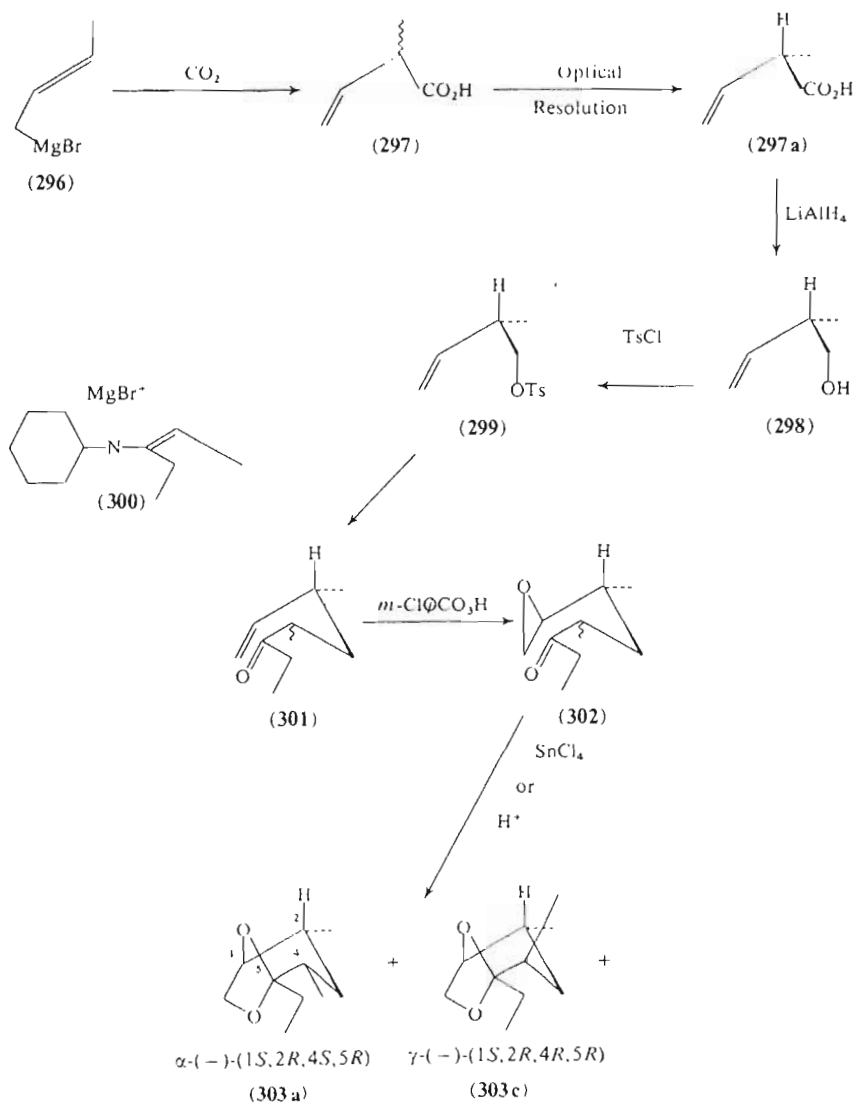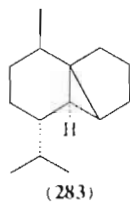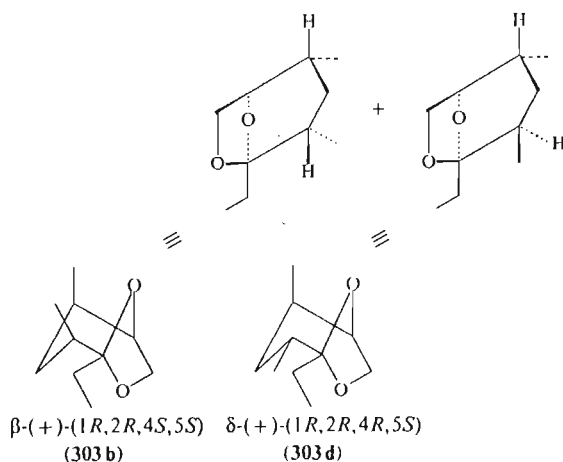
Scheme 55

yielded pure *cis*-alcohols (281), whereas reduction with lithium in liquid ammonia gave a mixture of *cis*- and *trans*-alcohols (281 and 282, respectively) (separable by preparative GC) plus verbenone. Flash-pyrolysis of these bicyclic alcohols stereospecifically afforded (R)- or (S)-ipsdienol.

(−)-(3S,4S)-4-Methyl-3-heptanol (294a, Scheme 54) and α-(−)-(1S, 2R,4S,5R)-multistriatin (303a, Scheme 55) are produced by the smaller European elm bark beetle *(Scolytus multistriatus)* and along with the host-produced α-cubebene (283) are responsible for pheromonal attraction in this species (46). Synthesis of racemic (294) was achieved by hydride reduction of 4-methyl-3-heptanone (146). MORI (147) synthesized the (3R,4R)-(+)-diastereomer (294b) from (R)-(+)-methyl citronellate (284) (Scheme 54). Resolution of (S)-(+)-2-methyl-3-butenoic acid (297a) by PEARCE *et al.* (49) yielded starting material for the synthesis of optically active multistriatin with the R configuration at C-2 (Scheme 55). Cyclization of keto epoxide (302) with SnCl₄ or H⁺ afforded a mixture of multistriatins (303a, 303b, 303c, and 303d) (34 : 1 : 7 : 58, respectively), which were separable by preparative GC. By starting with (R)-(+)-glyceraldehyde acetonide (269) MORI (148) obtained a mixture (in about
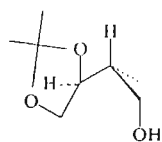
(307a)

the same ratio as PEARCE *et al.* (*49*)) of optically active multistriatins having the *S* configuration at C-1 (Scheme 56). In a similar synthesis, ELLIOTT and FRIED (*149*) were able to obtain a racemic mixture of (303a) and (303c) (85 : 15) in 73% overall yield from (*Z*)-2-butene-1,4-diol *via* (307a).
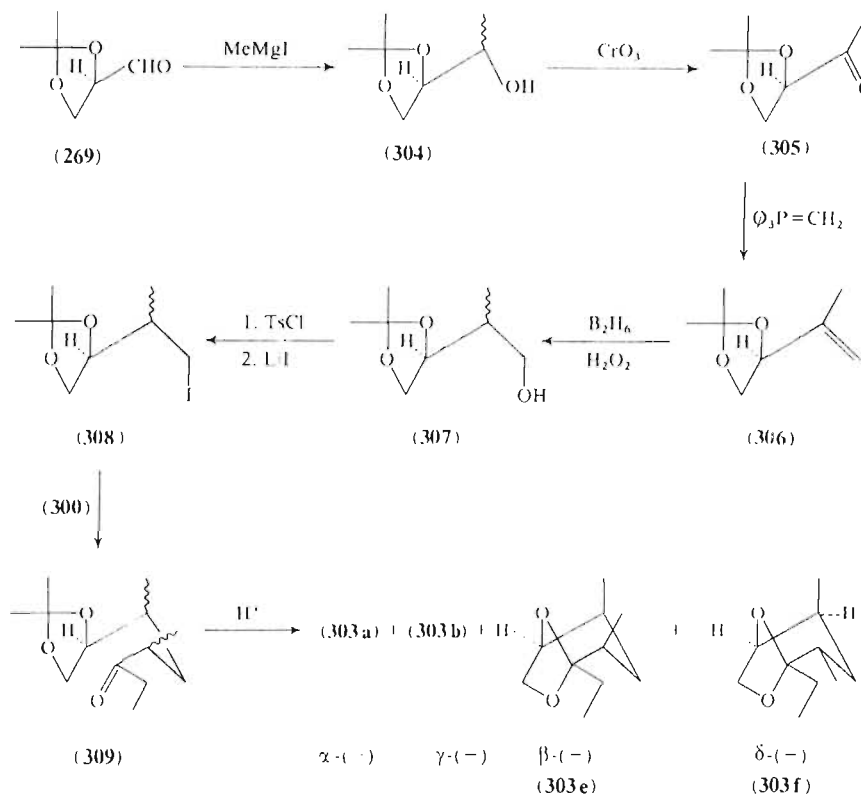
Scheme 56

B. Diptera

Synthesis of (*E*)-non-6-en-1-ol (315), the sex attractant of the Mediterranean fruit fly *(Ceratitus capitata)* was achieved by JONES *et al.* (*150*) through photochemical addition of propanal and 1,3-cyclohexadiene (311) and subsequent thermal cycloreversion of the bicyclic oxetane intermediate (313) (Scheme 57). The critical cycloreversion step was > 95% stereoselective. This synthesis is significantly shorter than that reported by JACOBSON *et al.* (*151*). ROSSI (*152*) has also reported a convenient synthesis.

4*
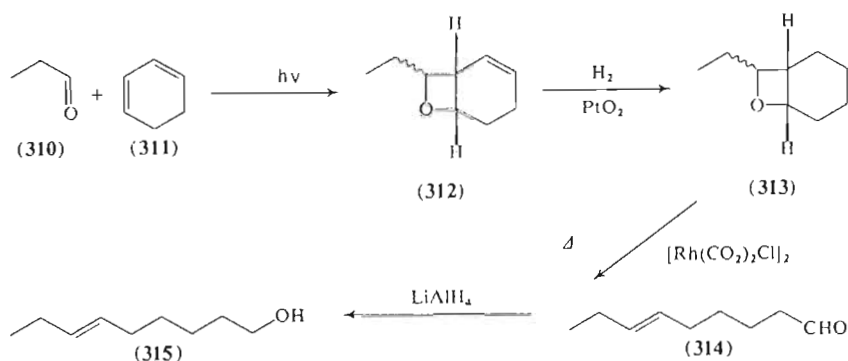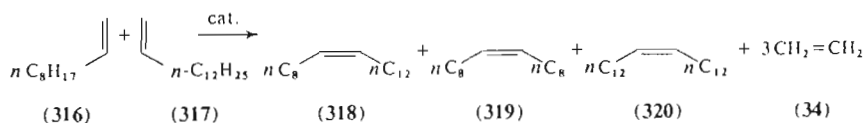
Scheme 57

Scheme 58

The housefly *(Musca domestica)* uses the hydrocarbon (Z)-9-tricosene (**318** in Scheme 58) as the major component of its sex pheromone. Simple syntheses involve Wittig coupling (*153, 154*) or alkylation of a terminal alkyne and subsequent reduction (*155, 156*) to afford the desired Z-isomer in *ca.* 95% purity: Erucic acid (*157, 158*) or oleic acid (*159, 160*) have served as starting material of known Z stereochemistry. The transition metal-catalyzed olefin cross-metathesis reaction has been applied by Rossi (*161*) to synthesize (**318**) as a mixture of E/Z-isomers together with the other possible C_{18} and C_{28} olefins (Scheme 58).

C. Homoptera

Females of the California red scale *(Aonidiella aurantii)* release a mixture of the branched unsaturated acetates (**332a**) and (**333**) to attract males. Synthesis of the R,Z component (**332a**) was achieved by Roelofs *et al.* (*74, 75*) starting from (S)-(+)-carvone (**321**) (Scheme 59). The final product consisted of a mixture of the Z and E isomers, which were easily separable by GC. The absolute stereochemistry of the 3-methyl group in (**333**) is unknown.

References, pp. 157—190

(321)
S-(+)

(322)

(323)

(324)

(325)

(326)

(327)

(328)

(329)

(330)

(331)

(332 a)

(332 b)

(333)
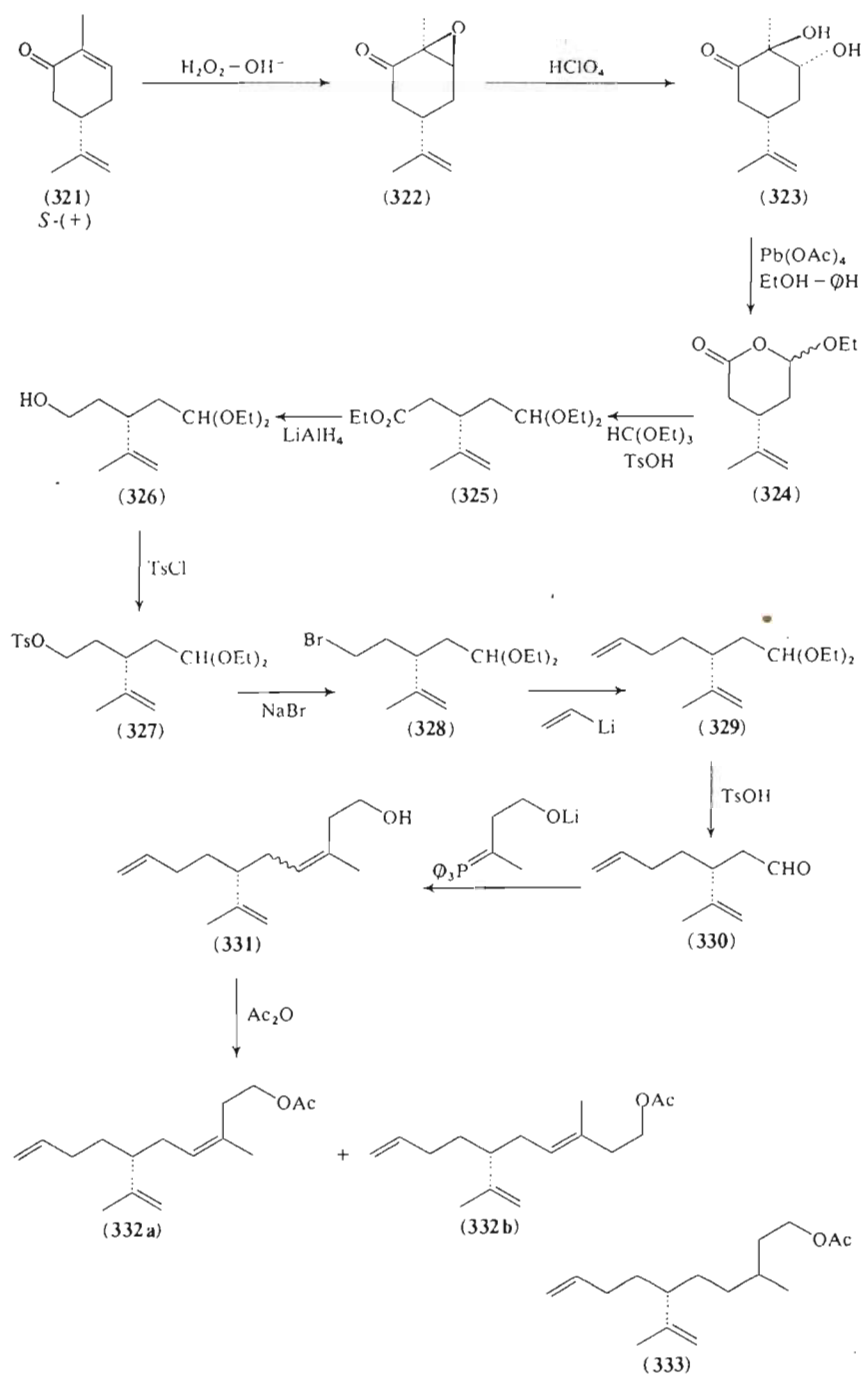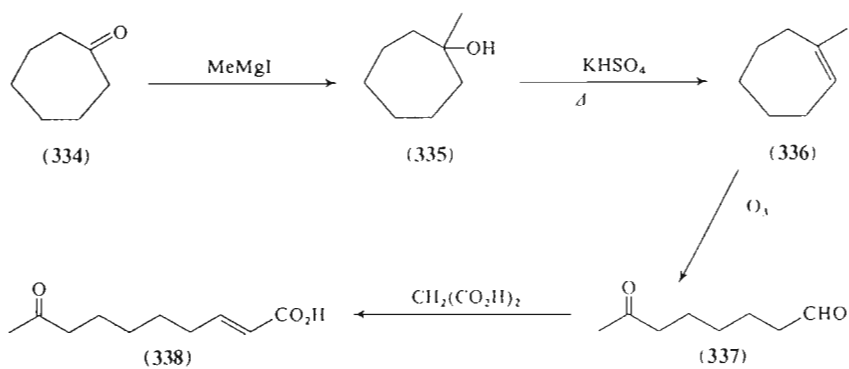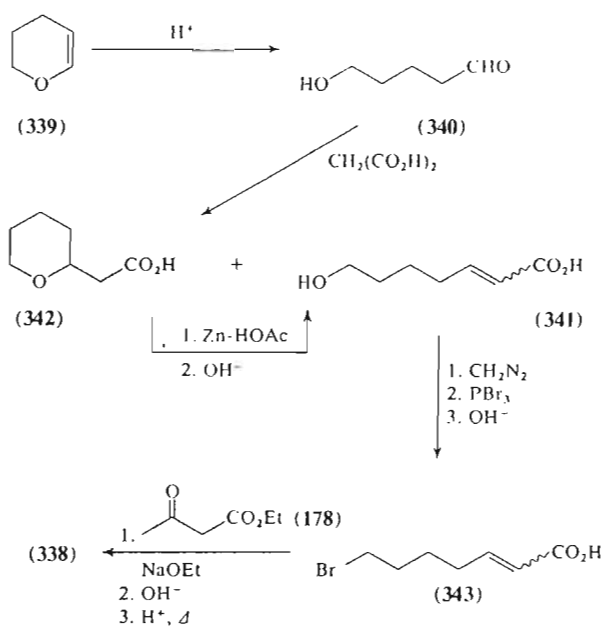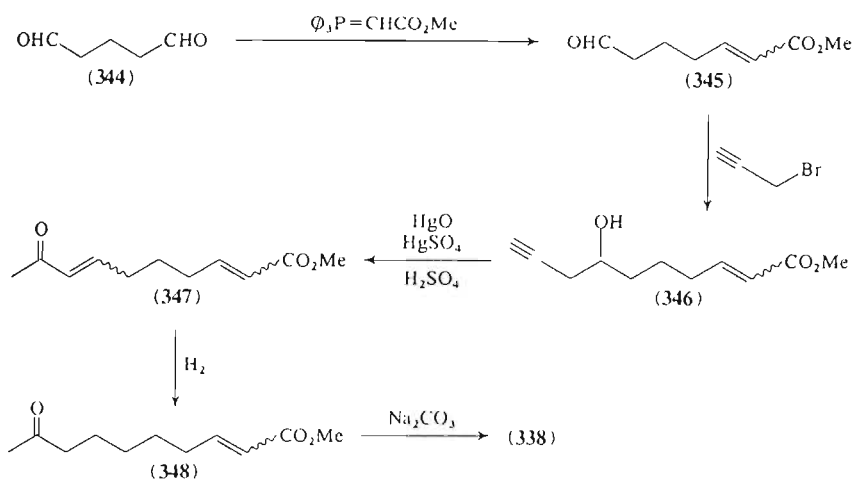
Scheme 60

Scheme 61

Scheme 62
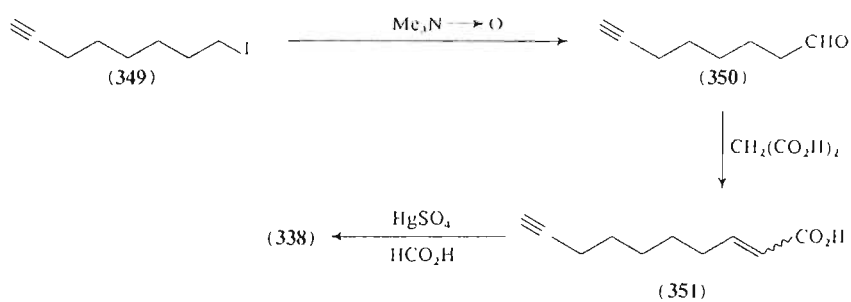
Scheme 63

D. Hymenoptera

1. Apidae

(*E*)-9-Oxo-2-decenoic acid (**338** in Scheme 60), the sex pheromone of the honey bee *(Apis mellifera)* has been synthesized by a variety of methods. In a lengthy sequence starting from azaleic acid, BUTLER *et al.* (*162*) dehydrohalogenated an α-bromomethyl ester and converted the other acid to a methyl ketone. Ozonolysis of 1-methyl-1-cycloheptene (**336**) by BARBIER *et al.* (*163*) afforded ketoaldehyde (**337**), which was condensed with malonic acid to give (**338**) (Scheme 60). JAEGER and ROBINSON (*164*) obtained (**337**) from 7-oxooctanoic acid *via* the corresponding acid chloride. Acidification of dihydropyran by KENNEDY *et al.* (*165*) and chain extension with malonic acid yielded the unsaturated

hydroxy acid (341) (Scheme 61). Bromination and alkylation completed the synthesis.

EITER (166) introduced the unsaturated carboxy moiety via a Wittig reaction with pentanedial and then chain extended to a β-hydroxyalkyne (346) (Scheme 62). Mercuric ion-catalyzed rearrangement and reduction afforded methylketo ester (348). In a relatively high yield synthesis, SISIDO et al. (167) oxidized iodoalkyne (349), chain extended and transformed the terminal alkyne to the methyl ketone (Scheme 63). In all of the above examples, the double bond is assumed to be in the more thermodynamically stable E form.

TROST and SALZMANN (168) were able to stereoselectively introduce an E double bond by their sulfenylation-dehydrosulfenylation method (Scheme 64). Palladium catalyzed telomerization of butadiene (358) by TSUJI et al. (169) afforded diene (360) (Scheme 65). Ketoacid ester (363), obtained by terminal olefin oxidation and subsequent reduction, was treated with diphenyldiselenide and the resultant phenylselenyl group oxidatively removed to give the E α,β-unsaturated keto ester (365).

LCIA = Lithium cyclohexylisopropylamide
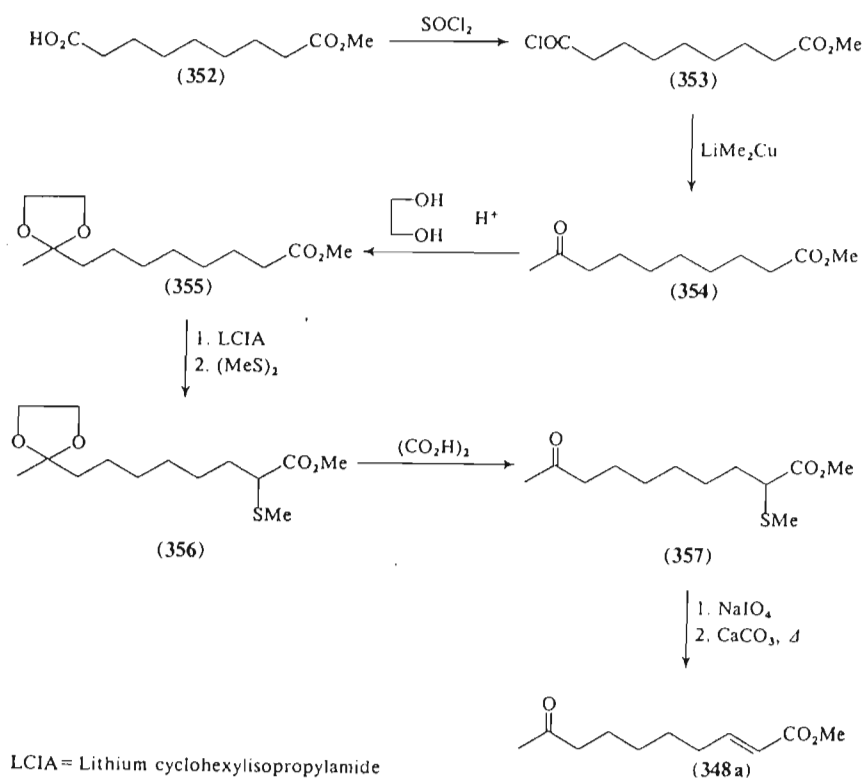
Scheme 64

References, pp. 157—190
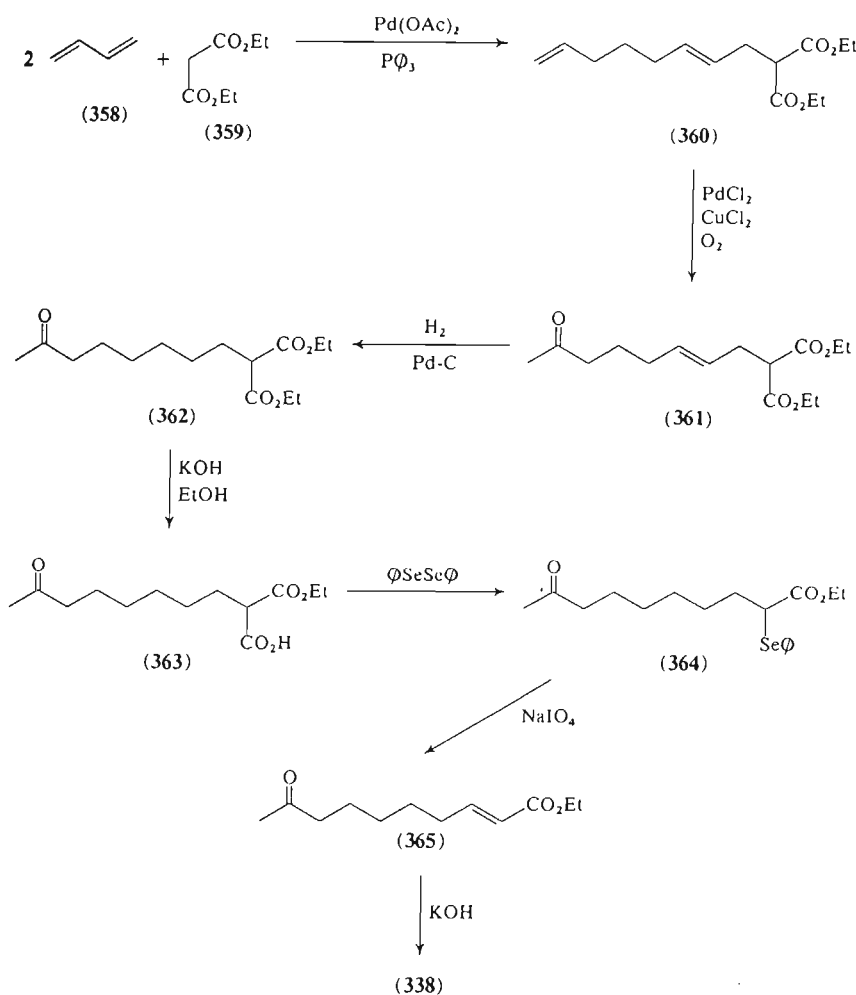
Scheme 65

2. Braconidae

Several saturated C_{32}, C_{33}, and C_{34} hydrocarbons with methyl branches in the C_{11-13} positions have been identified from the tobacco hornworm *(Heliothis virescens)* (Lepidoptera: Noctuidae) that elicit a host-seeking response of its parasitoid, *Cardiochiles nigriceps*. These kairomones have been synthesized by VINSON *et al. (170)* using appropriate methyl ketones and Wittig reagents followed by catalytic reduction.

DiBALH = Di-*t*-butoxyaluminium hydride
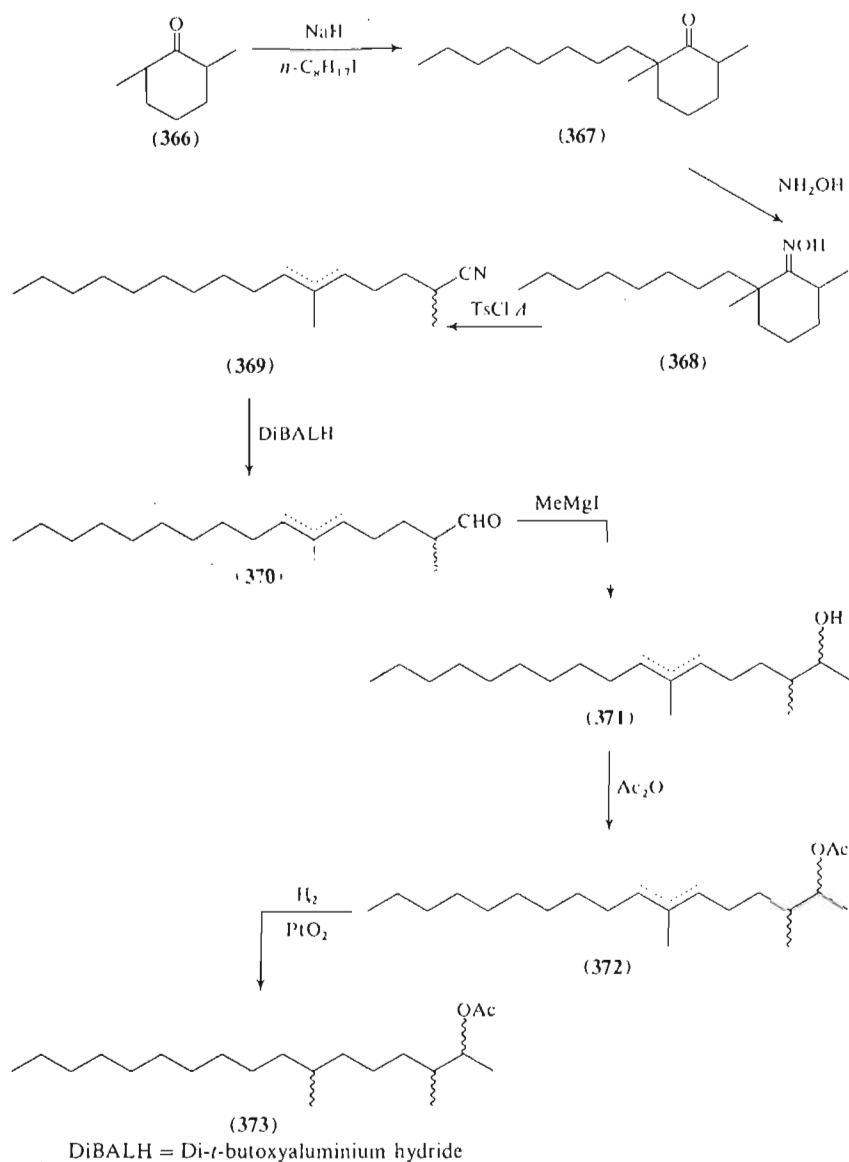
Scheme 66

3. Diprionidae

The sex pheromone (**373**) (Scheme 66) of the pine sawfly *(Neodiprion lecontei)* was synthesized by Kocienski and Ansell (*171*) as a mixture of epimers starting from 2,6-dimethylcyclohexanone. Alkylation and sub-
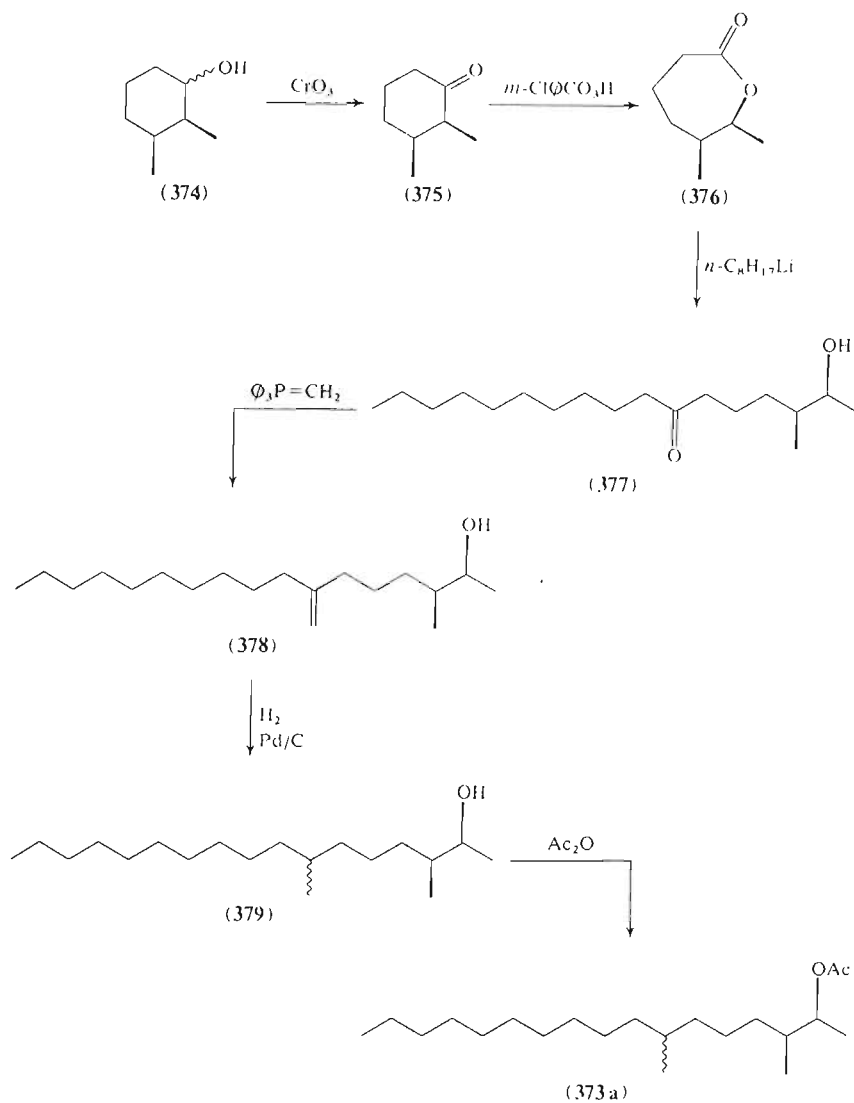
Scheme 67

sequent Beckmann rearrangement of oxime (368) yielded an acyclic un-
saturated nitrile (369), which was transformed to the desired acetate.
MAGNUSSON (*172*) utilized pure *trans*-2,3-dimethylcyclohexanone in his
synthesis of the *erythro*-isomer of the sex pheromone (Scheme 67).
Baeyer-Villiger oxidation and alkylation gave the acyclic hydroxy ketone
(377), which subsequently afforded (373a).

4. Formicidae

The alarm pheromones of ants are often simple readily available ketones. Syntheses of some of the less trivial pheromones are described below.

(S)-(+)-4-Methyl-3-heptanone (**386a**, Scheme 68), the alarm pheromone of the Texas leaf-cutting ant *(Atta texana)* was synthesized by Riley *et al.* (*173, 174*) and required resolution of the intermediate, racemic 2-methyl-4-pentenoic acid (**384**). A convenient synthesis of methyl 4-methylpyrrole-2-carboxylate (**392**), the trail pheromone of *A. texana,* has been reported by Sonnet (*175*) (Scheme 69). Formylation of ester (**390**) with Cl$_2$CHOMe and AlCl$_3$ gave predominantly the desired 4-formyl derivative (**391**).
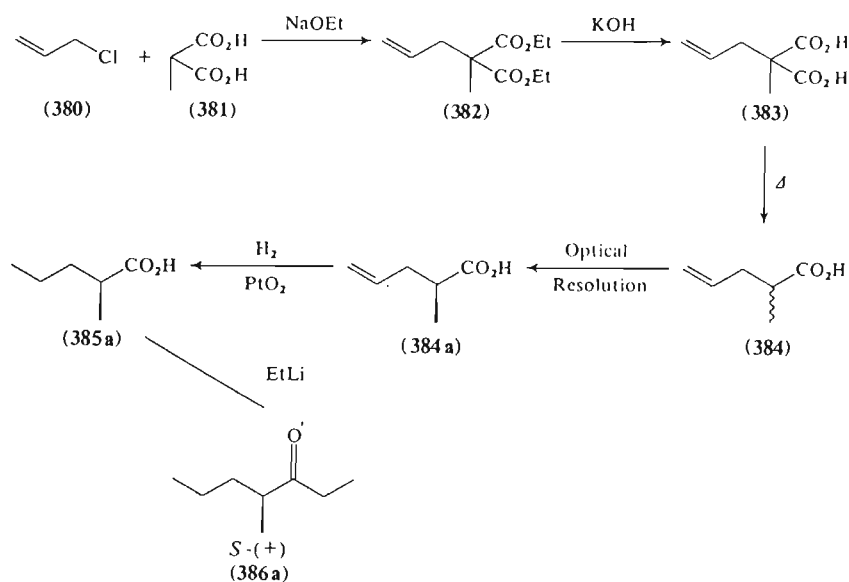
Scheme 68

Caste-specific compounds produced by some male carpenter ants *(Camponotus* spp.) include 2,4-dimethyl-2-hexenoic acid (**396**). Brand *et al.* (*176*) and Kocienski *et al.* (*177*) prepared (**396**) as a mixture of *E*- and *Z*-isomers (Schemes 70 and 71), whereas Katzenellenbogen and Utawanit (*178*) obtained the pure *E*-isomer (**396a**) by stereoselective dehydration of β-hydroxy ester (**402**) *via* a β-alanoxy enolate (Scheme 72).
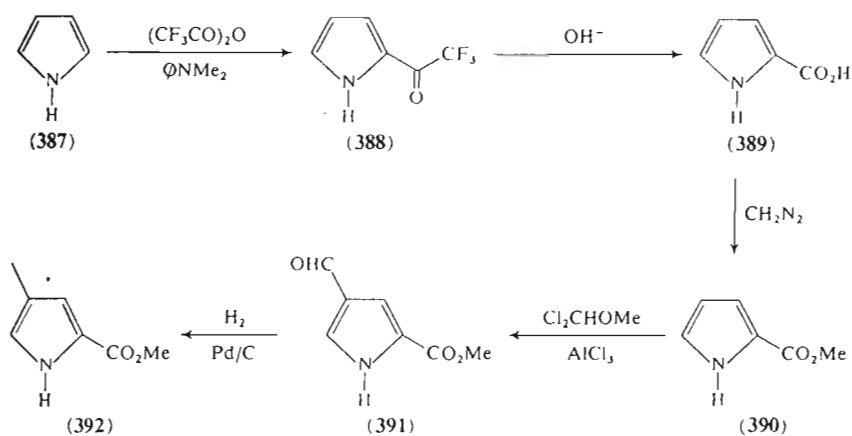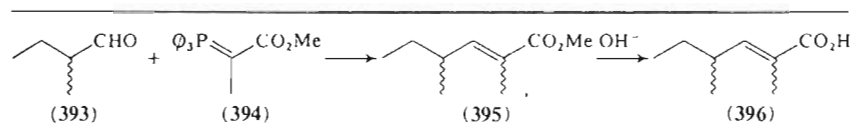
Scheme 69

Scheme 70
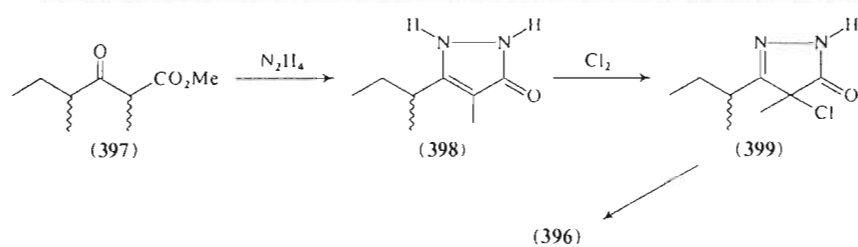
Scheme 71
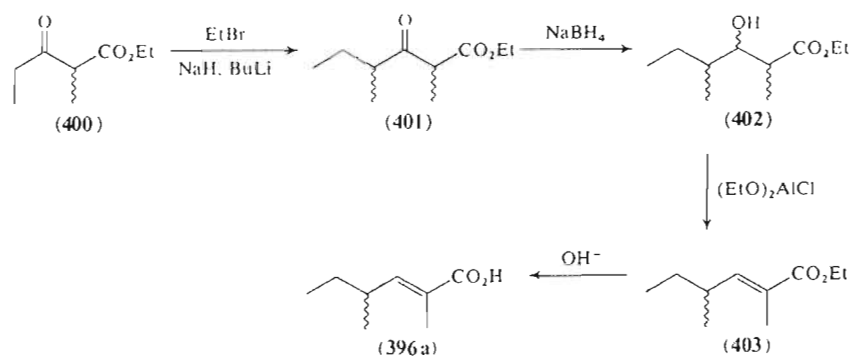
Scheme 72

Manicone, (E)-4,6-dimethyl-4-octen-3-one (**407**, Scheme 73), an alarm pheromone of *Manica* spp. was first synthesized by Fales *at al.* (*179*) by condensation of 2-methylbutanal (**393**) and 3-pentanone. Katzen-ellenbogen and Utawanit (*178*) and Kocienski *et al.* (*177*) transformed (**396**) into manicone by preparing the corresponding acid chloride and adding Et₂CuLi or Et₂Cd, respectively. The S-(+)-enantiomer was pre-pared by Banno and Mukaiyama (*180*) from (S)-(−)-2-methylbutanol (**404a**) (Scheme 73). Condensation of (**393a**) and silyl enol ether (**405**) in the presence of TiCl₄ gave diastereomeric (**406a**), which dehydrated stereospecifically to afford (6S,4E)-(+)-manicone (**407a**). Similarly (**404b**) yielded (**407b**).
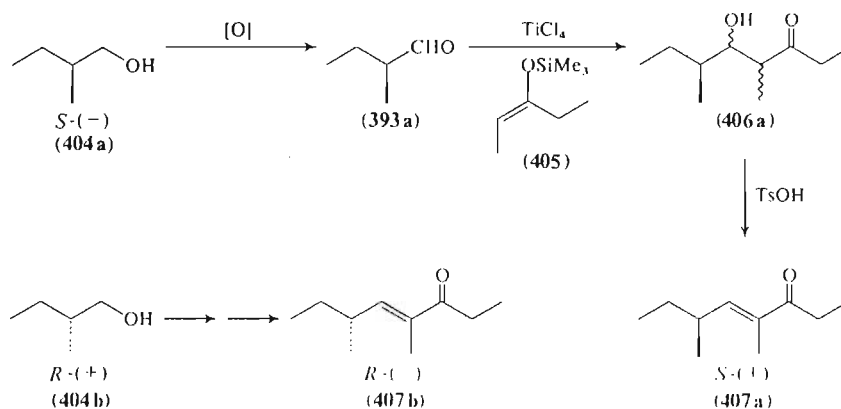
Scheme 73

The octahydroindolizine trail pheromone (**412**) of the Pharaoh ant *(Monomorium pharaonis)* was first prepared by Ritter *et al.* (*181*) as a mixture of stereoisomers, beginning with a termolecular condensation (Scheme 74). Oliver and Sonnet (*182*) then unambiguously synthesized the four stereoisomers from 2,6-lutidine (**413**) (Scheme 75) and 2-butyl-pyrrole (**418**) (Scheme 76).
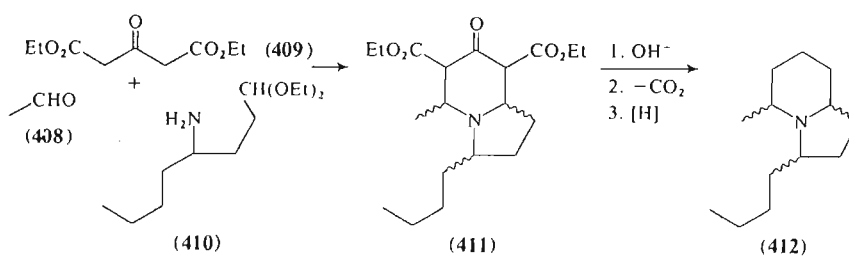
Scheme 74
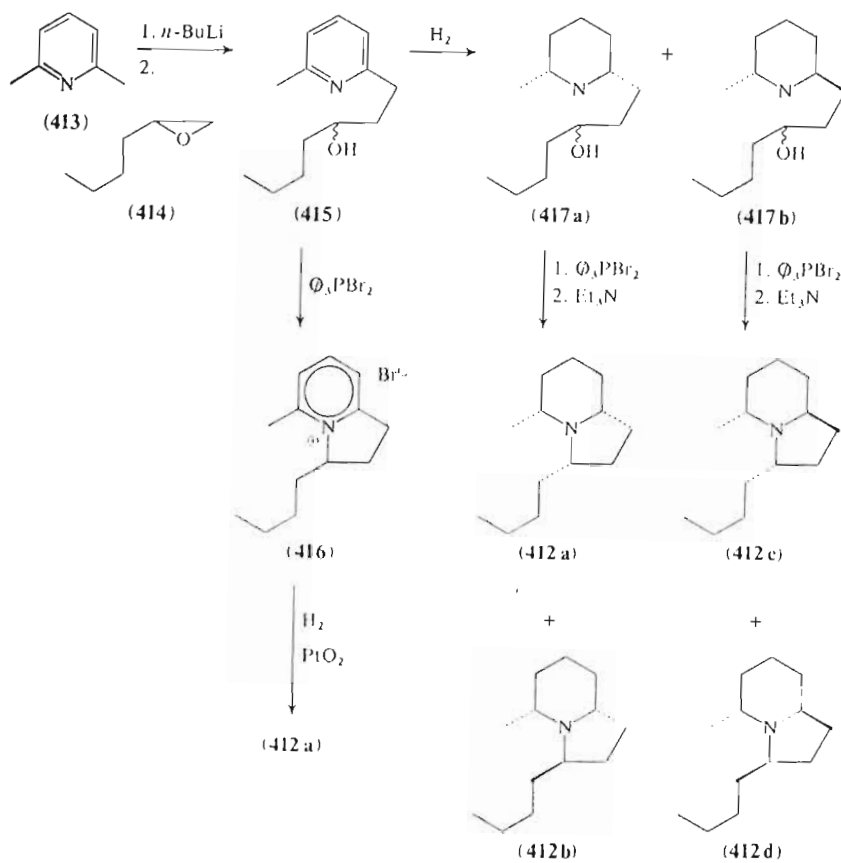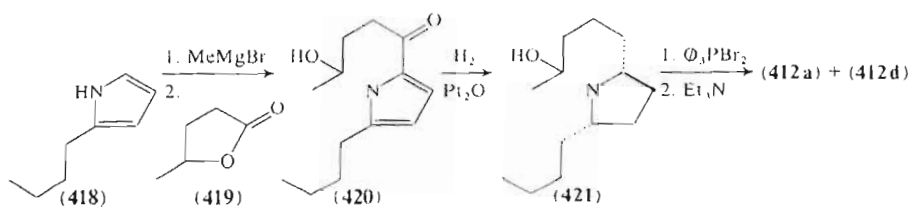
Scheme 75

Scheme 76

Vespa orientalis, the Oriental hornet, produces lactone (**428**) (Scheme 77), which is thought (*183*) to control some aspects of social behavior. The *R*-(+)- and *S*-(−)-enantiomers (**428a** and **428b,** respectively) were elaborated by COKE and RICHON (*184*) from 1-tridecene (**422**) *via* amino-alcohol (**424**), which was resolved into its enantiomeric forms by resolution of the optically active dibenzoyl tartarates (Scheme 77). Hofmann elimination followed by reaction with propiolic acid dianion (**425**) and reductive cyclization completed the synthesis.
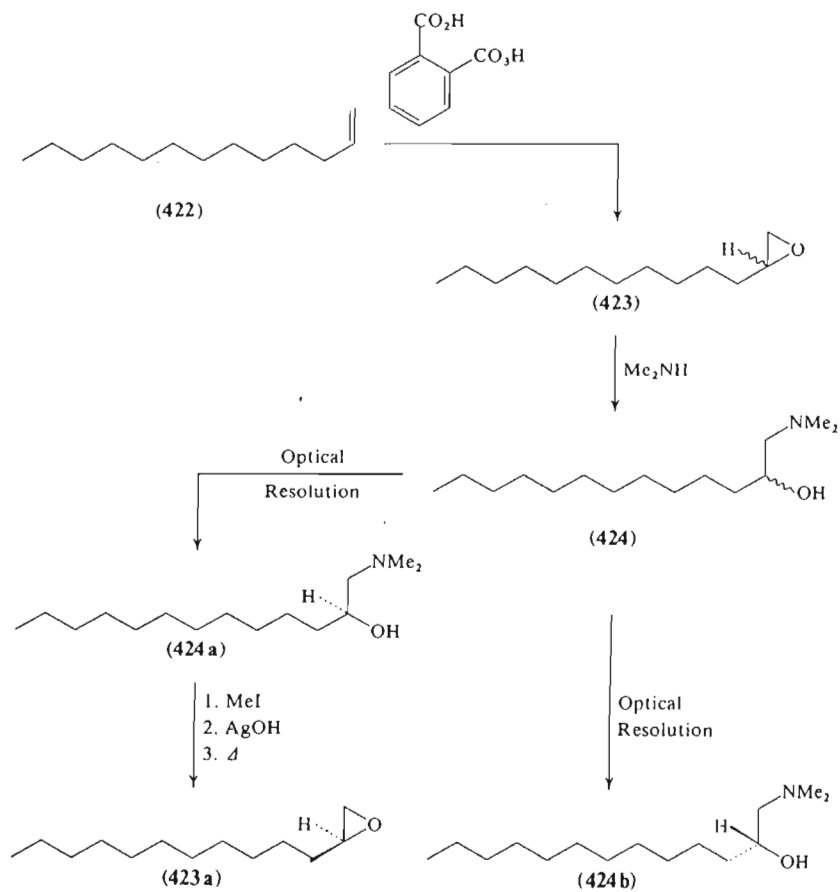
References. pp. 157--190
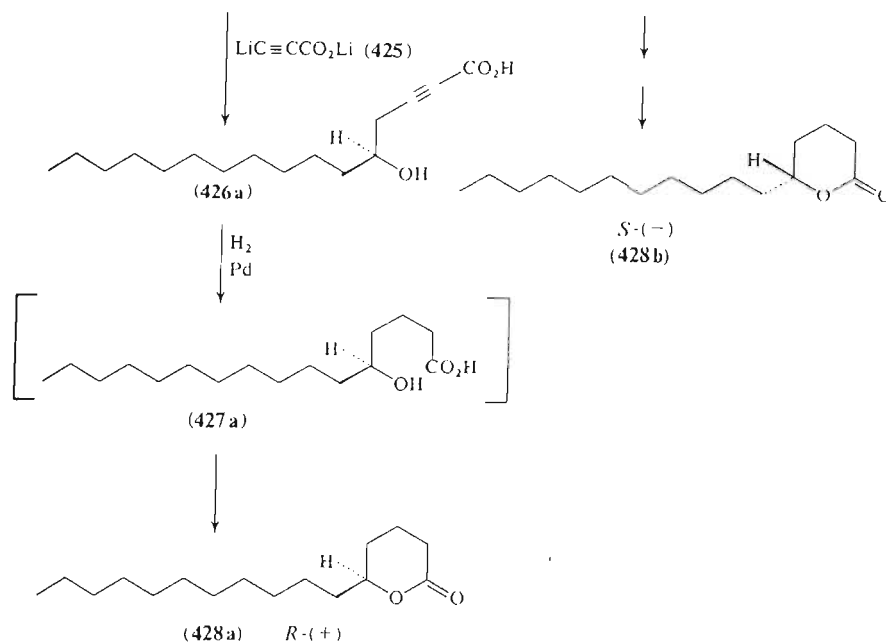
Scheme 77

E. Isoptera

Two syntheses of the trail pheromone, neocembrene (435), of *Nasu-titermes* spp. termites have been reported. KODAMA *et al.* (*185*) (Scheme 78) prepared the allylic phenyl thioether (430) from *trans,trans*-geranyl-linalool (429). Terminal epoxidation of (430) followed by intromolecular cyclization, desulfurization, and dehydration led to (435). KITAHARA *et al.* (*186*) cyclized *trans*-geranylgeranic acid chloride (436) with SnCl₄ to afford chloroketone (437) (Scheme 79). Dehydrohalogenation of (437) and subsequent reduction of ketone (438) *via* acetate (441) gave neo-cembrene (435).

(429)   (430)   (431)

(434)   (433)   (432)

(435)
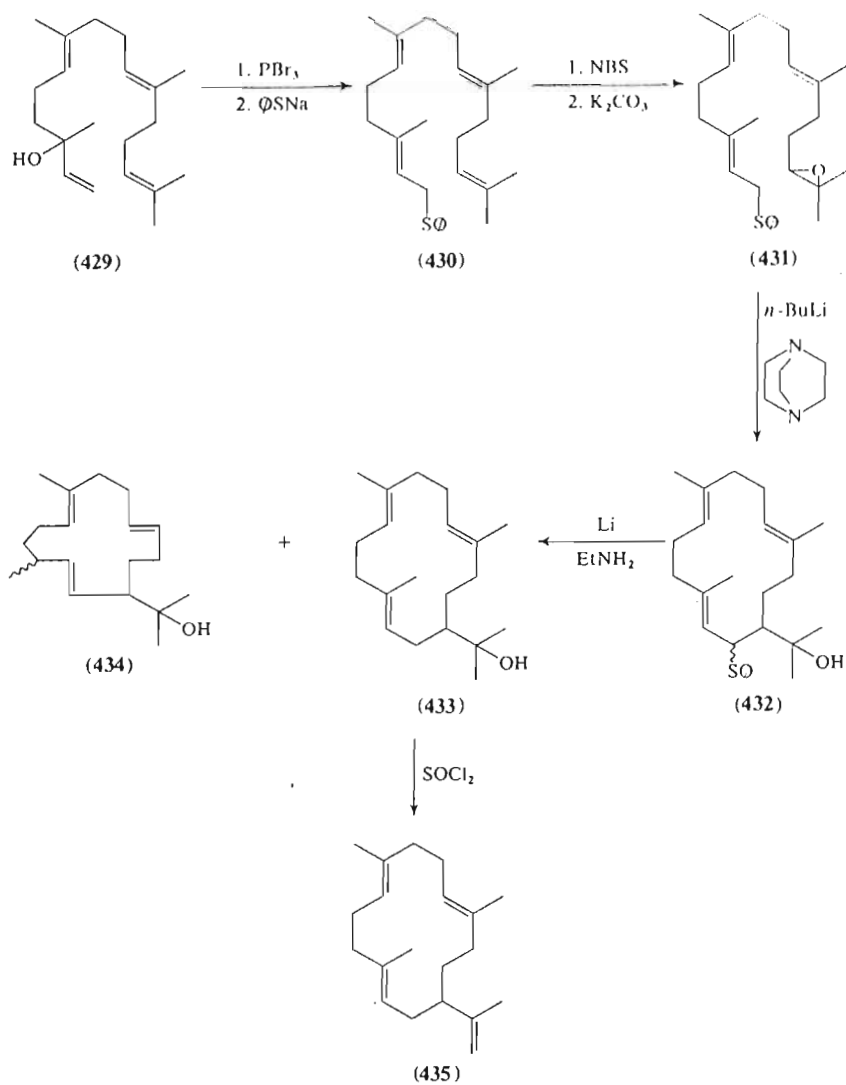
NBS = N-Bromosuccinimide

Scheme 78

References, pp. 157--190
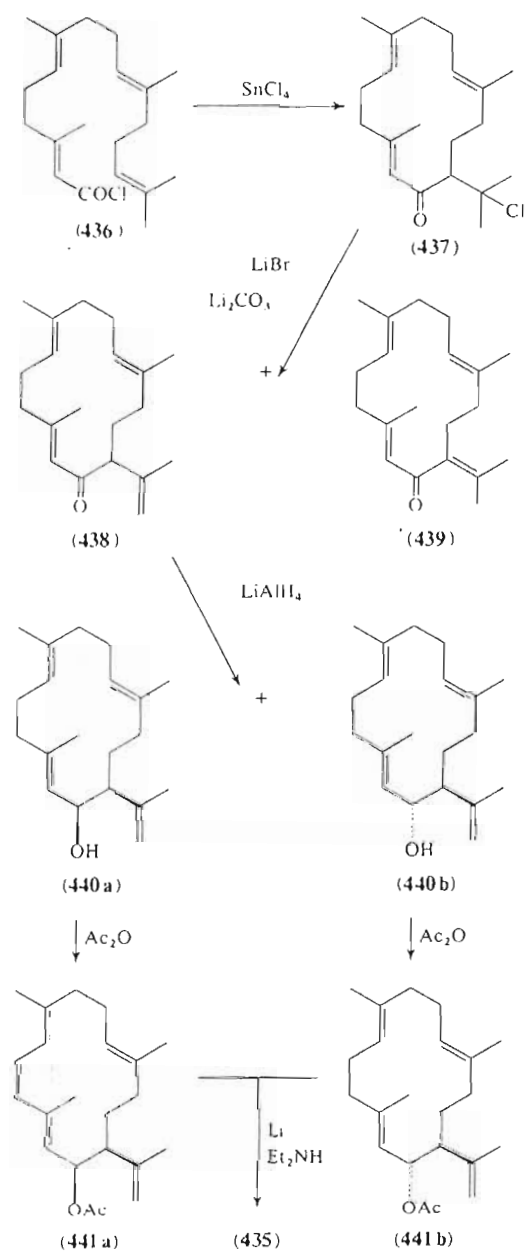
Scheme 79

F. Lepidoptera

With only a few exceptions, the majority of the Lepidopteran sex pheromones identified thus far are unsaturated, even-numbered, straight-chain acetates, alcohols or aldehydes, produced as a precise mixture of E- and Z-isomers. Thus stereospecificity in the formation of the desired isomer is the ideal, or at least one hopes for sufficient stereoselectivity to attain the desired blend. HENDRICK (90) has recently reviewed in great detail the syntheses of these compounds, so only a summary of the various methods will be given here.

1. Monoene Acetates, Alcohols, and Aldehydes

Terminal acetylenes are often used as precursors to the E- and Z-olefins. A typical synthesis is that reported by SCHWARZ and WATERS (187) (Scheme 80). The alkali metal salt of the acetylene is coupled with an appropriate alkyl halide to give the acetylenic tetrahydropyranyl ether (444). Sodium in liquid ammonia reduction of the ether stereospecifically affords pure E-olefins, which can be hydrolyzed to an alcohol and then acetylated. For Z-isomers, the protecting group must first be removed and the alcohol acetylated. Reduction over quinoline-poisoned

$HC \equiv C \cdot (CH_2)_n - OTHP$ in THF $R \cdot C = CH$ in THF (445)

(442)

1. n BuLi   1. n-BuLi

2. RX (443)   2. $Cl - (CH_2)_n - OTHP$
   in HMPT   in HMPT

                      (446)

$R - C \equiv C - (CH_2)_n - OTHP$ (444)

Na   AcOH

NH_3 AcCl

$R \diagup\diagdown (CH_2)_n - OTHP$ (447) $R - C = C - (CH_2)_n - OAc$ (449)

AcOH H_2
AcCl   Pd CaCO_3

$R \diagup\diagdown (CH_2)_n - OAc$ $R \diagdown\diagup (CH_2)_n - OAc$

(448)   (450)

HMPT = Hexamethylphosphortriamide
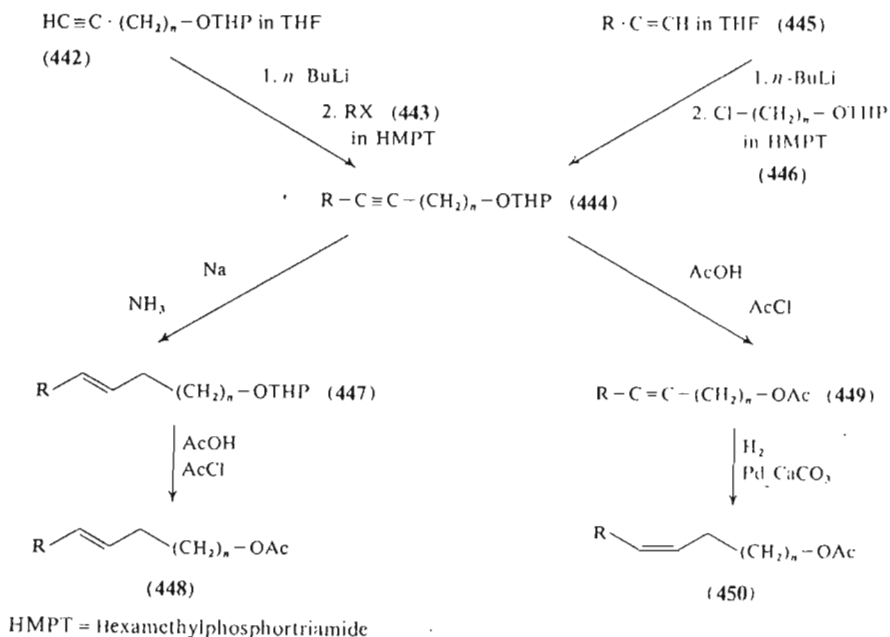
Scheme 80

Lindlar catalyst stereoselectively gives the Z-isomer contaminated with up to 5% of the E-isomer. Reduction of acetylenes to yield >98% Z-olefins has been accomplished by hydroboration with hindered reagents such as disiamylborane (*188*) or 9-BBN (*189*) followed by hydrolysis with acetic acid.

Wittig reactions between aliphatic aldehydes and phosphonium ylids generated with sodium bis(trimethylsilyl)amide in tetrahydrofuran ("salt free") have been used to prepare alkenes with a $Z:E$ ratio of 98:2, respectively (*190*).

HAYASHI and MIDORIKA (*191*) used a [3,3]-sigmatropic rearrangement of an allylic dithiocarbamate to stereospecifically generate E-olefins (Scheme 81). Reduction of alkylated allylic phosphonates with lithium aluminium hydride by KONDO et al. (*192*) exclusively gave E-alkenes (Scheme 82).
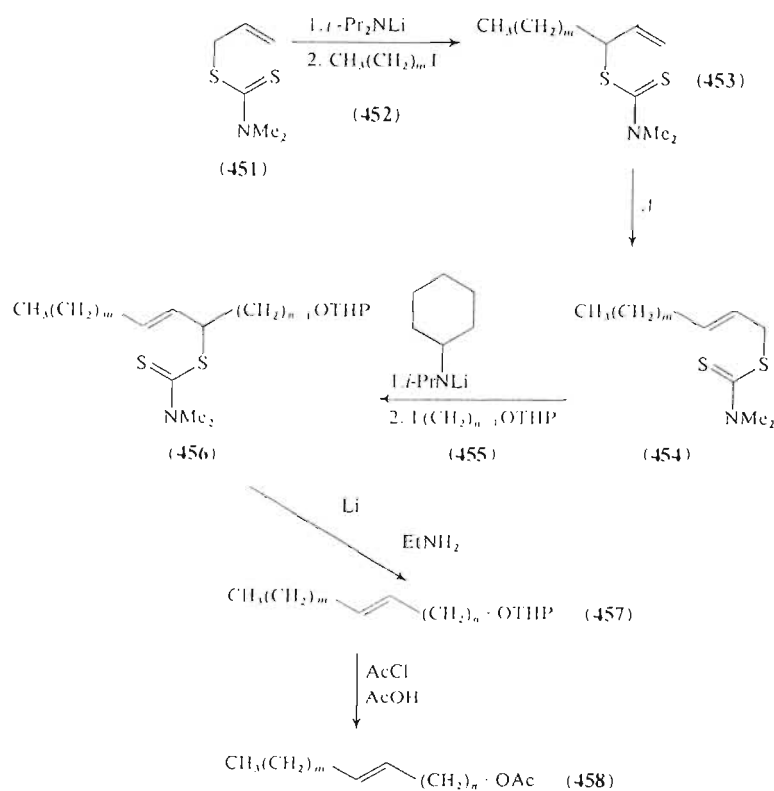
Scheme 81
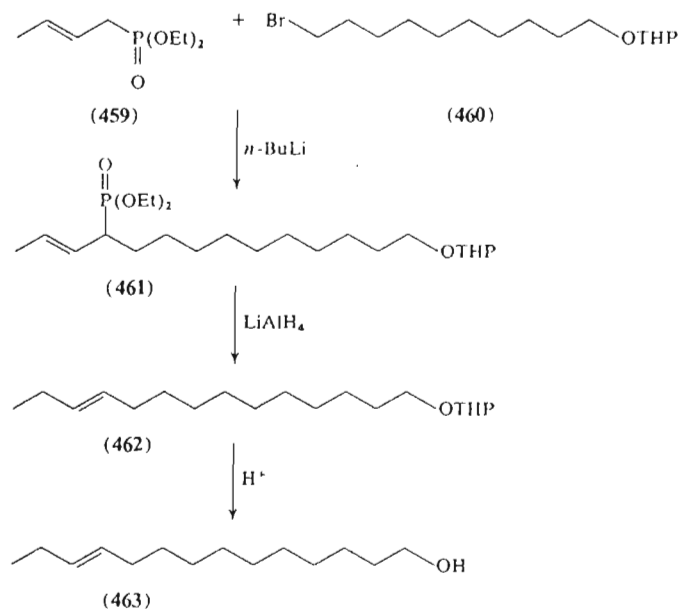
Scheme 82

Solid phase syntheses of alkynols and alkenols have been achieved by Leznoff *et al.* (*189, 193*) using polymer-bound symmetrical alkane diols.

Mori *et al.* (*194*) used the 2-alkylated cyclohexane-1,3-dione (**466**) as the source for the straight chain alkynoic acid (Scheme 83). Reduction of alkynol (**468**) with P-2 nickel catalyst afforded (*Z*)-alkenol (**469**).

Olefinic aldehydes have been synthesized by a variety of methods including oxidation of the corresponding primary alcohols with the chromium trioxide-pyridine complex (*195—197*) or N-chlorosuccinimide-dimethyl sulfide complex (*198*), heating a primary alken-1-yl mesylate with dimethylsulfoxide (*199*), or by alkylation of the lithium salt of 5,6-dihydro-2,4,4,6-tetramethyl-1,3-(4H)-oxazine with an alkynyl iodide followed by sodium borohydride reduction and acid hydrolysis (*200*).

Separation of mixtures of *E*- and *Z*-isomers and nearly quantitative recovery of the individual components has been accomplished by selective formation of *E* olefin-urea complexes (*201*).
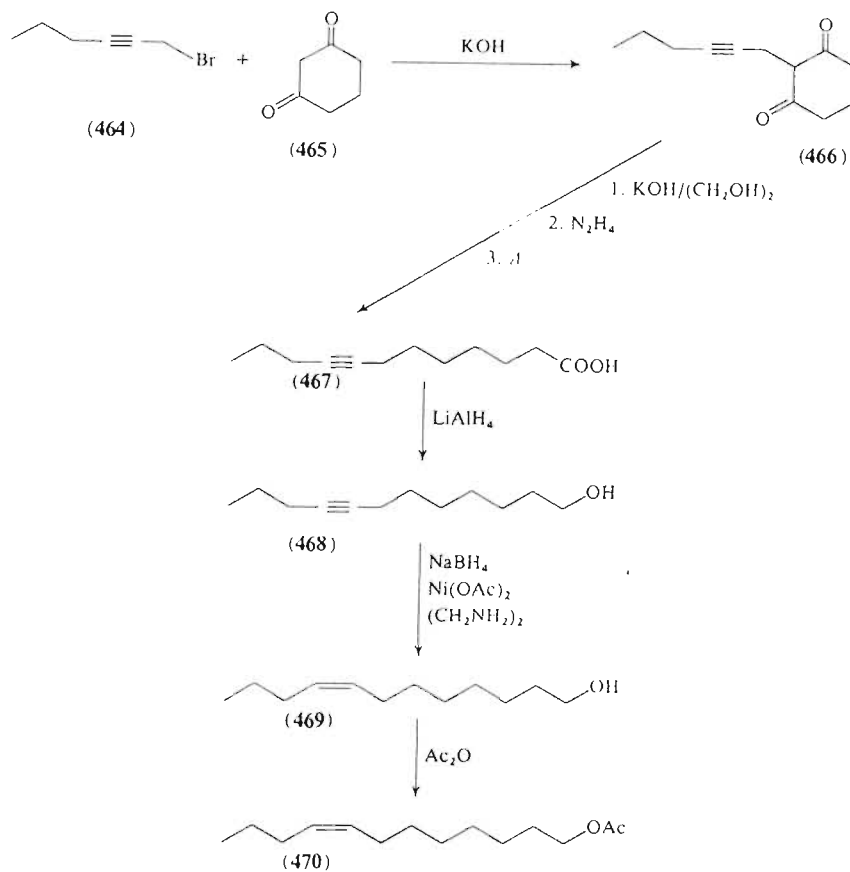
Scheme 83

2. Conjugated Dienes

A number of conjugated dienes have been identified as sex phero-
mones. Among these are $(8E,10E)$-8,10-dodecadien-1-ol (**475,** Scheme 84)
from the codling moth *(Laspeyresia pomonella)*, $(7E,9Z)$-7,9-dodecadien-
1-yl acetate (**490,** Scheme 86) from the European grapevine moth *(Lobesia
botrana)*, (E)- and (Z)-9,11-dodecadien-1-yl acetates (**518a** and **518b,**
respectively, see Scheme 90) from the red bollworm moth *(Diparopsis
castanea)*, and $(10E,12Z)$-10,12-hexadecadien-1-ol (**498,** Scheme 87) from
the silkworm moth *(Bombyx mori)*.

Reactions between an α,β-unsaturated aldehyde and a saturated Wittig
reagent *(71, 202—209)* or a saturated aldehyde and an olefinic *(202,
210—212)* or acetylenic *(213)* Wittig reagent have been utilized for

elaborating the conjugated dienyl system. By varying the solvent systems and reaction conditions, some control over E/Z ratios can be exercised. However, pure E- or Z-isomers are rarely obtained and some purification is necessary. Diels-Alder adducts of E,E dienes with tetracyanoethylene (204, 205, 211) or sulfur dioxide (206), urea complexes (213), or recrystallization (214) have been used to separate mixtures. Equilibration of isomeric diene mixtures to predominantly E,E dienes has been accomplished by heating without solvent in the presence of benzenethiol (215, 216) or in lower yield by photolysis in the presence of I_2 (210, 217).

VIG et al. (218) coupled an unsaturated phosphonate with a saturated aldehyde, to obtain an E,E diene of undisclosed purity.
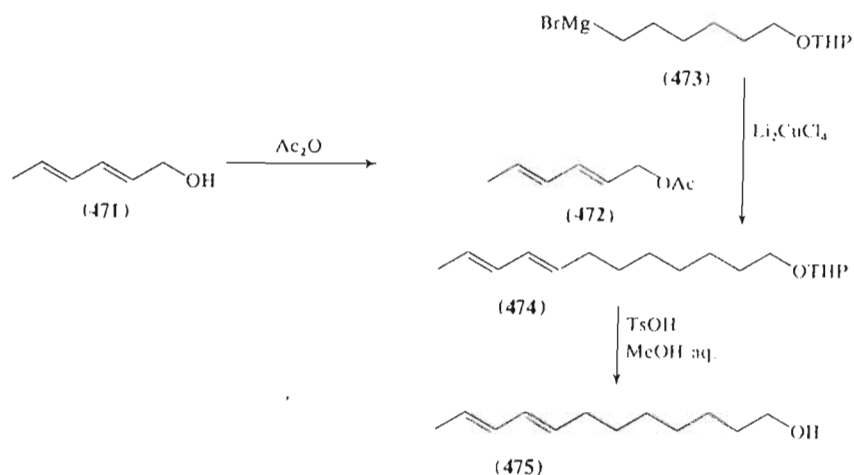
Scheme 84

In several syntheses, a fragment containing the diene system was first prepared and then elongated via a Grignard or alkali metal coupling reaction. Sorbyl alcohol (471) has been used by HENRICK et al. (219) (Scheme 84) and others (220—222) as an allylic diene precursor, which may be subject to some rearrangement during the coupling reaction. DESCOINS and HENRICK (214) used the Julia method to prepare the homoallylic bromide (479) (ca. 80% E,E) en route to (475) Scheme 85).
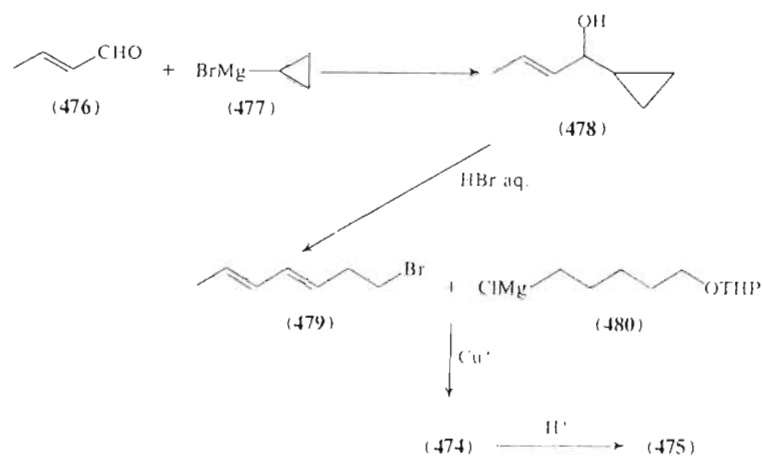
Scheme 85

Allylic nonconjugated enynols have been·rearranged (*206, 217, 223*) to prepare mixtures of *E* and *Z* conjugated enynes, which can be separated prior to further reaction, or as in one case (*223*) treated with the alkyl lithium reagent (**486**) in the presence of dilithium tetrachlorocuprate to give an *E*-enyne (Scheme 86). Conjugated enynes have also been prepared by dehydration of α-ynols (*202, 213, 217, 224*), by Wittig reaction (*213*), *via* vinyl copper intermediates (*225*) (Scheme 87), and from alkenyl boranes (see below). The acetylene can be reduced to a *Z*-olefin with Lindlar catalyst (*213, 217, 226*) or preferably *via* hydroboration-protonolysis (*223, 224, 227*), or to an *E*-olefin with lithium aluminium hydride (*225*).

(485)

Li$_2$CuCl$_4$ + Li⌒⌒⌒O⌒O⌒ (486)

(487)

1. H$^+$
2. Me$_3$SiCl

(488) OSiMe$_3$

1. $\left(\begin{array}{c}\\\\\end{array}\right)_2$BH

2. HOAc

(489) OH

Ac$_2$O

(490) OAc

Scheme 86

⌒⌒MgBr   1. CuBr
(491)   2. HC≡CH   ⌒⌒⌒Cu·MgBr$_2$
                       (492)

1. BrC≡CCH$_2$OSiMe$_3$ (493)
2. H$^+$

(494) OH

LiAlH$_4$

(495) OH

Ac$_2$O

References, pp. 157–190

(496)

1. Cu⁺
2. B₁Mg
3. H⁺

(497)

(498)
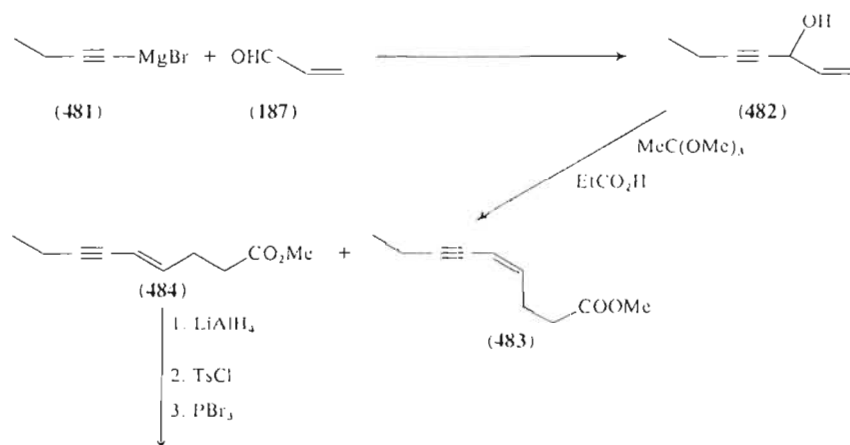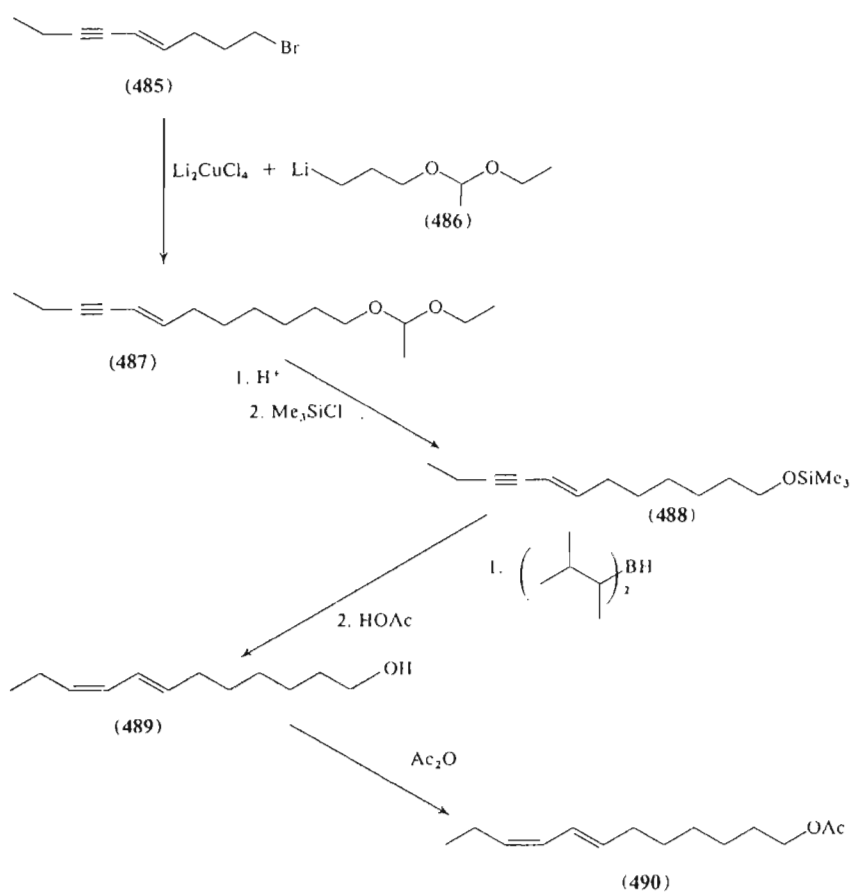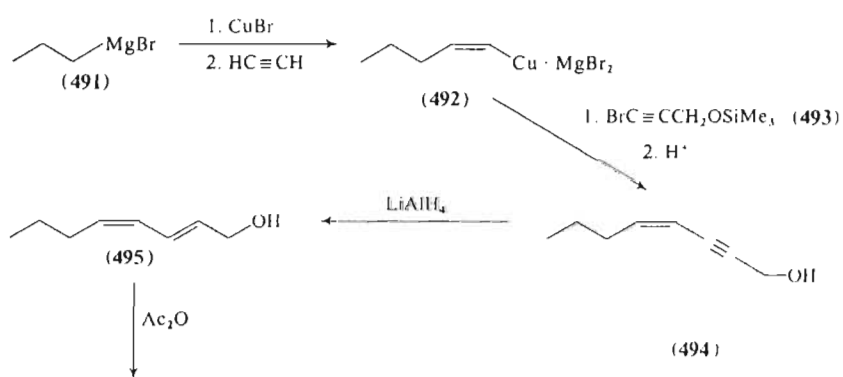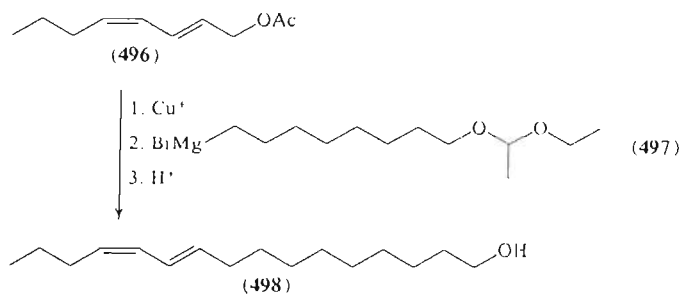
Scheme 87

Negishi and coworkers have developed a method for preparation of conjugated E-enynes *via* alkenylboranes. In one synthesis (*227*), acetylene (**501**) was treated with disiamylborane and then with the lithium salt of acetylene (**503**) to afford complex (**504**), which after treatment with iodine and sodium hydroxide stereoselectively afforded (**505**) (Scheme 88). Syntheses of other conjugated E,Z dienes have been achieved by this method (*228, 229*). By use of thexylborane and 1-bromo-1-alkynes, conjugated E,E dienes can be readily prepared (*230*) (Scheme 89).

(499) CO₂H — LiAlH₄ → **(500)** OH

Me₃SiCl

(501) OSiMe₃

(502) OSiMe₃

Li — ≡ — **(503)**

(504) OSiMe₃
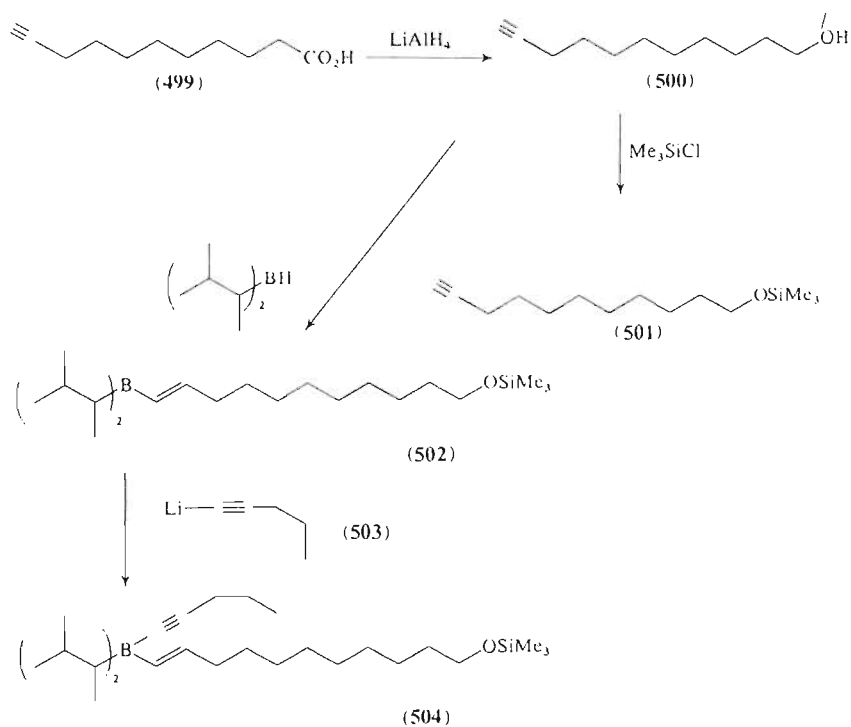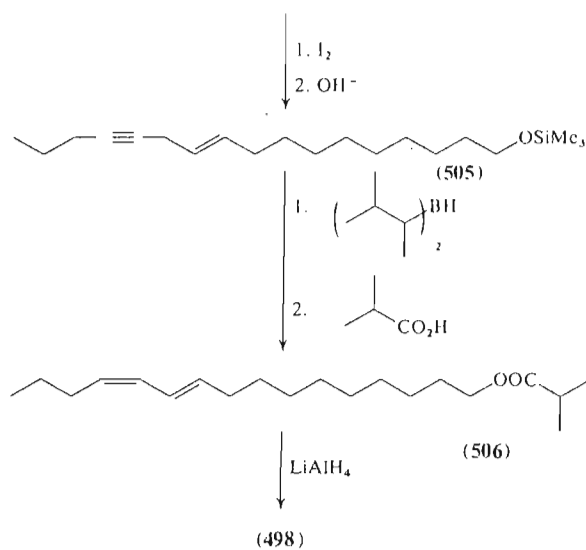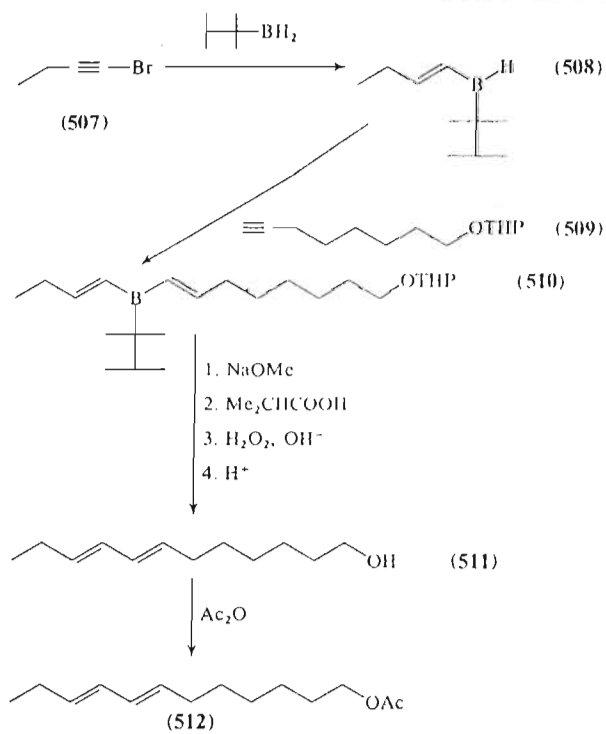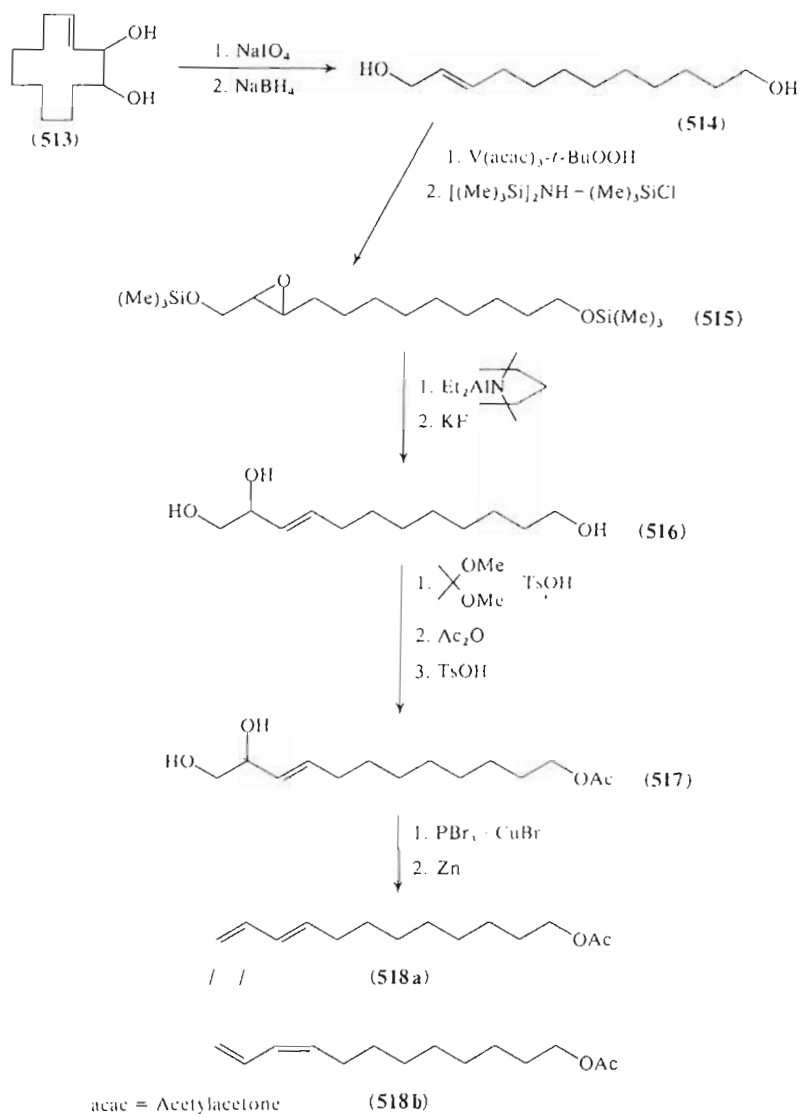
Scheme 88

Scheme 89

Scheme 90

Tanaka *et al.* (*231*) prepared (**518a**) from allylic alcohol (**514**) *via* the epoxy silyl ether (**515**), which was ring opened with diethylaluminum 2,2,6,6-tetramethylpiperidide to afford the 3-ene-1,2-diol (**516**) Scheme 90).
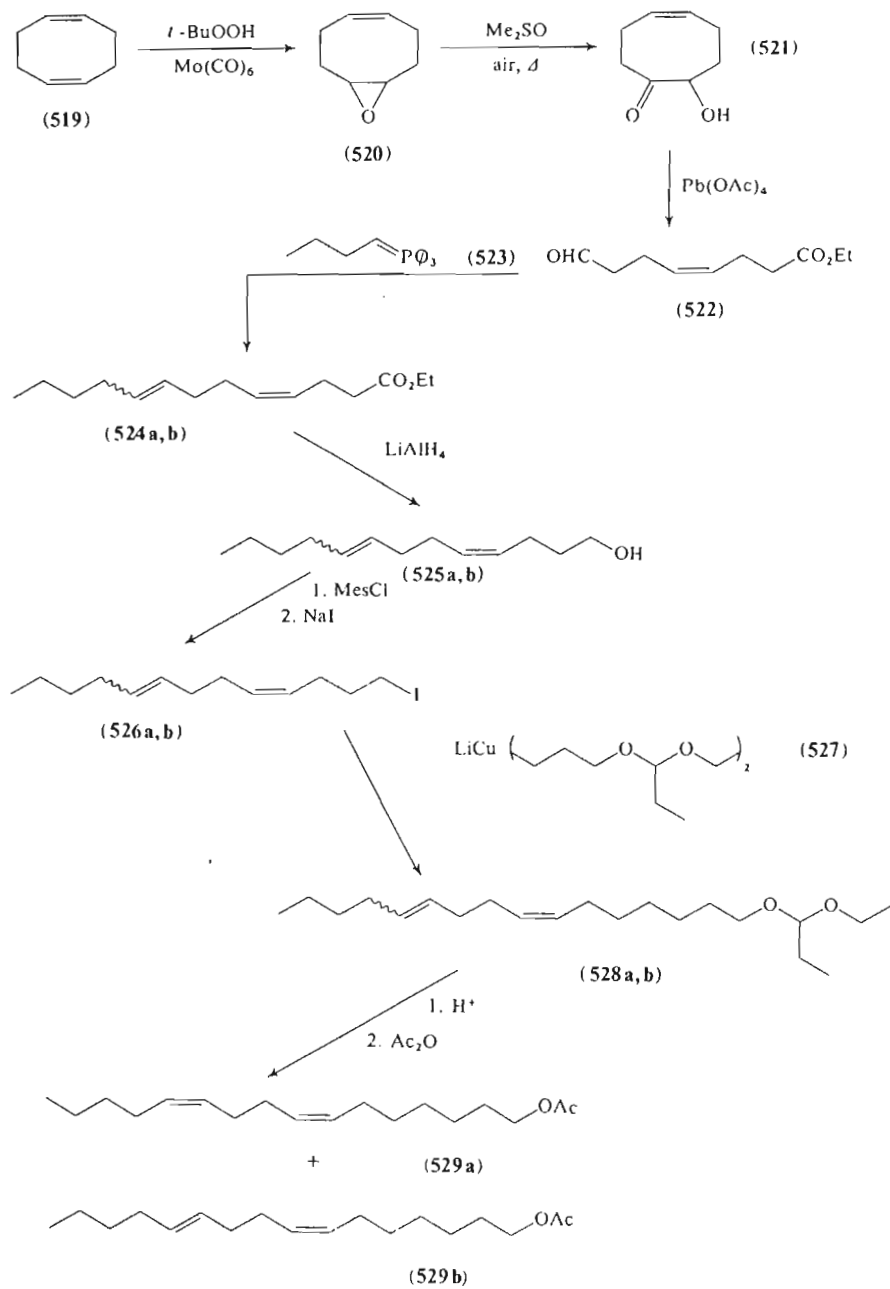
Scheme 91

3. Nonconjugated Dienes

Nonconjugated dienes have been identified as sex pheromones from a variety of species, including the pink bollworm moth (*Pectinophora gossypiella*). The initially assigned structure was that of the branched diene (**5**) (see p. 15). Numerous syntheses of this molecule have been reported and are reviewed by KATZENELLENBOGEN (*89*). The pheromone is now known (*79*) to be a 1 : 1 mixture of (7Z,11Z)- and (7Z,11E)-7,11-hexadecadien-1-yl acetate (**529a** and **529b,** respectively, see Scheme 91).

The methods employed in the synthesis of the individual components have tended to follow those for monoenes, namely *via* acetylenic intermediates (*232—236*), by allylic Grignard coupling (*232, 237*), and by Wittig reaction (*234, 238*). SONNET (*239*) isomerized the 7Z,11Z- and 7Z,11E-isomers (as their tetrahydropyranyl ethers) to the 7E,11E- and 7E,11Z-isomers, respectively, by treatment of the corresponding bis-epoxides with lithium diphenylphosphide and then methyl iodide.

A more convenient synthesis is that of ANDERSON and HENRICK (*240*) (Scheme 91). By starting with cyclooctadiene (**519**), the C-7 bond was fixed as *Z*, and the C-11 olefin was introduced as a 1 : 1 mixture of *E* : *Z* by careful choice of conditions for the Wittig reaction. This synthesis ultimately gave (**529a**) and (**529b**) in at least 99% purity.

4. Trienes

Larvae of *Cossus cossus* secrete from the mandibular glands a mixture of 5,13-tetradecadien-1-yl acetate, 3,5,13-tetradecatrien-1-yl acetate and small amounts of the corresponding alcohols (*241*). The function of these secretions is unknown. GARANTI *et al.* (*242*) synthesized the possible isomers of the acetates *via* acetylenic intermediates and Wittig reactions.

5. Epoxide

The gypsy moth (*Lymantria dispar* [formely *Porthetria dispar*]) uses epoxide (**536**) (disparlure, Scheme 92) as its sex pheromone. Essentially all syntheses have proceeded *via* *Z*-olefin (**535**), which is the presumed precursor used by the insect itself. This olefin has been prepared by Wittig reaction (*57, 243, 244*), with the greatest stereoselectivity achieved by BESTMANN *et al.* (*244*) (Scheme 92), by coupling bromide (**532**) with the lithium salt of 1-dodecyne and reducing the resulting acetylene over Lindlar catalyst (*245—248*); and *via* an organosilane intermediate (*249*) (Scheme 93). CHAN and CHANG (*250*) prepared a 1 : 1 mixture of the *E*- and *Z*-isomers by condensation of undecanal (**534**) with the triphenyl-silyl lithium salt (**543**) (Scheme 94). The olefin mixture has also been prepared in low yield by a transition metal-catalyzed olefin cross-metathesis reaction (*251*).

(530)

1. Mg
2. [epoxide]

OH　(531)

HBr

(533) =Pφ₃

1. Pφ₃
2. NaN(SiMe₃)₂

Br　(532)

OHC⟍⟍⟍⟍⟍⟍⟍⟍　(534)

(535)

$> 98\% \; Z$

$m\text{-ClφCO}_3\text{H}$

(536)

O

Scheme 92

OHC⟍⟍⟍⟍⟍⟍⟍
(534)

1. Me₃Si⟍Li　(537)
2. Ac₂O

Me₃Si⟍
OAc　(538)

MgBr
(539)　CuI

SiMe₃　(540)

HI

(535)
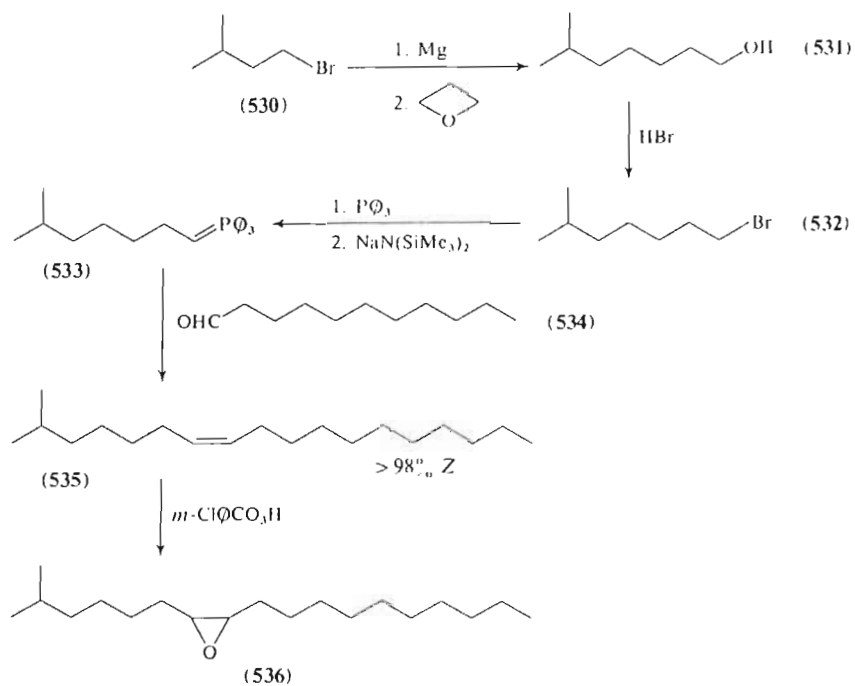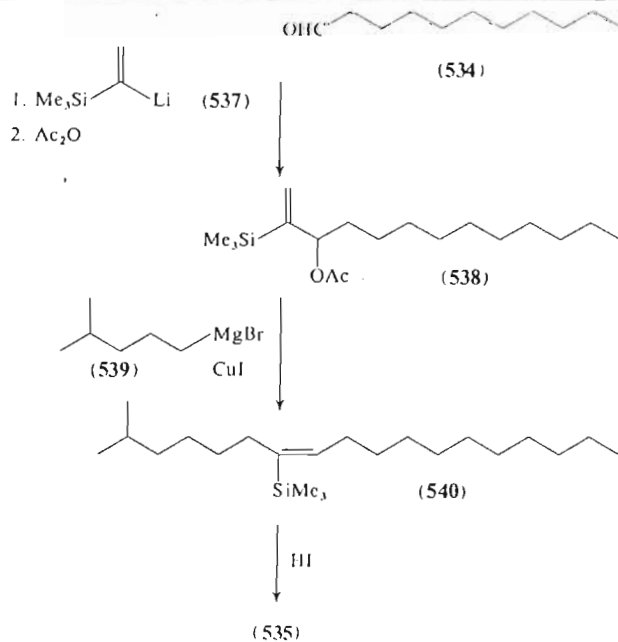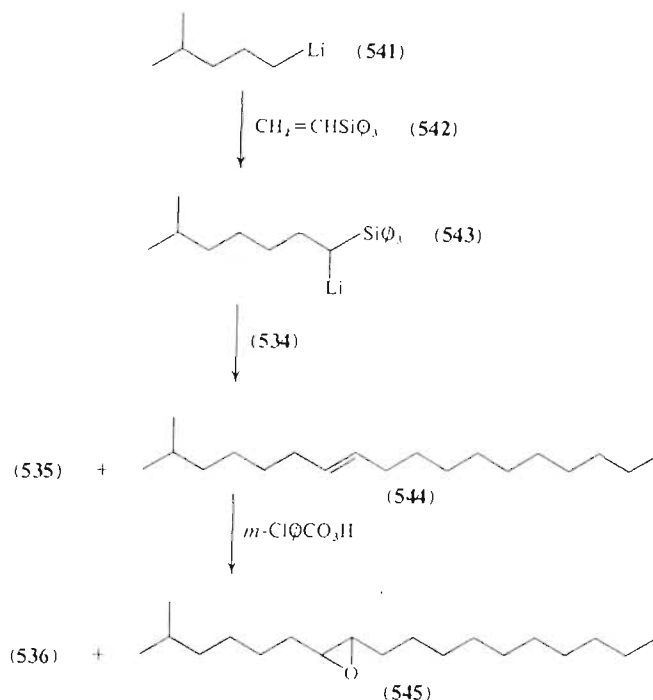
Scheme 93

Scheme 94

(*S*)-(+)-Glutamic acid (**140b**) served as the starting material for the synthesis of (7*R*,8*S*)-(+)-disparlure (**536a**) by IWAKI *et al.* (*252*) (Scheme 95). The final product was *ca.* 94% optically pure. MORI *et al.* (*253*) obtained (**536a**) in > 98% optical purity in their synthesis, which began with *L*-(+)-tartaric acid (**557**) (Scheme 96). A shorter stereospecific synthesis of (**536a**) (> 99% pure) has been reported recently by FARNUM *et al.* (*254*) from *L*-(−)-menthyl-*p*-toluene sulfinate (**566**) (Scheme 97).

NaBH$_4$

(548)

+

(549)

1. DHP

2. i-Bu$_2$AlH

(550)

(551)

(552)

H$_2$-PtO$_2$

(553)

1. TsCl

2. HOAc aq.

(554)

KOH-MeOH

O   (536 a)

(553)   7R. 8R-(+)

1. NaH - ⌀CH₂OH
2. H⁺

⌀CH₂O   OH   (555)

1. TsCl
2. H₂-Pd

HO   OTs   (556)

KOH-MeOH

(549)   (7S,8R)-(−)   (536 b)

O   (545 a)

+   7R. 8R-(+)

7S. 8S-(−)   (545 b)

Scheme 95

6*
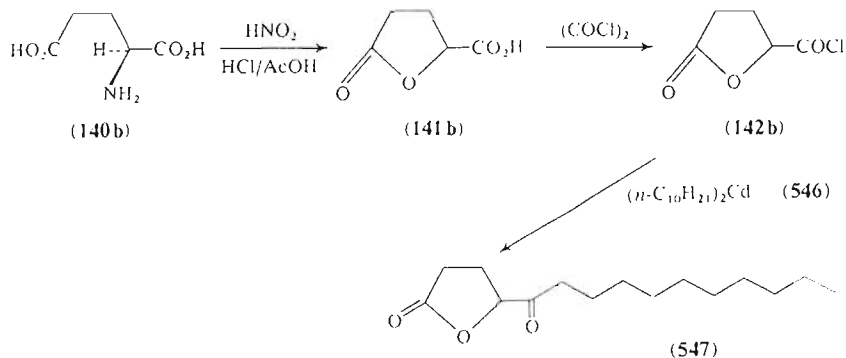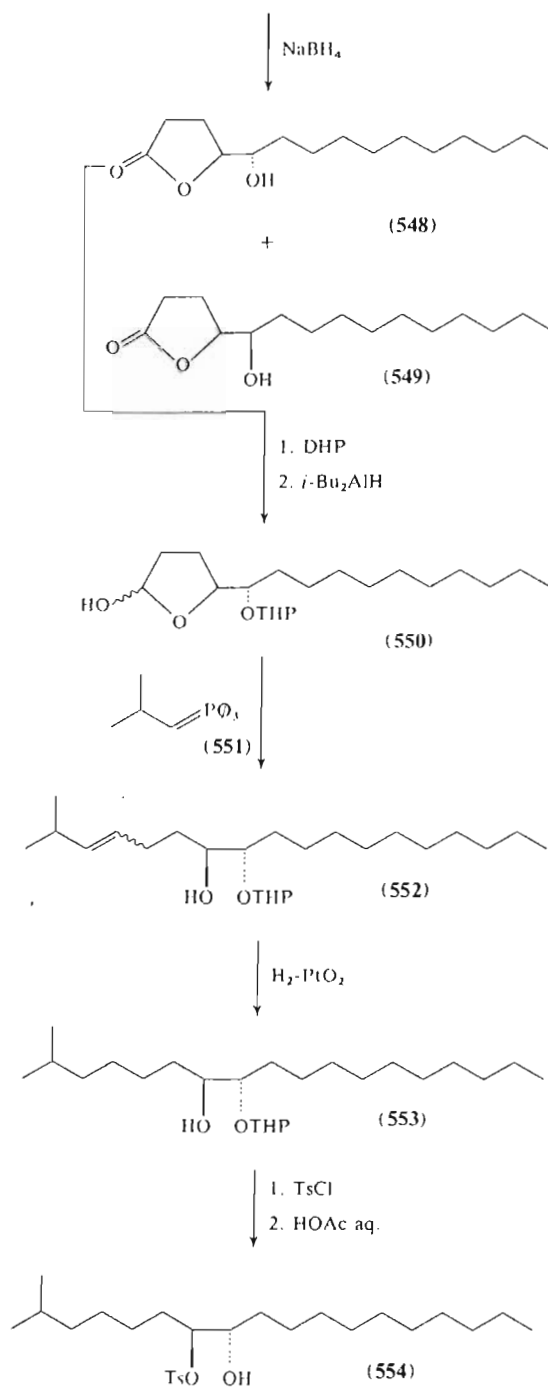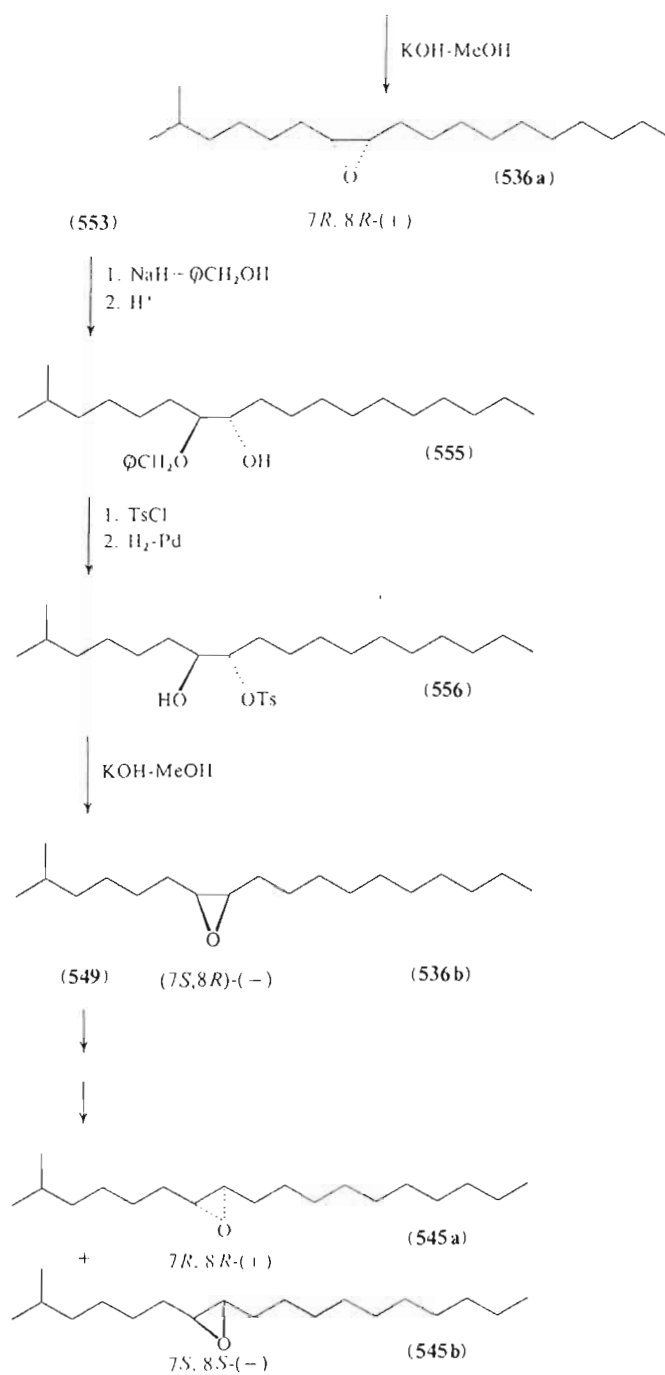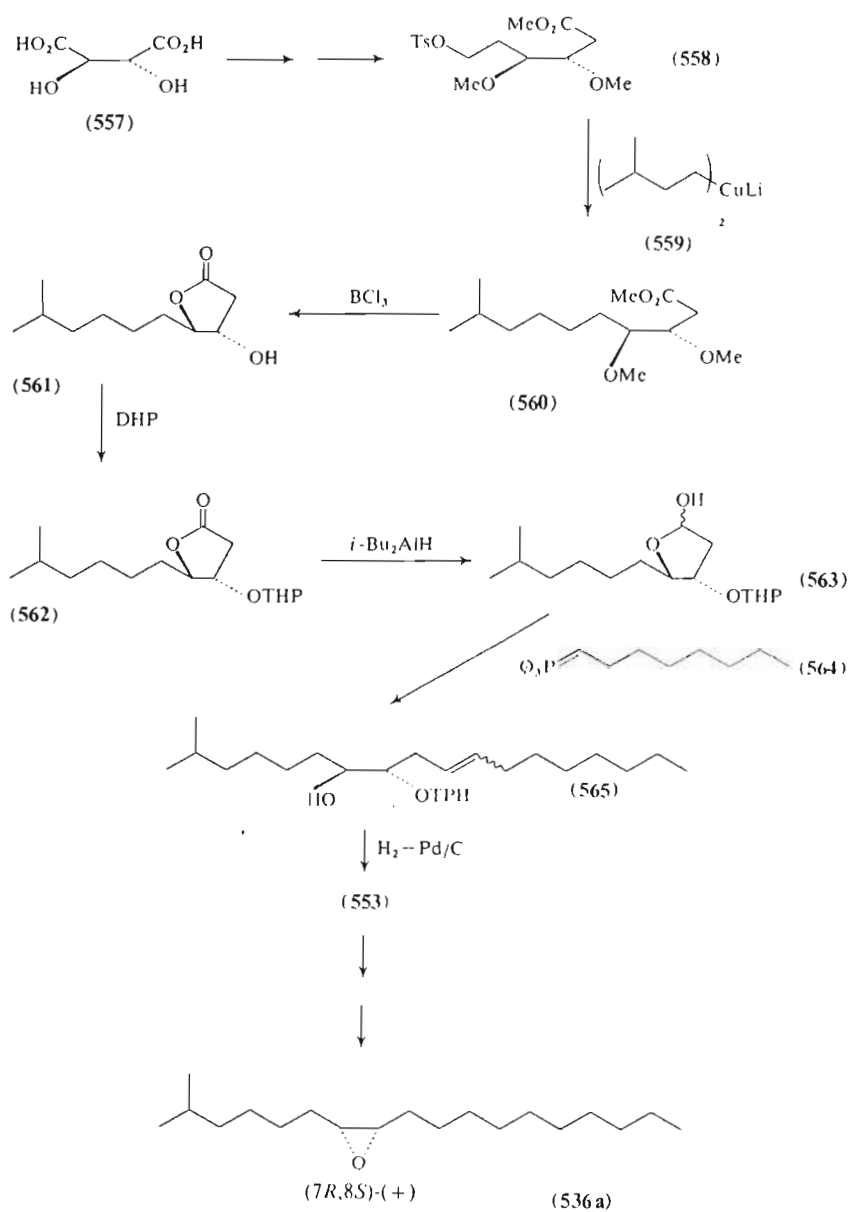
Scheme 96

References, pp. 157—190

(567)

(566)

Me₃CLi

(568)

(569)

(534)

(570)

1. SnCl₂
2. LiAlH₄

(571)

(572)

Me₃O⁺BF₄⁻

(545a)

(573)

NaOH

(536a)
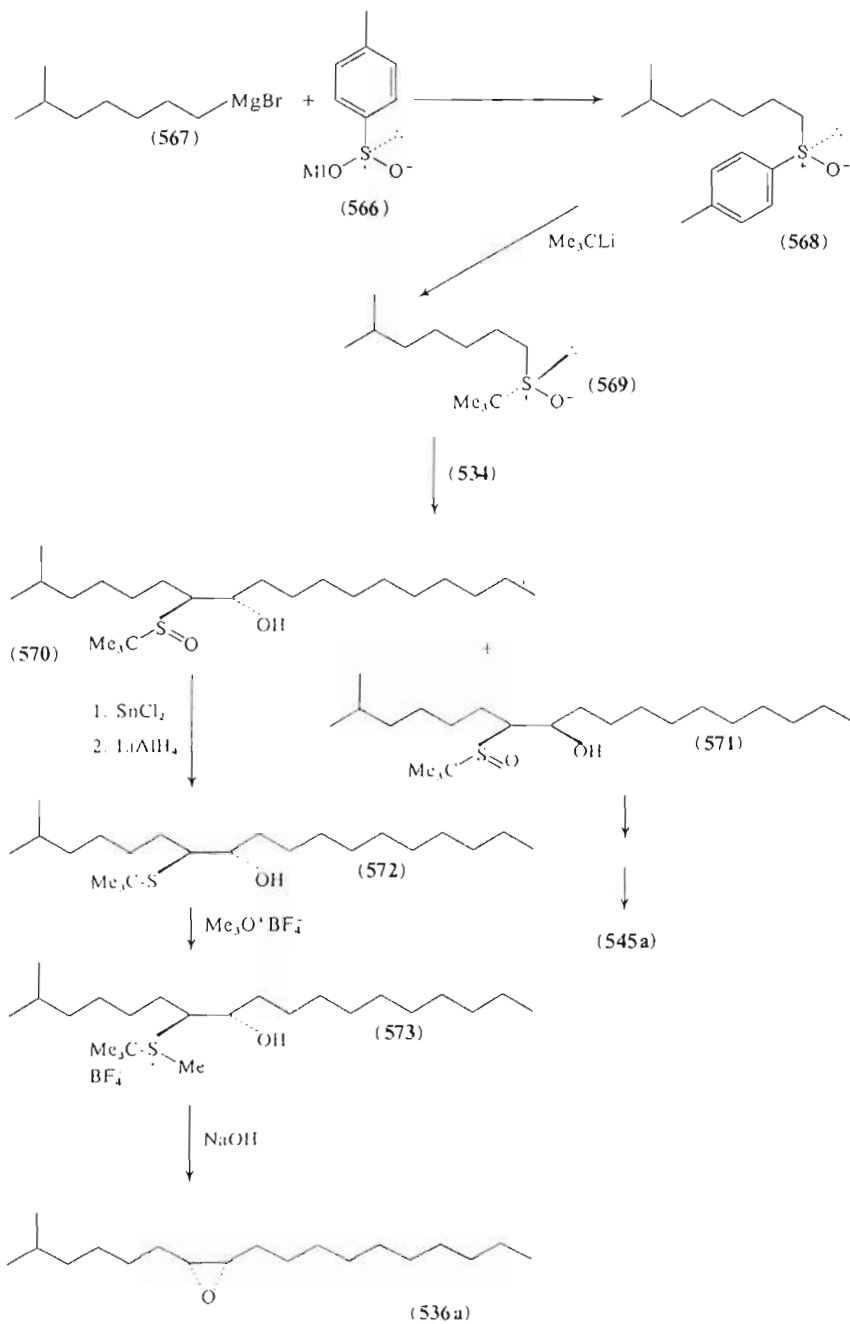
Ml is L-(−)-Menthyl
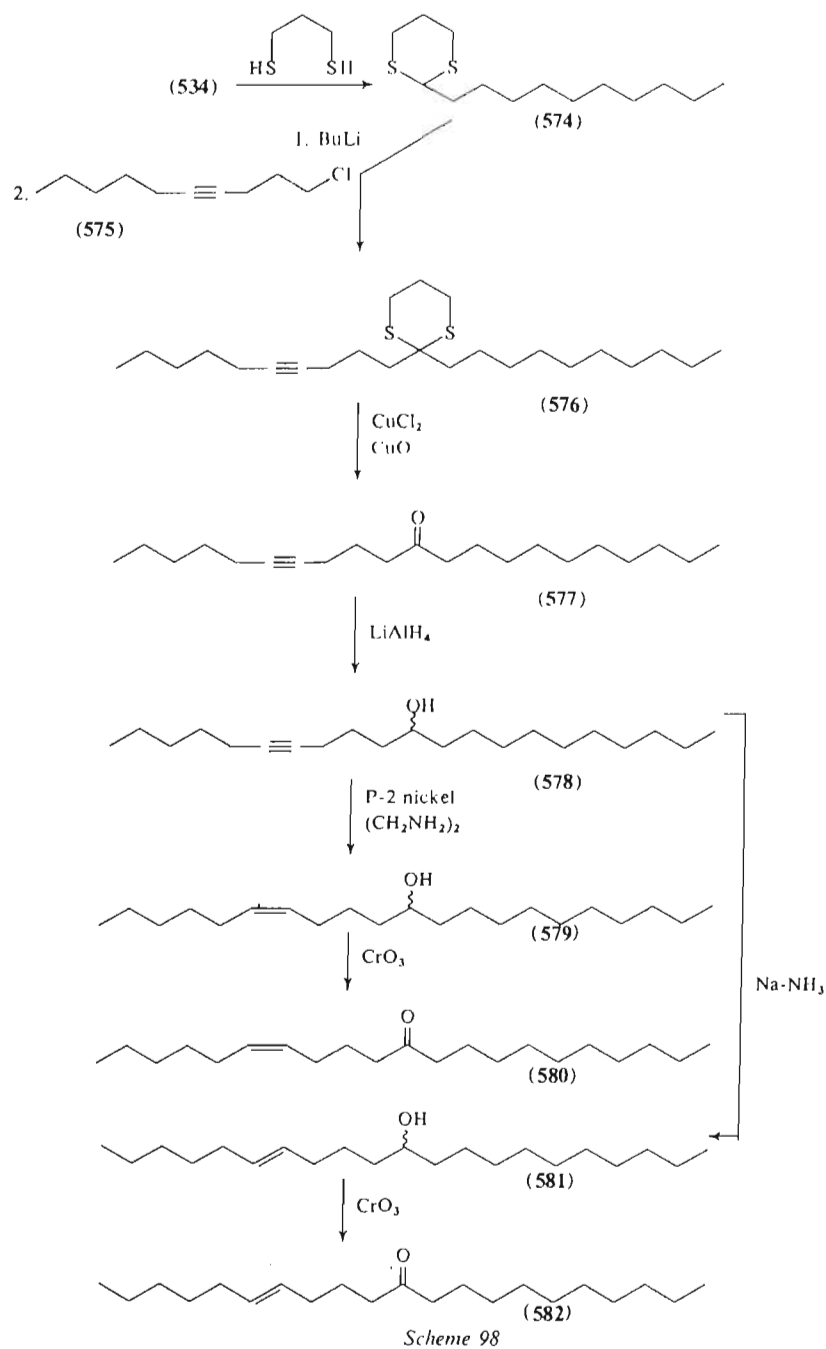
Scheme 97

Scheme 98

6. Ketone

Another atypical Lepidopteran sex pheromone is the Z unsaturated ketone (580, Scheme 98) produced by the Douglas-fir tussock moth (*Orgyia pseudotsugata*). The acetylenic ketone (577), produced *via* a dithiane intermediate, was used by SMITH *et al.* (*255, 256*) to prepare both the Z- and E-isomers (580 and 582, respectively) (Scheme 98). KOCIENSKI and CERNIGLIARO (*257*) and MORI *et al.* (*194*) used an Eschenmoser cleavage of an alkyl substituted α,β-epoxy ketone to obtain straight chain acetylenic ketones that were transformed into (580) (Schemes 99 and 100, respectively). Acetylenic alcohol (578) has also been prepared by Grignard reactions (*258, 259*).
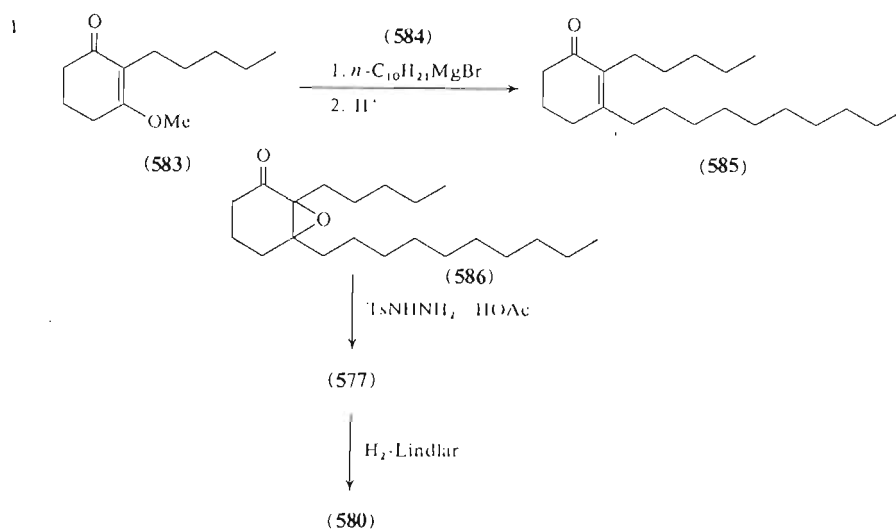
Scheme 99

G. Orthoptera

The German cockroach (*Blattella germanica*) uses the doubly branched methyl ketones (603, Scheme 101) and (615, Scheme 102) as sex pheromones. A number of syntheses of (603) have proceeded *via* bromide (600) or an analog unsaturated at the methyl branch. The branched methyl ketone moiety was introduced by alkylation of ethyl (*260*) or benzyl 2-methylacetoacetate (*261*) or of the lithium salt of (2-oxobutylidene)triphenyl phosphorane (*262*). ROSENBLUM *et al.* (*263*) coupled

$$(465) \longrightarrow (587) \xrightarrow[\substack{1.\ Li \\ 2.\ n\text{-}C_{10}H_{22}Br \\ (588)}]{} (589)$$

$$\downarrow H_2O_2$$

(590)

$$\downarrow TsNHNH_2 \cdot Ac_2O$$

(591)

$$\downarrow \substack{(CH_2OH)_2 \\ TsOH}$$

(592)

$$\downarrow \substack{1.\ n\text{-}BuLi \\ 2.\ n\text{-}BuBr \quad (593)}$$

(594)

$$\downarrow H^+$$

(577)

$$\downarrow H_2\text{-Lindlar}$$

(580)

Scheme 100

(595)

LiCu$\left(\begin{array}{c}\end{array}\right)_2$ (596)

(597)

$\varphi_3P=CH_2$

(598)

1. H$_2$-Pd/C
2. H$^+$

(599)

1. MesCl · Et$_3$N
2. LiBr

(600)

(601)

Li$_2$CuCl$_4$ + Li

(602)

1. H$^+$
2. CrO$_3$

(603)
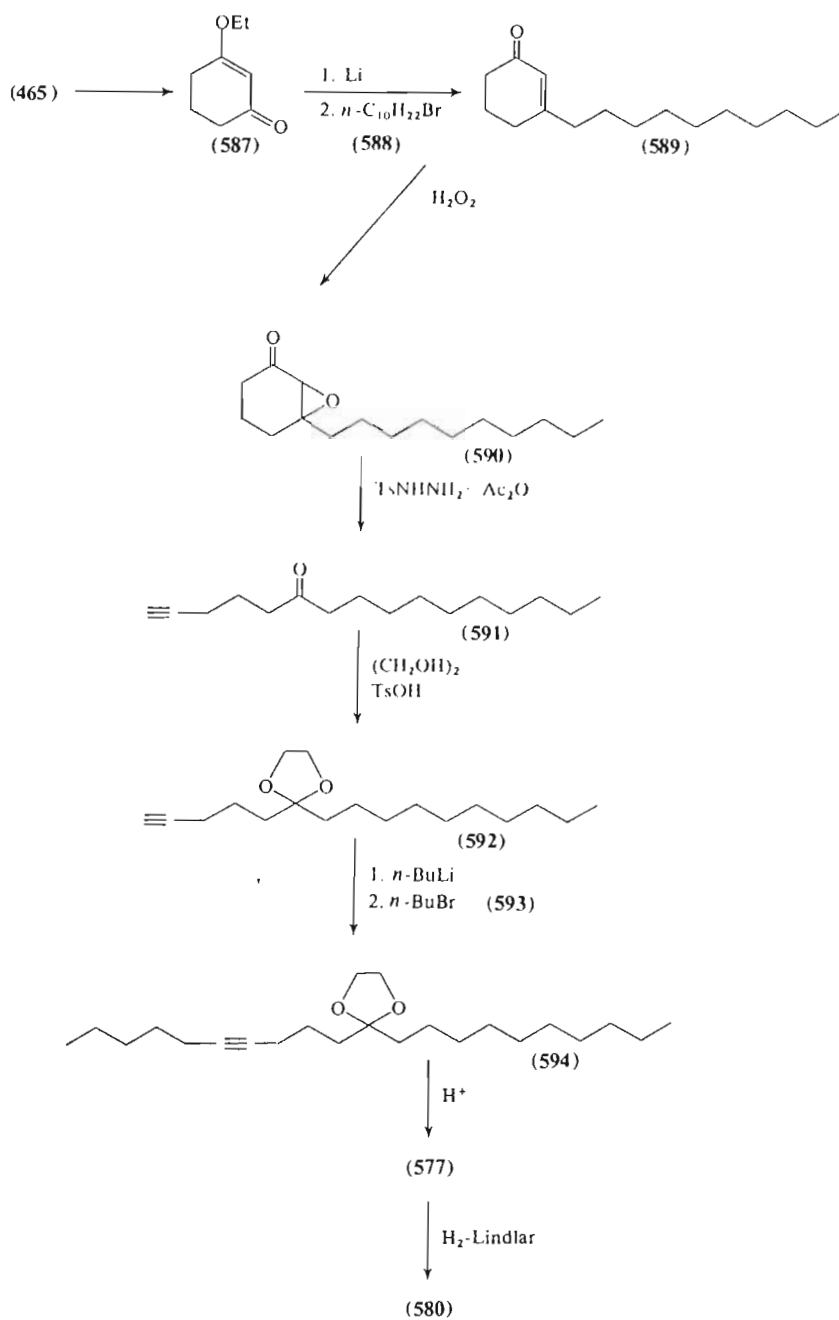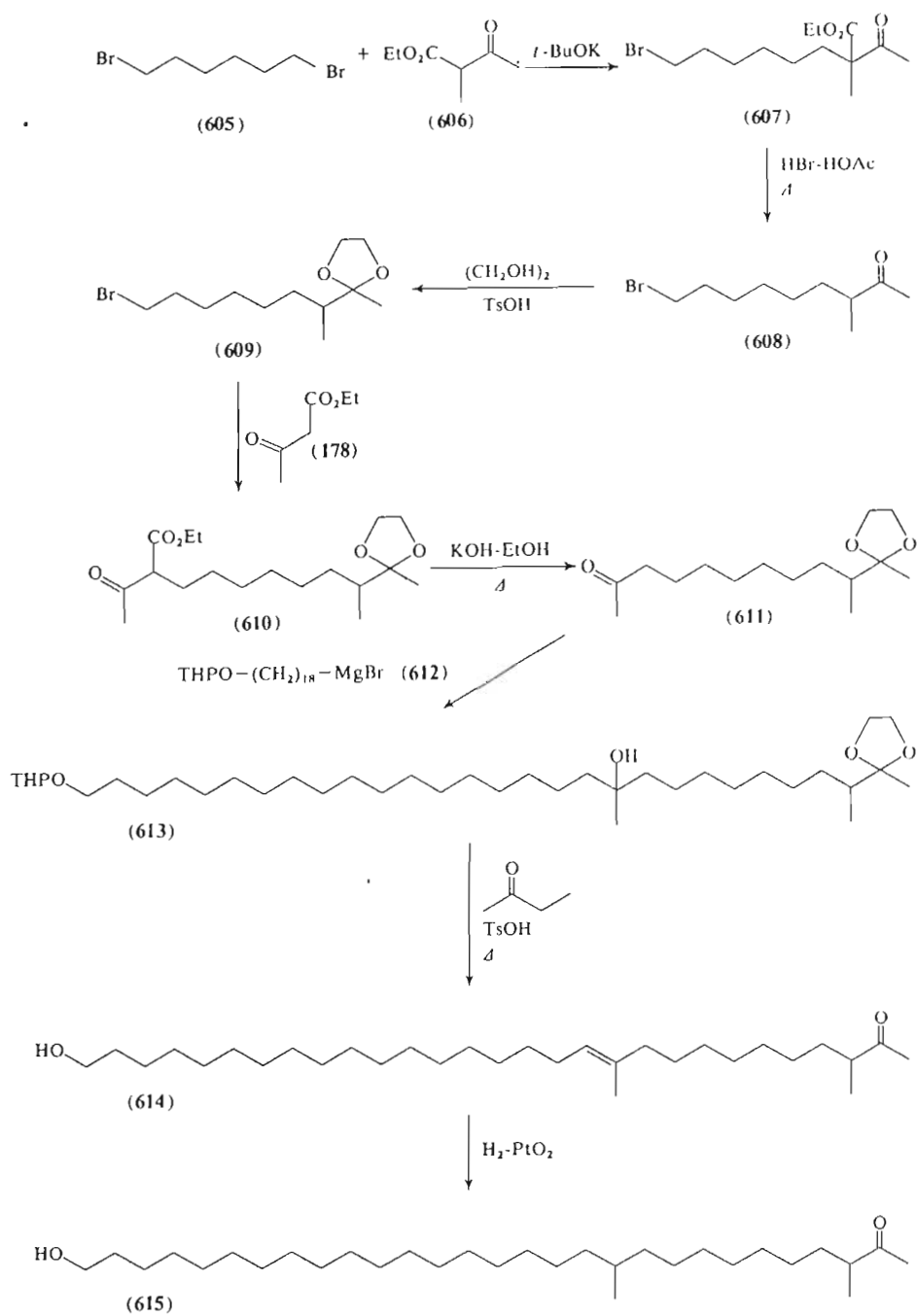
Scheme 101

Scheme 102

bromide (600) with lithium salt (601) (Scheme 101). To obtain (615), BURGSTAHLER *et al.* (*261*) used (604) as an alkylating agent and proceeded as above, whereas NISHIDA *et al.* (*264*) elaborated the methyl branched portion of the molecule (611) by a series of acetoacetate ester condensations and completed the carbon skeleton *via* a Grignard reaction (Scheme 102).

B/O ⟶⟶⟶ ≡ ⟶⟶⟶⟶⟶⟶⟶⟶⟶⟶Br

(604)
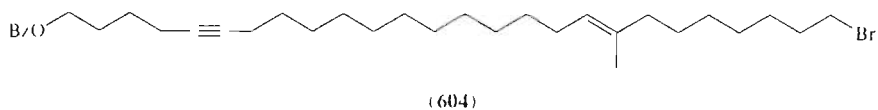
IV. Stereobiology

A. Geometric Isomers

It is now well accepted that pheromone blends are of general occurrence. Four recent reviews (*42, 43, 265, 266*) make this point clear in their thorough treatment of behavior-modifying chemicals of Coleoptera, Hymenoptera, Lepidoptera, and Diptera. For comprehensive lists of the ratios of *E*- and *Z*-isomers in lepidopteran pheromone blends as well as of other necessary components, the reader should consult the articles by SILVERSTEIN and YOUNG (*265*), TAMAKI (*42*), ROELOFS (*43*), ROELOFS and CARDÉ (*21*) and the annotated compendium of MAYER and MCLAUGHLIN (*41*). We intend to concentrate on some of the most recent findings concerning the ratios of *E*- and *Z*-isomers required for pheromone activity.

KLUN *et al.* (*267*), in studies at Ankeny, Iowa, on the European corn borer, found that males were only weakly attracted to highly purified (*Z*)-11-tetradecenyl acetate while the red-banded leafroller was not attracted at all to this compound. However, if small amounts of the *E*-isomer were added to the *Z*-isomer, both species were strongly attracted, yet neither species showed any response to the *E*-isomer alone. It was primarily after this study that it was realized that many lepidopteran sex pheromones are specific blends of the E- and Z-isomers of long chain acetate esters. The results on the Iowa European corn borer have recently been confirmed by more sophisticated separation techniques for chemically complex mixtures (*268*) and a number of specific ratios of the non-pheromone, (*Z*)- and (*E*)-11-tridecenyl acetate, have been studied in field trials on the red-banded leafroller (*269*).

Lepidopteran sex pheromones are typically esters, alcohols, aldehydes, a hydrocarbon and an epoxy-hydrocarbon (*270*). TAMAKI *et al.* (*259*) re-

cently added a ketone to this list; females of the peach fruit moth, *Carposina niponensis*, contain (Z)-7-eicosen-11-one (530) as a major component and (Z)-7-nonadecen-11-one as a minor component in their pheromone glands. A 20 : 1 ratio of the major to minor component proved most effective in field trials.

Abdominal tips of virgin female Asiatic leafroller moths, *Archippus breviplicanus* contained (E)-11-tetradecenyl acetate and (Z)-11-tetradecenyl acetate in a ratio of 70 : 30 (*271*). Individually these compounds were inactive, but a ratio of E to Z of 70 : 30 competed favorably with virgin females in field experiments. In addition, tetradecyl acetate was detected in extracts of virgin females and a slight synergistic effect of this compound in field trapping experiments was noticed. Dodecyl acetate showed no synergistic effect. The smaller tea tortrix (STT) and the summer-fruit tortrix (SFT) occur sympatrically in Japan (*272*) and their mating times are not significantly different under laboratory conditions (*273*). The sex pheromones of the two species have been identified as mixtures of (Z)-9- and (Z)-11-tetradecenyl acetate (*274—277*), yet field trapping experiments with virgin females and mate-choice experiments in the laboratory clearly indicate a significant sexual isolation (*278*). The ratio of Z9—14 : Ac to Z11—14 : Ac is 63 : 37 in STT and 82 : 18 in SFT (*273*) and the ability of males to discriminate between these ratios to a slight extent was demonstrated (*273*). In addition, there was no difference in the release rate of the pheromonal mixtures between STT and SFT females (*273*). On the basis of other data it was concluded that reproductive isolation could not be based solely on the difference in the mating time of the two species (*273*). Yet orientation of SFT males to virgin females was apparently species-specific. The presence of an additional factor involved in the attractiveness of females of both species is indicated (*273*).

The male oriental fruit moth, *Grapholitha molesta*, is maximally attracted to a ratio of (E)- to (Z)-8-dodecenyl acetate of 7 : 93 (*279—281*). Lower or higher ratios give reduced trap catches (*281*). As certain species are known to utilize variations in their pheromone blend to advantage (*282—284*) and as variation may be genetically controlled, Cardé *et al.* (*285*) investigated variation in response of males of *G. molesta* to three distinct Z : E ratios. A color-coded fluorescent dye was placed with each blend in nonsticky field traps so that attracted males were appropriately tagged. These traps were then replaced by sticky traps containing the three Z : E ratios in a randomized block design. The results indicated that in this insect the attraction to slight changes in the blend represented a normal distribution about the optimum mixture as males were reattracted to all blends in the same relative proportions as previous field tests (*281*) on these same blends.

References, pp. 157 –190

The problem of whether intraspecies pheromone polymorphism is genetically controlled at the biosynthetic level, or at the preception level, or neither, is basic to our understanding of its significance (see section on Biosystematics and Speciation). If we are to use pheromone blends in large scale field experiments, we need some understanding of whether we could be reducing one type of behavioral class or encouraging another; i.e., would the trapping of large numbers of a population affect the genotype that will remain?

The most attractive ratio of (Z,Z)- and (Z,E)-7,11-hexadecadienyl acetate (**529a** and **529b**; Scheme 91) for the pink boll worm, *Pectinophora gossypiella*, was claimed to be 1 : 1 (*79, 233*). ROTHSCHILD (*286*) found that three species, *P. gossypiella, P. endema* and *P. scutigera*, were maximally attracted to ratios of 1 : 1, 1 : 0.5 and 1 : 0.1 Z,Z, to Z,E and indicated that these ratios may provide a mechanism for reproductive isolation. FLINT *et al.* (*287*) have recently shown that the most attractive ZZ to ZE ratio for the pink boll worm varies with the time of year. In the early season a 2 : 1 ratio was considerably more attractive than a 1 : 1 ratio, and, as the season progressed, the response maximum was obtained with a much wider range of ratios. Various ratios of ZZ to ZE isomers have been claimed to be most effective (*287*).

EITER (*83*) has recently presented some critical comments on insect attractants and the work on the pink bollworm in particular. As sex attraction activity is brought about by so little material in the Lepidoptera and as it is difficult to obtain really pure configurations of olefins and polyolefins, EITER (*83*) questions the validity of some of the identifications. However, the two-component pheromone described in the preceding paragraph was independently identified by two groups (*79, 233*), and extensive field tests with the synthetic material have been very successful (Section VII D).

The sex attractant produced by the female clover cutworm, *Scotogramma trifolii*, is a mixture of (Z)-11-hexadecenyl acetate and (Z)-11-hexadecenol (*288*). A mixture of these two compounds containing about 10% Z11-16:OH has proved to be most attractive (*289, 290*) while 20—25% Z11-16:OH decreases the catch of males appreciably (*290*). Adult males of the glassy cutworm, *Crymodes devastator*, are attracted by traps baited with equal parts of (Z)-11-hexadecenyl acetate, (Z)-11-hexadecenal and (Z)-7-dodecenyl acetate (*291*). No other species were trapped by this mixture. The addition of small quantities of either (Z)-11-hexadecenol or (Z)-9-tetradecenyl acetate completely inhibited attractancy in the field. As a result of field tests, UNDERHILL *et al.* (*292*) have recently found that alkenals may be as important in lepidopteran sex attractants as the acetates and alcohols.

The sex pheromone components of the diamondback moth, *Plutella xylostella,* a pest of cruciferous crops, has been identified as (Z)-11-hexadecenal and (Z)-11-hexadecenyl acetate (*293*). More recent data show that (Z)-11-hexadecenol is a synergist of these two compounds in field trapping experiments (*294*).

The sex pheromone of the threelined leafroller, *Pandemis limitata,* is a 91 : 9 ratio of (Z)-11-tetradecenyl acetate and (Z)-9-tetradecenyl acetate (*295*). Field tests in Washington using a 94 : 6 ratio also caught large numbers of a sibling species *P. pyrusana.* Roelofs *et al.* (*296*) have shown that this ratio (94 : 6) of Z11-14 : Ac to Z9-14 : Ac is the actual ratio of these isomers in the pheromone gland of females of *P. pyrusana.* These two species therefore have very similar ratios of their sex pheromone isomers. The individual isomers are not active.

Males of the variegated leafroller moth, *Platynota flavedana,* are attracted in greatest numbers in the field to a ratio of 84 : 16 of (E)-11-tetradecen-1-ol to (Z)-11-tetradecen-1-ol (*297*). Female tip extracts contain these two alcohols in a 9 : 1 ratio (E : Z) as well as the corresponding acetates but the acetates do not appear to be used as pheromones. Trap catches of the alfalfa looper, *Autographa californica,* are greatly increased by the addition of (Z)-7-dodecen-1-ol formate to (Z)-7-dodecen-1-ol acetate (*298*), its proposed sex pheromone (*299*). It is not known whether the formate ester is an actual component of the natural pheromone.

Four species of *Trogoderma* beetles use methyl-branched alkenals as their major sex pheromones (*300*). Aeration of *T. granarium* and trapping of the volatiles on Porapak Q gave a 92 : 8 ratio of (Z)- to (E)-14-methyl-8-hexadecenal. The Z-isomer was the major component obtained from *T. inclusum* and *T. variabile* and the E-isomer was obtained from *T. glabrum.* In laboratory bioassays males could discriminate between the geometric isomers.

Females of the black carpet beetle, *Attagenus megatoma,* produce the sex pheromone (E,Z)-3,5-tetradecadienoic acid (*301*). An important related species, *A. elongatulus,* has also been shown to produce a sex pheromone (*302*) and a major attracting component has been identified as (Z,Z)-3,5-tetradecadienoic acid (*303*).

Several aphid species of economic importance use (E)-β-farnesene as an alarm pheromone (*304—307*). Bowers *et al.* (*308*) have studied the activity of various farnesene and nor-farnesene analogs in an attempt to define the structural requirements necessary for alarm activity. They concluded that the following properties were important: 1) there should be an E-configurational double bond in the central position of the molecule, 2) the double bond in the terminal isoprene unit acts as an activator, 3) a π-bond of 1.34 to 1.39 Å must be located ten carbon

units from the end of the terminal isoprene unit, and 4) this π-bond must have free rotation about a single bond.

Bombykol, (E,Z)-10,12-hexadecadienol, is usually considered to be the only female sex pheromone of *Bombyx mori*. However, it has recently been established that the abdominal gland of the virgin female contains both bombykol and the corresponding aldehyde, (E,Z)-10,12-hexadecadienal (bombykal) (*308a*). Bombykal inhibits behavioral responses of the male moth to bombykol but further investigation is necessary to elucidate its biological significance.

B. Enantiomers

Chirality is often associated with compounds of biological origin. Many identified pheromones can have enantiomeric forms and it appears, from what follows, that insects often will biosynthesize and utilize as a pheromone only one enantiomer or a specific ratio of enantiomers. It is only in the last few years that stereospecific syntheses of some pheromones have provided pure enantiomers in sufficient quantities for laboratory and field studies. A number of behavioral experiments have now shown that insects can often distinguish between enantiomers.

Employing measurements from single olfactory receptor cells, KAFKA et al. (*309*) showed that the honeybee and the migratory locust respond differently to each of the enantiomers of 4-methylhexanoic acid. Worker honeybees could be trained in the laboratory to associate certain enantiomers of carvone and 2-octanol with the availability of food (*310*). The female spruce budworm is stimulated to oviposit by (S)-$(+)$-α-pinene but not by the R-$(-)$-enantiomer (*311*). However, none of these compounds is a pheromone.

The principal alarm pheromone of *Atta texana* is (S)-$(+)$-4-methyl-3-heptanone (**386a**, Scheme 68) (*173*). Determination of threshold levels of both enantiomers showed that the naturally occurring $(+)$ enantiomer was about 100 times more active than the $(-)$ enantiomer (*173, 312*). It was also established that the $(-)$ enantiomer did not inhibit response to the $(+)$ enantiomer. However, the $(-)$ enantiomer contained 1.3% of the $(+)$ enantiomer, so it is not possible to determine whether the $(-)$ form has some slight activity of its own or not. It was concluded that the receptors on this ant must be chiral and respond maximally to the naturally occurring enantiomer. *Pogonomyrmex barbatus,* which also uses 4-methyl-3-heptanone as an alarm pheromone (*313*), responds to the S-$(+)$ enantiomer up to 10 times more than the R-$(-)$ enantiomer (*314*). It is not known which enantiomer is synthesized by this ant, but we would propose, as a general rule, that an organism responds optimally to its own enantiomer or ratio of enantiomers.

Gnathotrichus sulcatus produces 6-methyl-5-hepten-2-ol (sulcatol)
(229) as its aggregating pheromone, as a 65 : 35 mixture of *S*-(+) and
R-(−) enantiomers (*315*). BORDEN *et al.* (*316*) found that this ambrosia
beetle responded maximally to a racemic mixture (50 : 50) of the two
enantiomers. In fact, the response to the racemic mixture was significantly
greater than it was to a 65 : 35 ratio. More recent work, however, suggests
that *G. sulcatus* responds to some extent to *S*-(+)-sulcatol alone, that it
does not respond to the *S*-(−)-isomer, and that the range of active ratios
is much wider than previously indicated (*317*). In addition, BORDEN and
coworkers (*317*) have found that *G. retusus* responds to (*S*)-(+)-sulcatol
in an upwind laboratory bioassay and that the response appears to be
inhibited to some extent by the *S*-(−)-enantiomer. They speculate that
speciation may depend in part on the enantiomeric compositions of the
pheromone.

The response of the European elm bark beetle to isomers of its
pheromone blend was determined with two separate laboratory bioassays
and field tests (*93, 318*). A combination of 3 compounds that act syner-
gistically was found: (−)-4-methyl-3-heptanol **(294a)**, (−)-α-multistriatin
(303a), and (−)-α-cubebene **(283)** (for formulas, see pp. 48, 49) (*93*). The
absolute configuration of the (−)-α-multistriatin is 1*S*,2*R*,4*S*,5*R* (*49*)
and that of the (−)-4-methyl-3-heptanol is 3*S*,4*S* (*147*). Both the *A. texana*
alarm pheromone, (*S*)-(+)-methyl-3-heptanone, and the alcohol above
share 4*S* stereochemistry. Of interest is the fact that the reduction of
decalones by microbial enzymes is usually stereoselective, with optically
active alcohols of *S*-configurations being obtained (*319*).

WOOD *et al.* (*320*) studied the flight response of both sexes of *D. brevi-
comis* to a combination of *exo*-brevicomin, frontalin and myrcene. MORI
synthesized the enantiomers of frontalin (*133*, Scheme 43) and *exo*-
brevicomin (*122*, Scheme 34); STEWART *et al.* (*46*) established that the
natural enantiomers were (1*S*,5*R*)-(−)-frontalin **(195)** in males and
(1*R*,5*S*,7*R*)-(+)-*exo*-brevicomin **(150)** in females. WOOD *et al.* (*320*) claim
that these two enantiomers are the most active forms of these compounds.
Males of *D. frontalis* in a pedestrian type bioassay, are more responsive
to (−)-frontalin than to (+)-frontalin, while females are not responsive
to either enantiomer (*321*). The natural ratio of the frontalin enantiomers
in *D. frontalis* females is 85(−) : 15(+) (*46*) . Data on field tests using
optically pure isomers of brevicomin, frontalin and ipsenol indicate that
activity is associated with only one enantiomer of each compound (*322*).

Ips calligraphus responds to ipsdienol and *cis*-verbenol (*323*). (*S*)-*cis*-
Verbenol **(234)** is the active enantiomer and the response is not inhibited
by (*R*)-*cis*-verbenol when present at a 1 : 1 ratio but is inhibited when
the *R*-enantiomer is present at a 10-fold higher amount (*324*). *I. typo-
graphus* also responds to (*S*)-*cis*-verbenol (*325*). The lack of information

on the natural ratio makes it difficult to interpret these results, but we would presume that the *R*-enantiomer is not dominant.

Racemic disparlure, *cis*-7,8-epoxy-2-methyloctadecane (**536**, p. 80), is not as active in laboratory bioassays and electro-antennogram recordings as the 7*R*,8*S*-(+) (**536a**) enantiomer (*252*). The 7*S*,8*R*-(−) (**536b**) enantiomer is virtually inactive. Dosage-response effects of "inactive" enantiomers were therefore conducted by VITÉ *et al.* (*324*) on the gypsy moth. The response to low concentrations (10^{-3} to 10^{-5} dilution) of (+)-disparlure was not affected drastically by the addition of equal or lower concentrations of (−)-disparlure. However, (−)-disparlure in higher concentrations than the (+) antipode lowered the response of moths drastically. It was concluded that the "inactive" enantiomers appear to require a higher concentration in order to saturate the receptor sites for inhibitory effects on the pheromone to appear. A similar result has been obtained by PLIMMER *et al.* (*326*), but again, the natural enantiomer composition is not known.

BIRCH and coworkers (*327*) have shown in laboratory bioassays that female *Ips pini* from the western USA respond well to (−)-ipsdienol (**266a**, Scheme 52) but do not respond to either the (+)-isomer (**266b**) or a racemic mixture. This contrasts with the eastern *I. pini* which responds to the (+)-isomer and a racemic mixture but does not respond to (−)-ipsdienol (*328*). The mechanism for the intraspecific variation in pheromone systems of *I. pini* described by LANIER *et al.* (*329*) seems partially resolved. PLUMMER *et al.* (*45*) found that the natural enantiomer composition of ipsdienol for *I. pini* from Idaho to be 100% (−). The European fir engraver, *Pityokteines curvidens*, aggregates only in response to the *S*-(−)-isomer of ipsenol, and the *R*-(+)-isomer is inactive (*330*).

The sex attractant of the female Japanese beetle, *Popillia japonica*, which attracts males, is (*Z*)-5-(1-decenyl)dihydro-2(3*H*)-furanone (*73*). The pure synthetic *R*,*Z*-isomer (**144**, Scheme 27) was competitive with females, and male response was strongly inhibited by small amounts of the *S*,*Z*-isomer. The enantiomeric composition of the natural product has not been determined. The female also produces minor amounts of both the *E*-isomer and the saturated analog but the roles of these compounds has not been established. Females of the California red scale, *Aonidiella aurantii*, emit a pheromone that attracts males (*331*). Two compounds have recently been identified and synthesized: 3-methyl-6-isopropenyl-9-decen-1-yl acetate (**332a**) and (*Z*)-3-methyl-6-isopropenyl-3,9-decadien-1-yl acetate (**333**, Scheme 50) (*75*). The four enantiomers of the latter compound were synthesized and only the *R*,*Z*-isomer proved to be attractive to male red scale.

The use of the sesquiterpene, (*E*)-β-farnesene, as an aphid alarm pheromone is well established (*304*—*307*), and the spotted alfalfa aphid,

Therioaphis maculata, has been found to use another sesquiterpene hydrocarbon, (–)-germacrene A *(332)*.

The enantiomeric composition of several insect pheromones has been determined by employing a chiral derivative and chiral lanthanide shift reagents *(45)*. The amount of material required was of the order of 5—500 μg of substrate. As the enantiomeric composition of chiral pheromones is of fundamental importance to their efficacy and appears to play a role in speciation, one can expect its determination to become a standard procedure.

C. Chemorecognition

This discussion deals largely with the chemorecognition of a stimulus molecule (i. e., a releaser pheromone) by a receptor site. It also deals with how enantiomers may be distinguished. The interactive forces between a releaser pheromone molecule and a receptor site, or their binding, are considered to play a major role in chemorecognition.

The evidence that most insects use their antennae to locate the opposite sex has been reviewed by JACOBSON *(11)* and the nature of the olfactory receptors and chemosensory processes has been described by a number of workers *(333 338a)*. The concept of receptors was first introduced by EHRLICH *(339)* when he wrote that drugs do not act unless they bind. The best evidence for the existence of selectively sensitive receptors is the difference in the behavioral response to many stereoisomers. Therefore, in the study of the action of pheromones at the molecular level, it is necessary to consider both their conformation and their configuration. The importance of each and their influence on insect behavior has been pointed out.

It is generally accepted that a receptor is an elastic, three-dimensional entity, largely proteinaceous, with a specific region (receptor site) that can interact with the substrate and thereby generate a stimulus. An appropriate interaction results in a biological response. The interaction of the pheromone molecule and the receptor site is likely to involve weak forces such as ionic and dipole-dipole interactions, hydrogen bonding, and van der Waal's forces. The contribution of van der Waal's forces between a relatively non-polar volatile substrate and a non-polar receptor site is likely to be significant.

The binding of pheromone molecules by antennal proteins has been observed *(340, 341)* and transport of a pheromone molecule is considered by some as an integral part of the overall sequence leading to perception *(334, 342)*. In addition, antennae of males and females of *Trichoplusia ni* contain an enzyme capable of hydrolyzing its pheromone, (Z)-7-dodecenyl

acetate, and certain other isomers (342). The pheromone was generally degraded less than any of the other isomers and it was suggested that the decreased enzymatic hydrolysis of the pheromone could be the result of nonenzymatic binding to receptors or the absence of a cofactor. In microbial chemoreceptors responsible for chemotaxis, it appears that the binding proteins also serve as part of the transport system for these compounds (343—345). However, WRIGHT (346) does not consider the binding of an odorant molecule at a receptor site or its transport to be necessary for the information present in the molecule to be translated into nerve impulses, thereby transmitting a signal that elicits a behavioral response. The odorant molecule need only approach sufficiently closely to transfer energy before going on its way unchanged and free to act again and again.

A number of examples have been mentioned which illustrate that the receptor site can differentiate between enantiomeric forms of a pheromone. It is common knowledge that many enzymes are specific for only one of a pair of enantiomers. Also, many differentiate between enantiotopic groups of a single substrate molecule (347). If binding of a pheromone to a receptor takes place, it is probably analogous to the formation of an enzyme-substrate complex. The combination of each member of an enantiomeric pair with receptors of a given chirality results in the formation of two complexes that are physically and chemically distinct diastereomeric combinations. Therefore the differentiation between enantiomers may be dependent upon the creation of diastereomeric relationships. Should two enantiomers have equal activity, then the pheromone-receptor interaction may not involve the chiral centers of the enantiomers.

The concept that an enantiomeric pair forms a diastereomeric pair when bound, even if only transiently, to a chiral receptor, fits in with some of the results mentioned previously in which it is found that activity is brought about by one of a pair of enantiomers, and appears to be a plausible explanation of how an insect can discriminate between enantiomers. The initial olfactory process for each enantiomer is different.

Most identified pheromones are blends. In many cases, the response to the major pheromonal component is either synergized or inhibited by other compounds. Both phenomena may be accounted for by each of the components having specific receptor sites; inhibition could also be accounted for by competition for the same receptor site. If the receptor molecule were allosteric, then both inhibition and synergism could be accounted for by the presence of negative and positive modulators. The possibility that a synergistic effect could be due to an allosteric receptor protein has been suggested (316). The results reported by AIHARA and SHIBUYA (347a) may be pertinent. A better understanding

7*

may result if experiments similar to those in enzyme kinetics employing inhibitors and activators can be devised.

We can safely assume that the receptor site is chiral. It is also clear that there cannot be separate receptor sites for each and every odorous substance. Therefore, many receptor sites must be able to react with many, but not all, substances. A receptor site can distinguish both between a pair of enantiomers and between closely related achiral molecules, but it seems unreasonable to assume that there be two separate mechanisms for the chemorecognition of chiral and achiral molecules.

It has been demonstrated experimentally that when an achiral molecule approaches a chiral one, chirality may be induced in the previously achiral molecule (348—351). This molecular twisting occurs before the two molecules are close enough together to be bound by the formation of a donor-acceptor complex or by hydrogen bonding (348 351) and has been demonstrated by observing the induced circular dichroism of the n-π^* transition of various achiral substances in chiral solvents (348—352). This effect of the twisting of an achiral molecule into a helical form (i. e. into a chiral one), as revealed by the circular dichroism spectrum, has been termed "dispersion-induced circular dichroism" because the twisting occurs at a distance of several van der Waals radii from the chiral molecule (349, 352). At closer distances, (i. e. when binding occurs), "association-induced optical activity" will exist as decribed by SCHIPPER (353). These results emphasize that the solution conformation of a compound may be very different from its conformation at the active site.

To compare the extent to which chirality is induced in a given achiral molecule, HAYWARD (351) has measured the dichroic and isotropic molar extinction coefficients of the substance when dissolved in two different optically active solvents, and has plotted the ratio of these extinction coefficients in one solvent against the ratio in the other. This was done for a number of aliphatic ketones (351) which previously and independently had been tested as ant alarm pheromones (354). The points for a series of 2-alkanones fell approximately along a straight line and the points for the 3- and 4-alkanones fell along another straight line. The lines intersected in a region of the plot, and those alkanones that exhibit pheromone activity were found in or near the intersection. More recent studies have established the symmetry rules connecting sign and magnitude of the dispersion-induced circular dichroism with the molecular structure of the solutes (355).

It is for these reasons that HAYWARD (351) considers that the primary physical process in the olfactory detection of either a chiral or an achiral molecule by a chiral receptor involves some measure of induced chirality or modification of existing chirality, and that it is, in fact, this induction

or modification. In short, he regards the correlation between biological activity and the nature and amount of the dispersion-induced circular dichroism of a series of compounds to be compelling evidence that induced optical activity occurs at an early stage in the process of chemo-recognition, that it is intimately related to recognition-specificity, and that it is important in the process of olfaction as well as in other biochemical processes such as antibody-antigen reactions, drug action, allergies, etc. (355). If the phenomenon of dispersion-induced circular-dichroism is firmly established as being important in certain biochemical processes, it certainly will have predictive value.

WRIGHT (356) has proposed a vibrational theory of olfaction that has not hitherto attempted to explain differences in odor of enantiomers. However, in the light of the results discussed above, he now considers that if two enantiomers, on approaching a chiral receptor, are twisted, they will no longer be mirror images and will therefore have different vibrational spectra and may therefore have different odors (346).

PAYNE and DICKENS (357) have described a technique to elucidate the specificity of the receptor system of the southern pine beetle, D. frontalis. The technique employs the differential adaptation of the antennal olfactory receptors to various test compounds; either the single unit recording technique or the electroantennogram (EAG) technique is used. It is designed to determine whether different compounds are recognized by the same receptor site and is based on the exposure of the antennal preparation to one compound until the site is completely adapted, followed by exposure to a test compound. It is claimed that failure to show a response to a test compound after adaptation to another indicates that all the chemorecognition sites for the test compound are occupied by the first compound. As it had been shown that the receptors for both bicyclic ketals and host terpenes respond with equal intensites (358), DICKENS and PAYNE (321) calculated the percent of the acceptors (receptor sites) capable of interacting with the various compounds tested.

However, the components of the pheromone of a butterfly are known to give different amplitude EAG's (359). GRANT (360) found that phero-mones from different species elicit EAG's of similar amplitude in a common recipient. EAG's of similar amplitude were obtained when the antenna of the armyworm, Pseudaletia unipunctata, was stimulated with the male's known pheromone components, benzaldehyde, benzyl alcohol and benzoic acid, or with the related aromatic compounds, 2-phenyl-ethanol and benzyl acetate (361). The antennal response of male European corn borer moths, Ostrinia nubilalis, to their two pheromone components, (Z)- and (E)-11-tetradecenyl acetates has recently shown that repetitive stimulation with one of the isomers results in adaptation which affects the amplitude of response in subsequent tests to both compounds (362).

It was suggested that the receptors for the two isomers were either identical or highly interactive.

The differential adaptation technique was used by DICKENS and PAYNE (321) to establish the extent to which the known pheromone components of *D. frontalis* and host tree compounds interact with specific receptors. It was concluded that the bicyclic ketal, frontalin (195), could react with all the acceptors, and the oxygen-containing pheromones occupied a larger percentage of the acceptors than the host tree monoterpenes. In the female beetle antennal preparation, the attractant (frontalin), the inhibitors (*endo*-brevicomin (151) and verbenone (233)), and the synergists (*trans*-verbenol (232), 3-carene and α-pinene), formed three distinct groups. The inhibitors interacted to a greater extent with the receptor sites than did the synergists. However, DICKENS and PAYNE (321) claim that the previous suggestion that there are at least two classes of receptors for the bicyclic ketals and host tree terpenes (358, 363) is incorrect. It has also been pointed out that adaptation of receptor cells is a complex phenomenon and may have many origins (337).

Adaptation of the antennal preparation to *endo*-brevicomin (151) blocked response to *exo*-brevicomin (150), indicating that *endo*-brevicomin reacted with a greater percentage of the frontalin acceptors of both sexes of *D. frontalis* than did *exo*-brevicomin (321). The reverse order of presentation of these two compounds did elicit a response, and it was suggested that this difference may be due to steric limitations imposed by the *exo*-ethyl group (321). Molecular models illustrate quite clearly that there is a distinct difference between the *exo*- and *endo*-brevicomins with respect to the face of the ring containing the two oxygen atoms.

In the eastern spruce budworm, the differential adaptation of a receptor site by a pheromone and an inhibitor acting on a common receptor site probably indicates a different affinity of each of these molecules for the common receptor site (337). It seems reasonable to assume that the oxygen atoms of the bicyclic ketals will be involved in the interaction at the receptor site. For this reason, quantum calculations were carried out on all three of these bicyclic ketals in order to determine the relative negative charge on the oxygen atoms (CAPUTO and BRAND,

-1.9503   -1.9501   -2.0554

-1.9471   -1.9393   -2.0558

(151)   (150)   (195)
endo-brevicomin   *exo*-brevicomin   frontalin

References, pp. 157—190

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endo-brevicomin	*exo*-brevicomin	frontalin

References, pp. 157—190

V. Biosynthesis

A. Exposure of Bark Beetles to Pheromone Precursors

Many compounds have been isolated and identified from various bark beetle species and claims made as to their effects on behavior. A few of the claims are conflicting and some others are not well proved. It is therefore difficult to state categorically exactly which compounds do what and to whom. Also, the behavioral chemicals to which most bark beetles respond appear to be a complex blend of components and the actual behavioral contribution and meaning of each component part remain essentially unknown. However, it is known that the pheromonal blends, so far identified, to which beetles in the genera *Ips, Dendroctonus* and *Scolytus* respond, have a number of similarities.

Very little definitive work has been conducted on the biosynthesis of insect pheromones, and this is an area that could well be studied by the more biochemically minded. However, a number of investigations to determine the origin of many of the behavioral chemicals of bark beetles have been conducted. Most of these studies are a little different from the usual labelling experiments and we intend to concentrate the first part of this discussion on these studies.

In their natural habitat adult beetles of *Ips* and *Dendroctonus* either ingest, or are in intimate contact with, numerous host plant terpenes, three of the major ones being α- and β-pinene and myrcene. The roles of these and other host-tree monoterpene hydrocarbons have been summarized by BORDEN (367) and their function is generally considered to be synergistic in the overall aggregating pheromone complex. These monoterpene hydrocarbons are found in the insect, and their origin is considered to be the host tree, rather than *de novo* synthesis by the insect itself. However, certain termites (368, 369) and ants (370—372) can synthesize α- and β-pinene, as well as other monoterpene hydrocarbons.

It is the biosynthesis of the oxygenated monoterpenes that influence bark beetle behavior that provides us with an interesting and largely unresolved problem. The main compounds identified in this category are *trans*-verbenol (232) (373), *cis*-verbenol (234), 2-methyl-6-methylene-7-octen-4-ol (ipsenol) (240), and 2-methyl-6-methylene-2,7-octadien-4-ol (ipsdienol) (266) (see Chart 1) (136). The reaction for the biosynthesis of all these compounds seems to be the allylic hydroxylation of α-pinene and myrcene, both of which occur in abundant amounts in host pine trees.

Let us consider *cis*- and *trans*-verbenol (234 and 232). BORDEN (367) has summarized a number of species in which they occur, together with their probable functions. In general, these two compounds are found in

the hindgut and the frass and are considered to affect the behavioral response of adult beetles. To date, the precise site and mechanism of their production remain unknown. VITÉ *et al.* (*374*) analyzed the hindguts of 12 *Ips* species for the presence of *cis-* and *trans*-verbenol; they concluded that the major pheromones in the hindgut and frass arose by two distinct mechanisms. The synthesis of the verbenols did not require feeding; rather, they were formed on exposure of the insect to oleoresin, whereas ipsdienol production required prior feeding. All stages of bark beetles that attack pine trees will be in an atmosphere rich in monoterpenes, and the indication that exposure to, and contact with, oleoresin leads to the synthesis of the verbenols resulted in a series of experiments by the group at the Boyce Thompson Institute, in which beetles were exposed to the saturated vapors of various monoterpene hydrocarbons.

HUGHES (*375*) exposed both sexes of *D. ponderosae* to an atmosphere saturated with α-pinene, or with oleoresin obtained from an alternate host, *Pinus lambertiana*. Analysis of the volatiles present in the hindguts of treated beetles showed an increase in *trans*-verbenol content after exposure. HUGHES (*376*) then extended these experiments to include *D. frontalis, D. brevicomis, D. valens* and *D. pseudotsugae*. The results indicated an increase in the amount of *trans*-verbenol present in hindguts of all species after exposure to α-pinene. HUGHES (*376*) proposed that the production of certain substances may be under neural and/or hormonal control as first suggested by the work of BORDEN *et al.* (*377*). A more thorough study on the exposure of *D. frontalis* to α- and β-pinene was conducted by RENWICK *et al.* (*378*), and additional volatile components produced on exposure were identified. The major components in hindguts of males were *cis-* and *trans*-verbenol, 4-methyl-2-pentanol, pinocarvone (**616**), and *trans*-pinocarveol (**617**), while those in females were 4-methyl-2-pentanol and *trans*-pinocarveol.
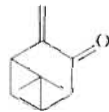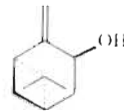
(616)   (617)

(618)

Renwick *et al.* (*379*) obtained interesting results on the biosynthesis of the geometric isomers and the enantiomers of the verbenols. All the previous experiments of the Boyce Thompson Institute group showed that α-pinene was converted to *cis-* and *trans*-verbenol and myrtenol (**618**) by all the species they studied. However, the α-pinene used in the experiments was always (±)-α-pinene. Since *cis*-verbenol is a component of the pheromone complex of *I. paraconfusus* (*136*), this species was chosen for experiments in which adult beetles were exposed to (+)- or (−)-α-pinene. Chromatograms of extracts of hindguts of adults of both sexes treated with (+)-α-pinene showed two prominent peaks corresponding to *trans*-verbenol and myrtenol. In contrast, both sexes produced predominantly *cis*-verbenol and myrtenol after exposure to (−)-α-pinene. The optical rotation of each purified product was measured in methanol. When (+)-α-pinene was used, (+)-*trans*-verbenol and (+)-myrtenol were obtained. When (−)-α-pinene was used, (+)-*cis*-verbenol and (−)-myrtenol were obtained. It was concluded that variations in the optical rotation of the α-pinene in trees under attack would strongly influence the ratio of *cis-* and *trans*-verbenol in this species. The implications of this finding are significant and may be important in other bark beetle species as within tree (*380*), and between tree (*381*), variations in monoterpene composition are known to occur.

Some confusion has arisen over the relationship between the sign of optical rotation and the absolute configuration, especially with regard to *cis*-verbenol. This problem has been resolved by Mori *et al.* (*48*). As mentioned above, (−)-α-pinene is converted to (+)-*cis*-verbenol by *I. paraconfusus* (*136*). In this case the optical rotation of the *cis*-verbenol was measured in methanol. Earlier syntheses (*51, 52*) of *cis*-verbenol from (−)-verbenone gave a product that was levorotatory in chloroform (*52*) and dextrorotatory in acetone (*51*). Both of these results are correct and both groups had in fact obtained (1*S*,4*S*,5*S*)-*cis*-verbenol (**234**). The sign of rotation of *S*-*cis*-verbenol changes between these two solvents (*48*). Also, the results of Renwick *et al.* (*136*) are quite in order but some of the structures in their publication are incorrect. For example, their structure for (+)-*trans*-verbenol is in fact (−)-*trans*-verbenol. However, their structure of (+)-*cis*-verbenol is correct (see *48*). Furthermore, no inversion of the bicyclic terpenoid ring system occurs during biological oxidation. Mori urged that the sign of optical rotation be used in conjunction with the *R* and *S* system of configurational nomenclature whenever possible to aboid confusion.

Renwick and Hughes (*382*) exposed *D. frontalis* adults to 1-methyl-1-cyclohexene, a non-terpenoid hydrocarbon, and identified 3-methyl-2-cyclohexen-1-ol (seudenol) and 3-methyl-2-cyclohexen-1-one (MCH) among other products. Both seudenol (*383*) and MCH (*384*) are identified

pheromones of the Douglas-fir beetle, *D. pseudotsugae*. While seudenol and MCH were synthesized from 1-methyl-1-cyclohexene (*382*), this compound is not a known component of the host tree of the Douglas-fir beetle. However, based on these and the previously discussed results, a general allylic oxidation and rearrangement mechanism was suggested in bark beetles.

It should be emphasized that all of these bark beetles are in intimate contact with many monoterpene hydrocarbons and that the normal detoxification mechanism for these substances is their oxidation. Many studies have confirmed the presence of numerous oxidation products of α- and β-pinene and myrcene in bark beetle hindguts. The presence of these oxidation products in the various species, albeit in differing proportions, should be considered biochemically inevitable. What must be stressed is that only certain of these compounds need be used as behavior-modifying chemicals by any one species, even though other related substances are present. The mechanism of perception by a species should be considered more discriminating than the mechanisms of biochemical oxidation. The release of, and the response to, a number of bark beetle pheromones, including MCH, by stridulation have been observed and discussed by RUDINSKY and coworkers (*385—390*). Results obtained by PITMAN and VITÉ (*391*) on the production of MCH by the Douglas-fir beetle led to a conclusion concerning the producing sex different from that of RUDINSKY and coworkers (*385, 386*).

HUGHES (*392*) showed that various *Ips* species produced ipsenol and/or ipsdienol on exposure to myrcene. *I. grandicollis* and *I. calligraphus* both required feeding before metabolizing myrcene, whereas *I. avulsus* and *I. paraconfusus* produced some products without prior feeding. Furthermore, the results indicated that ipsenol is produced by the reduction of ipsdienol obtained by the hydroxylation of myrcene. The biosynthesis of these pheromones appears to be under some form of control that is influenced by feeding in some species (*392*). BAKKE (*393*), studying *I. typographus*, found that ipsdienol and ipsenol were not detected in every male initiating galleries, and therefore presumably feeding, nor were they detected in fed males exposed to myrcene. CHARARAS (*394*), in experiments on the primary and secondary attraction in certain *Ips* species, concluded that pheromone production by males is influenced by nutritional factors such as carbohydrates of the host sap and the host plant terpenes.

HUGHES and RENWICK (*395*) have recently conducted additional exposure experiments with *I. paraconfusus* males. In this species it was found that no appreciable synthesis of *cis*-verbenol occurred in the absence of exogenous (−)-α-pinene. Therefore, unlike *D. frontalis* (*376*), newly emerged *I. paraconfusus* males do not appear to have an endogenous precursor of *cis*-verbenol.

Further experiments of this type by RENWICK et al. (396) established that D. brevicomis and D. frontalis oxidize camphene (**619**) to 6-hydroxy-camphene (camphenol, **620**). Both sexes of D. brevicomis transformed myrcene into 2-methyl-6-methylene-2,7-octadien-1-ol (myrcenol, **621**), whereas ipsdienol was a major product in males only.

(619)   (620)

(621)
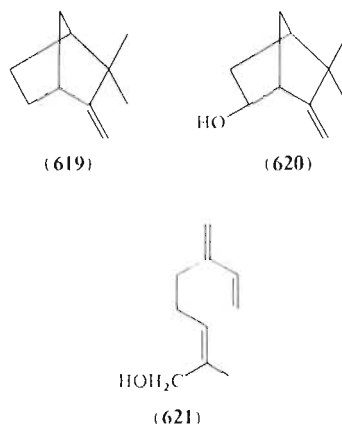
HUGHES (397) has attempted to elucidate the origin of α-pinene oxidation products by means of exposure experiments. Exposure of both larvae and emergent adults of D. frontalis to α-pinene resulted in their producing trans-verbenol, whereas exposure of pupae did not. In addition, adult males less than a week old did not contain appreciable quantities of verbenone while males a week old or more did, i.e. after the adult maturation, period. These results have been substantiated by BRIDGES (398). In summary, exposure of D. frontalis larvae to α-pinene results in the production of trans-verbenol, exposure of pupae does not, and exposure of emergent adults does. However, while pupae exposed to α-pinene do not produce trans-verbenol, this compound appears later in the adults that develop from the pupae. To continue with the puzzle, callow adults obtained from pupae removed from the bark do not contain trans-verbenol and verbenone, but these compounds can be detected after a week or so without any contact of these insects with α-pinene (397). Where did the compounds come from? HUGHES (397) suggested that the pupae conjugate some form of the terpene molecule with an unknown compound. Attempts by BRAND (unpublished data) to obtain trans-verbenol or ver-benone from pupae by mild hydrolysis of homogenates have proved unsuccessful. TASKINEN (399) has shown that cyclic allylic alcohols such as trans-verbenol can react with ethanol under acid catalysis to form ethyl ethers. This question remains unsolved and should be considered

of prime importance for the understanding of the biosynthesis of these oxygenated monoterpenes in these beetles.

B. Hormonal Influence on Pheromone Production

BORDEN *et al.* (*377*) showed that topical application of 10,11-epoxy-farnesenic acid methyl ester to male *I. confusus (I. paraconfusus* Lan.) improved the bioassay response to the hindgut region. In studies on the control of pheromone production in *I. paraconfusus,* the main findings of HUGHES and RENWICK (*395*) focus on neural and hormonal control of the synthesis of ipsdienol and ipsenol from myrcene. When myrcene is presented in the vapor form, feeding stimulates its metabolism to ipsdienol and ipsenol. This stimulation was also brought about by distension of the gut with air and by topical treatment with juvenile hormone (JH). Implantation of corpora allata into, or JH treatment of, decapitated males did not stimulate synthesis of the pheromones on exposure to myrcene. However, implanation of the corpora allata and corpora cardiaca together, or the corpora cardiaca alone, did stimulate synthesis. The following sequence for the neural-hormonal control of pheromone synthesis from myrcene was suggested (*395*). "1) Distension of the gut by feeding removes neural inhibition at the corpora allata, resulting in the release of JH; 2) JH acts through the brain neurosecretory cells and/or the corpora cardiaca to stimulate the production and/or release of brain hormone (BH); 3) BH stimulates the synthesis of enzyme(s); 4) the enzyme(s) converts myrcene or a myrcene-derived intermediate into ipsdienol, which is then reduced to ipsenol." GERKEN and HUGHES (*400*) have also reported that exposure of certain bark beetles to synthetic juvenile hormone analogs stimulates the biosynthesis of pheromones and therefore may have practical value in their isolation and identification.

HUGHES (*397*) confirmed that the gut of adult emergent *D. frontalis* males contained a considerable amount of verbenone. However, verbenone was essentially undetectable in either black adults enlarging the pupal chamber or in younger stages. In the *I. paraconfusus* study (*395*), corpora allata and attached corpora cardiaca from feeding males were implanted into callow males. Exposure of these males to myrcene did not result in the production of ipsdienol and ipsenol. Many insects cease the production of JH during the pupal and early adult stages, and, if this is the case in these bark beetles, it may provide part of the explanation for these findings. However, the implantation experiment of glands into callow adults suggests that more than a mere lack of JH and BH is occurring at this young stage as enzyme synthesis is apparently not stimulated by these two factors. In addition, it is suggested that feeding

removes neural inhibition at the corpora allata, resulting in the release of JH (395). Although synthesis of JH occurs in the corpora allata, its storage in appreciable quantities in this organ is thought not to occur. Cyclic changes in the amount of hormone secreted from the corpora allata of cockroaches control some aspects of reproductive behavior including pheromone production (401–408).

Hughes (409) has summed up his views on the significance and logic of the various exposure experiments as follows. "Chemically, scolytid pheromones identified to date may be placed into three categories: 1) bicyclic ketals, 2) terpene alcohols and a corresponding ketone, or 3) a group of simple cyclic or acyclic alcohols. Nothing is known about the synthetic mechanism for the production of the bicyclic ketals or the simple non-terpenoid alcohols except that, at least in the cases studied, their synthesis can be stimulated by juvenile hormone and/or host compounds with no requirement for exogenously supplied pheromone precursor. The precise synthetic pathways for the terpene-derived pheromones are also unknown, but a considerable amount has been published on their precursors and the control of production."

"Bark beetles invading conifers contact externally and/or ingest large quantities of monoterpenes known to be toxic to these insects. Alcohols and ketones of these terpenes have been identified from the hindgut contents and fecal material of all species examined, and more recent work has shown that these alcohols and ketones are produced by the metabolism of the host-derived terpenes. It is not clear at this time to what degree their production depends on microorganisms and to what degree it depends on the insect's own enzymatic system(s), but the mechanism is general for oxidation at allylic positions."

"The terpene alcohols and ketones identified to date are common to virtually all of the species studied, although the conditions under which they are found may vary from species to species and there may be both quantitative and, to a lesser degree, qualitative differences; sex-related differences in their occurrence have also been noted. Generally, these compounds appear to be innocuous with respect to the distance orientation behavior of the bark beetles, and their production depends primarily on the penetration of the terpenes through the integument or absorption by the intestine. However, a few of these compounds do function as aggregation pheromones or deterrents in certain species; the production of most of these terpene-derived pheromones is also passive, but active control of the mechanism for synthesizing pheromones from myrcene has been demonstrated in some *Ips* species."

"Other studies have shown that the absolute configuration of the precursor determines the activity of the pheromone both by fixing the absolute configuration and, in one case, the geometrical configuration

References, pp. 157–190

of the pheromone. It has also been demonstrated that adults can produce the pheromones from terpenes acquired in the pupal and teneral adult stages, suggesting a conjugated intermediate that is utilized by the adult at the time of maturation."

"The studies on the synthesis of the terpene-derived pheromones have led to the hypothesis that these compounds are waste products from the detoxification of host terpenes that, as a consequence of the timing and conditions of their production and release, have secondarily been utilized as chemical messengers. The nature of these oxidations, the large quantities of products formed, and the formation of the same products by other insects such as house flies when exposed to the terpenes led to the suggestion that the mixed-function oxidase system in the insect may well be involved."

We have covered these experiments on the biosynthesis of certain bark beetle pheromones by exposure to precursors and JH in some detail. These studies do not present us with a clear understanding of all the factors involved at this stage, but they do make us aware of the complexity involved in the regulation and control of pheromone synthesis in these economically important insects.

C. Possible Role of Mixed-Function Oxidases

The most likely reaction for the biosynthesis of the verbenols, as well as of ipsdienol and ipsenol, is allylic hydroxylation of α-pinene and myrcene. A reaction of this type could be carried out by microsomal mixed-function oxidases, a group of enzymes important in the degradation of insecticides and drugs. These enzymes are present in insect gut tissue, e. g. American cockroach (410), gypsy moth (411), and honeybee (412). These findings are significant in the biosynthesis of the bark beetle pheromones, particularly since the recent findings of BRATTSTEN et al. (413) in which mixed-function oxidases were induced in the midgut tissue of southern armyworm larvae by a variety of secondary plant substances, the most potent inducers being (+)-α-pinene and myrcene. However, only the enzyme activity was measured, and the oxidation products of these monoterpenes were not determined. The results of BAKER (414) which support a secretagogue mechanism for the control of digestive enzyme synthesis in insects, are also pertinent.

In the case of bark beetles invading a host tree, one would expect from the preceding that the ability to produce enzymes capable of oxidizing the monoterpenes would be rapidly induced. If this were the case then many experiments exposing various bark beetle species to α-pinene and myrcene are more understandable.

D. Possible Involvement of Microorganisms in Pheromone Synthesis

The natural habitat of most insects in their various stages of development dictates that they will come into intimate contact with numerous microorganisms. For example, many insects live in holes in the ground, in cavities in trees, in decaying logs, in leaf litter and compost, in sewers, and numerous other places all of which have an abundance of microorganisms. As might be expected, this constant exposure to microorganisms over the ages has led to many and varied associations between insects and microorganisms. Some associations may lead to disease while others may be of a beneficial and symbiotic nature (*415*).

Of the large number of insect pheromones identified in recent years, some are also known to be substances produced by microorganisms. For example, 3-octanone and 3-octanol produced in the mandibular glands of many ant species (*416*) are also produced by *Aspergillus flavus* (*417*). The typical defensive substance of ants in the subfamily Dolichoderinae, 6-methyl-5-hepten-2-one (*418*), recently also found in the ant genus *Formica* (*419*) and in a staphylinid beetle (*420*), is produced by *Endoconidiophora coerulescens* and *E. virescens* (*421, 422*) and a mycangial symbiont of *D. frontalis* (*423*). The monoterpene hydrocarbons, *dl*-limonene and α-pinene, produced by termites (*368—369*) and ants (*370—372*) also occur in algae (*424*), and the oxygenated monoterpenes neral and geranial which are widely distributed in the Hymenoptera (*425*) are also synthesized by *Ceratocystis variospora* (*426*).

The possibility that microorganisms associated with insects may synthesize insect pheromones has been demonstrated in a number of cases. The production of phenol in the colleterial gland of the New Zealand grass grub beetle is claimed to be due to a bacterium (*427*), and the termite trail pheromone, (*Z,Z,E*)-3,6,8-dodecatrien-1-ol (*428*), occurs in greater amounts in a wood-rotting fungus eaten by these insects than in the termites themselves. Recent attempts have been made to resolve the question of the origin of the trail pheromones of certain termites (*429*). While the authors found some degree of species specificity of termite trail pheromones, due possibly to secondary components, the biogenetic origin of the trienol was shown to be due to neither the termites alone nor fungal sources alone. Investigations are continuing to resolve these points (*429*). Matsumura *et al.* (*430*) have recently studied the production of (*Z,Z,E*)-3,6,8-dodecatrien-1-ol by the brown rot fungus, *Gleophyllum trabeum* (*Lenzites trabea*), on various carbon sources.

Larvae of the seedcorn maggot, *Hylemya platura*, damage certain crop species by feeding on the cotyledons and plumules. It has been assumed that the stimulus for oviposition was provided by the germinating seeds. However, Eckenrode *et al.* (*431*) found that significantly fewer eggs

were laid on microbe-free squash seeds than on regular non-sterile seeds. The most effective microbial elicitors of oviposition were a *Pseudomonas* sp. and the yeast, *Torulopsis aeria*. Ethanol is a common end product of anaerobic fermentation by yeasts. It has been identified in extracts of wood and bark which were attractive to the ambrosia beetle, *Trypodendron lineatum* (*432*) and it also has a synergistic effect on the aggregation pheromones of *D. pseudotsugae* (*433*). The response of males of the ambrosia beetle, *Platypus flavicornis*, is increased by the addition of ethanol (*434*).

In the case of certain bark beetles a number of their identified pheromones are derivatives of α-pinene (*136, 373, 435*), and it is probably correct to assume that these derivatives arise by enzymatic action on the α-pinene of the host plant. There are two possibilities for the origin of the enzymes concerned. Firstly, they could be secreted by the gut tissue of the insect and effect the transformation of α-pinene in this region. The induction of mixed-function oxidases in insect gut tissue by host plant monoterpenes has been mentioned. Secondly, they could be produced by microorganisms present either within the gut or in the host plant tissue itself or in the frass after excretion. It is the second possibility that concerns us now.

It is well known that many microorganisms are able to oxidize both nonactivated carbon-hydrogen bonds and allylic systems to produce alcohols (*436*). The fungus, *Aspergillus niger*, was shown to convert *dl*-α-pinene into (+)-*cis*-verbenol and (+)-verbenone (*437, 438*). Both of these α-pinene derivatives occur in the gut of various bark beetles and are known to be part of the pheromone system of these insects (*136, 373, 435*). The lower alimentary tract of insects usually contains a large number of microorganisms and it is therefore quite plausible that microorganisms associated with bark beetles could be responsible for the oxidation of some of the α-pinene present in the phloem of the host plant thereby forming behaviorally active substances. With this point in mind BRAND *et al.* (*439*) isolated, under aerobic conditions, various microorganisms from the gut of adult male and female *I. paraconfusus* and determined their ability to transform α-pinene into *cis*- (**234**) and *trans*-verbenol (**232**). One organism, identified as *Bacillus cereus*, was found which produced these two compounds, together with *trans*-pinocarveol (**611**) and myrtenol (**612**) in low yield. BRAND *et al.* (*439*) stated "while our preliminary results do not prove conclusively that *B. cereus* actually synthesizes the verbenols from α-pinene in the hindgut, our data clearly indicate that this is a distinct possibility and substantiate the hypothesis that microorganisms may play a significant role in the synthesis of certain pheromones occurring in the frass of these bark beetles." CHARARAS (*394*) has concluded from feeding experiments involving broad spectrum antibiotics that bacterial con-

version of ingested monoterpenes is possible but not essential for phero-
mone production in certain *Ips* species.

From these results we must conclude that certain gut-associated
pheromone components may be produced by the enzyme systems of the
insect itself and/or by microorganisms. If this is the case, then the resulting
blend occurring in the frass will be the result of the dynamic balance
between those substances that are most readily produced by both
microbes and insect and those that are most readily metabolized by both
microbes and insect. Furthermore, the types of microbes present in the
gut and the nature of the enzymes secreted by the gut tissue will be
affected by the diet of the insect. Therefore, the occurrence of frass-
associated pheromones should probably be considered the result of a
delicate equilibrium involving a number of biological species.

The female of *D. frontalis* has a mycangium, which usually contains
two fungi, *C. minor* var. *barrasii* (SJB-133) and a Basidiomycete (SJB-122),
and two yeasts, *Hansenula holstii* and *Pichia pinus* (*440, 441*). These
mycangial microorganisms are introduced into the phloem of host pines
upon attack by female beetles and result in rapid invasion of the gallery
system and surrounding phloem. Chemical transformation experiments
showed that the one mycangial fungus, SJB-133, could quantitatively
convert either *cis-* or *trans-*verbenol into verbenone (*442*). The *trans-*
verbenol used in these experiments had an enantiomeric ratio of (+) to
(−) of 60 to 40 (Brand, unpublished data). It was suggested by Brand
et al. (*442*) that SJB-133 growing in the phloem could produce verbenone
from *trans-*verbenol *in situ,* and, if this is the case, then a microorganism
external to the beetle would be responsible for part of the production of
at least one of its behavioral chemicals.

An increase in the verbenone concentration released from a success-
fully colonized tree has been proposed as an important factor in inhibiting
further attacks on the tree by both *D. frontalis* and *D. brevicomis* (*443*).
It is therefore possible that SJB-133, which is important in the beetle's
nutritional regime (*440*), could also play a significant role in regulating
response to the plant host. More recently *endo-*brevicomin has been
implicated in the shifting of attack from one tree to another (*444*).

Inquiries into the possible production of behavioral chemicals of
D. frontalis by microorganisms associated with it have been carried
further. As mentioned above, two fungi and two yeasts are associated
with the mycangium of the female (*440, 441*). The production of various
volatile substances, other than ethanol, by actively fermenting yeasts is
well established (*445*). Brand *et al.* (*446*) grew three yeasts obtained from
D. frontalis, namely *H. holstii, P. pinus,* and *P. bovis,* on Sabouraud's
dextrose broth, and identified isoamyl alcohol, 2-phenylethanol, isoamyl
acetate and 2-phenylethyl acetate as the main volatile substances (other

than ethanol) produced. The presence of 2-phenylethanol in the hind guts of emergent *D. brevicomis* males, and feeding *Ips paraconfusus* males has recently been reported (*447*). In field bioassays, the response of *D. brevicomis* was not affected by the addition of 2-phenylethanol to its known attractant, whereas the response of *I. paraconfusus* to male infested log sections was greatly enhanced by the addition of 2-phenylethanol (*447*).

The behavioral activity of the compounds isolated from the yeasts was tested in laboratory bioassays on pedestrian male and female *D. frontalis* (*446, 448*). In this bioassay procedure, a standard attractant mixture of frontalin : *trans*-verbenol : loblolly turpentine (1 : 1 : 12), referred to subsequently as the triplicate standard, was used. None of the yeast metabolites exhibited any activity alone. The two acetate esters were found to enhance the attractiveness, mainly of males, to the triplicate standard, especially at low concentrations of triplicate standard and ester. 2-Phenylethanol decreased the response of females to the triplicate standard. More recent results on the inhibitory effect of 2-phenylethanol on the response of females has shown that a concentration of triplicate standard that gives a response of 50—60% can be substantially decreased by the addition of 2-phenylethanol at concentrations up to 10^5 times lower than that of the triplicate standard (BRAND, unpublished data).

If it is assumed that these yeast volatiles are produced in the tree under active attack, it is very likely that they would be perceived by attacking beetles and could influence their behavior. *Scolytus multistriatus* often initiates attacks on bark directly over sapwood streaks, which are indicative of invasion of *Ceratocystis ulmi* (LANIER, *328*). The beetles' preference for attack at this site may be directed by odorants released through lenticels of the bark as it has been shown that *S. quadrispinosus* regularly initiates attack at the lenticels (*449*). Evidence has been obtained which indicates that host finding by the braconid, *Biosteres (Opius) longicaudatus,* involves attraction to specific fermentation products of fungi coming from rotting fruit, and not to compounds produced by host larvae (*450*).

We consider that thorough investigations on the origin of bark beetle pheromone blends will establish microbial systems as an important source. However, the real credibility of the examples cited remains to be firmly established; future results will modify or strengthen this idea. We hope that some of our statements will provoke discussion, criticism and experimentations among our colleagues.

E. Biosynthesis of the Bicyclic Ketals

The bicyclic ketals, frontalin (**195**) (*130*), *exo*-brevicomin (**150**), *endo*-brevicomin (**151**) (*62, 117*), and multistriatin (**303**), all shown in Chart 1,

8*

p. 8. have been identified from various bark beetles and appear to be of major importance in their pheromonal blends. No one has commented in any detail on the biosynthetic origin of these compounds. Let us consider frontalin. The immediate precursor of this bicyclic ketal may be either 6,7-dihydroxy-6-methylheptan-2-one (211, Scheme 43) or 6,7-epoxy-6-methylheptan-2-one (205, Scheme 42), both of which would have originated in 6-methyl-6-hepten-2-one (204). This latter compound is an isomer of 6-methyl-5-hepten-2-one. This methyl ketone is produced by one of the mycangial fungi (SJB-122) of female *D. frontalis* (*423*) but we do not wish to suggest at this time that the precursor of frontalin is produced by a mycangial fungus. However, it is food for thought. 6-Methyl-5-hepten-2-one is also produced by ants (*418, 419*) and certain other microorganisms (*421, 422*).

In a similar manner, the alicyclic precursor of multistriatin may be 4,6-dimethyl-7-octen-3-one (301, Scheme 55). Similar compounds have been obtained in alarm and defensive secretions of certain insects. Fales *et al.* (*451*) found that the mandibular gland secretion of the ant *Manica mutica* contained mainly 4,6-dimethyl-4-octen-3-one. Meinwald *et al.* (*452*) identified 4,6-dimethyl-6-octen-3-one in the defensive secretion of the daddy long legs, *Leiobunum vittatum*.

Gore *et al.* (*453*) recently analyzed extracts of emergent and boring females of *S. multistriatus* for 4,6-dimethyl-7-octen-3-one (301) and 4,6-dimethyl-6,7-epoxyoctan-3-one (302) but were unable to detect either compound. While these compounds are the most likely precursors of multistriatin, the possibility of a large pool of these intermediates is ruled out. Both the ant (*451*) and the daddy long legs (*452*) secretions also contained 4-methyl-3-heptanone (386a). This finding is pertinent as 4-methyl-3-heptanol (294) is a component of the pheromone complex of *S. multistriatus* (*146*). We must surely conclude that the biosynthesis of these various compounds is intimately related but no studies have been conducted in this area.

F. Boll Weevil Sex Attractant

Chemical studies on the sex attractant of the boll weevil led to the isolation (*454*) and identification (*38*) of four terpenoid compounds. Tumlinson *et al.* (*455*) suggested a hypothetical biosynthetic scheme in which all four compounds could be derived from a geraniol-like compound. Hardee (*456*) showed that male boll weevils required feeding for the synthesis of the attractant substances. Cotton squares proved to be the best diet, but pheromone production was demonstrated on a variety of diets. Mitlin and Hedin (*457*), using ^{14}C tracers, obtained evidence

that the biosynthesis of the pheromone compounds may be *de novo*. Adult males were injected with either $[1-^{14}C]$ acetate, $[2-^{14}C]$ acetate, $[2-^{14}C]$ mevalonic acid or $[U-^{14}C]$ glucose and the feces were steam distilled. Approximately 0.02% of the label was incorporated into the volatile fraction. While all four components accounted for only 39% of the volatile fraction they contained 57—80% of the radioactivity. It is assumed therefore that in spite of the boll weevil being an obligate insect of cotton, it does not seem to require any specific component in the cotton for the synthesis of the four terpenoid sex attractants.

HEDIN (458) has recently summarized factors that influence the biosynthesis of the boll weevil pheromone complex. Total synthesis was at a maximum during the summer and a minimum during the winter. In this study HEDIN (458) did two types of experiments. The first series exposed 10-day-old adult weevils to a saturated atmosphere of a number of terpene hydrocarbons. It was found that only myrcene and limonene produced detectable amounts of oxygenated substances and that males produced more than females. No compounds isolated suggested that the weevils are capable of cyclizing alicyclic terpenes or their pyrophosphates.

The second series of experiments conducted by HEDIN (458) involved the incubation of whole abdomen homogenates with added (+)-grandisol [(+)-*cis*-2-isopropenyl-1-methylcyclobutaneethanol, (33)] and (Z)-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol (110b, Scheme 18). After an overnight incubation at 37°, pentane extracts were analysed by GC-MS. Boiled homogenates and homogenates to which neither (33) nor (110b) were added served as controls. From the various identified products it was suggested that male abdomens possess three major enzymatic capabilities: (i) oxidation of the alcohols (33) and (110b) to aldehydes by a dehydrogenase, (ii) dehydration of the alcohols to hydrocarbons by a hydrase, and (iii) conversion to other alcohols by an isomerase. The dehydrogenase activity is the most significant as it can explain the oxidation of (110b) to (E)- (111a) and (Z)-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde (111b), two of the identified boll weevil sex attractants (Scheme 18).

The conclusion drawn by HEDIN (458) from these results is that male boll weevils possess enzymes that bring about the chemical transformations observed in the incubation studies. We suggest how this conclusion could be drawn with greater conviction. It has been reported by GUELDNER et al. (459) that the presence of a high bacterial load in the gut of boll weevils decreases the amount of pheromone produced, and BRAND et al. (439) have suggested that microorganisms may lead to the production or modification of certain pheromones in an insect gut. HEDIN (458) employed non-sterile abdomen homogenates for an overnight incubation at 37° in a pH 7.0 phosphate buffer. This medium would be suitable for

the growth of many microorganisms, and their enzymatic potential cannot merely be overlooked. Filter sterilization of the homogenates and incubation under aseptic conditions would make these experiments far more convincing.

G. Miscellaneous Labelling Studies

Biosynthetic studies usually imply the use of ^{14}C-labelled compounds, but the majority of studies on insect pheromones have dealt with their identification and synthesis and with field applications; very few have used ^{14}C-labelled compounds.

GORDON et al. (460) established that the defensive aldehydes, hexenal and decenal, are synthesized from [1-^{14}C]-acetate in the green vegetable bug, Nezara viridula var. smaragdula (F) and it has also been established that the ant, Acanthomyops claviger, synthesizes monoterpenes from acetate and mevalonate de novo (461). As mentioned previously, the boll weevil is capable of pheromone synthesis from labelled acetate, mevalonate and glucose (457). The pheromone components of the wax moth, Galleria mellonella, are n-nonanal and n-undecanal (462). Injection of labelled acetate, propionate and oleic acid into moths indicated that these aldehydes arise most readily from oleic acid (463). This insect contains a large amount of oleic acid (29.6—43%) in its total fatty acids, and its diet, is also a rich source (22.2% of the total fatty acids) (464). It was concluded from these studies that odd-numbered straight chain aldehydes are synthesized from many different precursors, both even- and odd-numbered.

The hairpencil secretion of the adult male bertha armyworm, Mamestra configurata, contains 2-phenylethanol (465, 466). Radioactive labelling experiments with male pharate adults indicated that it is synthesized from phenylalanine (466). The proposed biosynthetic pathway entails the irreversible loss of ammonia from phenylalanine to yield trans-cinnamic acid, decarboxylation to styrene, and hydration of the styrene to yield 2-phenylethanol (466). The production of 2-phenylethanol from phenylalanine is well established in microorganisms (467—470).

The ponerine ant, Paltothyreus tarsatus, produces various alkyl sulfides in its mandibular glands (471, 472). With the use of doubly labelled methionine, it has been established that the CH_3-S-group is incorporated intact but the origin of the third sulfur atom in dimethyl trisulfide remains unknown (473, 474).

H. Dietary Origin of Pheromones

The suggestion that the sex pheromone composition of the oak leaf roller was dependent to a large extent on diet was made (*82, 475, 476*) and refuted (*477, 478*). It is now generally accepted that the sex pheromone of this tortricid moth species is a specific blend (67 : 33) of (*E*)-11- and (*Z*)-11-tetradecenyl acetates (*477*). It is also accepted that in this species this ratio is not influenced by diet (*478*). However, the effects of diet on the amount of pheromonal compounds have been suggested for the summer fruit tortrix moth (*479*), the gypsy moth (*480*) and the smaller tea tortrix moth (*481*).

There are cases where the host plant supplies the precursor to compounds exhibiting pheromone activity. For example, adult male danaid and ithomiine butterflies are attracted to plants containing pyrrolizidine alkaloids which they modify into dihydropyrrolizines (*482—486*). These substances then occur in the hairpencil secretions.

The results obtained by RENWICK *et al.* (*379*) on the exposure of adult *I. paraconfusus* beetles to (+)- and (−)-α-pinene should be reiterated. (+)-*trans*-Verbenol and (+)-myrtenol were the major products in the hindgut after exposure to (+)-α-pinene whereas (+)-*cis*-verbenol and (−)-myrtenol were obtained after exposure to (−)-α-pinene. Therefore, variations in the enantiomeric composition of the α-pinene in trees under attack could influence the ratio of *cis*- to *trans*-verbenol occurring in the gut of this species.

The process of melanization in the desert locust, the tropical migratory locust and the brown locust is stimulated by crowded conditions and it was suggested that this process is influenced by an airborne pheromone (*487*). A pheromone, called locustol, was isolated and characterized as 2-methoxy-5-ethylphenol. It was postulated that this compound was formed by the degradation of lignin in ingested food in the crops of larvae (*488*) and a biosynthetic pathway has been proposed (*489*). The question has been raised as to whether microorganisms present in the crop may be responsible for the production of locustol (*490*) and recent evidence strongly suggests that they are (*491*).

The heteropteran, *Eurygaster integriceps*, uses ethyl acrylate and vanillin as components of the sex pheromone of the male (*492*). These compounds induce specific behavioral responses in sexually receptive females and may also act as short range attractants. Both compounds may be metabolic products from the degradation of ingested lignin (*492*).

VI. Chemosystematics and Speciation

The methodology employed in the identification of pheromones is usually rigorous and precise and not subject to personal opinion. How-

ever, the same degree of precision and impartiality cannot be applied to
the use of chemical data for solution of taxonomic problems. Chemo-
systematics would best be served by the detailed comparison of the enzy-
mology of the biosynthetic pathways. In addition, comparisons between
chemical compounds are best made by those familiar with the many
ramifications of metabolic pathways.

Some aspects of the value of pheromones in speciation of Coleoptera
have been discussed by Lanier and Burkholder (493). They concluded
that in spite of recent advances in the chemistry of pheromone systems
of beetles, our understanding of the role of pheromones in speciation is
limited, because the data are scattered among various groups of beetles
and good data on response specificity are lacking.

The interspecific attraction of males of certain *Trogoderma* species to
extracts of female beetles has been studied by Vick et al. (494) and by
Levinson and Bar Ilan (495). The extensive cross attraction between some
species and not between others suggests the involvement of several active
compounds. The degree of phylogenetic relatedness of seven *Trogoderma*
species has recently been studied by Greenblatt et al. (496). The most
important compound in the volatile fraction of four species is 14-methyl-
8-hexadecenal. The active isomers are *E* in *T. glabrum*, *Z* in *T. inclusum*
and *T. variabile*, and 92% *Z* : 8% *E* in *T. granarium*. This aldehyde is not
found in extracts of macerated females. The next most active compound
in these four species is the corresponding alcohol, 14-methyl-8-hexadecen-
1-ol, which is present only in trace amounts in the volatile fraction. How-
ever, this alcohol has been found in extracts of macerated females of
T. glabrum (*E*-isomer) (497), *T. inclusum* (*Z*-isomer) (498), and *T. variabile*
(*Z*-isomer) (499). The third most active compound is the corresponding
ester, methyl 14-methyl-8-hexadecenoate and is found in *T. inclusum*
(*Z*-isomer) (498) and *T. glabrum* (*E*-isomer) (497).

Greenblatt et al. (496) concluded that the response of males to
calling females is largely due to the aldehyde, and that the response to
extracts of macerated females is due largely to the alcohol. Strong
interspecific responses between *T. inclusum*, *T. variabile* and *T. granarium*
are a consequence of the presence of the *Z*-isomer of each pheromone
component and the different response of *T. glabrum* lies in its use of the
E-isomer. Neither *T. sternale* nor *T. grassmani* respond to the *Z*- or *E*-
aldehyde (496). *T. simplex* males respond strongly to extracts of *T. inclu-
sum*, *T. granarium* and *T. variabile* females which would indicate that
all four species share one or more pheromone components and that
T. simplex probably emits the *Z*-aldehyde.

On the basis of these and other data (morphological and interbreeding
responses) Greenblatt et al. (496) presented a cladogram of the possible
relationship between these seven species. *T. sternale* and *T. grassmani*

References, pp. 157—190

were placed together in one group and *T. simplex* was placed in a group on its own. *T. inclusum* and *T. variabile*, both of which have the Z-isomer of the aldehyde, are considered to be closely related, while *T. granarium* and *T. glabrum* were placed off a fourth main branch (*T. glabrum* has the *E*-isomer of the aldehyde and *T. granarium* has a mixture of *E*- and *Z*-isomers). Chemical investigations on the pheromones of *T. sternale* and *T. grassmani* are continuing.

Many sympatric tortricine moths possess overlapping chemical communication systems because at least one component of their pheromones is either a 14-carbon chain acetate, alcohol or aldehyde, with unsaturation in the C_{11-12} position. The species recognition of distinctive pheromone blends, together with non-pheromonal reproductive isolating mechanisms such as habitat preferences and differential mating times have been discussed by CARDÉ *et al.* (*500*). This article, together with the literature cited therein, should be read to obtain an informed view of pheromone specificity and its significance in tortricine moths.

(*Z*)-11-Hexadecenyl acetate is an important component of the sex attractant blend of several noctuid moths. Species specificity of blends containing common constituents can be attributed to the presence of different additional coattractant compounds, and each coattractant could be effective for a single species, or the ratio of components may be the deciding factor (*270*). Most laboratory and field bioassays are designed to show attraction. However, STECK *et al.* (*501*) have recently reported some experiments designed to show interspecies inhibition in a group of moths, all of which require (*Z*)-11-hexadecenyl acetate. Their results indicate that dual attractant/inhibitor roles are common occurrences among sex attractant compounds. The generality of this phenomenon is being investigated, as it could be of importance in the use of atmospheric permeation with pheromones for mating disruption in pest species.

The European corn borer (ECB), *Ostrinia nubilalis*, is known to employ different ratios of its sex attractants, (*E*)- and (*Z*)-11-tetradecenyl acetate, in various geographical areas. KLUN (*502*) has recently obtained some particularly interesting and significant results on this pest insect. (*E*)-9-Tetradecenyl acetate has always been observed in GC analyses of heptane surface washes of individual female ovipositors. This suggests that this compound is part of the insect's pheromone signalling system. However, in field and laboratory assays, this compound suppressed male attraction and precopulatory behavior. By the use of mixtures of (*E*)-9-tetradecenyl acetate and *Z* : *E*-isomer blends of 11-tetradecenyl acetate in bioassays, it was suggested that these positional isomers are perceived through separate sensory channels. The production of an "anti-sex substance" by the ECB is enigmatical. As (*E*)-9-tetradecenyl acetate does not deter the redbanded leafroller, a species that also

uses a $Z:E$-isomer blend of 11-tetradecenyl acetate as a sex pheromone, it does not serve a role in maintaining pheromonal specificity between these two species.

The likelihood of genetic control of the ratio of pheromone components has occurred to many. Some recent results provide an exciting beginning to what is bound to follow on this topic. Bioassays of hybrids of species in three groups of *Ips* bark beetles showed that hybrid males were intermediate in attractiveness to the parental types (*493*). The possibility that hybrid females were slightly more attractive to males of their own kind than to those of the two parental species was also suggested. However, in these bark beetles it was concluded that the genes controlling pheromone production and reception were not sex linked (*493*). The genetic basis of intraspecific pheromonal polymorphism in the ECB has been investigated more recently by Klun (*502*). The Iowa strain of the ECB responds maximally to a 97:3 ratio of $Z:E$-isomers of 11-tetradecenyl acetate. The reverse is true for the New York strain. Klun and coworkers have now shown that the geometric isomer composition of 11-tetradecenyl acetate in the female secretion is controlled by single Mendelian inheritance involving one pair of genes. The female AA genotype secretes an isomer blend of approximately 97:3 ($E:Z$) and the aa genotype secretes a blend of approximately 3:97 ($E:Z$). F_1 hybrid females from crosses between Aa and aa genotypes secrete an isomer mixture that approximates 65:35 ($E:Z$). Of particular significance is the finding that F_1 males from the same cross respond preferentially to the 65:35 $E:Z$-isomer combination rather than to either of the parental mixtures.

Therefore, both the isomeric composition of the sex pheromone secretions in female ECB and the mechanism of isomer-ratio perception in male ECB are genetically regulated. These studies by Klun and coworkers clearly demonstrate the need to know the genotypic profiles of females and the isomeric ratios of their sex attractants at any location where behavior-modifying chemicals are to be used for the suppression of this species. Cardé et al. (*502a*) have recently suggested that the E- and Z-strains of the ECB may be semi- or sibling species.

Many species of ants exhibit a synchronized swarming of male and female alates from many nests thereby ensuring that large populations of reproductives are available to each other at the same time. The coordination of this synchronized swarming in alates of the carpenter ant, *Camponotus herculeanus*, is governed by volatile substances secreted from the mandibular glands of the males, as well as by climatic factors (*503*). It has been reported that the males of a number of *Camponotus* species contain substances in their mandibular glands which do not occur in females or workers (*504, 505*). The quantitatively most

References, pp. 157—190

important compounds among those species studied were methyl 6-methylsalicylate, methyl anthranilate and mellein (3.4-dihydro-8-hydroxy-3-methylisocoumarin) (*504, 505*). The possibility that these caste-specific compounds may play a role in the regulation and coordination of swarming was inferred, but not established, and their application as an additional aid to the taxonomy of this large genus of ants was indicated.

The volatile products in the heads of a large number of Nearctic species of *Camponotus* have been studied more recently by DUFFIELD (*506*). In general, mandibular gland secretions were restricted to males although some species produce the same compounds in both sexes and castes. When a compound was produced by both sexes and castes it appeared to be a non-specific alarm pheromone, but the role of cast-specific compounds was not demonstrated. The results obtained by DUFFIELD (*506*) led to the preparation of a taxonomic key to the various species studied which is based primarily on chemical characters.

The identification of 6-methyl-5-hepten-2-one as the major detectable volatile substance in the mandibular gland secretions of workers of eight *Formica* species in the subgenera *Neoformica* and *Proformica* (*419*) indicates that this ketone may have some taxonomic value in this ant genus.

VII. Practical Applications of Pheromones: Status and Projections

A. Plea for Sanity and Integrated Pest Management

The era immediately following World War II saw the intensive use of organopesticides, starting with the archetypal DDT—the agricultural analog of the modern "magic bullet" of medicine, penicillin. The pesticides salesman prescribed his magical potions on some arbitrary schedule and vast amounts were applied worldwide. Extraordinary successes abounded. Crop yields were increased, pest populations declined and millions of lives were saved throughout the world, but the long-term effects of such indiscriminate application of pesticides were neglected. FLINT and VAN DEN BOSCH (*507*), and DETHIER (*508*) eloquently describe how preventive pest control practices were discarded and biological studies languished as reports on insecticide testing proliferated. Entomology, for a generation, suffered under the stigma of being the only branch of science dedicated to eradicating what should have been the object of its study. It should be noted, however, that basic studies of insect toxicology and metabolism flourished.

The world is never without prophets and during this era, several spoke; and they—in the usual manner of prophets—spoke in vain. Development of resistance to insecticides was the first theme of the

prophets' warnings, and as the inevitable happened, the predictable—and profitable—response was to increase the amount and frequency of application. Rebound of the target pest began to be noted as broad spectrum pesticides wiped out the natural enemies of the pest. In a very short time, new major pests were *created* as minor pest populations, freed of their natural enemies, exploded to devastating levels. Finally the problems of environmental contamination by persistent pesticides were brought home to the public by the appearance in 1962 of "Silent Spring" (*509*).

Since then the excesses of pest management seem to have peaked; starting with the ban on DDT in the United States in 1972, far-reaching, decisions have severely restricted the uses of other hard pesticides. The concept of "Alternatives to Hard Pesticides" was taking hold, and during the 1960s these efforts multiplied and resulted in a number of promising approaches. At the present time, we can list, in addition to sound agricultural practices, a fair number of such alternatives:

1. Biological control through manipulation of predators, parasites, and disease organisms.

2. Genetic selection of resistant plants.

3. Reproductive suppression by radiative or chemical sterilization.

4. Introduction of reproductively incompatible strains.

5. Use of hormones and hormone analogs.

6. Use of behavior modifying chemicals, of which pheromones and pheromone analogs are most important (*507, 510—517*).

Gradually the concept of Integrated Pest Management developed as an integration "of all of the factors impinging on the pest control decision so as to determine when control should be practiced and what would comprise the best method or combination of methods to employ" (*510*). Synthetic chemical pesticides would be relegated to an appropriate role within a holistic program that considers entire ecosystems.

In the following we will deal with the present and potential roles of behavior modifying chemicals (mainly pheromones) as one of the tools available to the manager of an integrated pest management system.

At the outset, we must state our own biases as participants in pheromone research. We subscribe to the statement from "Advancing toward Operational Behavior-Modifying Chemicals" (*518*): "Some observers have evidenced impatience with the various programs that have been directed toward the development of behavior-modifying chemicals (especially the pheromones) for insect pest management. However, it must be recalled that almost all of the practical research directed toward operational control programs has been initiated only during the past 5 years [written in 1975] and by a small number of investigators. Additionally, only limited resources have been made available for this

research. Thus, on any reasonable basis of expectation in science, progress should be considered quite remarkable." The lack of basic information on insect behavior and population dynamics of the important insect pests is obvious. The problems of determining reasonable economic thresholds are complicated by expectations—unblemished apples for example—that are themselves unrealistic. The basic problem of devising control situations against which to assess the effects of manipulating insect populations is a formidable one. Nonetheless, assessments of control strategies have been attempted, and as we shall see, with at least partial success. G. E. DATERMAN (private communication, *519*) believes that "for control purposes, the appropriate question is not 'will pheromones be used', but 'how soon'."

The availability of synthetic pheromones has provided a remarkable tool for investigation of insect behavior, ranging from studies of potentials generated in antennal receptors to studies of responses in the field over large distances. Practical applications can be categorized as follows:

1. Trapping to collect species that are otherwise difficult to obtain.

2. Trapping for monitoring and survey; timing of pesticide treatment can be based on results.

3. Luring to areas that are treated with pesticides.

4. Luring to areas that are treated with pathogens, which can then be spread by the infected individuals to the rest of the population.

5. Mass trapping for population suppression.

6. Disruption of communication by permeation of areas of insect population. In most cases, the most important function that can be disrupted is mating, the end result being population suppression.

Parapheromones (chemicals that mimic pheromones) or anti-pheromones (those that block responses), which are not part of the natural communication system, may also be used in some of these categories. A list of pheromones, parapheromones, and anti-pheromones active in the field has been compiled by INSCOE and BEROZA (*40*).

At the present time the first two categories are operational, although more research is needed to relate trap catches to insect populations. Use of pheromones for population suppression by the other techniques is now in the "highly promising" stage for many pests, and is operational for several; we shall consider the promises and problems on a crop-by-crop, insect-by-insect basis. Coverage will be selective and emphasis will be on the more advanced systems and those most familiar to the authors.

Although the U. S. Environmental Protection Agency (EPA) is constrained by law to treat pheromones as pesticides for registration purposes (*520*), it should be obvious that, operationally, there is a vast difference between spraying pounds of a liquid or solid, persistent, broad-spectrum pesticide to kill insects, and releasing a fraction of a gram of

a biodegradable, species-specific, natural product in the vapor phase to lure an insect to a trap or to disorient the matefinding process.

Certainly, individuals within the EPA will acknowledge these differences in private conversations and there is some indication that official policy will be modulated; at this writing, EPA is handling pheromone registration on a case-by-case basis. A Task Group convened by the American Institute of Biological Sciences is advising EPA on efficacy test procedures. The EPA response will be critical to the role of behavior modifying chemicals in control programs, because an unrealistic policy will stifle the already marginal interest of industry in these materials. The wary posture of EPA is motivated in part by past industrial practices resulting from lack of knowledge and from the inherent need for short-term benefits; because of this, the innovative industrialist is penalized by unrealistic restrictions (521).

For the present, at least, the impetus for promoting the use of behavior modifying chemicals within the context of integrated pest management must come from government agencies and private foundations. A vast amount of basic behavioral research is needed, but eventually the materials and methodology must be produced by industry under government regulation, and given the nature of the task, very likely under some form of government subsidy. Some cost estimates involved in bringing behavior regulating compounds from chemical synthesis to commercial use were presented by Siddall and Olsen (522), who conclude that not even a research-oriented concern can currently justify development of a pheromone for control of an insect pest by disruption of its communication system.

An interdisciplinary group has examined the economies from the industrial viewpoint of several components of pest insect control: bacteria, viruses, pheromones, conventional pesticides and a few miscellaneous agents. The following table from their report (511) summarizes the relative likelihood of pest suppression programs using pheromones:

	Relative Likelihood*	
Pest	*1980*	*1985*
Pink bollworm	3	3
Stored products pests	3	3
Western pine beetle	2	3
Boll weevil	1	2
Codling moth	1	2
Leaf roller complex	1	2
Cabbage looper	1	2
Tussock moth	0	1
Gypsy moth	0	1
Spruce budworm	0	1
European corn borer	0	1
Southern pine beetle	0	1

* Relative likelihood: 0 = negligible
1 = low
2 = medium
3 = high

This report also presents a detailed decision analysis for the manufacture of gossyplure, the pheromone of the pink bollworm. The material was to be used in permeation schemes to disrupt mating of the pink bollworm in the cotton fields of the Southwest. The conclusion was that such a venture "appears modestly attractive". Although some of the assumptions were based on solid research results, many of them were necessarily somewhat arbitrary. Note that the report is already dated by recent progress. For example, mass trapping of the ambrosia beetle *Gnathotrichus sulcatus* is now on a commercial basis (see below).

References, pp. 157—190

In the time frame of overall pest control we should realize that the first *field* test involving a correctly identified insect pheromone component was reported by GARY in 1962 (*19*), who showed that flying honey bee drones were attracted to (*E*)-9-oxo-2-decenoic acid (**338**), a component of the mandibular gland of virgin queens. BUTLER and FAIREY in 1964 (*20*) similarly showed that another component, (*E*)-9-hydroxy-2-decenoic acid, was also attractive. However, the honey bee is hardly a pest insect. In 1966, SILVERSTEIN, WOOD, and RODIN (*525*) reported that both sexes of *Ips paraconfusus* (formerly *I. confusus*), the California five-spined Ips, were trapped from a natural population by the 3-component pheromone isolated from the males. Results of further field tests by WOOD *et al.*, reported in 1967 (*526*), demonstrated the synergistic effects of the three components, and a masking or species isolation effect towards a sympatric species, *Ips latidens*. The extensive field studies of the early 1960s on the gypsy moth were carried out with a compound that had been erroneously described as the sex attractant.

As noted earlier, a small group of investigators has, in a very brief period, established behavior modifying chemicals as one of the more promising components of integrated pest management. We shall sample the opinions of several investigators active in developing this methodology. An earlier general review which includes some references from 1973 should be consulted (*527*). WOOD (*528*) recently discussed manipulation of several forest insect pests, and reviews by ROELOFS (*529*) and MITCHELL (*530*) are in press at this writing. A brief discussion of some of the variables in trapping is given by MINKS (*531*). BONESS *et al.* (*532*) have briefly summarized studies carried out by the Bayer AG group on a variety of insect pests in Europe. They describe several successful experiments, but overall results varied widely. BONESS (*533*) points out that pheromones are a potent tool, but do not readily lend themselves to manipulation by industry without involvement of official agencies.

This section will review selected field tests that have been carried out to establish monitoring or control systems with behavior modifying chemicals. Actually, four orders of insects, Lepidoptera, Coleoptera, Hymenoptera, and Diptera, contain most pests for which manipulation by behavior modifying chemicals has been attempted. (A few studies of tick pheromones have been reported.) It is convenient to divide them into pests of forest and shade trees, orchard trees and vines, field crops, stored products, and those that directly afflict humans or animals.

B. Forest and Shade-Tree Insect Pests

1. Coleoptera

Many coleopteran pests of forest and shade trees produce an aggregation pheromone that attracts both sexes of adults, which represent

the destructive stage. This is in contrast with many lepidopteran pests whose sex attractant is specific for the adult male and whose larvae are the destructive stage. Thus, manipulation of coleopteran populations, from this point of view, should have a greater impact than manipulation of lepidopteran populations.

a) Western Pine Beetle

The aggregation pheromone of the western pine beetle. *Dendroctonus brevicomis*, which kills stressed or even apparently healthy ponderosa pines, consists of three components, one produced by the male, one by the female, and one by the host tree (*266* and refs. therein). Wood (*528*) and Wood and Bedard (*534*) have summarized the results of large-scale attempts at population suppression based on mass trapping. Wood *et al.* (*515*) discuss integrated pest management of the western pine beetle; Bedard *et al.* (*535*) discuss the role of behavior modifying chemicals in the management of the western pine beetle.

Since the survival of the beetle depends on overwhelming individual trees in the aggregation phase mediated by the pheromone, control strategy depends on reducing the intensity of these attacks. Two approaches have been used. 1. Large sticky traps baited with the pheromone have been distributed throughout the area of infestation to reduce the number of beetles available to attack trees (trap-out method). 2. The forest canopy has been permeated either with the pheromone or with an anti-attractant.

In 1970, an experiment was carried out to suppress the western pine beetle population in plots within a moderately infested area of 65 km² at Bass Lake, California. Large baited sticky traps in a grid pattern captured approximately one million beetles—essentially the total population estimated for the area—and the tree mortality dropped from 283 ±89 before the experiment to 91 ±28 afterwards; tree mortality remained low for the following four years. A second experiment was carried out on a much larger scale in a more heavily infested region at McCloud Flats, California. Although about 7 million beetles were trapped, the total population was not decreased enough to affect tree mortality. However considerable redistribution of the population occurred in response to the pheromone. Results are still being analyzed. The following is a statement from Wood (*515*): "Evidence from these two experiments indicates that western pine beetle populations can be manipulated on a large scale. This strengthens our belief that an effective control strategy can be developed using methods similar to those employed in these experiments. ... Because chemicals used in this method are considered pesticides by the Environmental Protection Agency (EPA)

References. pp. 157—190

they are subject to the same scrutiny as insect toxicants in the areas of efficacy, toxicology, and production and environmental chemistry to insure that any recommended use will be safe and effective ... The compounds used in these field tests: 1. were nontoxic in our panel of tests; 2. occur in nature; 3. would be applied only in their normal environment; 4. would probably be applied at rates that do not exceed those found in nature and 5. are not introduced into plant, animal, soil, or aquatic systems (the trap-out method employs bait stations). We feel therefore that there is a high likelihood that these compounds can be registered under EPA requirements."

As part of both the Bass Lake and the McCloud Flats population suppression experiments, small survey traps (in contrast with the large suppression traps) were located on a grid pattern throughout the area to monitor the in-flight population through time and space. Studies to interpret and correlate the results are still in progress. Small survey traps for early detection of infestations are on extremely valuable contribution. If relationships between trap catch, insect population, and tree damage can be developed, this information will form an important basis for management decisions.

Permeation with the 3-component pheromone over a 0.81 hectare plot prevented the beetles from being trapped on traps baited with the same pheromone in the center of the treated area (536). When verbenone was released from formulations attached to ponderosa pine trees that were also baited with the pheromone, no mass attack occurred on these trees, in contrast to the mass attacks that occurred on trees that were simply baited with the pheromone. Further development of this approach is warranted to develop a method for protecting individual high-value trees.

One further feature that requires additional study is the effect of the pheromones on natural enemies. VITÉ (537) cites this effect as a disadvantage of trap-out; he prefers the use of baited trees when timely removal is feasible.

As a general summary statement, the following, taken from WOOD and BEDARD (534), will serve: "Attractant pheromones used over large areas have potential for estimating the size and distribution of WPB [western pine beetle] populations, and for manipulating populations as means of determining the interactions of adult WPB populations with its host and natural enemies. Further, these attractans can be used experimentally to redistribute populations so that we can study the dynamics of population behavior. At the same time, we can expect that the outcome of this research will have immediate practical benefits for the use of behavioral chemicals in pest management systems."

Wood (*538*) further suggests: "It is important to note that only very limited attempts have been made to develop the use of any of the available compounds in pest management programs. In a few cases where attempts have been made, the reasons for failure or limited success have not been made clear. Undoubtedly, the high cost of research following the identification phases and the inherent complexities of reasearch on the dynamics of highly mobile, widely distributed pest populations are the underlying causes for the current status of IBRs [Insect Behavior Regulators] in forest pest management."

b) Gnathotrichus sulcatus

The aggregating pheromone produced by the male ambrosia beetle, *Gnathotrichus sulcatus,* consists of a "single" compound, actually a 65/35 mixture of two enantiomers. In field tests, traps baited with the racemic mixture of this compound, sulcatol (**22g**), caught large numbers of both sexes of *G. sulcatus* in competition with natural host and beetle odors. Since infestation of freshly sawed lumber is of continuing concern to the forest industries of British Columbia, a survey to determine the population distribution and seasonality of flights within a commercial sawmill at Chemainus, B. C. was undertaken in 1974 (*539*). The results indicated that "sulcatol could be used as an inexpensive, reliable detection and survey tool that is considerably more accurate than currently used, visual methods". Furthermore, it was suggested that sulcatol and other scolytid pheromones might be used "as a sensitive detection tool at unloading and processing areas" in countries that import logs and un-seasoned lumber. Finally, it was proposed that "sulcatol should be tested as a means of intercepting *G. sulcatus* [beetles] before they are able to infest valuable lumber. Unlike programs directed at bark beetles in large tracts of forest ..., a sawmill-based program would challenge a more limited and potentially manageable population. This situation is particularly true for the Chemainus sawmill in which the only source of beetles appears to be infested logs transported to the mill site from distant logging operations."

Suppression experiments were carried out at the Chemainus sawmill in 1975. The conclusions (*540*) were that "sulcatol baited traps can capture most of a *G. sulcatus* population, that 2-to-4-week old lumber sawed from sapwood is attractive to flying beetles in sawmills, and that highest attack densities can be expected on loads [i.e. sawed lumber] adjacent to sulcatol-baited traps". The recommendation was made that piles of freshly sawed sapwood slabs be placed around the mill site and that "sulcatol-baited traps placed alongside each slab pile would attract and capture most beetles; those not caught could attack the slabbing, ..

which could then be removed and chipped . . .". These recommendations have been accepted and the procedure is in commercial operation at the Chemainus sawmill site "as part of their normal quality control operation" (*317*). BORDEN's general assessment is that "with lower beetle populations in limited areas in an uneven aged forest, I do believe that pheromone-based mass trapping, disruption, and tree-baiting techniques will find a useful place in forest pest management". However he feels that efficacy has not yet been demonstrated for general use, but sees immediate application for sawmills and dryland sorting areas for logs on the west coast. In these cases, "resident beetle populations, and the area to be protected, will be relatively small. Moreover, the prior investment in growing, surveying, harvesting, and hauling timber by the companies justifies a considerable additional investment in beetle control to protect a high value product . . .".

c) European Elm Bark Beetle

Introduced from Europe half a century ago, the European elm bark beetle, *Scolytus multistriatus* (together with its associated pathogenic fungus) has virtually eliminated the elm as a shade tree throughout most of the United States. Weakened or dying elms emit volatiles that are weakly attractive, but the addition of volatiles produced by boring females greatly increases the rate of attack by both males and females so that the tree is rapidly overwhelmed by the Dutch elm disease fungus. In 1970, efforts were initiated to mass-rear beetles in elm bolts and collect the volatile compounds for isolation and identification. The most effective procedure for collecting volatiles was by aerating the boring females and trapping the volatiles by passing the airstream through Porapak Q. (*541*). The isolation procedure was monitored by laboratory and field bio-assays, and three compounds were identified; two are produced by the female beetle and one by the stressed tree, each synergistic as components of the aggregation pheromone. The compounds were synthesized; a field test in 1974 confirmed that the mixture (multilure **283, 294a, 303a**) was a powerful aggregating agent (*146*). Since that time, baited traps have been used in many locations in attempts to detect, survey, and suppress populations (*542*).

In 1974 and 1975, large sticky traps were deployed in a grid pattern throughout heavily infested areas in Detroit, Michigan. In 1974, the approximately 1,000,000 beetles trapped in one section were estimated to represent only 20% of the population, and no significant impact on tree mortality was noted. In 1975, an improved trap and formulation resulted in trapping nearly 4,000,000 beetles, and in an actual increase in the number of attacked trees in the treatment area. Apparently the

grid deployment within a treatment area resulted in significant beetle immigration from adjacent areas.

Tests now in progress are based on a barrier trapping strategy. "Multilure-baited traps are deployed on utility poles or non-elms in one or more rows encircling an area containing high-value elms. The area may be an entire city with several thousand elms, or it may be a relatively small area having only a few (25—100) elms. In theory, the barrier traps will lure beetles out of the area within the barrier, and will intercept beetles flying in from outside of the barrier. The annihilation of these beetles should reduce the number of beetles within the plot that are available to feed on, or breed in, elms, thereby bringing about a corresponding reduction in the incidence of beetle-vectored DED [Dutch elm disease]." (543). Such a large-scale barrier trapping system is in effect at Ft. Collins, Colorado, and at Evanston, Ill. At Ft. Collins, the area involved is 3 × 5 km and contains 4,300 American elms *(Ulmus americana)* and 8,000 Asiatic elms *(U. pumila)*. The plot at Evanston is 346.5 km and contains about 15,000 elms, mainly *U. americana*. The elm population in Ft. Collins is virtually isolated, whereas the Evanston elms are contiguous with other elm forests to the north. Survey traps are deployed in both cities as well as in the control cities of Loveland and Greeley, Colorado.

In both test cities, a large drop in beetle population occurred, presumably as a result of the barrier traps. No significant effect on tree mortality, however, could be found during the single trapping season. The investigators propose to continue the study for a total of five years and they expect to see a decrease in tree mortality over this period. Such factors as "annual variation and lag between inoculation and symptom expression" require a longer test period.

In a complementary series, LANIER and collaborators (329) are conducting a number of barrier trapping tests by encircling 12 small discrete clusters of elms in 8 eastern states. Over the past three years, the "DED (Dutch elm disease) rate is down in every area. Whether or not, we can credit this effect to beetle trapping will depend on the accuracy with which we can determine historical rates in comparable areas where trapping is not done."

Another study was initiated in 1976 by BIRCH and collaborators (327) to survey and suppress the beetle population in the towns of Lone Pine, Independence, and Big Pine all in Owens Valley, California. These towns are separated from one another by at least 24 km of open, high elevation desert, and each contains a moderate number (300—500) of elms and beetles, but apparently no Dutch elm disease. Traps were deployed on the perimeter of Lone Pine, throughout Independence on a grid pattern and throughout Big Pine in four lines. Traps were also

placed between the towns and in outlying areas. A total of about 600,000 beetles were trapped in the three towns. Some of the outlying traps that were 8 or more km from any known elm also caught beetles. Apparently the beetles may disperse over greater distances than previously suspected. Some brood sources in dead branches in trees within the towns were located, but apparently the major source of beetles was elm woodpiles.

Further trapping was carried out during 1977 to correlate trap catches with beetle population and to assess the impact of the 1976 trapping. The first and second counts for 1977 were about 3% and 40% of the corresponding counts in 1976. It is tempting to ascribe these results to the trapping program, but obviously other factors could be involved. The major source on one town for 1977 appeared to be a stand of elms that died from lack of water. Since the disease is not present, it is obviously not possible to relate any population decrease to tree mortality.

BIRCH' assessment (327) for pheromones in general is: "Outlook good (some imminent successes) with integrative use and with increased biological input." On control of the Dutch elm disease by trap-out, he is dubious about the feasibility of attempts on the scale of the Detroit experiment, but feels that pheromones will certainly play a role as part of an integrated strategy applied to small discrete areas.

ARCIERO (544) has been trapping elm bark beetles throughout the Bay area counties in California to study the seasonal variation in population and to estimate how many beetles are carrying the Dutch elm disease fungus.

d) Douglas-Fir Beetle

The Douglas-fir beetle, *Dendroctonus pseudotsugae,* periodically builds up to epidemic levels mainly in windthrown Douglas-fir trees, and several attempts have been made to prevent this build-up with behavior-modifying chemicals in the Idaho-Montana region. The female produces two attractant compounds, which are synergized by host volatiles. However, a compound, 3-methyl-2-cyclohexen-1-one (MCH, **35**), produced mainly by the male, apparently functions to shut down the aggregation, and this "antiaggregative pheromone" was used in 1974 by FURNISS *et al. (545)* to reduce the level of attack on felled trees.

In 1975, FURNISS *et al. (546)* carried out a series of tests with several controlled-release formulations of MCH on 11 m × 43 m plots, each containing a single felled Douglas-fir tree. Attack density and brood density were significantly reduced by some of the MCH treatments. A pilot test is planned for 1978 if sufficient windthrown Douglas-fir becomes available during the previous winter. Plants (private communication, 547) call for application of formulated MCH by helicopter

on 4-hectare plots. "Evaluation would include measurement of dosage received, counts of frass on the windthrown trees in June, and measurement of number of egg galleries and progeny on bark samples in late summer. Resultant infestation of live trees near the plots may be evaluated also, depending on the presence of untreated windthrow outside the plots." Furniss points out the difficulties in utilizing aggregative or antiaggregative compounds in procedures for control of bark beetles in forests, but, on balance, he favors the use of anti-aggregative compounds: "The trick is going to be to see what level of attack density is required to obtain a lessening of damage to live trees by the subsequent beetle generation." He summarized as follows: "...Antiaggregative pheromones may still prove useful in disrupting attraction to beetles such as the pine engraver, Douglas-fir beetle, and spruce beetle, all of which depend on felled trees for massing numbers necessary to cause significant damage in the forest."

The Douglas-fir beetle has also been attracted with pheromones to "bait trees" that were scheduled for logging (548). No attempt at economic assessment was made, but the method is certainly limited to areas that are readily accessible and are scheduled for prompt logging.

e) Southern Pine Beetle

"Aggregation of the southern pine beetle, *Dendroctonus frontalis* ... on loblolly pine *(Pinus taeda)* under beetle attack was not disrupted by aerial application of frontalure, which is a mixture of the attractant pheromone frontalin (195) and the host terpene α-pinene (230). Instead, aerial saturation with the pheromone in a heavily beetle-infested pine forest resulted in a rapid increase in the aggregation of beetles on pine trees undergoing attack" (549). In this experiment, a ten hectare section of pine forest including 1.6 hectares of a *D. frontalis* infestation was treated twice by aircraft with rice seed soaked with frontalure. This formulation released virtually all of the frontalure within 24 hr (45 g/hectare for the first application and 450 g/hectare for the second).

"Reduced landing of beetles on host trees" was achieved by Payne et al. (549) with a mixture of *endo*- and *exo*-brevicomin (150, 151) from a controlled-release formulation in dispensers positioned throughout the test plot, which consisted of a 3×3 grid of 15 m^2 blocks. A total of 36 dispensers was used, each releasing ca. 1.5 mg/day of each compound (300 mg of each compound/hectare/day over a period of 30 days). Addition of verbenone (233) to the brevicomin mixture gave a 74% reduction in beetles landing on treated trees, which also showed a significant decrease in the number of galleries constructed (550).

Earlier studies (1971 and 1972) involved the use of frontalure to bait trees that were killed with cacodylic acid to reduce brood survival (551, 552). No further work in this direction has been reported.

f) Miscellaneous Bark Beetles

Spruce beetles *(Dendroctonus rufipennis* Kirby) were attracted to lindane-treated trees baited with frontalin (553), but baited trees could not compete with windthrown trees (554). FURNISS (547) reports the following studies in progress: tests of the Douglas-fir beetle pheromone components against the Eastern larch beetle *(Dendroctonus simplex* Le Conte) in Alaska; use of the antiaggregative compound MCH against the spruce beetle *(D. rufipennis)* in white spruce in Alaska, the anti-aggregative effect of ipsenol against the pine engraver, *Ips pini*, in ponderosa pine in Idaho, and similar studies involving both ipsenol (240) and ipsdienol (266a). The mating response of the European pine shoot moth, *Rhyacionia buoliana*, was disrupted by a controlled release formulation of the pheromone in small (3 m × 3 m) plots (555).

2. Lepidoptera

a) Gypsy moth, *Lymantria dispar* (formerly *Porthetria dispar)*

The gypsy moth, because of its highly visible defoliation of trees in populated areas and its rapidity of spread, has been the target of intensive efforts to eradicate, or at least control, this introduced pest. Use of massive amounts of hard pesticides for this has generated controversy, and alternative measures have also been unsuccessful, albeit less counter-productive. The pheromone was identified and named disparlure in 1970, and a number of promising field tests have since been carried out. In his review of the ecology and control of the gypsy moth, LEONARD (556) doubts that the "brush fire response" [present authors' term] to cyclical outbreaks has accumulated basic knowledge of the biology and ecology of the insect in proportion to the time and money spent.

In 1973, CAMERON (557) considered the decidedly mixed results of the 1972 trapping and confusant tests with disparlure, and stated: "There is reason for optimism." However, he pointed out the serious lack of behavioral studies and decided that "we do not now have the data which would justify any operational control programs in 1973; we need at least one more research field season for extensive testing." He concluded: "I seriously doubt that we ever will be able to manipulate well-established populations of the gypsy moth solely with disparlure (536). Its use is likely to be confined to fringe, newly infested, or isolated areas with very low populations, unless, of course, other agents can reduce outbreak pop-

ulations to levels at which disparlure may be effective. Under no circum-
stances can I see eradication of the gypsy moth from North America, and
the use of disparlure, in whatever manner, to eradicate local infestations
is still open to question. And, I feel that our attempts to establish
a barrier zone to confine the insect to the Northeast are still premature.
Evidence in hand simply does not support the practicality of this approach.
Disparlure is a potential tool for gypsy moth population manipulation.
We still have not been able to determine how to utilize its potential effec-
tively."

CAMERON and MASTRO (558) reported on the results of a 1974 ex-
periment designed to test whether subsequent application of disparlure
to an area treated with a pesticide would further reduce the population:
"With the technology and formulations available, we could not demons-
trate that disparlure further reduces a gypsy moth population sprayed
with Sevin 4 oil during the larval stage."

In 1974, CAMERON (559) felt that disparlure is operational for survey
and detection, but not for "population monitoring and/or prediction,
eradication of isolated infestations, the establishment of a barrier zone,
or use in a chronic infestation". In 1975, CAMERON et al. (560) reported
that the olefin precursor (535) of disparlure, which had earlier been
proposed by CARDÉ et al. (561) as a natural inhibitor of the attractant,
did not disrupt mate-finding or reduce trap catches to useful levels.

BEROZA (562) summarized the results of large scale disruption ex-
periments carried out in 1974: "Overall, the 1974 field results indicate
that air permeation with slow-release disparlure microcapsules applied
at the rate of 20 g lure/hectare is effective in reducing mating success in
low-level infestations of the gypsy moth or in infestations brought to
low levels with an insecticide." To date, there is no assessment of the
result of disparlure treatment on defoliation over a large area.

The state of knowledge of pheromone-mediated behavior of the
gypsy moth is discussed in a provocative paper by RICHERSON (563)
in which he comments that "most of the 'modes of action' of disruption
are symptomatic of the 'dogma of immaculate perception'". Obviously
RICHERSON takes issue with several of the neat formalizations of insect
behavior—at least as applied to the gypsy moth—and he points out
some of the limitations of mating-disruption by pheromones in high
populations.

In a recent presentation, CAMERON (564) once again summarized
the promises and problems of the field work with disparlure: "We do
need more time for testing--BUT—we do *not* need any large scale
tests in 1978. If this approach is to be pursued, emphasis in 1978 tests
should be directed to formulation development and rates of lure
release. . . ."

References, pp. 157—190

PLIMMER *et al.* emphasize the need for effective formulations, and discuss disparlure formulations and strategies of applications (*565, 566*). They agree with CAMERON (see above): "We [Agricultural Research Service] have considerable investment in this research and it is continuing. However, it would be a mistake to oversell until we have done much more homework. I would like to be able to define precisely and reproducibly where and when mating disruption will succeed and whether it can be effectively combined with insecticide treatment at the larval state (for Lepidoptera). Our formulations need much development before we can be sure of their economy and reproducibility." PLIMMER also notes (*567*) that even the basic question of responses to the enantiomers of disparlure still needs to be resolved.

BONESS (*568*) used disparlure in field trials against the nun moth, *Lymantria monacha*, which is closely related to the gypsy moth. After treating 5 hectares of pine forest with 5.4 g of microencapsulated disparlure, he distributed 40 small disparlure-baited traps in the central part of the plot. Catches were reduced by 99% in comparison with a control plot.   .

b) Eastern Spruce Budworm, *Choristaneura fumiferana*

This native defoliator of balsam fir and white spruce ranges from the Yukon through the northern Prairie Provinces into eastern Canada and the northeastern United States. Massive outbreaks have been countered with massive applications of hard pesticides with dubious long-range results. "There is some evidence that extensive aerial spraying operations using conventional pesticides have been successful in keeping trees alive, but there is mounting evidence that preserving the insects' food supply in this way also prolongs the outbreak." Furthermore, gravid females are dispersed in enormous numbers out of densely populated areas (*569*).

The male responds to one attractant pheromone component and is inhibited by two others. Surveys with small attractant traps have been shown to be "extremely effective in catching male spruce budworms in areas where conventional larval sampling has failed to locate any insects. Again, the potential use of the attractant is considerable, but implementation of the system for monitoring low density populations requires calibration of the catches with population density which must await the decline of the current extensive infestations to endemic levels." (*570*). SANDERS (*570*) has summarized the results of a semi-operational attempt at disruption of spruce budworm mating by the synthetic attractant in Ontario in 1977: "Semi-operational trials of the aerial dispersion of the synthetic attractant of the eastern spruce budworm from aircraft have

shown conclusively that mating behaviour is profoundly disrupted. In 1977 a release rate of 5 mg/ha/hr resulted in 99.4% reduction in catches in traps baited with virgin female moths. Assessment of the effects of this on population density in the following generation was complicated by late application of the attractant. However, apparently no further eggs were laid following the application. Further trials are planned for 1978 using different formulations and release rates with the assessment focusing on population regulation.

"The potential of the synthetic attractant for population regulation is therefore considerable. The major problems now are technological: inexpensive attractant and effective formulation. So far, formulations have been derived from the spin-off from various industries. There is an urgent need for custom formulations designed specifically for the slow release of synthetic attractants."

c) Other Forest Lepidopteran Pests

DATERMAN and his colleagues have been concerned with three western forest pests: Douglas-fir tussock moth *(Orgyia pseudotsugata)* and western spruce budworm *(Choristoneura occidentalis)*, both defoliators of Douglas-, grand-, and white firs; and the western pine shoot borer *(Eucosma sonomana)*, stunter of ponderosa and Jeffrey pines. DATERMAN summarizes these studies as follows (*519*):

"For tussock moth work, we have two major efforts underway; 1. to develop pheromone-baited traps for detecting critical increases in population density (early-warning system for impending outbreaks), and 2. to evaluate the pheromone for control purposes via the mating disruption approach. Our results to date have been promising in both areas. In 1977 we have gone an additional step with the control effort by applying aerial applications to test plots in New Mexico and Oregon. The dispenser system used in 1977 was the Conrel hollow fibers. Evaluation of 1977 tests is still taking place, but the initial returns are promising (*571, 572*).

"Our efforts on western spruce budworm have thus far been limited to survey applications. Specifically, we have been attempting to correlate moth captures (in pheromone-baited traps) with larval densities and/or defoliation on a series of test plots. We would like to expand this effort in the future, and also explore use of the budworm attractant for control purposes.

"The western pine shoot borer work dates only from 1976. We have a tentative structural identification, and using that material on very small scale ${}^1/_{10}$ acre) plots, were successful in demonstrating disruption of pheromone communication. We have plans to expand this effort in

References, pp. 157—190

1978, and have a very optimistic outlook for achieving damage reduction through control by pheromone application."

C. Orchard and Vineyard Insect Pests

1. Lepidoptera

Apple orchards in the eastern U. S. are massively afflicted by a variety of pests, mainly moths, and in consequence, the orchards are blanketed with insecticides during the entire growing season. Vineyards in the same area are considered to have only two major moth pests. After the pheromones of a number of these pests had been identified, the group at the New York State Agricultural Experiment Station at Geneva adopted the strategy of using small baited traps to monitor the population so that insecticide application for a particular pest could be timed to its arrival. Together with other information, moth catches can be used to predict population densities and egg hatch. Zoecon Corp. has been selling traps and lures for this purpose for the past few years, and several examples habe been reported of appreciably reduced insecticide application in conjunction with monitoring (*573—576*). Pheromone monitoring is an important component of the farm advisory pest management system set up in Wayne County, N. Y. Savings in pesticide costs are reported to range up to 50% of the costs under the previously used spray regime (*577*).

Population control by mass trapping in low populations has been demonstrated for the redbanded leafroller (RBLR), *Argyrotaenia velutinana*, in apple orchards and vineyards, and for the grapeberry moth (GBM), *Paralobesia viteana*, in vineyards. However it was concluded that the benefits relative to cost could not justify this means of control, and research was diverted to mating disruption programs.

Microencapsulated and hollow fiber formulations of the pheromone were shown to disrupt mating of both the RBLR and the GBM (*578*). However attempts to disrupt mating for the complex of moth pests were less successful; improved formulations with better control of release rates of individual components are needed. TUMLINSON *et al.* (*579*) and ROELOFS (*580*) have dealt with the problems involved in manipulating complexes of insect pests. ROELOFS and CARDÉ (*21*) have analyzed communication systems and their disruption by pheromones and parapheromones.

Throughout the apple and pear orchards of Canada and northwestern U. S., only a few major pests need be contained; population monitoring and control are especially promising. Monitoring, combined with visual inspection for the codling moth, *Laspeyresia pomonella,* is successfully

used to time sprays in British Columbia (581). Madsen et al. (582) have reported that, without the use of sprays, mass trapping of the codling moth resulted in population suppression and in the maintenance of fruit damage within acceptable commercial standards. Since the experiment was carried out in an isolated apple orchard, it is hard to predict the general applicability of a trap-out strategy.

An extensive study of the use of survey traps in the management of the codling moth was carried out in the Rhone Valley and in the vicinity of Paris, France, by Audemard and Milaire (583). They concluded that critical levels of trap catches in conjunction with temperature readings can be used to determine spray schedules.

Recently Audemard et al. (584) reported a successful mating disruption test against a natural population of the codling moth: "An attempt at controlling the codling moth *(Laspeyresia pomonella)* was made with the synthetic sex pheromone $8E,10E$-dodecadien-1-ol **(475)** in 1976 in a commercial apple orchard of 0.75 ha in Avignon. The technique of 'male disruption' was being used. The pheromone was being diffused in dispensers made of rubber tubing hung in trees. The quantity so used was 132 g/ha for the four series successively placed in the orchard. But only 0.55 g/ha/day have been effectively diffused, that is to say 84 g for the whole season. The results were very satisfying. The codling moth infestation at harvest was less than 0.5% injured apples. The codling moth population was drastically cut down by three times, thus making unnecessary the control of the first generation in 1977."

The pheromone of another major apple pest in British Columbia, the fruit leaf roller, *Archips argyrospilus*, has been used successfully to monitor activity and estimate populations so that sprays may be timed more effectively (585).

Moffitt (586), describing recent results on the codling moth in the Pacific Northwest, writes: "We have achieved season-long control of the codling moth on pears with three applications of the sex pheromone during the season." At this point, he is convinced of the technical feasibility of control of the codling moth with pheromones in conjunction with other tools.

Minks (587) has developed a system for determining the most effective time of spray against the summerfruit tortrix moth, *Adoxophyes orana*, in Dutch orchards; predictions are made on the basis of survey trap catches and temperature readings. Minks et al. (588) found that the geometric isomers of the pheromone of this moth blocked the attractant response (antipheromone), but large-scale attempts to reduce population by spraying a microencapsulated formulation of the antipheromone in apple orchards gave "variable results". Reasons for failure are discussed.

References, pp. 157—190

ARNE et al. (589) demonstrated that the mating disruption of the plum fruit moth *(Grapholitha funebrana)* was maintained throughout the growing season by permeating a plum orchard with the pheromone. Numbers of moths caught on survey traps and damage to fruit were comparable with the results in an orchard that was treated with the usual schedule of insecticides.

The number of sprayings against the apple maggot, *Rhagoletis pomonella*, in eastern Canada was reduced by coordinating the sprays with catches on baited traps (590).

2. Diptera

Several species of fruit flies have been controlled with a bait consisting of a carrier impregnated with an attractant (parapheromone) and a pesticide, and with attractant baited traps. Since the extensive literature has been recently reviewed by CHAMBERS (591), a brief summation here will suffice.

Food baits for fruit flies go back over 60 years, but an extensive, empirical, screening program for fruit fly attractants was carried out by the USDA during the 1950s. An interesting aspect of this work is that a male response was obtained to a whole series of natural fragrances and synthetic compounds. "... this olfactometer screening backed by field tests of promising candidates produced attractants in a relatively short time that have provided some of the greatest economic benefits yet demonstrated" (591). Attractants are now available for males of the following important pests: oriental fruit fly, mango fly, melon fly, Queensland fruit fly, Mediterranean fruit fly, and the Natal fruit fly. In addition, protein hydrolysate attracts both sexes of most fruit flies. CHAMBERS (591) gives a number of examples of population suppression and a few instances of apparent eradication from isolated areas.

D. Field Crops Insect Pests

1. Coleoptera

a) Cotton boll weevil, *Anthonomus grandis*

A recent thorough review of attempts to control the cotton boll weevil by means of pesticides (592) makes it unnecessary to go into detail here, but some perspective is presented, since the problem is massive and attempts to control this insect with pesticides have contributed to the difficulties alluded to in Section VII. A. One third of the agricultural pesticides used in the U. S. is dedicated to attempted control of the cotton boll weevil (592).

The pheromone consists of four compounds (**33, 110b, 111a, 111b**), produced by the male, that in combination attract the female and also serve in the spring and fall as an aggregant. The compounds (the mixture is grandlure) have been synthesized and are currently available is several formulations, and several effective traps were devised. A two-year experiment was started in 1971 to determine whether it was feasible to suppress or even eradicate a population from an isolated area. Grandlure baited traps were part of a program that included sound agricultural practices, insecticide treatment in the spring, and release of sterile boll weevil males. Despite the necessity to use supplemental sprays in peripheral area and despite evidence that mated female weevils had migrated into these areas from up to 40 km away, the technical guidance committee report concluded "that the boll weevil could be eliminated as an economic pest". The pheromone traps "improved the performance of the trap crop over unbaited trap crops" (*593*).

Subsequently, trapping tests and disruption tests have been conducted on a smaller scale. MITCHELL *et al.* (*594*) reported: "Grandlure-baited in-field traps at the rate of 10 per acre captured 76% of a population of overwintered *Anthonomus grandis* Boheman estimated to number ca. 25 per acre. ... A combination of in-field traps and insecticides captured or killed 100% of the emerging overwintered beetles ... the traps alone captured about 96% of a late emerging population ... These experiments were carried out on a well-isolated cotton planting totaling 108 acres ..." In contrast, HUDDLESTON *et al.* (*595*) reported disappointing results in an attempt to disrupt communication with grandlure.

KNIPLING (*593*) in 1974 discussed the use of population models to predict the feasibility of "suppression or eradication of the boll weevil through the use of insecticides, sterile insects, and the boll weevil sex pheromone employed alone, employed simultaneously or in sequence". Basic to the trapping strategy is the assumption that "the estimated effect of traps will be considerable only for low level populations". He concludes that "pheromone traps should come close to maintaining subeconomic populations in most boll weevil areas without the need for insecticide application if the populations are once brought down to a level of about 10 boll weevils per acre". "... If the population has been reduced to a level of about two per acre, which seems readily possible by the use of a thorough reproduction-diapause spray program, the in-field traps alone, based on the parameters, should achieve of the order of 98% capture of the females. ..." Two further uses for grandlure, KNIPLING points out, are to reduce the risk of developing insecticide-resistant strains, and to detect incipient infestations.

LLOYD (*596*) found that "the theoretical study of KNIPLING was validated by our [MITCHELL *et al.,* above] field research. The debate on

the feasibility of boll weevil eradication will be resolved by the Trial Boll Weevil Eradication Program which will begin in North Carolina and Virginia in 1978. The use of the boll weevil pheromone, grandlure, will represent a significant technical component in this Trial Program."

2. Lepidoptera

The communication system of each of the following important lepidopteran pests of field crops has been disrupted in field studies by either a pheromone, a parapheromone or both (597): pink bollworm *(Pectinophora gossypiella)*, cabbage looper *(Trichoplusia ni)*, alfalfa looper *(Autographa californica)*, soybean looper *(Pseudoplusia includens)*, corn earworm *(Heliothis zea)*, tobacco budworm *(H. virescens)*, yellow-striped armyworm *(Spodoptera ornithogalli)*, fall armyworm *(S. frugiperda)*, beet armyworm *(S. exigua)*, European corn borer *(Ostrinia nubilalis)*, Egyptian cotton leafworm *(S. littoralis)*.

With the exception of the corn earworm, fall armyworm, pink bollworm, and Egyptian cotton leafworm programs, these disruption tests were preliminary studies carried out on small plots without optimization of disruptant formulations and with little assessment of crop damage.

The corn earworm and fall armyworm are serious pests on corn crops. MITCHELL *et al.* (598, 599) have demonstrated disruption of mating for both insects in corn fields by permeation with either the pheromone or a parapheromone. Suppressions reported ranged from 85—97%. Furthermore, they showed that simultaneous mating disruption could be accomplished for both insects with a mixture of the pheromone and parapheromone. Disruption was in the range of 88—97%. A four-year program will be initiated in 1978 to develop the necessary technology for suppressing populations of these insects in corn, so that the large number of insecticide applications currently used on this crop can be reduced.

MITCHELL (597) has discussed the feasibility of using multicomponent formulations for control of several important pests of field crops. HENDRICKS *et al.* (600) showed that a mixture of looplure (622) and virelure (623, 624) were compatible and caught the cabbage looper *(T. ni)*, the soybean looper *(Pseudoplusia includens)*, and the tobacco budworm *(H. virescens)* on the same traps. It seems likely that pesticide applications to field crops could be greatly reduced by means of pheromone-baited monitoring traps, and such efforts will undoubtedly become a part of the integrated pest management projects now established by the Extension Service of the USDA.
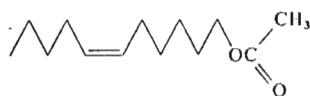
(622)

(623)

(624)

Because of the rapid spread of the pink bollworm through the cotton fields of the Southwest into California despite massive pesticides applications, and the realization that its establishment in the San Joaquin Valley of California would be a disaster, the impetus for developing control procedures was present. Between 1971 and 1973, TOSCANO et al. (601) showed that the number of pesticide applications could be reduced by monitoring the arrival of males by means of traps baited with the parapheromone, hexalure. In 1972, McLAUGHLIN et al. (602) reported disruption of the mating response by hexalure on small plots; SHOREY et al. (603) repeated the demonstration on larger fields, and also showed that permeation with hexalure throughout the growing season was as effective as treatments with the pesticide, carbaryl. Subsequent large-scale tests were conducted with the pheromone gossyplure, which was much more effective than the parapheromone. "We have now released [middle of May through early September, 1976] economically practical amounts of the pheromone into the air within three cotton fields [5, 6, and 12 hectares] throughout an entire growing season and have suppressed the population of pink bollworm larvae infesting the cotton bolls of those fields to an extent comparable to that achieved in ten comparison fields treated with conventional insecticide applications." The growers were instructed wo use insecticides in both sets of fields as counts of larvae in cotton bolls indicated. This resulted in a ninefold reduction in insecticide usage in the pheromone-treated fields. The technology used in this test was fairly primitive. Hoops of hollow plastic fibers containing the pheromone were attached by hand to the cotton plants in a grid pattern, and

References, pp. 157—190

were replenished at 3-week intervals to keep the release sources near the tops of the plants (*604*).

In 1976, BROOKS and KITTERMAN (*605*) treated 1,160 hectares of cotton in Arizona and California with a hollow fiber formulation of gossyplure, initially applied with ground equipment designed especially for this application; subsequent applications were made by air. "Efficacy of treatment was assessed by moth captures in monitoring traps, one trap per 10 acres [4 hectares], and twice weekly sampling of bolls for larval infestation. Since untreated control fields were not practical, the effectiveness of pheromone treatment was gauged by comparison of larval infestations in fields which were under conventional treatment regimes with insecticide." Pink bollworm levels were historically severe in one area and moderate to severe in two areas. In the severely infested area, "it is believed that early season suppression was helped by such [phero-mone] treatments". However, a rapid midseason buildup in a few problem fields coupled with a tobacco budworm infestation made it advisable to terminate the pheromone treatments and launch a regular insecticide treatment. The tentative conclusion is that "pheromone treatments postponed the onset of test field infestations by about one month". On a 132 acre farm in a moderately infested area, "boll infestation suppression ... indicates that pheromone treatment is almost directly comparable in effectiveness to insecticide treatment". Prophylactic insecticide treatment was used on several of the fields as a precaution against in-migration late in the season.

In 1977, a similar operation by Conrel Co. was carried out over 20,000 acres of large isolated blocks of 2,000—3,000 acres in Arizona and California. At this writing, the data have not been completely analyzed, but D. W. SWENSON states (*606*) that a large reduction in insecticide application was achieved, and that the economics are feasible for this type of operation. He points out however that timing is critical, and that in several fields, insecticide applications were needed to control the tobacco budworm, cotton leaf roller, and lygus bug. In general, the technology seems in place to make suppression by pheromone permeation a feasible component of an integrated pest management system for cotton crops in the southwestern part of the U. S. Further large-scale operations are scheduled for 1978.

Another cotton pest in several parts of the world is the red boll-worm, *Diparopsis castanea*. Its pheromone and an inhibitor have been shown to disrupt mating, but no crop damage assessment was made (*607*).

On the basis of their success in trapping *S. littoralis* at a distance of 3 km from cotton and alfalfa fields in Israel, NEUMARK *et al.* (*608*) proposed that "safety belts of baited traps be established around cultivated fields".

3. Hymenoptera

Several species of leaf-cutting ants do massive damage to a number of field crops in tropical countries. The trail pheromones of three species, *Atta texana, A. cephalotes,* and *A. sexdens rubropilosa* have been used in attempts to improve the pickup of poison bait. This was moderately successful for baits formulated with an inert substrate, but did not improve pickup in the field for baits formulated with such food materials as citrus pulp, grain, and bagasse fortified with molasses (*609*).

E. Stored Products Insect Pests

Stored food products are high-value items that have been planted, weeded, watered, fertilized, harvested, processed, transported, and stored in facilities, which also represent an investment. Since insect pests cause massive economic losses, strenuous efforts for detection and control of these pests are warranted and have been actively undertaken. Most of these pests are coleopteran or lepidopteran, and a number of pheromones have been identified in both orders (see reviews by Burk-holder (*610*) and Levinson (*611*)).

1. Coleoptera

Practical application of pheromone traps for detection and control is furthest advanced for several species of *Trogoderma* and *Attagenus,* which include some of the more notorious pests. In fact, small detection traps containing the pheromone and an insecticide are now in use in a number of storage facilities, and on ships for quarantine purposes. The very high levels of response make early detection in a sparse population much more feasible than previous procedures; the use of numerous small traps allows for pinpointing the sources of the infestation. Current studies are aimed at improved trap and dispenser design.

Although mating disruption of *Attagenus megatoma* by pheromones has been demonstrated, permeation of a storage facility is not considered a good tactic, principally because residues absorbed on the stored products or containers may attract insects after the material has left the warehouse.

Two concepts for population suppression with baited traps are under investigation. The first is simply the pheromone-insecticide trapping system. The second concept (*612*) involves the use of a pheromone trap treated with spores of a pathogenic protozoon. The males, which are attracted to the trap, are contaminated with the spores and transmit them to the rest of the population. Under simulated warehouse con-

References, pp. 157—190

ditions, "seminatural" populations of *T. glabrum* were effectively suppressed.

These systems may be very effective in mixed populations of *Trogoderma* because many species share some of the same pheromone components. One of the major targets is the khapra beetle, *Trogoderma granarium*, a notorious pest in warmer regions throughout Europe, Asia and Africa. Only a tight quarantine prevents its entry into the U. S. Ships and port facilities are being monitored with pheromone-baited traps.

2. Lepidoptera

Several members of the family Phycitidae share the same pheromone, and field studies involving several of these moths have been carried out. REICHMUTH *et al.* (*613*) showed the utility of pheromone traps for early detection of the tobacco moth, *Ephestia elutella*, and the Indian meal moth, *Plodia interpunctella*. SOWER *et al.* (*614, 615*) showed that mating frequency of the Indian meal moth and the almond moth, *E. cautella*, was effectively reduced at low population densities. WHEATLEY (*107*) and HAINES (*617*) also reported that mating frequency of the almond moth was reduced, and that detection and survey traps would be feasible and very useful for this pest and several related species.

F. Pests That Directly Afflict Humans or Animals

Pheromones have been identified and field-tested in only a few of the host of pests that directly afflict man or animals.

Muscalure (**318**), a weakly attractant pheromone of the common housefly, has been registered with the EPA (in fact, it is the only registered pheromone to date) and is available in a bait (Zoecon Corp.) containing sugar, a pesticide and the attractant. Both sexes are attracted in field use (animal barns), and enhanced catches of up to sevenfold over control traps have been reported (*618, 619*).

The sex attractant of several tick species have been identified, but only three examples of attempts to apply the attractant have come to our attention. SONENSHINE has demonstrated (*620*) mating disruption by dusting a tick-infested dog with a microencapsulated formulation of the pheromone, 2,6-dichlorophenol; the ticks used were the dog tick, *Dermacentor variabilis*, and the Rocky Mountain wood tick, *Dermacentor andersoni*. GLADNEY (*621*) applied a mixture of pesticide and male extrac. to a shaved area on the shoulders of cattle and found that the Gulf Coast tick *(Amblyoma maculatum)* could be attracted to the spots and killed

by the insecticide. RECHAV (622) described an aggregation pheromone in feeding males of *Amblyoma hebraeum* that attracts males, females, and nymphs. A pheromone-acaricide mixture painted on cattle was effective for four days.

G. Pheromone Formulations

Satisfactory formulations of pheromones that afford a constant release rate over a long period of time are not likely to be achieved without the cooperation of the chemical industry. Controlled release of pheromones is discussed in the Proceedings of a recent symposium (623). Fortunately, several organizations in the U. S. have been interested in the possibilities of the use of pheromone formulations on a commercial basis. One company (605) has supplied formulations for several investigators, and in addition has carried out extensive field tests with their own personnel. It has developed the use of hollow fibers, which are simply "microcapillary reservoirs that serve to contain a vaporizable material and mediate evaporation of the material into the atmosphere". The fiber wall is essentially impermeable. One end is sealed, and the release rate essentially depends on diffusion from the surface of the liquid-vapor interface to the open end of the hollow fiber. Fibers are supplied in two forms. A "tape form" consists of a parallel array of fibers on an adhesive tape; this form is used to establish point-source evaporators. The "chopped fiber form" is used for dissemination with a ground rig or from the air.

Another company (624, 625) supplies a controlled release dispenser, which is a three-layer plastic laminated sheet consisting of a bottom protective layer, a middle pheromone reservoir layer, and a top permeable layer through which the pheromone diffuses. The sheet can be cut into ribbons and applied over an area. Formulations have been field tested on a number of insect pests: gypsy moth, pink bollworm, peachtree borer, lesser peach tree borer, soybean looper, cabbage looper, European elm bark beetle, tobacco budworm, fall armyworm, tussock moth, eastern spruce budworm, and Mediterranean fruit fly.

Two companies (626, 627) provide microencapsulated formulations of pheromones. This is a very flexible system in that encapsulating materials and particle size can be varied over a wide range. The microcapsules can be applied as solid particles or sprayed as a slurry. A study with microencapsulated disparlure (536) on the gypsy moth showed that the field life of disparlure could be extended to approximately six weeks (628).

References, pp. 157—190

VIII. Conclusion

As we move from passive dependence on the environment to technological advances that permit both abuse and control of the environment, the consequences of our decisions become awesome. Given a reasonable level of cooperation and commitment by government agencies, the research community, farmers, and industry, integrated pest management will become the modus operandi of the future. Surely, pheromones will play an important role in such programs, if the basic research, on which such roles are based, can be strengthened and the momentum for development, mainly by governmental agencies, can be increased.

Addendum *

II. Structure Elucidation

A. Isolation

1. Collection

A method for trapping disparlure (536) from air with type 4A molecular sieves and subsequent quantitative analysis of a brominated derivative by electron-capture GC has been developed by CARO et al. (629). It has been successfully applied in the field to disparlure air concentrations as low as 0.2 ng/m³.

Tenax GC has been used to collect volatile compounds from fungi that are attractive to cheese mites (630).

B. Identification

2. Examples

Female ambrosia bark beetles (Trypodendron lineatum) boring in Douglas fir produce a substance, named lineatin, that attracts males. On the basis of spectral information and the results from hydrogenolysis, MACCONNELL et al. (631) proposed structures (625) or (626) for lineatin. The cyclobutane portion was assigned after it was observed that hydrogenolysis of grandisol (33) gave essentially the same products. Synthetic studies to distinguish between these structures are underway.

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* Material in the Addendum was compiled as of October 1, 1978 and is arranged under the headings used in the main section.
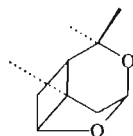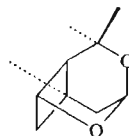
(625)   (626)

FRANCKE *et al.* (*632*) identified spiroacetal (627) as an aggregation pheromone in the bark beetle *Pityogenes chalcographus*.
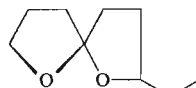
(627)

III. Synthesis

The synthesis of chiral pheromones has been recently reviewed (*633*).

A. Coleoptera

1. Bruchidae

By synthesizing both enantiomeric forms of the dried bean beetle (*Acanthoscelides obtectus*) sex pheromone, PIRKLE and BOEDER (*634*) determined that the natural pheromone had the *R*-(−) configuration (11 a).
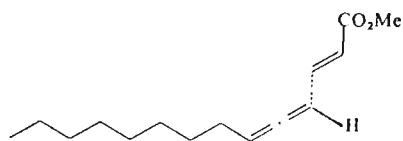
(11 a)

2. Curculionidae

WENKERT *et al.* (*97, 98*) have recently published (*635*) details of their synthesis of racemic grandisol (33), one of the sex pheromone components

References, pp. 157—190

of the boll weevil *(Anthonomus grandis)*. Both enantiomers of (33) were synthesized by MORI *(636)*, DE SOUZA and GONÇALVES *(637)* reported conversion of (106) into the three cyclohexane sex pheromone components (110b, 111a, and 111b).

B. Diptera

An acetylenic intermediate was used by KOVALEV *et al.* *(638)* in their synthesis of muscalure (318), the sex pheromone of the house fly *(Musca domestica)*.

C. Homoptera

A synthesis of the diene component (333) of the California red scale *(Aonidiella aurantii)* sex pheromone has been achieved by SNIDER and RODINI *(639)*.

D. Hymenoptera

1. Apidae

Queen substance (338), the multi-purpose pheromone of the honey bee *(Apis mellifera)*, was obtained from substituted 5-bromothiophenes by TAMARU *et al.* *(640)*.

All four possible diastereomers of 2-methyl-5-hydroxyhexanoic acid lactone (628) have been synthesized by PIRKLE and ADAMS *(641)* and await comparison with natural material to determine which is the pheromone of the carpenter bee.
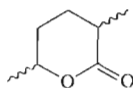
(628)

3. Diprionidae

A number of new syntheses of the sex pheromone (373) of the pine sawfly *(Neodiprion lecontei)* have been reported by MORI *et al.* *(642, 643)*, PLACE *et al.* *(644)*, and TAI *et al.* *(645)*.

F. Lepidoptera

1. Monoenes

Stereoselective reduction of β- or ω-alkynols to corresponding (E)-alkenols has been achieved by Rossi and Carpita (646) with lithium aluminum hydride. Fyles et al. (647, 648) have reported further applications of polymer supports in the synthesis of sex attractants.

5. Epoxide

Racemic disparlure (536), the sex pheromone of the gypsy moth (Lymantria dispar), has been prepared by Klünenberg and Schäfer (649) and Tolstikov et al. (650), and its geometric isomer (545) by Okada et al. (651).

6. Ketone

(Z)-1,6-Heneicosadien-11-one (629), an attractive analog of the Douglas fir tussock moth (Orgyia pseudotsugata) sex pheromone (580) has been identified and synthesized by Smith et al. (652).

(629)
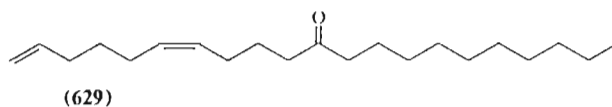
Tamada et al. (653) have reported a synthesis of the unsaturated ketones (630) and (631), which are components of the peach fruit moth sex pheromone.

(630)

(631)
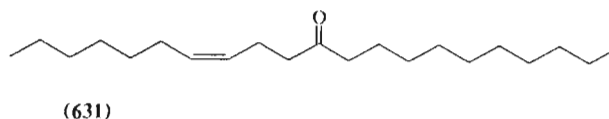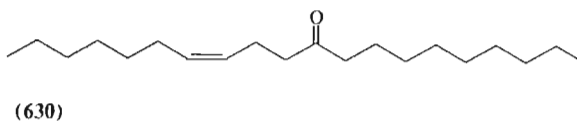
References, pp. 157—190

IV. Stereobiology

A. Geometric Isomers

Confirmation of the chemical structure and the natural occurrence of bombykal in *Bombyx mori* has recently been described (*654*).

Data on the attractiveness of pheromone blends and of isomer blends in field trials have been presented for the male peachtree borer (*655*) and the carpenterworm (*656*) respectively.

(*Z*)-11-Hexadecenal has been isolated from the female moth, *Heliothis armigera*, and is a potent olfactory stimulant for males in laboratory and field tests (*657*). The olfactometer response of laboratory-reared males of *H. virescens* to its pheromones, (*Z*)-11-hexadecenal and (*Z*)-9-tetradecenal and the inhibitor (*Z*)-9-tetradecen-1-ol formate has been studied (*658*).

B. Enantiomers

Trapping and behavioral tests on the attractiveness of disparlure racemates and the antipodes indicated that the (+) enantiomer is more attractive than racemic disparlure, and the (−) enantiomer exhibits an antagonistic effect (*659*). The effect of (−)-disparlure was much more apparent on in-flight than on pre-flight behavior (*660*).

The response of the bark beetle, *Ips pini*, to the attractant blend produced by conspecific males boring in ponderosa pine is inhibited by *S*-(−)-ipsenol (*661*).

C. Chemorecognition

A review relating the mechanism of pheromone perception to perception and behavior of the pheromone-stimulated insects has appeared recently (*338a*).

Many lepidopteran species produce a very precise blend of pheromone components. In addition, many male moths are captured with a considerably broader range of ratios, and, in some tests, with ratios quite different from that produced by their corresponding females. It is basically these phenomena that have led ROELOFS (*662*) to propose a hypothesis that employs threshold diagrams of binary mixtures of geometrical or positional isomers and their concentrations to illustrate activity relationships between these factors.

WRIGHT (*663*), employing the results of HAYWARD mentioned in the main review, has proposed how optical isomers may be perceived differently according to his vibrational theory.

i

The major trail pheromone of the ant, *Atta texana*, is methyl-4-methyl-pyrrole-2-carboxylate (*664*) and trail-following activity studies of synthetic analogs of this compound have been conducted by Sonnet and Moser (*665, 666*). It was concluded from the bioassay studies that, for activity, a molecule must have a particular shape and substitution pattern. It seems likely that the pyrrolic nitrogen atom is important in the process of chemorecognition by the receptor site and calculations of the charge of the N-atom of many of the substituted pyrroles bioassayed showed that the most active compounds all have an identical charge on the pyrrolic nitrogen (*667*). It is suggested that if behavioral activity could be related to electronic parameters then quantum calculations on as yet unsynthesized and untested structural analogs of pheromones may have predictive value for new compounds with activity.

The European corn borer (ECB) and the red banded leafroller (RBLR) both employ (Z)-11-tetradecenyl acetate as a sex pheromone. The racemate and pure enantiomers of 9-(cyclopent-2-en-1-yl)nonyl acetate were synthesized and have been found to mimic certain biological properties of the natural pheromone (*668*). The ECB responds to the *S*-(−)-enantiomer while the RBLR responds equally to both the *S*-(−)- and the *R*-(+)-enantiomer. Because of the behavioral response of the RBLR it is suggested that this species has two stereospecific chemoreceptors that may have different conformational requirements for the achiral phero-mone. The use of this type of stereochemical probe for the study of neuro-chemical receptor systems of achiral molecules offers an exciting metho-dology for the investigation of chemical sensing.

An interesting review on taste receptors and their specificity by V. G. Dethier (*669*) is well worth reading.

Renwick (*363*) reported on the activity of a number of frontalin analogs in field tests on *D. frontalis*, the Southern pine beetle. The only analog which showed any additive activity was 5,7-dimethyl-6,8-dioxabicyclo[3.2.1]octane when presented together with frontalin and α-pinene. The 5,7-dimethyl substitution pattern is the same as the 5-methyl-7-ethyl substitution pattern of *exo*- and *endo*-brevicomin. Renwick (*363*) noted this and also pointed out that while brevicomin is an active component of the attractant of *D. brevicomis*, it is inactive or suppresses response in *D. frontalis*. It has since been established that (−)-frontalin is the active enantiomer in this species (*46*).

The Western pine beetle, *D. brevicomis*, responds maximally to a ternary mixture of (1*R*,5*S*,7*R*)-(+)-*exo*-brevicomin, (1*S*,5*R*)-(−)-frontalin, and the host-produced alicyclic terpene, myrcene (*320*). Mix-tures containing the antipodes of *exo*-brevicomin and frontalin were much less attractive.

References, pp. 157—190

Could (+)-*exo*-brevicomin and (−)-frontalin both interact with the same receptor? Let us assume that the receptor site(s) for the bicyclic ketals recognizes that face of the molecule which contains the oxygen bridges. As mentioned earlier in this review, the charge on the oxygen atoms of frontalin and *exo*- and *endo*-brevicomin are essentially the same within any one molecule, but differ slightly between molecules. Therefore (−)-frontalin may be rotated so that the oxygen atoms at positions 6 and 8 correspond to those at positions 8 and 6 of (+)-*exo*-brevicomin with the equivalence of the oxygen atoms remaining unchanged.

(+)-*exo*-brevicomin   (−)-frontalin
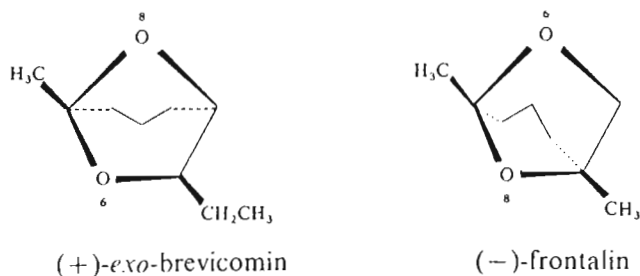
On a stereochemical basis it seems likely that a single receptor site could recognize both of these molecules. It will be interesting to establish whether the bicyclic ketals interact with a single receptor site. If they do, then it is possible that when (−)-frontalin displays activity, activity (either synergistic or inhibitory) might also be displayed by (+)-*exo*- or (+)-*endo*-brevicomin.

V. Biosynthesis

HUGHES and RENWICK (*670*) have extended their studies on hormonal and host factors that stimulate pheromone synthesis to include female Western pine beetles, *Dendroctonus brevicomis*.

The biosynthetic significance of enzymes in the defensive secretion of a bug, *Leptoglossus phylopus*, has been demonstrated (*671*), and the biosynthesis of formic acid in a formicine ant poison gland has been described (*672*).

The biosynthesis of the aggregation pheromones of the European fir engraver beetles, *Pityokteines curvidens, P. spinidens* and *P. vorontzovi* has recently been described in some detail by HARRING (*673*).

VII. Practical Applications

Progress in practical applications of behavior-modifying chemicals during the 1978 season was summarized at the Advanced Research Institute (ARI) on Chemical Ecology: Odour Communication in Animals, at Leeuwenhorst, Holland, September 25—29, 1978; the Proceedings will be published (674). Brooks et al. reviewed the successful commercial operation against the pink bollworm in cotton fields in 1977 and gave a preliminary report on the 1978 studies. In addition to crop protection, permeation with gossyplure with concomitant reduction by 50--80% in pesticide use resulted in lint yield improvements of as much as 20—50%. The 1978 experiments were carried out with material registered by EPA in February, 1978; this represents the first registration of a sex pheromone for protection of a field crop. Brooks has also submitted a manuscript for publication as a chapter (675) which describes the application of hollow fiber technology for controlled release of pheromones. As target insects for commercial exploration, he lists the pink bollworm, grape berry moth, codling moth, spruce budworm, and tussock moth.

Siddall's chapter in the ARI Proceedings is entitled "Commercial Production of Insect Pheromones: Problems and Prospects". This paper argues that industry in the USA is deterred from developing pheromones for insect control mainly because of the unrealistic and uncertain requirements of EPA — the very agency charged with the development of alternatives to hard pesticides. The participants in the ARI meeting resolved to request EPA to establish firm, realistic guidelines within one year. Siddall's chapter contains a number of recommendations that could form the basis for reasonable policy decisions.

Despite the usual number of inaccurate statements inherent in "popular" writings, a recent article in Harper's magazine (676) points up the ironies involved in the attempts to introduce new procedures for insect control and the frustrations of scientists who have tried to deal with EPA. At the same time, the "sympathetic" observation is made that a bureaucratic agency, faced with an option to assume a risk and possibly incur the ire of the "environmentalists" or to do nothing, will do nothing. There surely are risks involved in all of the options to hard pesticides, but the article concludes that scientific evidence and common sense rank these risks as more acceptable than continued dependence on hard pesticides by default.

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Thoughts on the Improvement of Microbial Cellulase Production

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ABSTRACT

Certain microorganisms produce a blend of enzymes capable of degrading cellulose into fermentable sugars. The existing assays that are generally employed to determine the extent of hydrolysis of cellulose give no real quantitative idea of the extent of production of individual enzymes, and, therefore, of rate limiting enzymes in the blend. It is suggested that overcoming catabolite repression in, and successful genetic manipulation of, a cellulase producing organism requires critical assay procedures that enable the estimation of individual enzymes in the blend.

The world's appetite for liquid fuel continues to increase, and in a quest for nations to become less dependent on imported oil, scientists are looking to alternate sources of suitable liquid fuels. The production of ethanol from renewable biological materials, such as cellulose, is one possibility that is receiving serious attention. In order to make the process economically acceptable from the standpoint of industrial chemistry one must overcome a major difficulty, namely, the conversion of cellulose to fermentable sugars. Once one has fermentable sugars a variety in products can be made by standard fermentation technology e.g. ethanol and single cell protein. The choice of cellulose as starting material is dependent upon the fact that this natural polymer is the most abundant organic compound on earth and is made up entirely of glucose.

Cellulose can be hydrolysed by both chemical and enzymatic means. Commercial enzymatic processes could have a number of advantages i.e. cheaper installations, no requirement for harsh and toxic chemicals, and low temperatures and pressures. The most promising enzymatic procedure at this time is based on the fungal *(Trichoderma viride)* production of a number of enzymes capable of hydrolysing cellulose to fermentable sugars. The nature and production of this enzyme complex, generally termed the cellulase complex, is the subject of study in many laboratories throughout the world.

Cellulose is a natural high polymer of variable molecular weight and exists in both crystalline and amorphous phases. Also, it is associated in various ways with

protein, hemi-celluloses and lignin. Because of these facts its hydrolysis to glucose is carried out by a medley of enzymes working in concert. This enzyme complex is generally divided into 3 categories depending on the substrate used.

(a) β - Glucosidase is that enzyme(s) capable of hydrolysing the disaccharide, cellobiose, to glucose.

(b) Carboxymethyl cellulase (CMCase) is the complex of enzymes that hydrolyses CMC to reducing sugar monomers.

(c) Filter paper cellulase (FP cellulase) is the complex that can produce glucose from filter paper, a particular source of microcrystalline cellulose (other sources that have been used include cotton, Avicel, and Solka-Floc).

These various enzymes and enzyme complexes are therefore rather loosely defined and certain aspects of the assay procedures deserve further comment. The assays become increasingly complex as the substrate used increases in structural complexity from cellobiose to CMC to microcrystalline cellulose. This is brought about by, (i) the substrate becoming less well defined, and, (ii) the number of enzymes involved in the degradation to monomeric products increasing. With the assay of ill-defined polymeric substrates being dependent on a number of enzymes, the possibility of an unknown enzyme in the sequence becoming rate-limiting increases.

The limitation of the present enzyme assays based on the measurement of reducing sugar produced does not allow one to detect rate-limiting enzymes directly. Any rate-limiting step will appear as a decrease in the overall enzyme activity, even though some enzymes involved in the hydrolytic sequence are in excess. The problem is complicated further by the fact that there are an unknown number of enzymes involved in hydrolysing microcrystalline cellulose, each with its own affinity for different sizes or types of oligomer. Until such time as these are isolated free from other cellulase components, specific assays developed for them, and their kinetic properties determined, their interaction with one another will remain obscure and the detection of rate-limiting steps in the cellulase complex will remain unattainable.

The enzymes that hydrolyse cellulose are subject to a phenomenon termed catabolite repression. This means that when the final products of the reaction sequence, e.g. glucose, accumulate to levels higher than that required for optimum growth, the production of certain enzymes of the cellulase complex is repressed. A similar phenomenon also occurs if any other readily utilisable carbon source becomes available to the organism. In most organisms this is an economy measure, since it is wasteful to the cell to produce enzymes for which it has no need. Furthermore, it could be suicide for a slow growing organism to produce a readily utilisable carbon source that can be used by faster growing organisms in its immediate environment. The rate of the overall reaction sequence is therefore carefully controlled and an excess of all the necessary enzymes of the complex is never produced. For industrial applications such a situation is obviously undesirable.

Many fermentations of a mixture of biopolymers proceed in a sequential manner initially utilising that polymer which is most readily hydrolysed. In general, natural, sources of cellulose are in fact mixtures of various biopolymers. The monomeric

products of the easily hydrolysable polymer are likely to inhibit enzyme production of the most refractory polymer by catabolite repression. If one could somehow overcome this natural regulatory effect of catabolite repression a much more rapid overall hydrolysis may be possible.

Two problems are then faced by researchers wishing to modify and improve microbial cellulase production:

(i) overcoming catabolite repression, and,

(ii) increasing yields generally.

Minimising catabolite repression in an existing strain of an organism has not been done successfully although attempts have been made to provide conditions where compounds involved in catabolite repression are eliminated. A hyper-producing mutant (*T. reesei* NG-14) has been developed which is less subject to catabolite repression (MONTENECOURT and EVELEIGH, 1977). This department has recently obtained results that suggest it may be possible to decrease the effect of catabolite repression in shake-flask cultures of an existing strain of *T. viride*, namely strain 92027. It is considered that such an achievement could be of great utility to industry without a thorough understanding of the basic mechanisms involved. Research on this topic is continuing.

With the present successes of genetic manipulation stirring the imagination, a number of research groups have advocated the introduction of certain cellulase enzyme genes into suitable procaryotes. This is a very plausible approach but there are pitfalls. At this time, a major problem with this approach is the inability to assay specific enzymes of the cellulase complex. Results obtained in this laboratory, as well as the results of others (MANDELS, 1975), show that the ratio of FP cellulase to CMCase in *T. viride* varies during growth. This also is true of the fungus, *Sclerotium rolfsii* CPC 142 (SADANA *et al.*, 1979). These results suggest that these two enzyme systems are not coordinately induced. Therefore, the gene regions coding for the synthesis of these two enzyme systems are likely to be under separate control. As it is now known that eucaryotic genes are often not contiguous in a particular region on the chromosome, further problems of genetic manipulation will be encountered. The DNA coding for a particular eucaryotic protein is often separated by spacers of inactive DNA called introns and only after transcription of the DNA into RNA are these redundant regions removed (ABELSON, 1979). Thus, even if the genes could be identified, transfer of the intact gene to a procaryote could be difficult.

Previous genetic manipulations have concerned single genetic elements coding for assayable proteins. The task of transferring spacially removed multiple genes coding for an unknown number of enzymes for which there are no known specific assays is formidable. Such a shot-gun approach may be lucky and prove successful. However, it is felt that a more methodical approach to the problem of assaying changes in enzyme levels should also be adopted in order to complement, and possibly explain, successful modifications.

In any genetic manipulation study, or even in the formation of mutants, it is the limiting enzymes of the hydrolytic sequence (i.e. those which are carefully regulated) that one would like to be able to monitor with a view to their modification.

As mentioned previously, the removal of one rate-limiting step may produce another step in the hydrolytic sequence which is limiting. If all the enzymes are assayable, then the monitoring of their modification is relatively easy. However, if the enzymes are not easily monitored, then a hyper-producing mutant of a particular component of the cellulase complex may be discarded. One should therefore give high priority to an assay procedure that can detect the enzymes which hydrolyse cellulose, in particular those that initiate the breakdown of crystalline cellulose.

At the present time mankind is utilising photosynthetically reduced carbon for solid and liquid fuels. Secondarily, these are used for chemical feedstocks. Ethanol is one form of reduced carbon that can be used in many areas where oil is now used while embracing our present technology. As cellulose is the most abundant renewable form of stored solar energy that we have, we must learn to use it, and there is no doubt that in the future cellulose will be hydrolysed on an economic industrial scale.

A point that should be borne in mind is that the combustion of our present sources of fossil fuel is adding to the carbon dioxide content of the atmosphere. It is considered very likely that if the present increase in CO_2 continues at the predicted rate by the use of oil and coal, the earth's climate will begin to change noticeably and irreversibly 50 years from now. In contrast, the combustion of ethanol derived from renewable biological sources will merely recycle atmospheric CO_2 removed by green plants during growth.

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Regulation of β-Galactosidase and Pectic
Acid Lyase in
Erwinia chrysanthemi

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ABSTRACT

Both β-galactosidase (EC 3.2.1.23) and pectic acid lyase (PAL, EC 4.2.2.2) were found to be inducible enzymes in a local isolate of Erwinia chrysanthemi. Glucose repressed the synthesis of both enzymes. When the organism was grown in a medium containing both lactose and sodium polypectate (NaPP), the inducers of β-galactosidase and PAL respectively, PAL was synthesised in preference to β-galactosidase. This occurred even when lactose-grown cells were used for inoculum. This suggests that NaPP, or its breakdown products, behave as catabolite repressors of β-galactosidase. These results also suggest that cyclic AMP (cAMP) levels required for PAL synthesis are lower than those required for the synthesis of β-galactosidase.

We conclude that a 'pecking order' exists for the expression of inducible operons, and that this is possibly controlled by intracellular cAMP levels.

INTRODUCTION

Erwinia chrysanthemi causes soft rot of plant tissues by secreting pectic enzymes that degrade pectic substances in the cell wall (STARR and CHATTERJEE, 1972; BATEMAN and BASHAM, 1976). The most important pectolytic enzyme produced by this organism is pectic acid lyase (PAL, EC 4.2.2.2) which cleaves the α-1,4 bond of galacturonic acid polymers to produce a reducing group and a β-4,5 unsaturated uronide (BATEMAN and MILLAR, 1966).

A plant pathogen of this type necessarily encounters a variety of sugars and polysaccharides during growth in the cell wall region. If inducible enzymes are synthesised in order to degrade polysaccharides for growth, one may question whether the induction of certain enzymes is favoured over others, even though the substrates (and inducers) of all the enzymes are present. It appears reasonable to assume that the effect of the various substrates on the regulatory

mechanisms of the pathogen is one factor that may be of fundamental importance in determining the success of the pathogen. A study of the interacting factors regulating the synthesis and secretion of these enzymes is therefore economically and academically important.

Both *E. chrysanthemi* and *Escherichia coli* are classified in the Enterobacteriaceae (STARR and CHATTERJEE, 1972). This is particularly fortunate as the genetics and metabolic regulation of *E. coli* has been studied in more detail than any other organism. *E. coli* transports only two classes of protein across the plasma membrane, viz., the hydrophobic proteins of the outer membrane and the soluble periplasmic proteins (RANDALL *et al.*, 1980). In contrast, *E. chrysanthemi* produces several enzymes that also are secreted into the external medium. Because there is evidence that the genetics of genera in the Enterobacteriaceae is similar (CHATTERJEE and STARR, 1977), the mechanisms of metabolic regulation also may be similar. For these reasons *E. chrysanthemi* is an excellent model for studies on the regulation and secretion of proteins.

Cyclic AMP is a positive modulator of gene expression, and, when the levels in the cell are low, those genes that require it for expression are transcribed less frequently (PASTAN and ADHYA, 1976). Rapidly metabolised carbon sources, such as glucose, depress cAMP levels, thereby explaining the phenomenon of catabolite repression. Inducible enzymes are not synthesised until the rapidly metabolised carbon source is depleted and the cAMP level rises. This explanation is satisfactory when one of the carbon sources is metabolised by constitutive enzymes and the other is metabolised by inducible enzymes.

A logical extension of these studies is to investigate what occurs when the two carbon sources are metabolised by enzymes that themselves are both inducible and catabolite repressible. Are both enzyme systems induced simultaneously, or does induction of one take precedence over induction of the other?

Previous work on the lactose, arabinose and tryptophan operons in *E. coli* (LIS and SCHLEIF, 1973; PIOVANT and LAZDUNSKI, 1975; PAVLASOVÁ *et al.*, 1976, 1977, 1980) provides convincing evidence that these operons have differential cAMP requirements for expression. In all instances, the cAMP levels required for expression of the lactose operon are lower than the cAMP requirements for expression of the tryptophan and arabinose operons.

In this paper we present data on the induction and catabolite repression of PAL and β-galactosidase in *E. chrysanthemi* and show that there is a 'pecking order' in the production of inducible enzymes in the presence of their substrates.

MATERIALS AND METHODS

Culture Conditions

A local isolate of *E. chrysanthemi* pathogenic to maize (MILDENHALL, 1974) was maintained on nutrient agar plates at 20°C, and stored at −20°C in 40% (w/v) glycerol. The medium contained the following ingredients (g l^{-1}) : $Na_2HPO_4.2H_2O$, 1,77; KH_2PO_4, 0,27; $MgSO_4.7H_2O$, 0,24; NH_4NO_3, 0,40 ; and either 0,10 g l^{-1} yeast extract (low yeast medium) or 0,9 g l^{-1} (high yeast medium). The pH was adjusted to 7,4 before autoclaving. The carbon source was either lactose or sodium

polypectate (NaPP). They were autoclaved separately and added to the cooled basal medium as specified in the legends to the figures.

The inoculum was prepared from cells passed through two transfers on high yeast medium containing 0,18% (w/v) lactose. The cells from the second transfer were harvested during exponential growth by centrifugation (4°C, 5 000 g, 20 minutes) and resuspended in low yeast medium containing no carbon source.

All experiments were performed at 30°C.

Assays

Growth was monitored at 620 nm.

Pectic Acid Lyase (PAL, EC 4.2.2.2) activity in culture supernatants was assayed at 30°C on 0,4% (w/v) NaPP in 50 mM Tris-HCl buffer, pH 8,5, containing 1 mM Ca^{++} at 230 nm. One millilitre samples of culture were contrifuged in a Beckman Microfuge for 1 minute, and stored at 4°C until they were assayed. One unit of activity is defined as the production of 1 $u$mole of unsaturated uronide min^{-1}. A molar extinction coefficient for the unsaturated uronide of 4 600 $M^{-1}cm^{-1}$ (NAGEL and ANDERSON, 1965) was used to calculate enzyme activity.

β-galactosidase (EC 3.2.1.23) was assayed at 30°C using o-nitrophenyl- β-D-galactopyranoside (ONPG) as substrate. Samples (1 ml) were added to 20 ul of sodium deoxycholate and 20 ul of toluene. After shaking at 37°C for at least 30 minutes to remove the toluene, the samples were transferred to a 30°C waterbath and equilibrated for 10 minutes. Substrate (0,1 ml of 0,013 M ONPG in 0,25 M phosphate buffer, pH 7,0) was added and after a measurable quantity of product appeared (indicated by a yellow colour), the reaction was stopped by the addition of 3 ml of 1 M Na_2CO_3, and the time of incubation noted. The resulting absorbance of the solution was read at 420 and 550 nm against a water blank. Turbidity in the solution was corrected for by subtracting 1,71 x A_{550} from the 420 nm reading. One unit of activity is defined as the production of 1 $u$mole of o-nitrophenol (ONP) min^{-1} at 30°C. ONP has a molar extinction coefficient of 21 300 $M^{-1}cm^{-1}$ (JONES, 1969).

RESULTS AND DISCUSSION

Both β-galactosidase and PAL are inducible enzymes with only basal levels of these enzymes being produced, in the absence of their respective inducers, when grown on either lactose or NaPP (see Figs. 1 and 2). Synthesis of both enzymes is repressed by glucose (Fig. 1) and in this respect they resemble enzymes that are catabolite repressible in *E. coli* (PASTAN and ADHYA, 1976). Although NaPP, or the breakdown products, represses the production of β-galactosidase (Fig. 2A), there is no reciprocal repression of PAL synthesis by lactose (Fig. 2B). β-Galactosidase synthesis is repressed by NaPP, even if cells preinduced on lactose are used as inoculum. As the cells grow on lactose a functional permease must be present, yet enzymes degrading NaPP are induced in preference to enzymes responsible for lactose utilisation. The data in Figs. 1 and 2 clearly demonstrate that there is a 'pecking order' of induction of inducible enzymes in the cell. Enzymes metabolising glucose are produced constitutively by the cell, and, consequently, glucose is usually the preferred carbon source for growth.
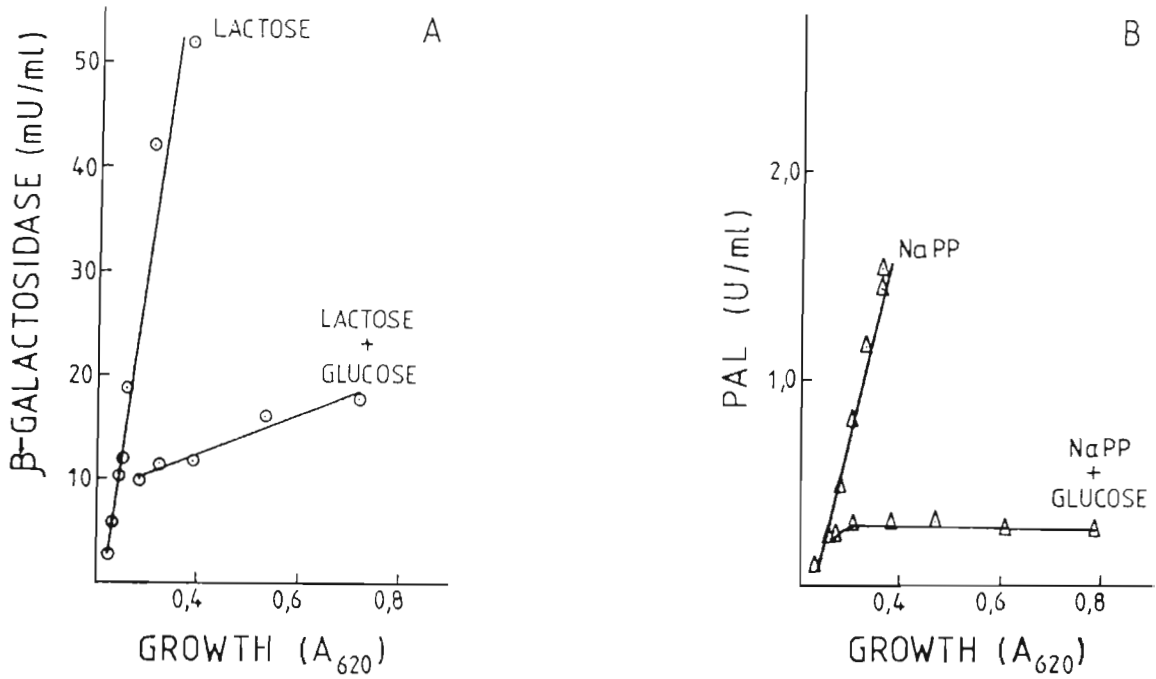
FIG. 1.   Repression of β-Galactosidase (A) and PAL (B) in *E. chrysanthemi* by Glucose.

    Flasks (250 mℓ) of low yeast medium (25 mℓ) containing either 0,18% (w/v) lactose (o—o)or 0,18% (w/v) NaPP (Δ—Δ) were inoculated with 1,0 mℓ of suitably diluted inoculum and placed on a shaker at 30○C. One hour later, glucose was added to each flask to give a final concentration of 10^{-3} M. The control flasks received an equivalent volume of sterile distilled water. Growth and enzyme levels were monitored as described in Materials. and Methods

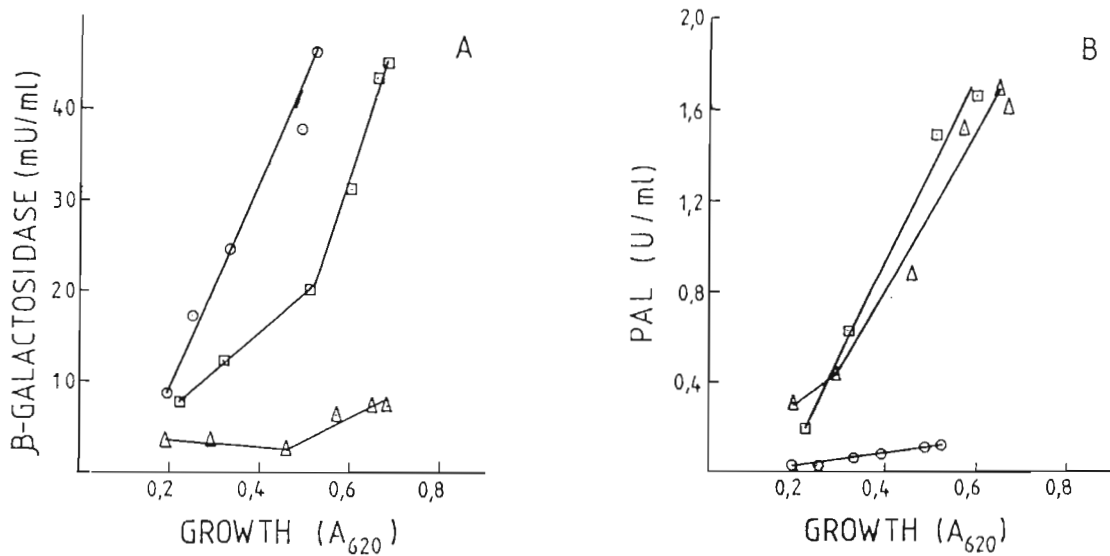FIG. 2.   Effect of NaPP and Lactose on β-Galactosidase (A) and PAL (B) Synthesis in *E. chrysanthemi*.

    Flasks (250 mℓ) of low yeast medium (25mℓ) containing either 0,18% (w/v) lactose (o—o), 0,18% (w/v) NaPP (Δ—Δ), or 0,09% (w/v) lactose +0,09% (w/v) NaPP (□—□) were inoculated with 1,0ℓ of suitably diluted inoculum. Growth and enzyme activity were assayed as described earlier.

The lactose operon in *E. coli* requires lower cAMP concentrations for expression than does either the arabinose operon (LIS and SCHLEIF, 1973) or the tryptophan operon (PIOVANT and LAZDUNSKI, 1975; PAVLASOVA *et al.*, 1976, 1977, 1980). This was determined by finding conditions that allowed expression of the lactose operon but not of the arabinose or tryptophan operons, and then determining the cAMP levels. Our results suggest that a similar situation may exist in *E. chrysanthemi* for the production of *B*-galactosidase and PAL. Cyclic AMP is known to be involved in gene expression in the closely related species *E. carotovora* (MOUNT *et al.*, 1979) and presumably is involved in *E. chrysanthemi*. Therefore, it must be concluded that NaPP, or its breakdown products, acts as a catabolite repressor of *B*-galactosidase. Unpublished data have established that the synthesis of this enzyme is not resumed until the NaPP is depleted in the medium.

Assuming that cAMP is involved in the expression of these two inducible enzymes in *E. chrysanthemi*, it can be argued that the cAMP concentrations ([cAMP]) in the presence of the various substrates can be ranked in the following order

$$[cAMP] \quad > \quad [cAMP] \quad > \quad [cAMP]$$
$$\text{lactose} \qquad\qquad \text{NaPP} \qquad\qquad \text{glucose}$$

A number of mechanisms can be offered to account for the requirement of different cAMP concentrations for the expression of different operons. Possibly the most economical to the cell is for the promoters of the different operons to display different affinities for the cAMP-Cyclic AMP Receptor Protein (cAMP-CRP) complex. Those promoters displaying high affinity for this complex are transcribed more efficiently at lower cAMP levels than operons having promoters with lower affinity for this complex. Each operon has the property of determining its own sequence of expression and rate of transcription.

Other possibilities exist that might explain these results and cannot be ignored. For example, each operon may code for a unique CRP and the resulting cAMP-CRP complexes could have different affinities for the promoters of their respective operons. There is no evidence for such a mechanism and it does not appear to be an economical proposition for the cell. Finally, there may be some factor that is involved in modifying the affinity of cAMP for CRP as has been proposed by ULLMANN *et al.*, (1976) and DESSAIN *et al.*, (1978a,b) in *E. coli*.

However, a serious problem exists in accepting that cAMP may be the only factor, other than the inducer, that regulates PAL and β-galactosidase synthesis. The results in Fig. 1 show that, quite contrary to the cAMP concentration sequence established above, PAL synthesis is more severely repressed by glucose than is the synthesis of β-galactosidase.

The effect of glucose on the cAMP levels in the cell should be considered in two ways. Firstly, glucose may lower the intracellular levels of cAMP to the same extent in the presence of both lactose and NaPP. This would imply that β-galactosidase requires a lower cAMP level for synthesis than does PAL. However, the data in Figs. 1 and 2 refute this order. A second possibility is that glucose lowers

the levels of cAMP in the presence of NaPP to a greater extent than it does in the presence of lactose, thereby accounting for the more severe repression of PAL. If the first possibility is correct, then further regulatory mechanisms imposed upon induction and catabolite repression that involve inducer uptake and protein secretion must be considered.

Work is currently underway to determine if other inducible enzymes exhibit a similar 'pecking order' of synthesis. Also, alternative mechanisms of control operative at levels other than induction and catabolite repression, viz., inducer uptake and protein secretion, are being investigated.

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o-Aminoacetophenone: Identification in a primitive fungus-growing ant *(Mycocepurus goeldii)*

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Summary. o-Aminoacetophenone is the major volatile product present in the mandibular gland secretion of the primitive fungus-growing ant *Mycocepurus goeldii*. This novel arthropod natural product is biosynthetically far removed from the aliphatic ketones and alcohols found in those genera of the tribe Attini that represent the main line of evolution. The divergent phylogenetic position of *Mycocepurus*, and possibly of other closely related genera, is emphasized.

Recent investigations[2-6] on the natural products chemistry of attine ants have demonstrated that these hymenopterans are a rich source of 2- and 3-alkanones, the corresponding alcohols, and in some cases, oxygenated monoterpenes. The ethyl ketones, which are the main releasers of alarm behavior in species in the more highly evolved genera[2,5], are generally the major compounds produced in the mandibular glands. An investigation[5] of the mandibular gland products of fungus-growing ants in several genera that reflect the accepted phylogeny of the tribe Attini[7] indicates that the distribution of these exocrine compounds is in accord with the recognized evolution of the genera in this taxon. However, whereas this chemosystematic study utilized species in genera whose established relationships clearly defined them as pivotal taxa in the phylogeny of the tribe[8], it did not include species in the small genera that appear to have diverged from the general attine stem[7]. We now wish to report that the mandibular gland chemistry of *Mycocepurus goeldii*[9], a species in one of these divergent genera, is dominated by o-aminoacetophenone, an exocrine compound unique to the tribe Attini or for that matter, to any other arthropod species.

Materials and methods. Colonies of *M. goeldii* were collected near Presidente Prudente, Brazil. Crushed heads of workers possess a strong grape-like fragrance and in southern Brazil these ants are sometimes referred to as the 'formica perfume' (perfume ant). Extracts were prepared either by dissecting mandibular glands or by crushing heads in spectrograde *n*-pentane; volatile compounds were resolved gas-chromatographically on both 1% OV-1, programmed from 100-250 °C at 5 °C/min, and 10% Carbowax 20 M, isothermally at 180 °C. Eluting compounds were collected on graphite and their mass spectra obtained by direct insertion into the ion source of a Bell and Howell 21-490 mass spectrometer.

Behavioral studies were undertaken on either field or laboratory colonies by exposing the ants to mandibular gland extracts, crushed heads, or pure compounds applied to filter paper squares (1 cm^2) or to the tips of wood applicator sticks. The activity of compounds as alarm releasers for another attine species, *Atta texana*, was determined as described previously[2,10].

Results. Four compounds, all present in mandibular gland extracts, were detected by gas chromatography, the major and final eluting one possessing the grape-like odor associated with *M. goeldii*. The mass spectrum of this compound was characterized by a molecular ion and base peak at m/z 135, with major fragments being present at m/z 120 (loss of CH_3), m/z 92 (further loss of $-COCH_3$), and m/z 65 (aromatic ring). The mass spectrum and retention times of this substance were completely congruent with those of authentic o-aminoacetophenone.

Three minor constituents, eluting earlier than aminoacetophenone, were not conclusively identified. The mass spectrum of the 1st of these compounds, possessing a strong molecular ion at m/z 150 and a base peak at m/z 135, was similar to o-methoxyacetophenone, but differed in minor respects. The other 2 compounds possessed molecular ions at m/z 218 and m/z 232, and their mass spectra were very similar to homofarnesene and bishomofarnesene[11]. Insufficient quantities of these compounds prevented their complete characterization.

Workers of *M. goeldii* are attracted to a crushed mandibular gland or head and respond similarly to 1 µg of o-aminoacetophenone placed on filter paper squares or tips of wood applicators. One the other hand, workers of the highly evolved attine species *Acromyrmex nigra*, *Atta laevigata* and *A. sexdens* do not appear to react to a crushed head of *M. goeldii*. High concentrations of o-aminoacetophenone are repellent to workers of *M. goeldii* whereas workers of the *Acromyrmex* and *Atta* species are slightly attracted to high concentrations of this compound. o-Aminoacetophenone was completely inactive as a releaser of alarm behavior for workers of *Atta texana* when compared at all concentrations to their natural alarm pheromone, 4-methyl-3-heptanone[1,10].

Discussion. The production of o-aminoacetophenone by workers of *M. goeldii* demonstrates that the exocrine chemistry of this fungus-growing ant differs considerably from those in genera representing the main line of attine evolution. Whereas aliphatic compounds such as 3-octanol and 4-methyl-3-heptanone are typical of the mandibular gland products identified in a variety of attine genera[4,5], aromatic natural products are clearly atypical of species in

817

taxa identified with the phylogeny of the Attini[7]. On the other hand, *Mycocepurus* is one of a series of small genera whose members cultivate specialized fungi that differ considerably from those produced by polymorphic attines such as *Acromyrmex* and *Atta*[7]. If the mandibular gland chemistry of *M. goeldii* is typical of other attine genera that have diverged from the main stem of attine evolution in not emphasizing simple aliphatic ketones and alcohols, then a large potential treasure-trove of natural products remains to be characterized. This could be particularly true for species in the genera *Mycetophylax* and *Mycetarotes,* two divergent taxa that are considered to be closely related to *Mycocepurus*[7].

Although *o*-aminoacetophenone exhibits demonstrable pheromonal activity for workers of *M. goeldii,* it possesses no pronounced activity for workers of attine species in more specialized genera. Species of the latter, such as *Atta texana,* utilize 4-methyl-3-heptanone as an alarm pheromone and in view of the great olfactory acuity they manifest for their own alarm pheromone[1], it is not surprising that they can readily distinguish this minty ethyl ketone from the unrelated grape-like aromatic ketone produced by *M. goeldii.* It is worth noting that another ant pheromone, methyl anthranilate[12], also is characterized by a powerful grape-like odor, and it may not be insignificant that both *o*-aminoacetophenone and methyl anthranilate possess similar shapes. Since insect pheromones have been utilized as paradigms for studying the relationships of molecular shape to odor quality[13], it would appear that these natural products, because they can be evaluated behaviorally, may be particularly useful for studying olfactory theory. Finally, as the degradation of tryptophan could possibly lead to the synthesis of *o*-aminoacetophenone, this species may provide a particularly useful model for a worthwhile biosynthetic study.

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Relation of Water Activity to Growth and Extracellular Pectate Lyase Production by *Erwinia chrysanthemi*

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Abstract

The growth of *Erwinia chrysanthemi* pv *zeae* was investigated in a glucose-yeast extract-salts (glucose-YS) medium adjusted to various water activities (A_w's) with either NaCl or mannose. The specific growth rate (n) was similar between 0.998 A_w and 0.990 but declined rapidly thereafter. Extracellular pectate lyase (PL) production decreased significantly between 0.998 and 0.980 A_w when either mannose, sorbitol, or D-arabinose was used to adjust A_w of the sodium polypectate (NaPP)-YS medium.

A similar reduction in enzyme production occurred when *E. chrysanthemi* was grown in galacturonic acid-YS medium adjusted with NaCl. PL activity declined rapidly in cell-free shake culture when the NaPP-YS medium, only, was adjusted to 0.990 (lactose) or 0.980 (mannose) but not at 0.990 (NaCl). Under stationary conditions, however, PL was stable in these media. When E. chrysanthemi was grown in these media under stationary culture, PL production was similar to that observed in shake culture. Decrease in PL production with bwered A_w could not be ascribed to enzyme deactivation in viscous media. The significance of these findings to pathogenicity will be discussed.

Introduction

Studies on the ecology of the soft rot erwinias have shown that free water is invariably required for the development of soft rot (9). The role of free water in the infection process has been ascribed to its effect on the turgidity of tissue and to the depletion of oxygen under saturated conditions. The availability of water, however, may also influence the growth and physiology of the pathogen itself. Although the importance of

water in soft rot development has been documented, there have been few quantitative studies on the moisture requirements of phytopathogenic bacteria (1, 15).

Bacterial stalk rot of maize (*Zea mays* L) caused by a pathovar of *Erwinia chrysanthemi* (Burkholder, McFadden and Dimock) characteriscally occurs on sprinkler irritated maize (4, 6, 7). The association of free water with the development of stalk rot prompted us to investigate the water relations of this pathogen.

The term water activity (A_w) as defined by Scott (14) has been widely accepted for determining the effect of solute or water removal on the growth and physiology of micro-organisms. The A_w is directly related to the relative humidity (RH):

$$A_w = \frac{P}{P_o} = \frac{\% \, RH}{100}$$

where P = vapor pressure of solution
Po = vapor pressure of pure water

Water activity is also directly related to water potential, a term that has been used in several studies on fungi (3).

$$\psi = \frac{-RT}{\bar{V}} \ln A_w$$

where ψ = water potential
R = ideal gas constant
T = temperature (°K)
$\bar{V}$ = mole volume of water

We selected liquid media for the investigation of the water relations of *E. chrysanthemi* because, first, the results appear to be similar to those obtained on solid surfaces (15) and, second, liquid media facilitate quantitative measurements of growth and enzyme production.

Pectate lyase (PL) or polygalacturonic acid trans-eliminase E.C 4.2.2.2 (2) is an important enzyme in tissue maceration. The growth *in vivo* of *E. chrysanthemi* may be profoundly influenced by factors which affect the production of PL. The objective of this study was to determine the effect of A_w upon growth and extracellular PL production by *E. chrysanthemi*. Growth and extracellular PL production are profoundly influenced by the A_w of the medium.

Materials and Methods

Organism

A local isolate of *E. chrysanthemi* (7) was used. This has been deposited in the collection of Prof. A. Kelman, Department of Plant Pathology, University of Wisconsin, Madison, U.S.A.

236

Inoculum Preparation

In all experiments, late logarithmic phase cells from a culture which had been seeded with log phase cells were used. One millilitre of inoculum was added to each flask. In all experiments the inoculum was grown on the same carbon source as that being investigated except for galaturonic acid-yeast extract salts (YS) medium where the inoculum was cultured in sodium polypectate (NaPP)-YS medium.

Medium •

The YS medium was similar to that described by Scott (14) and contained the following ingredients (g/1000 g H$_2$O): Na$_2$HPO$_4$, 1.42; KH$_2$PO$_4$, 0.27; MgSO$_4$7H$_2$O, 0.24; NH$_4$NO$_3$, 0.40; yeast extract, 0.90; carbon source, 1.80. This yielded a medium of pH 7.6 and no further adjustment was necessary. Either D-galacturonic acid, glucose or NaPP was used as carbon source. The galacturonic acid-YS medium was titrated with 1 N NaOH to pH 7.5 and filter sterilized. D-galacturonic acid was obtained from Sigma Chemical Corp., NaPP from Nutritional Biochemical Corp., yeast extract from Difco Laboratories and mannose and D-arabinose from Riedel de Haën, Hannover, W. Germany. All other chemicals were obtained from E. Merck, Darmstadt, W. Germany.

Water Activity Adjusters

The A$_w$ for growth studies was adjusted with either NaCl or mannose. NaCl was autoclaved in the glucose-YS medium. Mannose and glucose were filter sterilized into the autoclaved YS medium. Water lost during autoclaving was replaced. The amount of solute required to prepare media of various A$_w$'s (Table 1) was determined for NaCl from data of Robinson and Stokes (13) or by using Wecor psychometer (10).

Table 1. Water activity of solutes at 25°C.

| A$_w$ | Molality | | | | |
	Lactose (monohydrate)	Mannose (anhydrous)	NaCl	D-arabinose	Sorbitol
0.995	0.35	0.26	0.15	0.20	0.28
0.990	0.70	0.52	0.30	0.45	0.56
0.985	1.04	0.81	0.46	0.72	0.84
0.980	1.38	1.10	0.61	1.00	1.13
0.975	1.72	1.40	0.76	1.24	1.41
0.970	2.08	1.70	0.91	1.50	1.69

Growth and Enzyme Studies

All cultures were grown in 250 ml side-arm Erlenmeyer flasks and growth was measured on either a Klett-Summerson colorimeter with a no.

64 filter or at 620 nm on a Spectronic 20 spectrophotometer (Bausch & Lomb). One millilitre samples removed at suitable intervals for extracellular PL assays were assayed on a Beckman model 35 spectrophotometer with a recorder. Details of growth conditions and enzyme substrate preparation have been described elsewhere (8).

Enzyme Stability Studies

The stability of PL in the media adjusted to various A_w's using either NaCl (0.990 A_w), lactose (0.990 A_w), or mannose (0.980 A_w) was investigated in three different ways. First, 2 ml of a sterile dialyzed supernatant fluid ($\approx$20 units PL/ml) was added to 25 ml cell-free medium and shaken as described previously (8). Second, the organism was grown (shake culture, 8) in double strength NaPP-YS medium to early stationary phase (absorbance 0.60) and then the A_w adjusters were added. Third, flasks were prepared as in the first instance but were not shaken. At suitable intervals samples were removed and assayed for PL. The cell-free dialyzed supernatant fluid was prepared as previously described (8).

Results

Evaluation of A_w Adjusters

Difficulties were encountered in selecting a suitable A_w adjuster that is freely soluble in water, is not used as a nutrient, neither represses nor induces PL production, and is not toxic to *E. chrysanthemi*. NaCl was selected as an ionic adjuster and among the organic solutes, sorbitol, D-arabinose, mannose, and lactose were used (Table 2).

Effects of A_w on Growth

The growth rate of *E. chrysanthemi* in glucose-YS medium was similar at 0.998 A_w and 0.990 A_w but declined rapidly when the A_w was lowered to 0.970 (Figs. 1-2). *E. chrysanthemi* failed to grow at 0.970 A_w (NaCl, Fig. 1) within 16 h but upon prolonged incubation growth was observed in some flasks. At 0.970 A_w mannose was less inhibitory to growth (Fig. 2) than NaCl.

Effect of A_w on Extracellular PL Production

Lowering the A_w of the NaPP-YS medium with either sorbitol (Fig. 3) or D-arabinose (Fig. 4) resulted in a significant decline in PL levels although the final cell concentration was similar (Table 3). A similar trend was observed in galacturonic acid-YS medium adjusted with NaCl (Fig. 5).

Effect of A_w on Enzyme Stability

Pectate lyase was unstable in cell-free shaken media (Fig. 6A) but not under still conditions (Fig. 6C). The enzyme was most stable in the medium containing NaCl (0.990 A_w) and least stable in the medium containing mannose (0.980 A_w) and lactose (0.990 A_w) (Fig. 6A, C). Addition of these A_w adjusters to an early stationary phase culture resulted in a similar

pattern of enzyme inactivation (Fig. 6B). When *E. chrysanthemi* was grown to an absorbance of 0.25 in shake culture and subsequently incubated in still culture, extracellular PL production declined with a lowering of the A_w (Fig. 7).

Table 2. Evaluation of solutes for adjusting the A_w of growth media for *Erwinia chrysanthemi*.

A_w adjuster	Growth rate as a o/o of the rate on NaPP	Remarks
NaCl	0	NaPP but not galacturonic acid precipitates below 0.990 A_w
Lactose	55	Poorly soluble at 0.985 A_w PL not repressed
PEG 400	0	Toxic
Sucrose	57	Metabolized PL repressed
Mannose	29	Slowly metabolized PL not repressed
D-arabinose	0	Not metabolized PL not repressed
Glycerol	71	Rapidly metabolized
Sorbitol	0	PL not repressed

Fig. 1. Effect of A_w (NaCl) on growth of *Erwinia chrysanthemi* in glucose-YS medium at 0.998 A_w, 0.990 A_w, and 0.970 A_w. Each point represents the mean of six determinations. The specific growth rates (h-1) at each A_w were 0.63, 0.58, 0.35, and 0.24, respectively.

Table 3. Maximum specific activities of extracellular pectate lyase attained by *E. chrysanthemi* during exponential growth at various water activities.

Adjuster	Water activity					
	0.998	0.995	0.993	0.990	0.985	0.980
Lactose[a]	11.05[c]	7.40	7.48	3.06	ND[d]	ND
Mannose[a]	5.98	1.66	ND	0.37	ND	0.43
D-arabinose[a]	5.52	0.83	ND	0.27	0.24	0.14
Sorbitol[a]	5.46	1.08	ND	0.47	ND	ND
Sodium chloride[a]	13.81	5.29	11.60	20.44	ND	ND
Sodium chloride[b]	1.76	ND	ND	1.32	0.70	0.70

[a]Sodium polypectate as principal carbon source
[b]Sodium galacturonate as principal carbon source
[c]Specific activity expressed as micromol unsaturated uronide released per min per mg cell protein
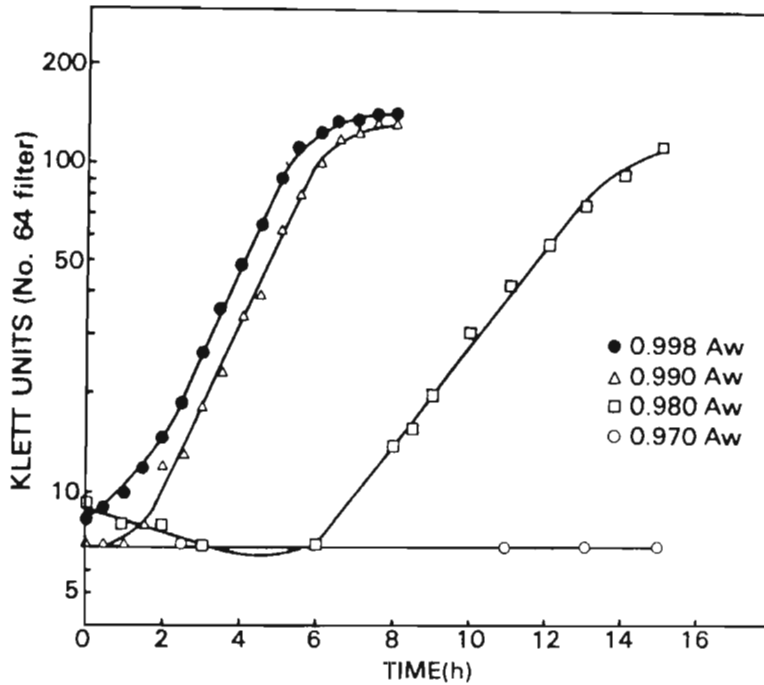[d]Not determined.
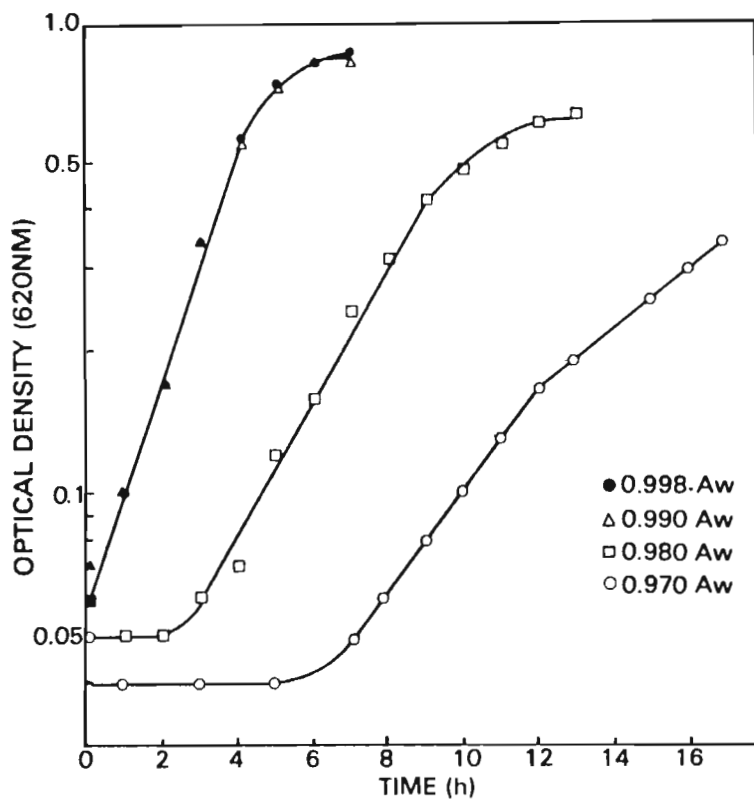
Fig. 2. Effect of A_w (mannose) on *Erwinia chrysanthemi* in glucose-YS medium at 0.998 A_w, 0.990 A_w, 0.980 A_w, 0.970 A_w. Each point represents the mean of three determinations. The specific growth rates (h^{-1}) at each A_w were 0.55, 0.32, and 0.24, respectively.
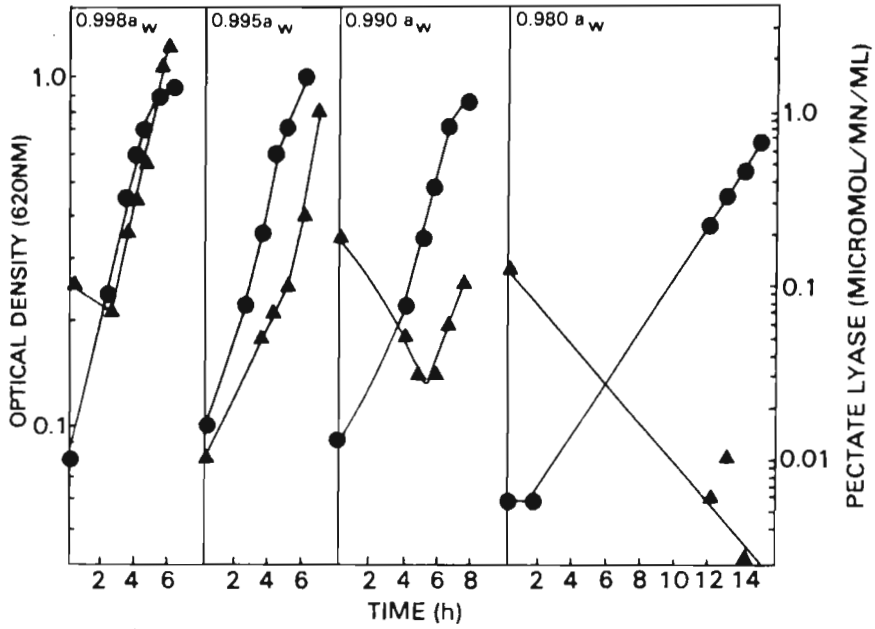
Fig. 3. Relation of growth (●) to pectate lyase production (▲) by *Erwinia chrysanthemi* in NaPP-YS medium at various A w's adjusted with sorbitol. Each point represents the mean of three determinations.
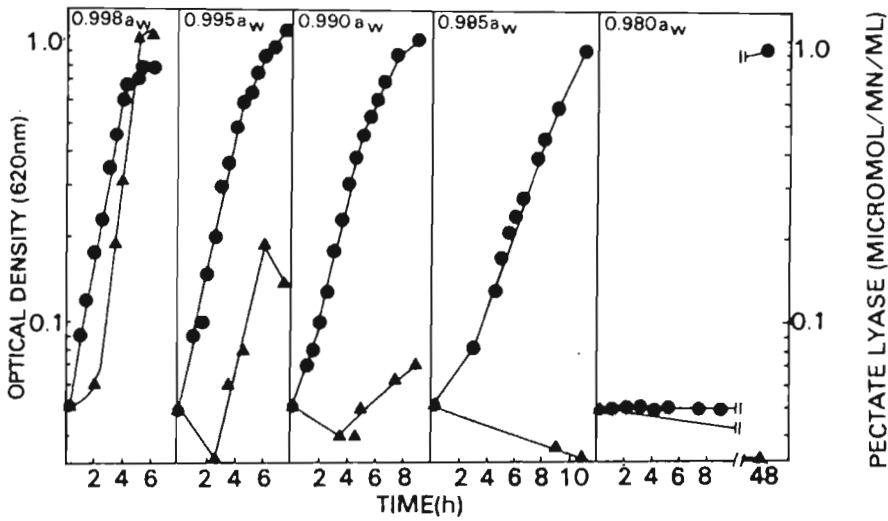
Fig. 4. Relation of growth (●) to pectate lyase production (▲) by *Erwinia chrysanthemi* in NaPP-YS medium at various A w's adjusted with D-arabinose. Each point represents the mean of three determinations.
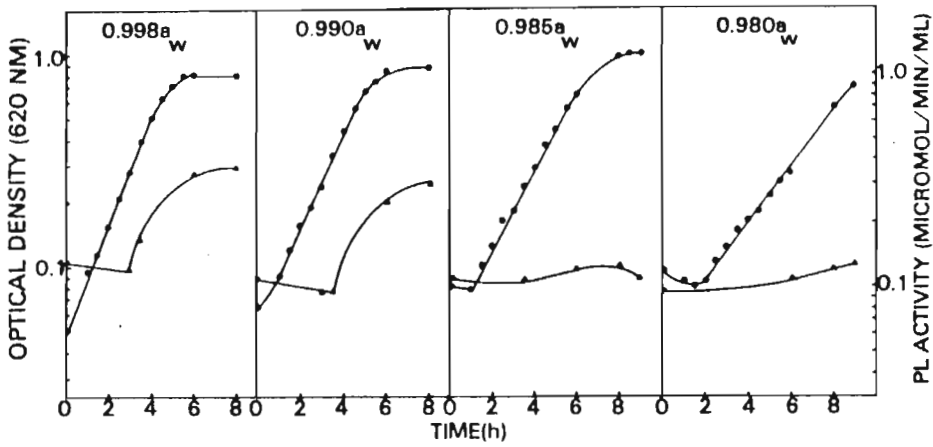
Fig. 5. Relation of growth (●) to pectate lyase production (▲) by *Erwinia chrysanthemi* in galacturonic acid—YS medium at various A_w's adjusted with NaCl. Each point represents the mean of three determinations.
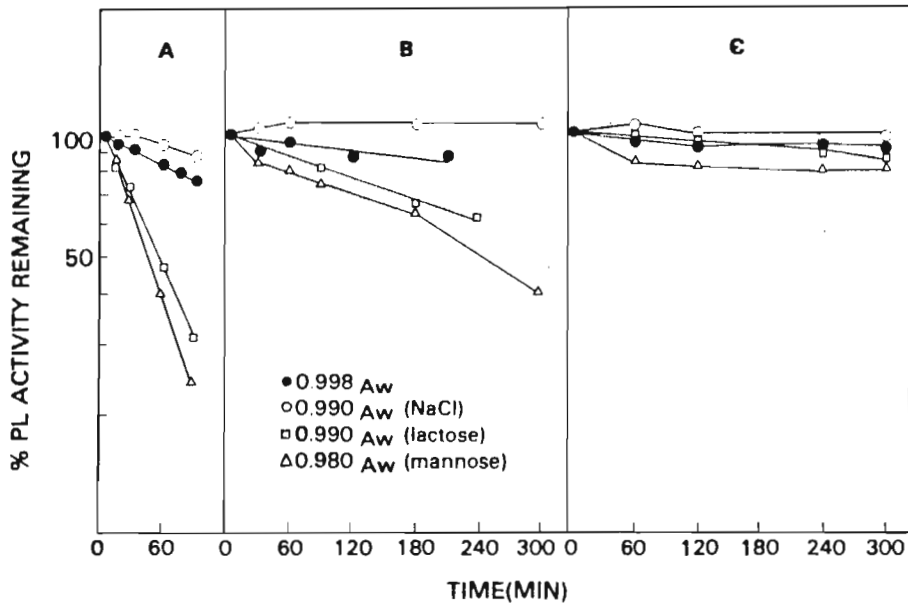
Fig. 6. Stability of pectate lyase of *Erwinia chrysanthemi* at 0x998 A_w, 0.990 A (NaCl), 0.990 A_w (lactose), and 0.980 A_w (mannose). A=cell-free shake medium; B=organism grown to early stationary phase, adjuster added to desired A_w, and culture shaken; C=cell-free still medium.

243
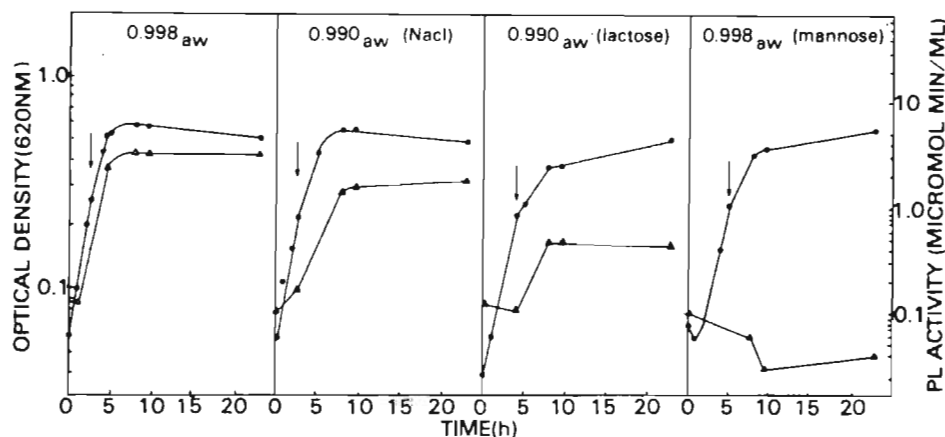
Fig. 7. Effect of agitation on growth (●) and pectate lyase production (△) by *Erwinia chrysanthemi* at various A_w's. Arrows indicate when shaking of cultures ceased.

Discussion

Growth and extracellular PL production by *E. chrysanthemi* are profoundly influenced by lowering the A_w of the medium. The specific growth rate (n) was similar at 0.998 A_w and 0.990 A_w but further lowering of A_w resulted in a rapid decrease of n. A decline in PL production was associated with the lowering of the A_w of both the NaPP-YS medium, adjusted with either sorbitol or D-arabinose (Figs. 3 and 4) and the galacturonic acid-YS medium adjusted with NaCl (Fig. 5). Similar results were obtained when either mannose or lactose was used as A_w adjusters of the NaPP-YS medium (8). An anomaly of these results was the higher levels of PL observed in the NaPP-YS medium adjusted with NaCl (Table 3). Because NaCl precipitated NaPP at A_w's less than 0.990 (Table 2), we were unable to investigate this effect at lower A_w's.

The data presented in Figs. 6 and 7 may be interpreted as follows: First, the addition of NaCl (0.990 A_w) to the NaPP-YS medium stabilizes the enzyme (Fig. 6). Second, organic solutes inactivate the enzyme in shaken media (Fig. 6A, B) but not in still media (Fig. 6C). Similarly, other enzymes are inactivated by shaking in viscous media (12). Third, the decrease in PL production associated with lowered A_w when the organism was grown in still culture (Fig. 7) suggests that the results obtained in shake culture (Figs. 3 and 4) are not solely due to enzyme inactivation from shaking but also to an A_w effect.

These studies, however, do not explain whether the lowering of the A_w affected the synthesis or the excretion of PL. Previous studies have shown that A_w affects the intracellular levels of enzymes in *Pseudomonas fluorescens* (11) and membrane permeability in *Staphylococcus aureus* (5). Elucidation of the mechanism whereby A_w affects PL production demands further study.

244

Shaw (15) found the *Erwinia amylovora* failed to grow *in vitro* below 97% RH, both in liquid and on solid media. Pear fruits and shoots maintained in environments of different RH's failed to develop disease below 97% RH when inoculated with *E. amylovora*. Therefore the minimum RH at which growth occurred *in vitro* corresponded to the minimum RH in which disease developed. Studies on the infection of maize at different RH's by *E. chrysanthemi* are in progress.

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A SIMPLE STAINING-DESTAINING APPARATUS FOR CYLINDRICAL POLYACRYLAMIDE GELS

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ABSTRACT

Construction details of a cheap and effective stainer-destainer for cylindrical polyacrylamide gels are presented.

The detection of proteins separated electrophorectically in cylindrical polyacrylamide gels usually requires staining the protein bands, followed by destaining the gel matrix. The staining procedure invariably relies on diffusion, but destaining of the gels can be accomplished either electrophoretically (DAVIS, 1964) or by diffusion (SERGREST and JACKSON, 1972). Electrophoretic destaining is rapid but poorly fixed proteins may be lost and protein bands may be displaced. Diffusion destaining has the advantage that protein bands are not displaced (CHRAMBACH *et al.*, 1976).

A particular study of ours concerns the quantitative comparative analysis of proteins in the culture filtrates of the cellulolytic fungus, *Trichoderma reesei*. Gels were stained and destained by diffusion in a nylon block with slotted holes that accommodated the gels. Destained gels looked perfectly satisfactory, but, on scanning in a Beckman 35 spectrophotometer fitted with a gel scanner, we found that the absorbance of protein bands varied considerably if the gels were rotated 90° and rescanned. This presumably was due to two sides of the gel being more exposed during staining and destaining. Diffusion destainers are commercially available that allow even diffusion from the entire gel surface (e.g. Bio-Rad model 172A, Catalogue F, p. 108) but are expensive.

We have designed, made, and used a stainer-destainer that is cheap (about R25 for materials) and produces evenly stained gels. We must point out that at this University an individual department is not required to pay for the time of machine shop personnel. The apparatus and construction details are presented in Fig. 1A and B. The various plates are plexiglas, glass rods (2.75 mm diameter) hold the gels, spacer rods (80 mm) and screws are stainless steel. The 3 large plates have an O.D. of 165 mm and the central holes have a diameter of 65 mm. Details and dimensions of the holes for the glass rods are given in Fig. 1B. Two drill jigs were constructed for drilling the holes in the top plate; the first was used to drill the four 2.75 mm diameter holes, and the second, with two flattened locator pins, was used to drill the 7 mm hole. These dimensions give a distance of 6 mm between the glass rods to accommodate the 5 mm diameter gels.

After running gels in the usual manner each gel is removed from its tube and rinsed into a numbered position. With the gels inserted (any number can be used) the whole apparatus is placed in a large beaker containing the appropriate stain and the solution stirred with a magnetic stirring bar. The diameter of the beaker

A
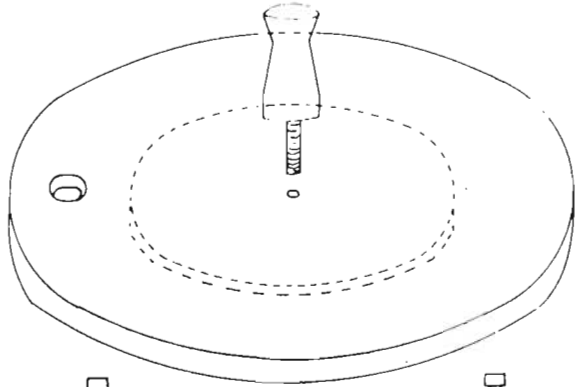
DIAL
PLATE
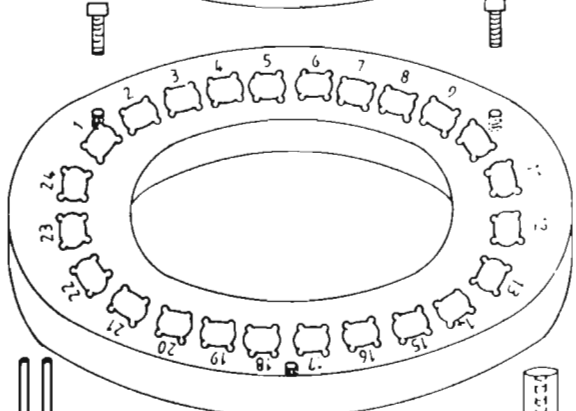
TOP
PLATE

GLASS
RODS
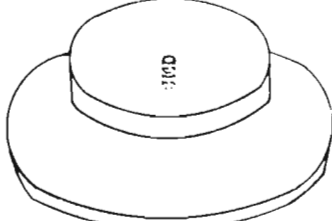
SPACER
ROD

DIAL PLATE
HOLDER
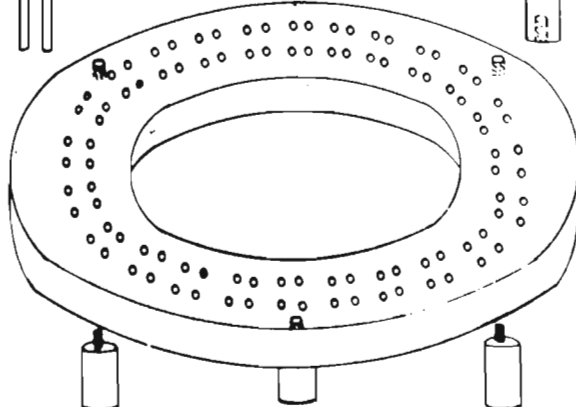
BOTTOM
PLATE

FEET

B

6.88 mm

6.88 mm

7.0 mm

2.75 mm
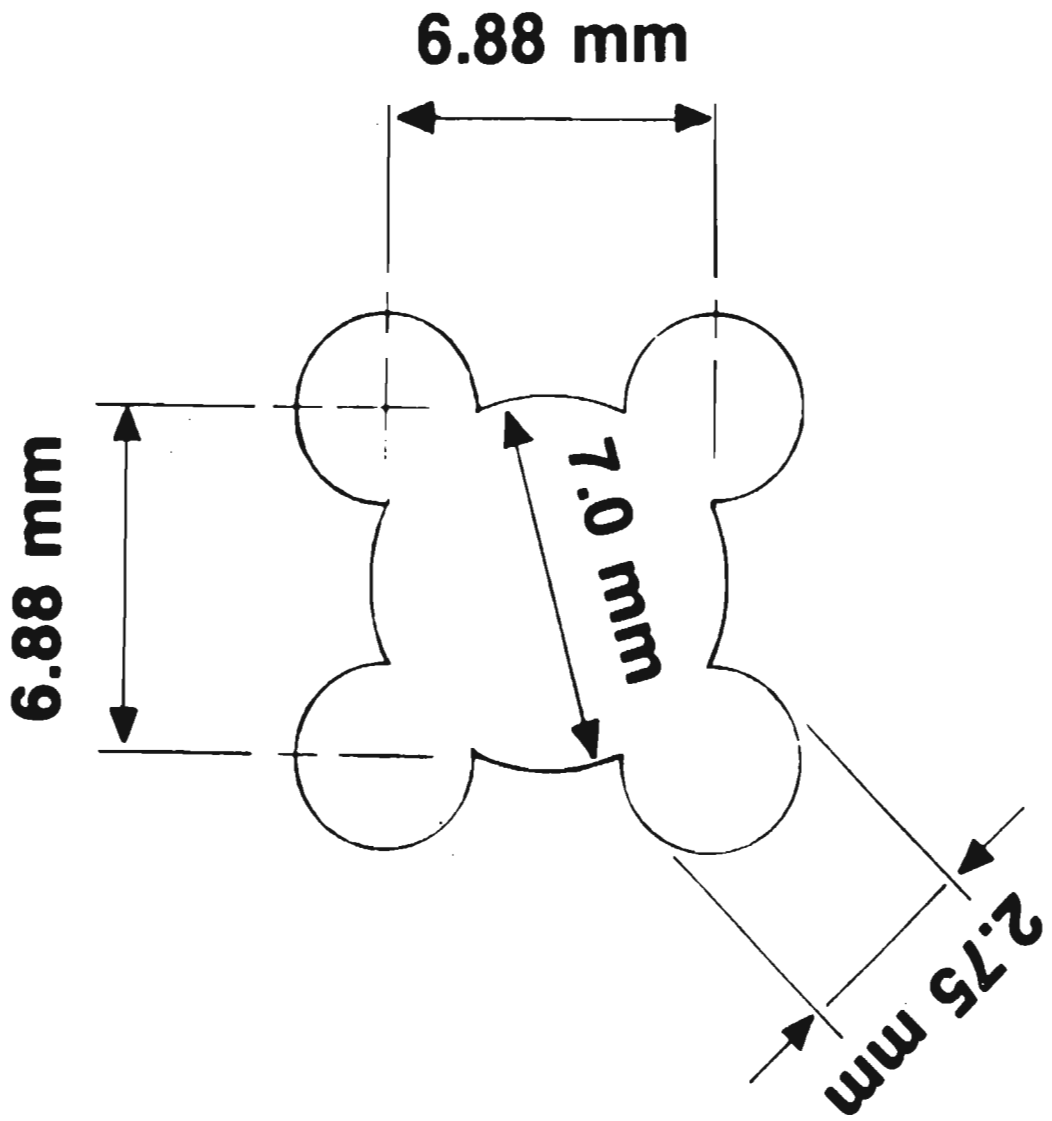
FIG. 1a and b. Diagrammatic representation of the gel stainer-destainer apparatus

must be at least 20 mm greater than the apparatus. Alternatively, gels may be stained individually in tubes and inserted in the apparatus for destaining only.

Destaining is carried out against the appropriate solution in a similar beaker with a magnetic stirrer without having to remove the gels. The stirring bar creates a current that moves upwards around the outside of the glass rods, passes through them and around the gels, and returns down the middle. With a gentle vortex formed, the gels wobble slowly in the 1 mm clearance they have in the glass rod cage. When destaining is complete, a plastic tube is inserted in the hole in the dial plate, the whole apparatus inverted, and the gel rinsed into the tube with a jet of water from a squeeze bottle. Gels stained and destained with this apparatus are found to be uniformly stained and suitable for scanning.

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SOME THERMODYNAMIC CONSIDERATIONS FOR ESTABLISHING COCULTURE CONDITIONS FOR H₂-PRODUCING ACETOGENIC BACTERIA WITH H₂-UTILIZING BACTERIA

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ABSTRACT

Short chain fatty acids (other than acetate and formate) produced by the primary fermenters are metabolized to acetate and H_2 by a group of strict anaerobes. Certain species of this H_2-producing acetogenic group recently have been grown in coculture with H_2-utilizing methanogens and desulfovibrios by other workers. Propionate utilizers proved particularly difficult. We show how a cursory look at some fundamental thermodynamic considerations may improve the success rate of attempted cocultures by establishing necessary conditions, and illustrating parameters to be avoided, such as a high percentage of CO_2 in the gas phase.

Many microorganisms produce short chain fatty acids as end products of anaerobic metabolism. These fatty acids must in turn be broken down further, finally yielding CO_2 and CH_4. A group of strict anaerobes is now known to exist that degrades fatty acids, longer than acetate and formate, to H_2 and acetate (Bryant, 1979; McInerney *et al.* 1979; Boone and Bryant, 1980; McInerney *et al.*, 1981a; McInerney *et al.*, 1981b). This syntrophic association between species is thermodynamically possible only if the H_2 produced by this group of anaerobes, as well as by the primary fermenters, is maintained at a very low partial pressure by associated H_2-utilizing organisms. The successful growing of syntrophic cocultures of H_2-producing acetogenic species with H_2-utilizing species has confirmed the thermodynamic constraints imposed on this association (McInerney *et al.*, 1979; Boone and Bryant, 1980). This control of the emphasis of anaerobic metabolic end products by the H_2 partial pressure has been described by Wolin (1974, 1976) and Kaspar and Wuhrmann (1978a, 1978b).

Recently, Bryant (1979) has outlined the important ecological role of this group of H_2-producing bacteria. They are considered to metabolize the short chain fatty acids (longer than acetate) produced by the primary fermenters thereby maintaining the open steady state condition of many important microbial ecosystems. This group of organisms is not considered to function during a flux of readily fermentable substrate due to the considerable amount of H_2 produced by the primary fermenters under these conditions (except, of course in non-representative isolated ecological pockets). It is only when the H_2 partial pressure has been reduced to a low level by terminal fermenters, such as H_2-utilizing methanogens and desulfovibrios, that the thermodynamics of the conversion of short chain

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H_2 producing reactions is the low partial pressure of H_2 maintained by

fatty acids to acetate and H_2 will yield energy for the H_2-producing acetogenic group.

Difficulties encountered in the attempted isolation of cocultures of the H_2-producing acetogenic group with species of either methanogens or desulfovibrios, none of which can use acetate, have been explained by considering the thermodynamics of the conversion of butyrate to acetate and H_2, and of propionate to acetate, CO_2, and H_2 (McINERNEY et al., 1979; BOONE and BRYANT, 1980). Under natural conditions the driving force of H_2-utilizing organisms. It is primarily this low partial pressure that makes ΔG differ considerably from $\Delta G^{o'}$ in cultures of H_2-forming bacteria (THAUER et al., 1977). In general, H_2 may be the major consideration, but conditions for coculture isolations must also take note of the effect of any additional factors imposed by the experimental conditions.

Previous workers have considered the thermodynamics of the conversion of short chain fatty acids to acetate and H_2 under biological standard state conditions, i.e. $\Delta G^{o'}$ (McINERNEY et al., 1979; BOONE and BRYANT, 1980). In order to obtain a more realistic view of the energetics of these reactions in natural ecosystems, one should consider the actual partial pressures that are likely to occur. An organism can only obtain energy from a reaction under conditions for which ΔG is negative. One must therefore establish conditions under which this would be fulfilled in any coculture isolation attempts. We believe that these calculations can lead to a better understanding of difficulties encountered in coculture isolations of H_2-producing acetogenic bacteria with H_2-utilizing bacteria. This appears particularly important in establishing coculture conditions for those organisms that convert propionate to acetate, CO_2 and H_2. In this case the CO_2 concentration is also of prime importance in setting up conditions to provide a realistically favourable thermodynamic situation for the two organisms.

Consider the following reactions:

1. H_2-producing acetogenic bacteria

$$\Delta G^{o'} \text{ kJ/reaction}$$
$$\text{(THAUER et al., 1977)}$$

(a)   Butyrate$^- + 2H_2O = 2$ Acetate$^- + 2H_2 + H^+$ $+48.1$

(b)   Propionate$^- + 3H_2O =$ Acetate$^- + HCO_3^- + 3H_2 + H^+$ $+76.1$

2. H_2-utilizing methanogens and desulfovibrios

(c) $4H_2 + HCO_3^- + H^+ = CH_4 + 3H_2O$ -135.6

(d) $4H_2 + SO_4^{2-} + H^+ = 4H_2O + HS^-$ -151.9
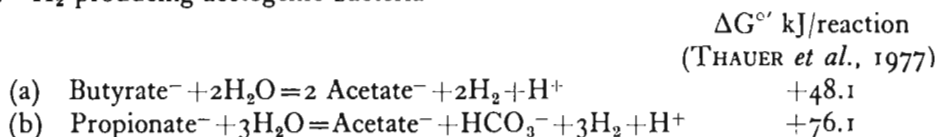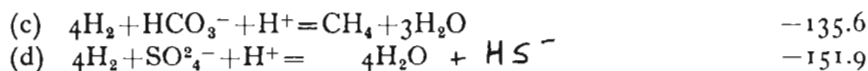
It suggested that either of the organisms in 2 above could drive either of the reactions in 1 above to the point of having a negative ΔG by lowering the partial pressure of H_2 in their environment (BOONE and BRYANT, 1980).

The concentrations of acetate, propionate, and butyrate in the rumen and ceca of some animals is usually in the order, acetate > propionate > butyrate (WOLIN, 1974). MACKIE and BRYANT (1981) found that the order in digesters fermenting cattle waste was approximately 2 (acetate) = propionate = 8 (butyrate). However,

in certain gut systems the concentration of butyrate may be greater than propionate (see WOLIN, 1974). From the published data it appears that under the steady state conditions that may actually prevail in the natural environment when these overall reactions occur, the concentrations of the various fatty acids will be of the order of 1 mM. In the following calculations we make the assumption that [acetate] = [propionate] = [butyrate] = 1 mM as ΔG is not affected appreciably by small changes in their concentrations.

At equilibrium, $\Delta G = 0$, and we can calculate $[H_2]$ for the butyrate reaction from the equation, $\Delta G^{\circ\prime} = -RT \ln K$ (T = 298°K).

$$48.1 = -5.708 \log \frac{[\text{acetate}]^2 \, [H_2]^2}{[\text{butyrate}]}$$

Therefore,

When [butyrate] = [acetate] = 1 mM, then $[H]_2 = 1.9 \times 10^{-3}$ atm.

BOONE and BRYANT (1980) state that the isolation of a propionate degrader in coculture with a second organism would be more difficult than the isolation of a butyrate degrader because the thermodynamics of the reactions would require a lower partial pressure of H_2 for the propionate degrader. The $\Delta G^{\circ\prime}$ value for the butyrate reaction is +48.1 kJ/reaction and +76.1 kJ/reaction for the propionate reaction (THAUER et al., 1977).

In the roll tubes used by BOONE and BRYANT (1980) for coculture isolations for both the butyrate and propionate degraders, the gas phase composition was 80% N_2 and 20% CO_2. As the propionate degrader produces CO_2 as a product of the reaction, the $[HCO_3^-]$ must be calculated and substituted in the equation in order to calculate H_2 partial pressure. Using the appropriate converion equations from Umbreit et al. (1959), for a pH of 7, 40°C, and 1 atm pressure, the $[HCO_3^-]$ for 20% CO_2 in the gas phase is 2.37×10^{-2} moles 1^{-1}. Under these conditions, the H_2 partial pressure of the propionate reaction at equilibrium is 1.25×10^{-4} atm, which is about an order of magnitude lower than that of the butyrate reaction at equilibrium.

As CO_2 is a product of the propionate reaction, one can calculate the CO_2 concentration at equilibrium for a H_2 partial pressure of 1.9×10^{-3} atm, i.e. equal to the butyrate reaction. Because CO_2 is not a product of the butyrate reaction, cocultures with either a butyrate degrader or a propionate degrader under these conditions should grow equally well. At a gas phase CO_2 percentage of 5.7, the H_2 partial pressures should be equal for both cocultures.

It appears that if roll tube coculture conditions were set up such that the CO_2 content of the gas phase for both the butyrate and propionate degraders was about 1 — 2%, then cocultures with a methanogen should be equally succeessful. Alternatively, if it is the $[HCO_3^-]$ that is important, as we are assuming, then this concentration could be influenced by buffering the medium at a pH lower than 7.

Our calculated values of H_2 partial pressure at equilibrium indicate that for the butyrate degrader to obtain energy it must be almost 10^{-4} atm or lower, and for the propionate degrader to obtain energy in 20% CO_2 (pH of 7, 40°C, and 1 atm pressure) it must be almost 10^{-5} atm or lower. Can these values lead us to say

anything about whether the choice of methanogen or sulfate-reducer would be equally successful? The difficulty of obtaining a coculture of a propionate degrader and a methanogen has been stated (BOONE and BRYANT, 1980).

Methanogens generally grow only at H_2 electrode potentials more negative than -0.330 V (SMITH and HUNGATE, 1958; HUNGATE, 1972), while desulfovibrios can grow at electrode potentials of -0.150 V (POSTGATE, 1959; BROWN *et al.*, 1973). Using the following equation, the H_2 partial pressures corresponding to -0.330 V and -0.150 V can be obtained.

$$E = E^\circ + \frac{RT}{nF} \ln \frac{[H^+]^2}{[H_2]}$$

$$\text{e.g. } -0.330 = 0 + 0.0296 \log \frac{[10^{-7}]^2}{[H_2]}$$

$$[H_2] = 1.41 \times 10^{-3} \text{ atm}$$

One may therefore assume that most methanogens will not grow at a H_2 partial pressure much lower than 1.41×10^{-3} atm and that the desulfovibrios will not grow at a H_2 partial pressure much lower than 1.2×10^{-9} atm.

The ability of the desulfovibrios to lower the H_2 partial pressure more than the methanogens, thereby increasing the negative value of ΔG for the reaction of the H_2-producing acetogenic bacteria, would make available more energy to the fatty acid degraders and allow a more rapid growth rate. Possibly of greater significance, the H_2 partial pressure window for which either coculture can succeed with a methanogen, is narrow with only a little energy available. In the case of the propionate degrader in a roll tube containing 20% CO_2 the window may be so narrow that the coculture, in fact, cannot succeed.

This factor may be a prime consideration in obtaining the correct conditions necessary for obtaining a successful coculture, especially for a propionate degrader that produces both CO_2 and H_2. The choice of an appropriate methanogen, and the necessary culture conditions, should be far more critical than the choice of an appropriate desulfovibrio and associated culture conditions, due mainly to the width of the H_2 partial pressure window in which the fatty acid degraders can obtain energy.

In the natural environment, (where methanogens dominate), when the terminal fermenters have reduced the H_2 partial pressure to a more or less steady state level, the gas phase composition is about 60% CO_2 and 40% CH_4 (M J KLUG, personal communication). While the $[HCO_3^-]$ will be dependent on the pH of the ecological microenvironment, it is almost certain that the propionate degraders will require a lower H_2 partial pressure for growth than the butyrate degraders in these situations. However, before setting up experimentally determined coculture conditions, a cursory look at thermodynamic considerations may be a most valuable asset.

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Activation Energy: A Coherent Approach for Undergraduate Teaching

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A concept central to the understanding of enzyme action is that of activation energy, yet many introductory textbooks treat activation energy in a cursory and, we feel, misleading fashion. Figure 1, a typical textbook representation of the free energy change during an enzyme-catalyzed reaction, conveys the distinct impression that the sum of the free energies of E and S is lower than the free energy of the activated complex, ES*. In reality, were this so, E and S would never react to produce ES* since ΔG^* for the reaction is positive.

No doubt anticipating this objection, a number of authors resort in the accompanying text to arguments of a statistical nature. Seen in these terms, the various E + S pairs exhibit a range of free energies with an average somewhat below that of an ES* molecule. A minority of pairs however possesses a free energy greater than the average by an amount of ΔG^* or more. It is only these more energetic pairs that react spontaneously to produce ES*.

Whereas the statistical argument just outlined is probably quite acceptable on its own, its relevance to Fig 1 is highly questionable. For example, if only those E + S pairs react whose combined free energy is either equal to or greater than that of a molecule of ES*, why is not the free energy level of E + S in Fig 1 either on a level with or higher than the level of ES*? The rejoinder might well be that the free energy level of E + S represents the average of the E + S pairs and is, by the statistical argument, naturally lower than ES*. Such an answer is illogical since by the same argument it is not the average E + S pairs that react, but rather it is the more energetic ones that do so. Further, if free energy in Fig 1 really only refers to the average free energy per molecule, why should the free energy level of E + P be necessarily lower than E + S?
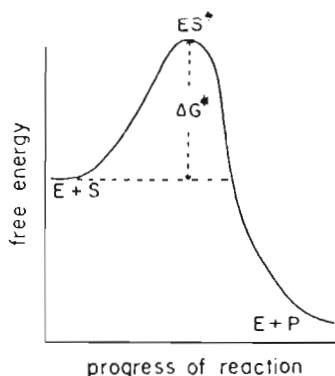
Figure 1 A typical activation energy diagram. E, S and P represent enzyme, substrate and product, and ES* symbolises the activated enzyme-substrate complex. The positive value of ΔG^* is misleading since it implies that the reaction E + S → ES* cannot take place.

The question arises as to whether Fig 1 should be altogether eliminated, retaining only the statistical argument, or whether Fig 1 should be retained in a more meaningful form. We suggest the latter course for two reasons. Firstly, a compact diagram is often more readily assimilated than a paragraph (or more) of text. Secondly, Fig 1 can be converted readily into an unambiguous macroscopic thermodynamic argument involving no concepts other than the familiar ones of standard free energy change and equilibrium constant. This transformation is achieved by substituting 'Standard Free Energy' for 'Free Energy' on the ordinate to yield Fig 2.
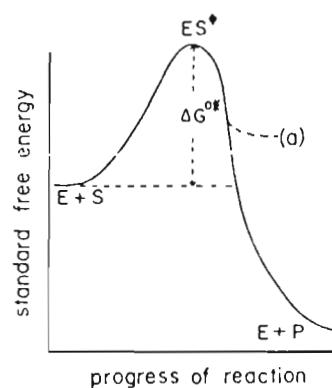
Figure 2 An improved activation energy diagram. 'Standard free energy' has replaced the 'Free energy' of Fig 1. Note that since standard free energies are being considered, the energy level of E + P may be either lower than E + S, as generally depicted, or may be higher than E + S as indicated by the dashed line at (a). Similarly, $\Delta G^{\circ}* > 0$ implies no thermodynamic contradiction.

The revised diagram may now be taken quite literally since a positive $\Delta G^{\circ}*$ for the reaction E + S → ES* implies no thermodynamic contradiction. In fact, as indicated in Fig 2, ΔG° for the overall reaction (E + S → E + P) may also be positive. In the text accompanying Fig 2 it might be pointed out that according to transition-state theory, (a) E + S and ES* are in equilibrium (and thus $\Delta G^* = 0$ for the reaction E + S → ES*!), and (b) the rate of product formation is directly proportional to the concentration of ES* It should be clear that a large equilibrium constant

$$K^* = [ES^*]/[E][S]$$

implies a comparatively high reaction rate and from the familiar equations, $\Delta G^{\circ}* = -RT \ln K^*$ or $K^* = e^{-\Delta G^{\circ}*/RT}$, the inverse relation between $\Delta G^{\circ}*$ and reaction rate should be perfectly understandable.

In conclusion it is worth stressing that whereas the overall standard free energy change (ΔG°) has no influence on reaction rate, the same cannot be said of the standard free energy of activation ($\Delta G^{\circ}*$). Although there is nothing contradictory in this, it is interesting to find an example in which a standard free energy change ($\Delta G^{\circ}*$) *does* influence reaction rate.

LACTOSE METABOLISM IN *ERWINIA CHRYSANTHEMI*

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ABSTRACT

Lactose metabolism in a local isolate of Erwinia chrysanthemi, *pathogenic to maize was investigated. It was found that, although the wild type* E. chrysanthemi *possessed a functional β-galatosidase, it was unable to metabolize lactose, and grew on lactose-containing agar media as small, clear colonies. A strain of* E. chrysanthemi *was isolated from the wild type parent that was able to metabolize lactose. It was characterized by a spreading slimy growth on lactose containing media, due to the production of an extracellular polysaccharide from lactose. The wild type and mutant* E. chrysanthemi *strains were called EC-C and EC-S respectively, due to their appearance on lactose agar media (clear and slimy). Both strains were characterized as regards their behaviour on a range of differential media, general physiology, and pathogenicity. These characteristics were found to be similar, except for those concerning the metabolism of lactose.*

Both strains produced constitutive levels of β-galactosidase, that could be further increased by the gratuitous inducer, isopropyl-β-D-thiogalactoside (IPTG). Although lactose could induce elevated levels of β-galactosidase in EC-S, it was inactive as an inducer in EC-C. The β-galactosidase enzymes from both strains appear to be similar as regards their activity on both o-nitrophenyl-β-D-galactopyranoside and lactose. It was concluded from this work that the failure of EC-C to grow on lactose was not due to the absence of a functional β-galactosidase, but to the absence of a lactose transport system.

Introduction

Phytopathogenic bacteria cause enormous losses annually in food crops and the elucidation of the mechanisms involved in pathogenesis is therefore important. However, it is only recently that a study of the metabolism and genetics of various phytopathogenic bacteria has been undertaken (Chatterjee and Starr, 1980; Lacy, 1979; Starr and Chatterjee, 1972). *Erwinia chrysanthemi*, one of the soft rot bacteria, is a member of the *Erwinia carotovora* group (Dye, 1969; Kelman and Dickey, 1980; Leliot, 1974) which forms part of the Enterobacteriaceae and shares a close genetic relatedness to *Escherichia coli* (Chatterjee and Starr, 1980), the most widely studied member of this family.

Possibly the best studied system in *E. coli* is the regulation of the lac operon. This operon codes for three proteins; ß-galactoside permease, ß-galactosidase and thiogalactoside transacetylase (Zabin and Fowler, 1980). The first two proteins are responsible for the transport and hydrolysis of ß-galactosides, but the physiological function of the third enzyme is not known. The lac operon is often used as a model for the study of the regulation of other catabolic operons, both in *E. col.*

and in other microorganisms. For this reason, a study of lactose metabolism in an organism provides a good basis for the study of the regulation of other operons in that organism.

Dickey (1979), in a study of 322 strains of *E. chrysanthemi*, reported the presence of ß-galactosidase in all strains, although most of these strains could not grow on lactose as a carbon source. Other authors indicate that growth on lactose is delayed (Goto, 1979; Lelliot, 1974). It is not clear from the limited experimental evidence available whether *E. chrysanthemi* can hydrolyse lactose and use it as a sole carbon source. We investigated certain aspects of lactose metabolism in a local isolate of *E. chrysanthemi*, and found that the presence of a functional ß-galactosidase is an insufficient criterion for lactose metabolism.

Materials and Methods

Reagents. Isopropyl-ß-D-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-Gal), and *o*-Nitrophenyl-ß-D-galactopyranoside (ONPG) were purchased from Sigma (St. Louis, Missouri, USA). Merckotest Glucose (GOD-PAP method), triphenyl tetrazolium chloride, eosin yellow, and methylene blue were obtained from Merck Chemicals (Darmstadt, W. Germany). Hexokinase, glucose-6-phosphate dehydrogenase, ß-galactosidase, adenosine-5'-triphosphate, (ATP), and nicotinamide adenine dinucleotide phosphate (NADP) were purchased from Boehringer Mannheim (Mannheim, W. Germany). All media components were obtained from Difco Laboratories (Detroit, Michigan, USA). Polygalacturonic acid (PGA) for enzyme assays, and sodium polypectate (NaPP) for growth media were purchased from Nutritional Biochemicals (ICN Pharmaceuticals, Inc., Life Sciences Group, Cleveland, USA). The API 20 Enterobacteriaceae (Apparaeils et Procedes d'Identificion) for the identification of bacteria was purchased from API Systeme, Montalieu-vercieu, France. All other chemicals were of analytical standard.

Media. Both Eosin Methylene Blue (EMB) agar and McConkey agar were made up according to the manufacturer's instructions. EMB agar, when it was to be supplemented with a sugar other than lactose, and Tetrazolium (TCZ) agar, were made up according to Miller (1972). High yeast broth (HYB) contained (g/l): $Na_2 HPO_4 .2H_2O$, 1,77; KH_2PO_4, 0,27; $MgSO_4.7H_2O$, 0,24; NH_4NO_3, 0,4; yeast extract (Difco), 0,90. The pH of the medium after autoclaving was 7,3 — 7,4. HY agar (HYA) contained 15g agar (Difco) per litre HYB. All media were supplemented with 0,5% carbon source, autoclaved seperately, and added to the cooled media, unless specified otherwise. Medium containing X-Gal (40 mg/l) was prepared from HYA supplemented with glycerol (0,5%) and either IPTG (0,5 mM), or lactose (0,2%). Crystal violet-pectate (CVP) medium was prepared according to Schaad (1980).

Organism. A local isolate of *E. chrysanthemi* pv. *zeae* pathogenic to maize was used in all these studies. This isolate has been deposited in the collection of Prof. A. Kelman, Department of Plant Pathology, University of Wisconsin, Madison, USA.

A strain of *E. chrysanthemi* capable of producing slime on lactose was selected from wild-type *E. chrysanthemi* (called EC-C), by streaking on HYA plates supplemented with 0,5% lactose and incubating at 37 C. On this medium EC-C normally appears as small clear colonies. However, after about three days growth a certain proportion of the clear colonies sector into a slimy morphology type (Fig 1). This slimy varaint (called EC-S) was purified, and found to consistently give rise to slimy colony morphology in 24 hours of growth on HYA-lactose. In contrast, EC-C is still clear after 24 hours growth on this medium. EC-S produces copious quantities of slime on this medium, with greater quantities being produced at 20 C than at 37 C.

Fig. 1. Appearance of slimy sectors during growth of wild type *Erwinia chrysanthemi* on HYA-lactose medium.

Both EC-C and EC-S were maintained on nutrient agar (NA, Difco) plates at 15 C.

Preparation of Cell Free Extracts. EC-C and EC-S were grown in shake culture at 30 C in HYB containing glycerol (0,5%) with either IPTG (0,5 mM) or lactose (0,2%) for 16 hours. The cells were washed by centrifugation in 10 mM Tris-acetate buffer, pH 7,5, containing 10 mM NaCl. After suspension in the same buffer, containing 10 mM 2-mercaptoethanol (2-ME), the cells were disrupted by subjecting them to three 30 second bursts in a MSE 150 W sonicator at 4 C, with a 24 to 26 micron amplitude. The suspension was cooled for two minutes between each burst. The cell debris was removed by centrifugation (48 000 g, 15 minutes, 4 C), and the supernatants were assayed for ß-galactosidase activity and protein content.

Pathogenicity Trials. The pathogenicity of EC-C and EC-S to two week old

maize plants *(Zea mays* L., cv. SSM_{42}) was determined by filling the whorl of the plant with washed cells of EC-C and EC-S that had been grown overnight in Nutrient Broth (NB), and resuspended in 0,1% Tween-80 (Hartman and Kelman, 1973). The ability of *E. chrysanthemi* to macerate potato slices was also tested according to the method described by Kelman and Dickey (1980).

Assays

(i) *β-Galactosidase assay on ONPG.* Cells were permeabilized for ß-galactosidase assays by a modification of the method of Putnam and Koch (1975). The 10% sodium dodecyl sulphate (SDS) was replaced by an equal volume of 10% sodium deoxycholate in the lysis mixture (LM), as this was found to be equally effective in permeabilizing the cells to the substrate without the disadvantage of inhibiting enzyme activity. The assay mixture contained: 875 μl Z-Buffer (0,1 M phosphate buffer, pH 7,0, containing 10 mM K^+, 1 mM Mg^{2+}, and 50 mM 2-ME), 25 μl LM, and 100 μl cells. After 5-8 minutes at 30 C, the reaction was started by the addition of 200 μl 13,2 mM ONPG, and stopped by the addition of 1,0 ml 1 M Na_2CO_3 after the appearance of a definite yellow colour. The absorption at 420 nm was corrected for turbidity by the subtraction of 1,711 x A_{550} (Miller, 1972). A molar extinction coefficient for *o*-nitrophenol of 21 300 m^{-1} cm^{-1} (Jones, 1969) was used to calculate enzyme activity, which was defined as the hydrolysis of 1 nmole ONPG per minute.

ß-Galactosidase in cell free extracts was assayed in imidazole hydrochloride buffer, pH 7,0, containing 10 mM NaCl, 1,0 mM $MgSO_4$, and 100 mM 2-ME. The reaction contained 950 μl buffer and 200 μl 13,2 mM ONPG. After equilibration for 30 minutes at 30 C, the reaction was started by the addition of 50 μl suitably diluted enzyme, and stopped by the addition of 1,0 ml 1 M Na_2CO_3 after the development of a definite yellow colour. The absorbance of the solution was determined at 420 nm against an enzyme blank.

(ii) *β-Galactosidase assay on lactose.* The reaction mixture contained 50 mM imidazole hydrochloride buffer pH 7,2, containing 10 mM NaCl, 6,7 mM $MgSO_4$, and 10 mM lactose in a final volume of 1,4 ml. After temperature equilibration (30 C, 5-8 minutes), the reaction was started by the addition of 100 μl enzyme. The reaction was stopped by the addition of 150 μl of 33% perchloric acid, and held at 4 C for 30 minutes for the protein to precipitate completely. The precipitated protein was removed by centrifugation (10 000 g, 2 minutes, Beckman Microfuge). The supernatant was neutralized with 5 M K_2CO_3, and the insoluble $KClO_4$ was allowed to precipitate at 4 C for 10 minutes. Aliquots of the neutralized supernatant were removed for the determination of the liberated glucose by either the glucose oxidase method, or the hexokinase method. It was found that the ß-galactosidase time-course assay was non-linear if the liberated glucose was assayed by the glucose oxidase reagent. This appeared to be a property of the glucose assay procedure, since a purified commercial preparation of ß-galactosidase also gave non-linear results when assayed by this method. Assay of the liberated glucose by the hexokinase method gave linear time-course assays for both the *E. chrysanthemi* enzymes, and the commercial *E. coli* enzyme.

Glucose. Glucose was assayed by either the glucose oxidase method using a commercial kit (Merckotest Glucose), or by the hexokinase/glucose-6-phosphate dehydrogenase method (Bergmeyer *et al.,* 1974).

Protein. Protein was extracted from washed cells by heating (95 C) in 1 N NaOH for 10 minutes. The Lowry (Lowry *et al.,* 1951) procedure was used to analyse for protein, with bovine serum albumin as standard.

Results

Pathogenicity and strain characteristics. Both EC-C and EC-S appear to be identical in their:

(i) physiological characteristics (API tests), including the ability to hydrolyze ONPG, indicating the presence of enzymes capable of hydrolyzing ß-galactosides,

(ii) pathogenicity to maize, typical disease symptoms being produced in 48 hours,

(iii) pathogenicity to potato slices, decay and a black discolouration being produced in 6 hours, and,

(iv) growth on and liquifaction of the pectate in CVP medium.

Even when grown in the absence of lactose, the slimy characteristic of EC-S appears to be stable. After 15 transfers of EC-S on NA no clear colony types were obtained when re-inoculated onto HYA-lactose. Similarly, the strain is stable at −20 C in 40% glycerol.

The two strains were tested on a variety of differential media containing different sugars (Table 1). EC-C showed up as Lac⁻ on EMB-lactose, MacConkey agar, and TCZ-lactose, whereas EC-S was found to give typical Lac⁺ results on these media. The results obtained on the media containing X-Gal show that enzymes capable of hydrolyzing ß-galactosides are present in both EC-C and EC-S. The result on X-Gal + glycerol indicates that constitutive levels of these enzymes are produced by both strains.

Table 1. *Reactions of EC-C and EC-S on differential media.*

Medium	Colony Type	
	EC-C	EC-S
HYA-lactose	small, clear colonies	slimy, spreading colonies
HYA-glucose	slimy, spreading colonies	slimy, spreading colonies
HYA-galactose	very slimy, spreading colonies	very slimy, spreading colonies
EMB-lactose	light colonies, no dye change	dark colonies, green sheen dye change
EMB-glucose	dark colonies, green sheen, dye change	dark colonies, green sheen, dye change
EMB-galactose	dark colonies, green sheen, dye change	dark colonies, green sheen, dye change
McConkey Agar	colourless colonies	red colonies surrounded by precipitated bile salts
X-Gal-glycerol	blue colonies	blue colonies
X-Gal-glycerol + IPTG	blue colonies	blue colonies
X-Gal-glycerol + lactose	blue colonies	blue colonies
TCZ-lactose	dark red colonies, thin light border	light, spreading colonies
TCZ-glucose	light, spreading colonies	light, spreading colonies
TCZ-galactose	light, spreading colonies	light, spreading colonies

The results on EMB and TCZ media containing either glucose or galactose indicate that both EC-C and EC-S ferment these two sugars (Table 1). Slime production also occurs on these two sugars when they are incorporated into HYA (Table 1). Therefore, slime production appears to be a growth characteristic of EC-C and EC-S on glucose and galactose, the two monomers of lactose.

β-Galactosidase production by EC-C and EC-S. Both EC-C and EC-S produce constitutive levels of ß-galactosidase. The gratuitous inducer, IPTG, was able to further increase the levels of ß-galactosidase in both EC-C and EC-S, with higher levels of enzyme being induced in EC-S (Table 2). Lactose was only active as an inducer in EC-S but not in EC-C (Table 2). The failure of EC-C to grow on lactose was shown not to be due to an absence of ß-galactosidase induction, since EC-C grown on EMB-lactose, MacConkey, and TCZ-lactose media in the presence of IPTG (to ensure induction of ß-galactosidase) were still Lac⁻. Therefore, factors other than the presence of a functional ß-galactosidase are required for lactose metabolism.

Table 2. *β-Galactosidase levels in whole cells and cell-free extracts of* **Erwinia chrysanthemi** *after induction by IPTG or lactose.*

Inducer	ß-Galactosidase (U/mg protein)			
	EC-C		EC-S	
	Cells	CFE[1]	Cells	CFE
IPTG	76,4	169,5	156,7	234,1
Lactose	13,5	2,6	238,2	257,4

[1]Cell Free Extract

Hydrolysis of ONPG and lactose. The possibility that the ß-galactosidase prepared from IPTG induced cells being unable to hydrolyze lactose was discounted by the experimnent reported in Table 3. The ratio of hydrolytic activity of ß-galactosidase from EC-C and EC-S on ONPG and lactose was shown to be similar (about 18:1), but to differ from that obtained with *E. Coli* ß-galactosidase (about 9:1). This indicates that the *E. coli* enzyme hydrolyzes lactose more readily than the *E. chrysanthemi* enzyme.

Table 3. *Comparison of β-galactosidase activities on ONPG and lactose.*

Source of ß-Galactosidase	Activity (mU per ml)		Ratio
	ONPG	Lactose	
EC-S + glycerol + lactose	1848,9	99,2	18,6
EC-S + glycerol + IPTG	105,1	6,1	17,2
EC-C + glycerol + IPTG	190,3	10,2	18,7
E. coli Boehringer	1266,2	145,0	8,7

Discussion

The metabolism of lactose by *E. chrysanthemi* is not well documented in spite of it being used as taxonomic parameter in a number of studies (Dickey, 1979; Goto, 1979; Lelliot, 1974). This study shows that although enzymes capable of hydrolyzing ß-galactosides are present in a wild-type strain of *E. chrysanthemi*, its presence is not the only criterion for growth on lactose, confirming the results of Dickey (1979) on 322 different strains. The two strains of *E. chrysanthemi* are similar in their general physiological characteristics and pathogenicity to both maize plants and potato slices. It appears that they are similar in all respects except for their ability to utilize lactose.

The gratuitous inducer, IPTG, induces ß-galactosidase activity in both strains of *E. chrysanthemi*, although the level in EC-C is considerably lower than in EC-S (Table 2). In contrast, both lactose and IPTG induce high levels of ß-galactosidase in EC-S, but lactose is ineffective as an inducer in EC-C. The failure of lactose to induce ß-galactosidase in EC-C is however not the cause of its inability to grow on lactose, because EC-C grown on differential media in the presence of both lactose and IPTG, the latter of which is an effective inducer, are still phenotypically Lac⁻. Although the level of ß-galactosidase induced by IPTG in EC-C is lower than that induced in EC-S, sufficient enzyme should be present to support growth, albeit at a slow rate.

Strains of *E. coli* have been isolated that no longer produce ß-galactosidase coded for by the lac operon due to a deletion in the lac-Z gene. Nevertheless, low residual levels of activity are still retained by the organism due to the presence of a second gene coding for a ß-galactosidase (Campbell *et al.*, 1973; Hartl and Hall, 1974) that is more active on artificial substrates than on lactose (Hartl and Hall, 1974; Hall, 1976). The second ß-galactosidase is coded for by the ebg (evolved ß-galactosidase) gene, and is regulated independently of the lac operon (Campbell *et al.*, 1973; Hartl and Hall, 1974; Hall and Hartl, 1975; Hall and Clarke, 1977). The activity of ebg on lactose is too low to support the growth of *E. coli* on lactose (Hall and Hartl, 1974; Hartl and Hall, 1974). However, certain lac-Z deletion strains can be selected for growth on lactose, due to mutations in the ebg gene allowing far greater activity on lactose (Campbell *et al.*, 1973; Hall, 1976; Hall and Hartl, 1974; Hall and Clarke, 1977). Provided that a functional lactose transport system is retained by the cell, strains of *E. coli* containing this mutation can grow on lactose as a sole source of carbon (Campbell *et al.*, 1973; Hall and Hartl, 1974; Hall and Clarke, 1977).

As lactose is not a normal component of plant material, the ß-galactosidase produced by EC-C may be similar to the ebg enzyme and have greater activity on ß-galactosides more common to plant material. In addition, the inducer of ß-galactosidase in EC-C may be different from that in EC-S. The possibility that the ß-galactosidase was similar to the ebg gene was eliminated by comparing the activities of the EC-C and EC-S ß-galactosidases on ONPG and lactose (Table 3). No difference in the relative rates of ONPG and lactose hydrolysis could be determined for the different preparations. Both ß-galactosidases hydrolyzed ONPG about 18 times more rapidly than lactose. Thus, enzymes with different

specificities for lactose are not induced by IPTG and lactose in the two strains of *E. chrysanthemi.*

The ratio of the rate of hydrolysis of the *E. coli* ß-galactosidase on ONPG and lactose differ significantly from the *E. chrysanthemi* enzyme. The *E. coli* ß-galactosidase hydrolyzes ONPG 9 times more rapidly than lactose, whereas in *E. chrysanthemi,* the rate on ONPG is 18 times higher than on lactose. Further, the *E. coli* ebg system and the *E. chrysanthemi* system differ with respect to the effect of inducers on the production of the enzymes. IPTG is ineffective as an inducer of the ebg enzyme (Hartl and Hall, 1974; Hall and Hartl, 1974; Hall and Hartl 1975; Hall and Clarke, 1977), whereas it is a good inducer of the *E. chrysanthemi* enzyme.

Factors other than enzyme induction and enzyme specificity appear to be responsible for the failure of EC-C to grow on lactose. A plausible explanation for the inability of lactose to support growth in EC-C is that it is unable to enter the cell due to the absence of a lactose transport system. This would also explain the lack of induction in EC-C by lactose. Messer (1974), working with *E. coli,* found that certain strains with a defective lac permease system could transport lactose by means of the arabinose permease system. A similar mechanism may be operative in *E. chrysanthemi.*

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ENANTIOMERIC COMPOSITION OF AN ALARM PHEROMONE COMPONENT OF THE ANTS *Crematogaster castanea* AND *C. liengmei*

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Abstract—The enantiomeric composition of 3-octanol produced by the ants. *Crematogaster castanea* Forel and *C. liengmei* F. Smith, as a component of their alarm pheromone complex, has been determined to be S-(+)-3-octanol by gas chromatography of the diastereomeric S-(+)-3-octanyl R-(+)-*trans*-chrysanthemates.

Key Words—Crematogaster castanea, C. liengmei, Hymenoptera, Formicidae, ant, alarm pheromone, enantiomers, 3-octanol.

INTRODUCTION

A number of species of the myrmecine ant, *Crematogaster*, secrete an alarm pheromone from the mandibular glands that contains 3-octanone and 3-octanol as major components (Crewe et al., 1969, 1970). As biological activity is often dependent on the enantiomeric composition of a chiral compound, the ratio of enantiomers of the 3-octanol in two species of *Crematogaster* has been determined by gas chromatography of the 3-octanyl R-(+)-*trans*-chrysanthemates. The use of R-(+)-*trans*-chrysanthemoyl esters for the resolution of a number of synthetic chiral alcohols has been described (Brooks et al., 1972) and the method has been applied recently to the determination of the enantiomeric composition of 3-octanol from three species of *Myrmica* ants (Attygalle et al., 1983).

METHODS AND MATERIALS

Collection of Ants. A nest of *Crematogaster castanea* F. Smith was cut from a branch of a tree in Nylsvlei, Transvaal, and a number of colonies of *C.*

177

liengmei Forel were obtained from cracks in old fence poles at Margate, Natal. These colonies were maintained in the laboratory on a diet of water, honey, crickets, and grasshoppers.

trans-*Chrysanthemic Acid.* A natural pyrethrum extract was hydrolyzed with base, extracted three times with ether, acidified to pH 2 with HCl and reextracted three times with ether. These latter ether extracts were pooled and concentrated on a rotary evaporator. The yellow-brown oil was separated on a Florisil column by eluting first with *n*-hexane, followed by *n*-hexane–ether (9 : 1), and ether. Collected fractions were monitored by development on silica gel TLC chromatoplates in *n*-hexane–ether (1 : 1) and staining in I_2 vapor. The presence of *trans*-chrysanthemic acid at an R_f of about 0.6 was checked against authentic *cis*-chrysanthemic acid. Rerunning the Florisil column on pooled fractions of the partially purified *trans*-chrysanthemic acid resulted in a pale yellow oil that gave one single spot on TLC with I_2 vapor. Comparison of proton and carbon-13 NMR spectra of this oil in $CDCl_3$ against spectra obtained for authentic *cis*-chrysanthemic acid confirmed its structure and purity.

Preparation of Derivatives. Worker ants of *C. liengmei* were steam distilled and continuously liquid–liquid extracted with *n*-pentane. Heads of worker ants of *C. castanea* were extracted directly with *n*-hexane. Solvent was removed carefully, under a stream of N_2, and the residue immediately treated with the acid chloride of the *trans*-chrysanthemic acid, obtained by reaction with oxalyl chloride. Authentic *R*-(−)-2-octanol, *S*-(+)-2-octanol, and racemic 3-octanol were derivatized in the same way.

Gas Chromatography. Three different capillary columns were used for each analysis: 12-m SE-30 (180°), 25-m BP-1 (J&W) (170°), and 25-m BP-20 (S.G.E.) (180°).

RESULTS

3-Octanone and 3-Octanol. The solvent extracts of *C. castanea* and *C. liengmei* gave chromatograms on all three columns having two major peaks eluting at retention times corresponding to 3-octanone and 3-octanol. Treatment of a portion of the *C. liengmei* extract with $NaBH_4$ resulted in the loss of the peak corresponding to 3-octanone and an increase in the relative area of the peak corresponding to 3-octanol. These results, together with the published data of Crewe et al. (1969, 1970), confirm the presence of 3-octanone and 3-octanol in these species.

trans-*Chrysanthemic Acid.* NMR established the purity and structure of the *trans*-chrysanthemic acid. Reaction of the acid chloride of the *trans*-chrysanthemic acid with either *R*-(−)- or *S*-(+)-2-octanol gave gas chromatograms on each column with a single peak. Previously obtained mass spectral data on different columns had confirmed that these peaks were due to 2-octanyl *trans*-chrysanthemates. The retention times of these peaks on two of the columns were

as follows: S-(+)-2-octanyl *trans*-chrysanthemate: BP-1 (170°) 9.80 min, BP-20 (180°) 9.26 min; and R-(−)-2-octanyl *trans*-chrysanthemate: BP-1 (170°) 10.14 min, BP-20 (180°) 9.55 min. The appearance of these two resolved peaks in these chromatograms established the *trans*-chrysanthemic acid to be enantiomerically pure.

3-Octanol and Ant Extracts. Racemic 3-octanyl R-(+)-*trans*-chrysanthemate gave two peaks on the BP-1 column (170°) at 9.19 and 9.41 min and on the BP-20 column (180°) at 8.56 and 8.69 min. Derivatized extracts from both ant species gave only one peak corresponding to the earlier of the two 3-octanyl peaks. This peak was well resolved from any other peaks in the chromatogram. Coinjection of the racemic 3-octanyl R-(+)-*trans*-chrysanthemate and the derivatized ant extract resulted in an increased peak height for the first of the two peaks. Therefore, the chirality of the 3-octanol in both ant species corresponded to that of the 3-octanol in the earlier eluting diastereomer.

DISCUSSION

Workers of both *C. castanea* and *C. liengmei* produce 3-octanone and 3-octanol as major components of their alarm pheromone complex. The gas chromatographic results reported here confirm the earlier work of Crewe et al. (1969, 1970) on *C. castanea* and add the species, *C. liengmei*, to the list of *Crematogaster* species producing these related compounds.

The *trans*-chrysanthemic acid purified from the pyrethrum extract was shown to be pure from its NMR spectrum. As it gave two distinct peaks on reaction with the enantiomers of 2-octanol, it was concluded to be the R-(+)-*trans* enantiomer known to occur in an esterified form in pyrethrin I. As (+)-2-octanol is the S enantiomer, and (−)-2-octanol is the R enantiomer (Brooks et al., 1972), the results obtained illustrate that the R–S diastereomer elutes before the R–R diastereomer as previously shown by Brooks et al. (1972). Furthermore, Attygalle et al. (1983) found that the R–S diastereomer of the 3-octanyl derivative elutes before the R–R diastereomer. Therefore, these two species of *Crematogaster* produce S-(+)-3-octanol exclusively. This finding is in contrast to the results of Attygalle et al. (1983) for three species of *Myrmica*, which produce a mixture containing mainly R-(−)-3-octanol. Even though species of both genera produce 3-octanol, there is nevertheless a high degree of specificity in their biosynthetic mechanism and, most likely, in their receptor mechanism. The enzymatic reduction of 3-octanone requires *Si*-face addition of a hydride ion to form S-3-octanol, and *Re*-face addition to form R-3-octanol. The pure enantiomers of 3-octanol are not available to the author at this time for any behavioral studies, but their enzymatic synthesis is being attempted.

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Pheromonal Blends: Certain Components May Only Convey Gross Information

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Abstract—Certain insect secretions contain 3-octanone and 3-octanol, or 2-heptanone and 2-heptanol. It is shown that the vapour phase composition of each of these binary mixtures may be very different from the liquid phase composition with which it is in equilibrium. It is suggested that there may be little need for biosynthetic exactness in the ratio of some components of pheromone blends and, further more, that certain components of a pheromone blend may be able to convey only gross information rather than subtleties in communication.

Introduction

It is generally assumed that each component of a pheromone blend may influence the behavioural response of the receiving organism and that a sequential message is produced from the active spaces of the various substances [1]. The most volatile components will have the most rapidly expanding active space and qualitative changes may be felt at different distances from the source [2]. This explains, in part, the phenomenon of long- and short-range behavioural responses [4]. However, it should be noted that if the vapour pressures of the components differ significantly, the vapour phase composition can differ appreciably from that in the liquid phase [5]. Commentary in this paper is directed at an aspect of this latter point.

As pheromone blends and defensive secretions of insects are often mixtures of volatile compounds, their chemical composition is usually determined by gas chromatographic analysis of solvent extracts. These extracts are generally made from the glandular source or body parts of the emitting organism, and analyses present us with a quantitative estimate of the various substances present in the biological source. A particular ratio of the substances may be species specific, account for species recognition, have chemotaxonomic value, or account for a certain

behavioural response (see for example refs [2, 6–11].

The composition of the vapour above a liquid blend will generally be similar to that of the liquid if the substances making up the blend have similar vapour pressures and functionality. If, however, the substances in the liquid differ appreciably in vapour pressure and functionality, then the vapour phase composition can differ widely from that of the liquid from which it arises. This was shown for components of the *Heliothis virescens* sex pheromone [11]. The physicochemical principles involved will not be dealt with here.

3-Octanone and 3-octanol are major components of the mandibular gland secretions of certain species of the ants *Crematogaster* [9, 12] and *Myrmica* [7]. 2-Heptanone and 2-heptanol occur in various ants and bees [13] and may produce alarm or trail-following behaviour. The composition of the vapour above known liquid blends of the two ketone–alcohol mixtures (3-octanone–3-octanol and 2-heptanone–2-heptanol) has been determined and it is suggested that certain components of a blend may be able only to convey gross information.

Results and Discussion

The ketone–alcohol ratios in both the liquid and the equilibrated gas phase are presented in Figs 1 and 2. In both cases the ratio of ketone to alcohol is very different in the two phases, with the ketone dominating the gas phase due to its greater vola-
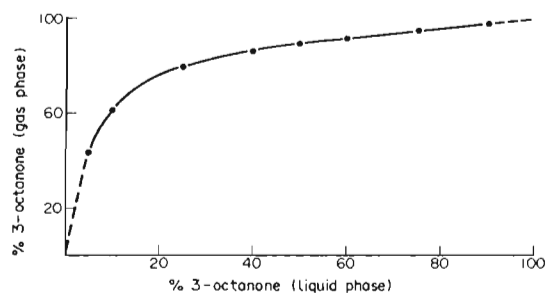
FIG. 1. VAPOUR–LIQUID EQUILIBRIUM COMPOSITION DIAGRAM OF 3-OCTANONE–3-OCTANOL AT 22°.
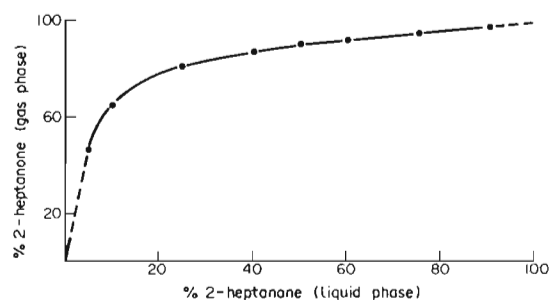

FIG. 2. VAPOUR–LIQUID EQUILIBRIUM COMPOSITION DIAGRAM OF 2-HEPTANONE–2-HEPTANOL AT 22°.

tility. It should be noted that the vapour phase composition remains relatively constant over a wide range in liquid phase composition. Due to the lower vapour pressure of the alcohol, the total amount of vapour over a 10:90 (ketone–alcohol) mixture is ca. 30% of that over a 90:10 (ketone–alcohol) mixture, based on integrated area measurements.

The curves in Figs 1 and 2 show that, even though the liquid ratio varies from 90% to 30% ketone, the vapour phase ratio only varies from 97.6% to 85.3% ketone. One would, therefore, expect that the informational content of the gas phase will not change appreciably between relatively close liquid phase ratios. A quantitative verification of this suggestion probably falls within the realm of psychophysics [14].

In view of the common assumption that minor variations in the liquid phase ratios of components of a blend can play an important role in species recognition and behavioural response, the above consideration seems particularly relevant. For example, it seems unlikely, at least for the two binary mixtures reported here, that varying the glandular composition from 80:20 to 60:40 (ketone–alcohol) would contribute significantly to the ability of species to discriminate between each other. The above ratios of 3-octanone–3-octanol have actually been obtained for pooled samples from Crematogaster castanea and C. liengmei, respectively. In addition, considerable individual variation exists and the ranges for these two species overlap (unpublished data). Cammaerts et al. [7], in a comparision of compounds present in the mandibular glands of M. rubra and M. scabrinodis, found the 3-octanone–3-octanol ratios to be 60:40 and 73:27, respectively. While such results from pooled samples may have chemotaxonomic value, they may have little behavioural significance.

Many blends of different chemical classes of compounds with differing volatilities will be deposited on adsorptive surfaces, such as sand, and thereafter be slowly released. The more polar a compound is the more strongly it will adsorb to such materials (consider TLC on silica gel) and this phenomenon might exaggerate the type of effect presented in Figs 1 and 2. Therefore, the vapour composition produced from a liquid blend could remain relatively constant with respect to certain components, the main compositional change in the gas phase occurring only when the more volatile component(s) has largely evaporated. Conversely, deposition of a blend on a hydrophobic surface, such as insect cuticle or a leaf surface, should have contrary effects. Changes in the gas phase composition can be expected to influence the organism's behaviour, not only as it moves along the track, but also as the freshness of the track diminishes.

It is concluded that, within certain limits, there is little need for biosynthesis exactness in these ketone–alcohol blends, the same 'message' may be conveyed by mixtures of rather different composition. In addition, certain components of a blend may be best suited to convey gross information only, while subtleties in communication are left to other components. These suggestions may merit consideration when attempting to determine the behavioural roles of the various components of a blend.

Experimental

Chemicals. 2-Heptanone, 2-heptanol and 3-octanone were

purchased from Aldrich Chemical Co. and 3-octanol from Chemical Samples Co.

Gas chromatography. Screw top tubes (15 ml) with black septum tops were used for equilibrating the liquid and gas phases. Liquid mixtures of each ketone and its corresponding alcohol were added to each tube to a final vol of 1 ml. All tubes were equilibrated at 22° for 48 h prior to analysis. A gas-tight syringe was used for obtaining 0.5 ml samples of headspace from each tube and duplicate analyses were carried out on each tube. A 2 m × 3.2 mm o.d. stainless steel column packed with 5% Carbowax 20 M at 120° was employed for all analyses and the percentage composition of samples was obtained from area measurements using a Hewlet–Packard 3390A integrator. The detector response was shown to be equal for each ketone–alcohol pair and no calibration factors were required.

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ENZYMATIC STEREOSPECIFICITY AND BARK BEETLE PHEROMONES

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ABSTRACT

The enantiomers of α-pinene in the host trees of certain bark beetle species are stereospecifically hydroxylated to specific enantiomers of cis- and trans-verbenol. The stereospecific replacement of the pro-S and pro-R hydrogens of (+)- and (−)-α-pinene to form the verbenols is discussed and the complexity of their enzymatic stereoselective synthesis is illustrated.

Various bark beetles use the host tree monoterpene α-pinene as a component of their pheromone blend. Also, the two hydroxylation products of α-pinene, *cis-* and *trans-*verbenol, are produced in varying yields by exposure of certain beetles to the vapours of α-pinene (for references see Wood, 1982) and can be important components of their pheromone blend. A careful look at the stereochemistry of the α-pinene substrate and its hydroxylation products has been undertaken in only two studies.

Some years ago, Renwick *et al.* (1976) studied the production of the two geometric isomers *cis-* and *trans-*verbenol from the two enantiomers of α-pinene by the bark beetle, *Ips paraconfusus.* They found that (−)-α-pinene gave (+)-*cis*-verbenol and (+)-α-pinene gave (+)-*trans*-verbenol. Since *cis-*verbenol is the active geometric isomer for *I. paraconfusus,* the enantiomeric ratio of the α-pinene in the host tree could influence the amount of pheromone produced by a beetle.

Recently, Byers (1983) conducted similar experiments with *Dendroctonus brevicomis* and found that both sexes convert (−)-α-pinene to (−)-*trans*-verbenol and (+)-α-pinene to (+)-*trans*-verbenol. Very low amounts of *cis-*verbenol were detected with this species. Since (−)-*trans*-verbenol inhibited the response of females, but not of males, and since it is the females of this species that initiate the attack on a tree, the enantiomeric ratio of α-pinene could influence pheromone production and behavioural response.

This conversion of α-pinene to the verbenols is best visualized from a consideration of the structures shown in Fig. 1.

(+)-α-pinene

I. paraconfusus
D. brevicomis

(+)-*trans*-verbenol

(−)-α-pinene

I. paraconfusus

(+)-*cis*-verbenol

D. brevicomis
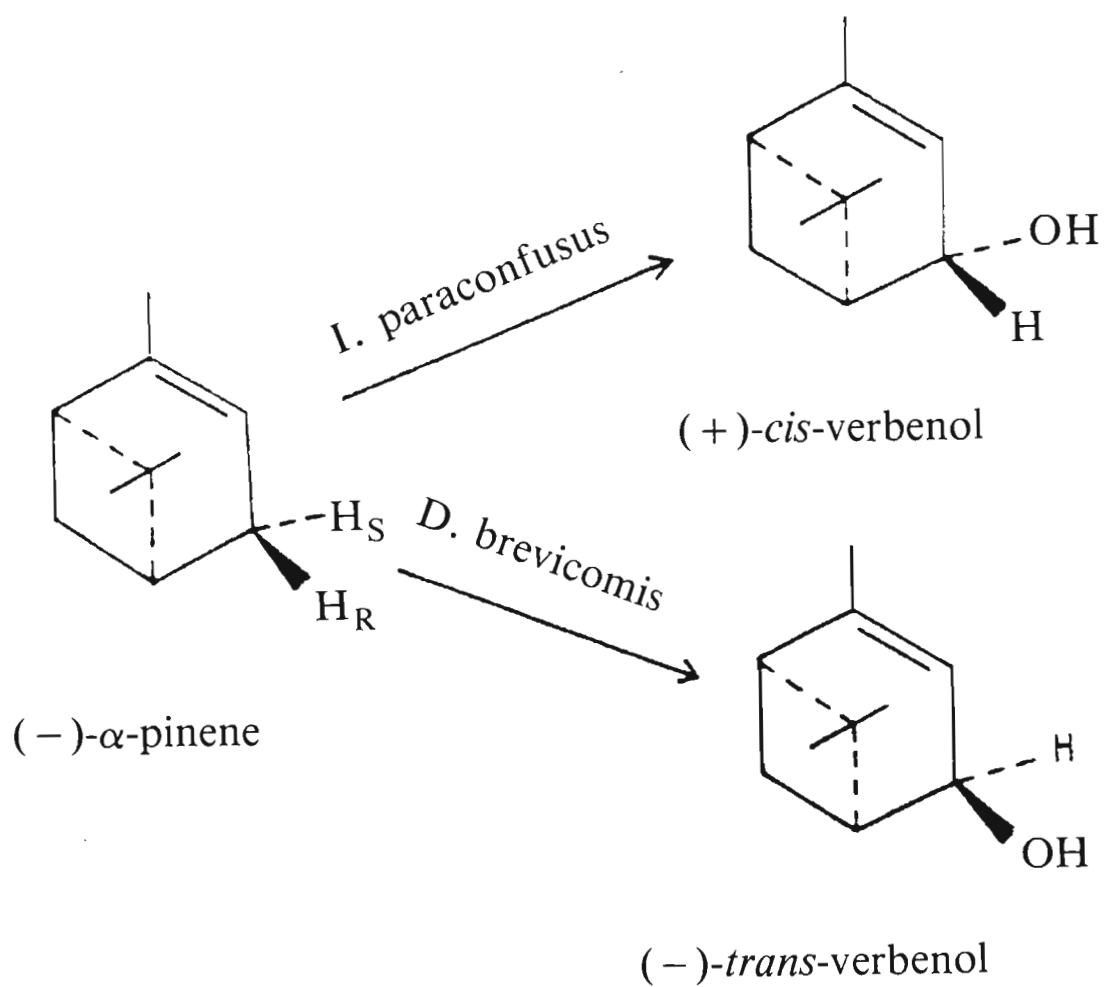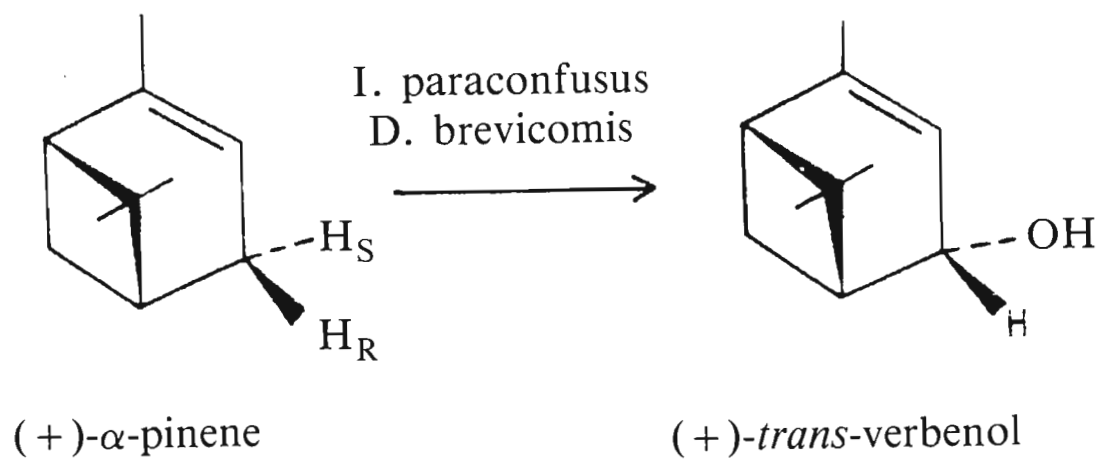
(−)-*trans*-verbenol

The enzyme(s) of *I. paraconfusus* catalyses the stereospecific replacement of the *pro-S* hydrogen of both (+)- and (−)-α-pinene to form (+)-*trans*-verbenol and (+)-*cis*-verbenol respectively. The α-pinene enantiomers apparently are oriented by an active site that primarily recognises the position of H_R and H_S at the prochiral center relative to the double bond, the vinyl methyl, and the bridging carbon centers. Thus H_S is always stereospecifically replaced.

In contrast, the enzyme(s) of *D. brevicomis* apparently orients the α-pinene enantiomers by an active site that primarily recognizes the relative position of the bridging carbon chain containing the geminal dimethyl group. The diastereotopic hydrogen stereospecifically replaced by *D. brevicomis* is always *trans* to this bridging structure.

A detailed investigation of the enzymatic synthesis of the verbenols from α-pinene by these two bark beetles offers an interesting topic for study. Closely related taxa are more likely to synthesize the same compound by the same route than are widely separated taxa. An understanding of the biosynthetic steps for the synthesis of a pheromone component may prove valuable in the ultimate utilization of pheromone blends for integrated management of an insect pest.

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PACKED COLUMNS AND THE SOLVENT EFFECT

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ABSTRACT

The utilization of the solvent effect on a packed bed immediately before the coated solid support of a regular packed gas chromatographic column is described. The technique allows the injection of large volumes of dilute solutions by concentrating and focusing the solutes coupled to their subsequent chromatographic separation.

The gas chromatographic separation of solvent-focused solutes is usually done using capillary columns in order to exploit the high effeciency of these columns. However, an early paper describing the solvent effect explained how the sample can be used as its own stationary phase on an uncoated Teflon 6 packed column (DEANS, 1971). A general description and theoretical treatment of solute-focusing in GLC, using the solvent effect, has been described by PRETORIUS *et al.* (1983a, 1983b and references therein) and the basis for this effect is now well understood.

The application of solute focusing in an uncoated packed bed placed immediately before the coated solid support of a regular packed column is illustrated here. Readers may find the simplicity of application to packed columns worth considering for certain separation problems.

A solid support (ANAKROM Q, 110/120) was coated with Carbowax 20M (5% w/w) and packed into a glass column (2m × 3mm i.d.). Uncoated Chromosorb G HP (100/120) was packed immediately above the coated solid support at the inlet end for a distance of 7 cm and held in place by a small plug of silanized glass wool in the usual manner. An on column injector was used and the distance from the septum to the glass wool plug was the same as the length of the syringe needle. Any crushing of the Chromosorb G in the bed by the needle during injections will expose undeactivated support and cause severe tailing of polar compounds.

Standard solutions of either *n*-tridecane or of 3-octanone and 3-octanol (1 ppm of each) were made up in *n*-hexane (Riedel de Haen, Spectranal grade). An injection volume of 1 μl would therefore contain 1 pl of each compound (i.e. about 0,8 ng). This amount of material would not normally be detectable on a packed column by the average chromatographer. However, about 80 ng of each compound should be detectable without much trouble. Therefore, 100 μl of the solutions were injected onto the packed bed at a flow rate of 25 ml/min and an inlet and column temperature of 65°C. When the solvent had evaporated from the packed bed and the vapor had passed through the column, as indicated by the falling detector response, the column temperature was programmed from 65°C to 110°C at 15°C per min, and thereafter held at 110°C. Even though the solvent took some minutes to evaporate from the packed bed, the readily detectable peaks were no wider than peaks obtained from

more concentrated solutions injected in the regular manner on a packed column. In addition, provided that the same volume is introduced, retention times are highly reproducible.

These results raise the following possibilities:

1. Some workers using regular packed columns will be able to introduce more sample where the dilution of the solutes was proving to be a limiting factor in their detection.

2. Plumbing changes at the interface of the packed bed and the coated solid support can be designed to vent the majority of the solvent so that it does not enter the column and pass through the detector. In this way, beds that can hold any injection volumes of solvent could be used to focus the solutes before allowing their introduction to the column.

The solvent-focusing of solutes from dilute solutions at a low temperature, without significant loss of solutes, in a process coupled to their subsequent chromatographic separation is attractive and offers the potential for exploitation in both preparative and analytical applications.

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Appendix

Example of text which could be used for teaching or testing purposes For teaching, the underlined words could be given separately. For testing, it may be required to supply them.

Vitamins and Trace Elements in the Function of Enzymes[2]

The vitamins are trace organic substances which are . . . 1 . . . in the function of most forms of life but which some organisms are unable to . . . 2 . . . and must obtain from exogenous sources. Most of the water-soluble vitamins function as components of different . . . 3 . . . or prosthetic groups of enzymes important in cell metabolism. Thiamine (vitamin B_1) is the active component of thiamine pyrophosphate, a . . . 4 . . . required as a transient carrier of . . . 5 . . . in the enzymatic de-carboxylation of pyruvate, a major product of . . . 6 . . . breakdown in cells. . . . 7 . . . (vitamin B_2) is a component of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which function as hydrogen-carrying prosthetic groups of certain oxidative enzymes. . . . 8 . . . acid is a component of the nicotinamide adenine dinucleotides (NAD^+ and $NADP^+$), which serve as transient carriers of . . . 9 . . . ions in the action of certain . . . 10 . . . Pantothenic acid is an essential component of coenzyme . . . 11 . . ., which functions as a transient acyl-group carrier during the enzymatic oxidation of . . . 12 . . . and . . . 13 . . . acids. Vitamin B_6 (pyridoxine) is an essential precursor of pyridoxal phosphate, the prosthetic group of . . . 14 . . . and other amino acid-transforming enzymes. Biotin functions as the prosthetic group of certain . . . 15 . . .; it serves as a carrier of carboxyl groups. Folic acid is the precursor of tetrahydrofolic acid, a coenzyme functioning in the enzymatic . . . 16 . . . of one-carbon compounds. Vitamin B_{12}, as its 5'-deoxyadenosyl derivative, functions in the enzymatic exchange of . . . 17 . . . atoms and certain substituent groups between adjacent carbon atoms.

The . . . 18 . . .-soluble vitamins serve other important roles. Vitamin A is a precursor of a light-sensitive pigment in the visual cycle of . . . 19 . . . cells in vertebrates. Vitamin D_3, or . . . 20 . . . formed from 7-dehydrocholesterol by exposure of the skin to . . . 21 . . ., is the major biological precursor of 1,25-dihydroxycholecalciferol, which has a hormone-like action in regulating . . . 22 . . . metabolism in the small intestine and bones. Vitamin . . . 23 . . . is a cofactor in the enzymatic formation of the γ-carboxyglutamyl residues of prothrombin, a Ca^{++}-binding plasma protein important in blood clotting. Iron, copper, zinc, manganese, cobalt, molybdenum, selenium, and nickel are essential components of a variety of different . . . 24 . . . In addition, a number of other elements including vanadium, tin, chromium, and silicon, are essential in the . . . 25 . . ., but their precise functions are not known.

Original words: (1) essential (2) synthesize (3) coenzymes (4) coenzyme (5) acetaldehyde (6) glucose (7) riboflavin (8) nicotinic (9) hydride (10) dehydrogenases (11) A (12) pyruvate (13) fatty (14) transaminases (15) carboxylases (16) transfer (17) hydrogen (18) fat (19) rod (20) cholecalciferol (21) sunlight (22) Ca^{++} (23) K (24) enzymes (25) diet.

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Editor's Note A Spanish version of this manuscript is available from Dr Argilés.

Thermodynamic Prediction of Steady-State Reaction Direction

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The direction of chemical reactions in closed systems, at constant temperature and pressure, can be determined from the sign of the associated Gibbs free energy change (ΔG). Is the same true for steady-state reactions in open biological systems?

The approach to the determination of reaction direction found in most biochemistry textbooks is that appropriate for isolated reactions in closed systems, yet these books are really dealing with steady-state reactions. We will show that if strict account is taken of the steady-state condition, the ΔG obtained for a steady-state reaction is of no use in determining reaction direction. Paradoxically, if the steady-state is ignored, the non-steady-state, closed system ΔG that is calculated does predict reaction direction in the steady-state.

Closed systems allow no exchange of matter across their boundaries. When a spontaneous chemical reaction occurs in such a system, at constant temperature and pressure, the system undergoes a negative ΔG by virtue of a change in its composition, according to the relationship,

$$dG = \sum_i \mu_i \, dn_i \qquad (1)$$

where μ_i is the chemical potential and dn_i is the change in moles of the ith reactant. Living systems also normally operate at constant temperature and pressure but are open in that they exchange matter with their surroundings. As a consequence, many *in vivo* reactions are in steady-state and are not accompanied by an appreciable change in the amounts of the reacting species even though, in metabolic pathways, the reactants are turning over continuously.

124

It is often possible to view an open biological system, such as a cell, as part of a larger, closed system incorporating a source of reactants (eg a reservoir of glucose and oxygen) and a sink for products (eg carbon dioxide and water). The ΔG of this large closed system reflects the changing levels of source and sink. However, the contribution of a steady-state reaction to the ΔG is evidently zero, by equation (1), since in such a reaction the dn_i terms are all zero. Therefore the ΔG of steady-state reactions (ie their contribution to the ΔG of the system in which they occur) is of no use in predicting reaction direction.

This is an unfortunate state of affairs for the ability to predict the direction of steady-state reactions from a knowledge of the reactant concentrations, via ΔG, would have been useful. This simple approach now appears to be possible only for non-steady-state reactions. Fortunately the situation can be redeemed by linking thermodynamic and kinetic concepts. Consider the following relationships applicable to non-steady-state reactions in a closed system at constant temperature and pressure:

$$\Delta G < 0 \text{ implies, and is implied by,}$$
$$V_f/V_b > 1 \text{ (reaction feasible)} \quad (2)$$

$$\Delta G > 0 \text{ implies, and is implied by,}$$
$$V_f/V_b < 1 \text{ (reaction not feasible)} \quad (3)$$

$$\Delta G = 0 \text{ implies, and is implied by,}$$
$$V_f/V_b = 1 \text{ (equilibrium)} \quad (4)$$

where V_f and V_b refer to the forward and back reaction rates respectively. The direction of a reaction is determined by the actual kinetic ratio V_f/V_b and this applies whether the reaction is in steady-state or not. Hence, according to (2) and (3), the sign of the corresponding non-steady-state ΔG also determines the direction of a reaction in or out of steady-state.

Therefore, if the ΔG of a metabolic reaction is calculated employing actual physiological concentrations, as if the reaction took place in isolation in a closed system, the sign of this ΔG apparently can be used to predict the direction of the reaction in the steady-state. This way of linking thermodynamics and kinetics might find general application to steady state processes.

Comment on the paper by Lindner and Brand by C John Garratt (*Department of Chemistry, University of York, York, UK*)

There is no doubt that the application to open systems of classical thermodynamics derived from the study of closed systems is complex. However, the important point which should emerge from Lindner and Brand's note is that, should anyone wish to do so, it is rigorously correct to use ΔG values derived from closed system thermodynamics to give information about the reaction direction in open systems in a steady state. I suggest that this can be justified by a more simple method than the one they have chosen, and I disagree that the justification is paradoxical.

The difficulty with applying conventional thermodynamics to open systems in a steady-state stems from the fact that the measurement of free-energy changes depends on changes in state actually occurring. Thus, changes in free energy are measured or defined by changes in the composition of a closed system. It is important to note that any free-energy change (or absence of it) refers to the whole system and not to any individual part of it. A strength (and arguably a limitation) of thermodynamics is its ability to deal with an overall system and to ignore the fact that the system may have many individual constituents. An open system must be a part of a larger closed system: for example, a cell can be part of a closed system which provides a (changing) reservoir of glucose and oxygen and a (changing) sink for CO_2; an enzyme converting reactants (R) into products (P) can be part of a closed system which provides a reservoir of (precursors of) R and sink for (products of) P. Thermodynamics can tell us something about the free-energy changes in the closed system: thus, it gives clear information about the free energy available from a defined system oxidising glucose to CO_2 and water. It gives no information about the individual steps in which the free energy is made available, and many other interesting questions. Because thermodynamics is not concerned with individual steps within a system, it is not self-evident (despite Lindner and Brand's assertion) that a reaction in a steady state within a closed system contributes nothing to the overall free-energy change of the whole system; it is better to say that, under these conditions, ΔG for that reaction has no meaning because the concept of free-energy change applies to the whole (ie closed) system of which the reaction is a part.

For many practical purposes (but not all, as I indicate above) this does not matter because another strength of thermodynamics is that measurements made on one system can be applied to another. Thus, if we know (from the study of closed systems) the values of the standard free-energy changes for the hydrolysis of ATP and of glucose phosphate, then we know what is the standard free-energy change for the transfer of phosphate from ATP to glucose. We know this independently of a mechanism providing a route for this transfer. Using this principle, it is possible to calculate the standard free-energy change for a large number of chemical reactions or processes even though the processes themselves may be difficult to study in a closed system. From this it is a trivial matter to use the equation

$$\Delta G^\theta = -RT \ln K$$

to calculate the equilibrium constant for the reaction and hence the ratio of the activities of products to reactants at equilibrium. If a kinetically-possible reaction is not at equilibrium, it must proceed towards it. This conclusion is absolutely independent of whether the system is closed or open. We can therefore use it in considering steady-state reactions in open systems.

If the actual concentrations of products and reactants of such a reaction are known, and if it can be assumed that these are negligibly different from their activities (and either condition is likely to be unsatisfied), then it is a simple matter to determine the direction in which that reaction must proceed in order to get closer to equilibrium. The conclusion does not involve the kinetic argument used by Lindner and Brand, though the principle on which it is based is the same as theirs.

Of course, another way of arriving at the same conclusion is to use the equation

$$\Delta G = \Delta G^{\theta} + RT \ln [P]/[R]$$

where $[P]$ and $[R]$ represent the physiological concentrations (activities) of products and reactants respectively. Technically the value of ΔG obtained gives the change in free energy which would occur if the reaction being considered were isolated in a closed system. The fact that it is not so isolated makes no difference to the fact that the reaction is displaced from its equilibrium and therefore is bound to proceed towards equilibrium (if a kinetic pathway is available). Thus, a negative value of ΔG calculated for a closed system gives a direct indication of the reaction direction, but no information about any free-energy change which may occur in the actual closed system of which the reaction concerned is a part.

The calculation, though correct, is unnecessary for the determination of the reaction direction since this information can be obtained more directly from the data needed to calculate ΔG. Furthermore, as I have written elsewhere (In TIBS, March 1979, p N52), biochemists rarely need to use thermodynamics to determine the direction of a reaction: this is usually established by some other means.

The Estimation of the Apparent Standard Free Energy Change ΔG°pH of a Biochemical Reaction from the Standard Free Energy of Formation and Apparent Free Energy of Ionization of the Participating Molecules and its Application to the Reactions of the Purine Metabolism

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Introduction
An understanding of the dynamics of metabolism requires information about the possible directions of the reactions. A reaction will not proceed in a direction of increasing free energy and for this reason knowledge of the change in free energy of a reaction will allow us to put 'one-way traffic signs' on certain metabolic routes. This is what thermodynamics can do for us. Unfortunately it tells us nothing about the traffic density and its regulation, which are controlled by the kinetic properties of the enzymes and the availability of substrates. A calculation of the change in Gibbs free energy accompanying a reaction between neutral molecules is straightforward when the free energies of formation from the elements of the participating molecules are known. It is, however, more difficult to calculate such a change when ionized molecules are involved. It is the purpose of this article to explain how the free energy change is calculated in such cases when the free energies of formation of the neutral compounds are known together with the dissociation constants. First it is shown how the apparent free energy of ionisation can be calculated. This calculation is straightforward for simple acids and bases but becomes more complicated for amphoteric ions. [As an example the calculation of the apparent free energy of ionisation of AMP is given in the **Appendix**. It has little practical value, because the dissociation constants involved in the equilibria can only be estimates.]

Next the standard free energy change of a reaction between ionic species is calculated at a specified pH using these results. The reasoning is then reversed to show how to calculate the standard free energy of formation from the elements of a compound participating in an equilibrium reaction.

Free energies of formation and acid dissociation constants of the common purines are compiled from the literature and presented together with the calculated free energies of ionisation. These data are used to calculate the standard free energy changes accompanying the reactions of the purine metabolism, at constant temperature and pressure (Table 1).

Calculation of the apparent free energy of ionisation: ΔG_i
Suppose it is possible to prepare a solution of a monobasic acid AH at a specified pH and the dissociation of the acid into A^- and H^+ can be inhibited. The concentration of AH in this 'hypothetical state' is $[A_o]$. The Gibbs free energy of one mole AH is now $G_o = \mu^{\theta}_{AH} + RT\ln[A_o]$. When one mole AH is now allowed to dissociate an equilibrium mixture will result.

When one mole AH dissociates into α moles A^- and α moles H^+, $(1 - \alpha)$ moles AH will remain in the undissociated form. The free energy of this mixture is given in the next formula:

$$G_{pH} = [\mu^{\theta}_{AH} + RT\ln[AH]_{eq}] + \alpha\{\mu^{\theta}_A + RT\ln[A^-]_{eq}$$
$$+ \mu^{\theta}_{H^+} + RT\ln[H^+] - \mu^{\theta}_{AH} - RT\ln[AH]_{eq}\} \quad (1)$$

Where μ^{θ}_{AH} etc is the standard Gibbs free energy of species AH and $[AH]_{eq}$ is the concentration of AH in the equilibrium mixture. At equilibrium:

$$\Delta G^{\circ}_{i(A)} \equiv \mu^{\theta}_{A^-} + \mu^{\theta}_{H^+} - \mu^{\theta}_{AH}$$

$$= -RT\ln [A^-]_{eq}\cdot[H^+]/[AH]_{eq} \equiv -RT\ln K_a \quad (2)$$

dard samples at 410 nm. The standard curve is constructed by plotting $A_{410 \text{ nm}}$ versus [p-nitrophenoxide].

Kinetic Assay Procedure. The following is a general procedure for the kinetic assay of BCA. To each cuvette add 0.5 ml of an appropriate dilution (to be determined below) of the stock enzyme solution (solution 3) and 4.0 ml of 0.04 M phosphate buffer, pH 7.5. Prepare a "blank" by adding an additional 0.5 ml of acetonitrile. Use this tube to "zero" the instrument at 410 nm. For each tube in turn, initiate kinetic runs by adding 0.5 ml of $1.00 \times 10^{-2} M$ PNPA in acetonitrile (solution 4), rapidly mix, and place the tube in the instrument. Note the exact time to the second when the substrate is added. This is taken as the kinetic zero. Record the $A_{410 \text{ nm}}$ at 30-s intervals over the period of time needed to reach ~0.5 absorbance units.

Determination of Specific Activity.[2] Using the above assay procedure, assay 4.5-ml reaction solutions containing respectively, 0.1, 0.2, 0.3, 0.4, and 0.5 ml of the stock enzyme solution (solution 3). Again, the final volume becomes 5.0 ml when the kinetic runs are initiated with the addition of 0.5 ml of $1.00 \times 10^{-2} M$ PNPA. In each case make a plot of the absorbance versus time. Using the present kinetic assay method, our students found that determinations can be made comfortably when the most rapid reactions result in increases in absorbance ~0.5 units over a 10-min period. If more rapid rates are observed, dilutions should be made accordingly. If considerably slower rates are observed, a more highly concentrated stock enzyme solution may be warranted.

Having determined an appropriate enzyme concentration range, run 4 dilutions in this range to establish enzyme dependency. Also, since the hydrolysis of PNPA is catalyzed to a moderate extent by the buffer components, make one kinetic run in the absence of enzyme.

Calculations of Results

Calculation of the Extinction Coefficient of p-*Nitrophenol.* The slope of the best straight line resulting from the plot of $A_{410 \text{ nm}}$ versus [p-nitrophenoxide] gives the molar extinction coefficient for p-nitrophenol.

Calculation of Initial Reaction Velocities. The hydrolysis of PNPA is a pseudo-first-order process for which

$$v = \frac{d[p\text{-nitrophenoxide}]}{dt} = k[\text{PNPA}]$$

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[2] Specific activity determinations made throughout the entire sample of such commercial enzyme preparations are always reproducible. Furthermore, the ratio of esterase activity to hydrase activity is constant (see ref. (2)). Thus, such commercial samples of BCA are homogeneous and, indeed, BCA possesses both hydrase and esterase activities precluding the possibility of contamination by nonspecific esterase activity.

[3] It should be noted that the kinetic assay procedure described in this paper utilizes saturating substrate concentrations (see ref. (2)). Under these conditions, what we have defined as the molecular activity is identical to the turnover number.

[4] The pK_a of p-nitrophenol is around neutrality. Thus for pH-rate profile determinations, it is easiest to follow reaction rates at the isobestic wavelength for the p-nitrophenol/p-nitrophenoxide system ($\lambda = 348$ nm).

[5] Again, since our kinetic assay utilizes saturating substrate conditions, results of such experiments would reflect variations in the turnover number rather than the Michaelis constant.

Although semilogarithmic plots may be used to deduce the first-order rate constants for the reactions, the reaction rates are slow enough so that initial reaction velocities may be determined directly from the initial slopes of the lines which result from plots of $A_{410 \text{ nm}}$ versus time:

$$v_{\text{initial}} = \frac{\text{slope}_{\text{initial}}}{[p\text{-nitrophenoxide}]}$$

Calculation of Enzyme Activity. The initial reaction velocities are the sum of enzymatic and buffer catalyzed components

$$v_{\text{initial}} = v_{\text{buffer}} + v_{\text{enz}}$$

$$= v_{\text{buffer}} + k_{\text{enz}}[\text{enz}]$$

Thus, a plot of v_{initial} versus [enz] should give a straight line from which the molecular activity[3] of BCA can be calculated: slope = mol PNPA min^{-1} mol$_{\text{BCA}}^{-1}$. The specific activity (in units of mol PNPA min^{-1} mg$_{\text{BCA}}^{-1}$) can be determined from the slope of the corresponding plot of v_{initial} versus mg$_{\text{BCA}}$ (remember, $MW_{\text{BCA}} = 30,000$ g/mol).

Additional Experiments

Our students also designed projects which were to reflect some characteristic of the mechanism of the action of carbonic anhydrase. They used "Advances in Enzymology" (3) and "The Enzymes" (4) as literature sources to explore the possibilities and then found suitable journal articles that would aid them in setting up the appropriate experimental protocol for their chosen project. Some of the projects included catalytic versatility studies using various carboxylate esters of p-nitrophenol, determinations of pH-rate profiles,[4,5] solvent deuterium isotope effects,[5] inhibition constants for sulfonamides and for various anions, activation energies[5] for the enzyme-catalyzed reaction, and studies of the effects of added water-soluble organic solvents on BCA activity. The students wrote journal-style reports and shared their results with their peers by means of oral presentations. The laboratory activity, taken was a whole, served to demonstrate to our students how enzyme mechanisms can be deduced from the interpretation of kinetic data.

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Drying Silica Gel in a Microwave Oven

Like many laboratories, we often have to dry exhausted silica gel, and this usually takes many hours in a drying oven. The other day we were in a hurry, so a layer of crystals was placed in a beaker and put in the microwave oven. After one minute on the high setting most of the pink crystals had turned a brilliant blue. This raises the possibility of drying other solid drying agents or activating silica gel TLC plates in the same way, but we have made no systematic study.

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Trimethylamine: The Substance Mainly Responsible for the Fishy Odor Often Associated With Bacterial Vaginosis

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The vaginal discharge of women with bacterial vaginosis often has a prominent fishy odor. Intensification of this fishy odor by the addition of strong base to the vaginal discharge suggests that it could be due to trimethylamine, the substance responsible for the characteristic odor of spoiling fish. Samples were collected from 11 women with a vaginal discharge having a fishy odor and from 10 women with no detectable odor. Gas chromatographic analysis of headspace samples of alkalinized vaginal discharges indicated the presence of trimethylamine in all 11 samples with the fishy odor but not in the other samples. The chemical identity of trimethylamine was confirmed by gas chromatography-mass spectrometry of headspace samples from two vaginal discharge samples. It is concluded that trimethylamine is the primary cause of the fishy odor associated with bacterial vaginosis. (Obstet Gynecol 68:682, 1986)

It has been known for many years that the characteristic odor of spoiling fish is due primarily to trimethylamine.[1] The prominent fishy odor of the vaginal discharge from many women with bacterial vaginosis, and the intensification of this fishy odor by the addition of a strong basic solution to the vaginal discharge, led us to suspect this amine was the primary cause of the odor. The presence of methylamine and isobutylamine, and the less volatile amines, putrescine, cadaverine, histamine, tyramine, and phenethylamine, in the vaginal discharge from patients with bacterial vaginosis has been reported[2] and these substances may also contribute to the malodorous nature of the discharge. However, the odor of trimethylamine is far more characteristic of the fishy odor emitted from this discharge than is that of any of these other amines.

Several analytic methods have been developed for the detection and determination of trimethylamine.[1,3] The choice of gas chromatography for this study was based on personal expertise with headspace analysis of

odors and the compatability of this technique with mass spectrometry for positive chemical identification. Our primary objective was to establish whether or not the fishy odor of the vaginal discharge from patients with bacterial vaginosis was correlated with the presence of trimethylamine in the discharge.

Materials and Methods

Eleven patients who had a clinical diagnosis of bacterial vaginosis and ten normal patients were evaluated. The diagnosis of bacterial vaginosis was based upon the finding of a characteristic discharge, a vaginal wall pH of 5 or more, presence of clue cells and typical bacteria microscopically, and an increase in odor with the addition of KOH. Vaginal wall pH was determined by placing a strip of pH Hydrion papers pH 3–5 (Micro Essential Laboratory, Brooklyn, NY) against the epithelium of the anterior fornix until development occurred. Cultures were not performed, as our experience has been that this problem can be accurately diagnosed from the described clinical findings.

After the clinical diagnosis of bacterial vaginosis, each vaginal discharge was collected on a swab which then was squeezed out in 0.5 mL 0.5 N HCl in a 6-mL screw-top vial. All samples were stored frozen. Urine samples (1 mL) were also acidified and frozen after collection.

For the detection of trimethylamine, the urine (1 mL) and vaginal discharge (approximately 0.5 mL) were made alkaline with 4 N KOH saturated with K_2CO_3 to a final volume of 4.5 mL in the 6-mL screw-top vial. A teflon-faced gas chromatographic septum was used as an insert in an open hole screw-top cap. All samples were equilibrated in an oven at 55C for at least 18 hours before headspace analysis.

For the detection of trimethylamine oxide, samples of urine (0.1 mL) or vaginal discharge (0.1 mL) were treated with 0.5 mL 10% $TiCl_3$ at 55C in vials (6.0 mL)

sealed with a teflon-faced septum for at least 18 hours. All vials were made alkaline with 4 N KOH saturated with K_2CO_3, to a final volume of 4.5 mL.

Gas chromatography studies were accomplished using a Pennwalt 231 column (12 ft × 4 mm inside diameter glass) with a helium flow of 10 mL/minute at 70C was used for all gas chromatographic analyses. Headspace samples (0.2 mL) from equilibrated vials were injected with a Pressure Lok gas syringe into the on-column inlet of a Varian 3700 gas chromatograph. An electrometer setting of 10^{-11} amps/mV and an attenuator setting of zero (Hewlett Packard 3390A integrator) were used for all analyses.

To be certain of the correct identity of the compounds, mass spectrometry was performed using a Finnigan 1015 quadrupole mass spectrometer operated at 70 eV with a 28% Pennwalt 223 + 4% KOH on gas Chrom R (80/100, Applied Science, 12 ft × 4 mm inside diameter, glass) column was used for all GC-MS analyses. The column was temperature programmed from 50C to 150C at 10C/minute. The total ion current was monitored, as well as ion chromatograms for m/e 44 and 58. Mass spectra were recorded for authentic dimethylamine, trimethylamine, and acetone. Mass spectra were recorded from urine and vaginal discharge samples for compounds eluting at the same time as dimethylamine, trimethylamine, and acetone.

Results

Preliminary gas chromatographic analyses on the headspace of alkalinized urine and vaginal discharge samples indicated that two major compounds were readily detected on a 28% Pennwalt 223 + 4% KOH column. GC-MS analysis of a urine sample, monitoring ions at m/e 44 dimethylamine and 58 trimethylamine and acetone, showed that one peak in the total ion current was in fact two unresolved compounds. Examination of the mass spectra of these two unresolved compounds, using the background subtract facility, gave spectra corresponding to dimethylamine and trimethylamine. Acetone eluted after trimethylamine on this column.

The Pennwalt 231 column completely resolved dimethylamine and trimethylamine, but as the maximum temperature of this phase was only 120C, it was used for gas chromatographic analyses only. The 28% Pennwalt 223 + 4% KOH column, with an upper temperature limit of 220C and lower bleed characteristics, was used for all GC-MS analyses.

Headspace gas chromatographic analyses of urine and vaginal discharge samples from the 11 symptomatic patients and from 10 normal patients were conducted. Results of the presence or absence of trimeth-

Table 1. Summary of Analyses of Vaginal Discharges for pH, Presence or Absence of a Fishy Odor, and Presence or Absence of Trimethylamine in the Discharge, and Urine

Vaginal pH	Fishy odor	Trimethylamine	
		Discharge	Urine
5.5	+	+ + +	+ + +
5.0	+ +	+ + +	+ + +
5.0	+	+ + +	+ +
5.0	+	+	+ + +
5.0	+ +	+ + +	+
5.0	+ +	+ +	+ +
5.0	+	+ + +	+ + +
5.5	+	+ + +	+
5.0	+	+ +	+ + +
5.0	+	+ +	+
6.0	+	+	+ + +
4.0	−	−	+ +
4.0	−	−	+ +
4.5	−	−	+ + +
4.0	−	−	+ + +
4.0	−	−	+ +
4.0	−	−	+
3.5	−	−	+
4.5	−	−	Trace
3.0	−	−	Trace
4.0	−	−	−

Minus sign indicates absence, plus sign(s) indicates presence and relative amount.

ylamine in the gas chromatogram, together with the vaginal pH and presence or absence of a fishy odor, as determined by one of the authors (RPG), are presented in Table 1. All of the patients with a detectable fishy odor had vaginal pH 5–6 and easily detectable quantities of trimethylamine. All of the normal patients had a vaginal pH 4.5 or less and no dectectable trimethylamine.

GC-MS analyses of urine and vaginal discharge samples from two of the symptomatic patients were conducted. The urine samples showed the presence of dimethylamine, trimethylamine, and acetone, while the vaginal samples showed only trimethylamine and acetone. The recorded mass spectrum of authentic trimethylamine and that of trimethylamine from a vaginal sample are presented in Figure 1 and shows that the two compounds are identical.

No correlation was evident between the presence of trimethylamine in the urine and trimethylamine in the vaginal discharge (Table 1). Furthermore, reduction of trimethylamine oxide by $TiCl_3$ in urine and vaginal discharge, followed by analysis for trimethylamine, indicated that variable amounts of trimethylamine oxide were present in the urine and little, if any, was present in the vaginal discharge. An increase in the amount of acetone detected after this treatment was
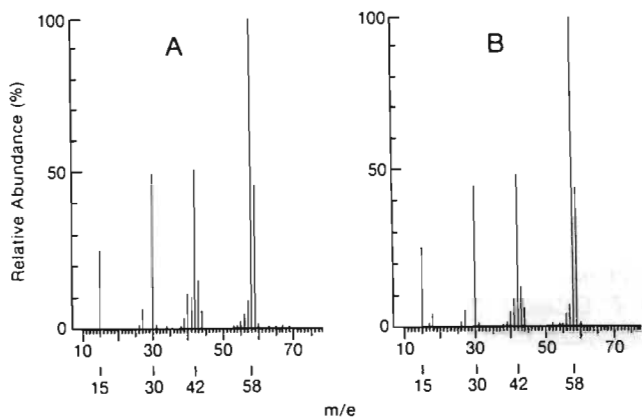
Figure 1. Mass spectra of authentic trimethylamine (A) and trimethylamine from a vaginal discharge (B).

due to decarboxylation of acetoacetic acid present in the sample.

Discussion

Several methods have been published for the detection and determination of trimethylamine, especially in fish and urine.[1,3] As our particular goal was the detection and chemical identification of this compound in a vaginal discharge with a fishy odor, we decided on gas chromatography and GC-MS as the methods of choice. These techniques are particularly sensitive and discriminating for very volatile compounds, and mass spectrometry offers positive identification.

Preliminary analyses indicated that trimethylamine was detected on chromatograms only from vaginal discharges perceived to have a fishy odor. Because the amount of vaginal discharge collected on a swab and the amount of discharge squeezed from the swab into the sample vial varied from patient to patient, quantitative analyses on these samples would be of little value and was not attempted. Although our main objective was the identification of trimethylamine gas chromatographically in samples of vaginal discharge having a fishy odor, the integrated area on the chromatogram is a rough measure of the relative amount of the amine present.

Our study confirmed the finding of Lowis et al,[3] that, for headspace analysis of trimethylamine, the solution must be strongly basic and saturation with K_2CO_3 improved our ability to detect it. Further, our results showed that it was detectable only from patients with vaginal pH 5–6 and whose discharges were perceived to have a fishy odor. As trimethylamine is known to be the compound responsible for the typical odor of fish,[1] we concluded from our findings that it was also the compound primarily responsible for the typical odor of the vaginal discharge in bacterial

vaginosis. Under acidic conditions, trimethylamine is protonated and tends to remain in solution. It is only under strongly basic conditions that it occurs as the free amine (pK_a 9.8) and readily goes into the vapor phase. The presence of trimethylamine, a fairly strong organic base, in vaginal samples of pH 5.0 and higher might be responsible, in part, for the elevated pH. However, these analyses also indicate that it was not the slightly higher pH of the discharge that made the odor more perceptible, as the compound was not present in sufficient quantities in the lower pH discharges to be detected.

While the presence of trimethylamine in vaginal discharge from patients with bacterial vaginosis and a fishy odor was established, the biosynthetic origin of this substance remains unknown. The characteristic odor of spoiling fish is due to trimethylamine produced by reduction of trimethylamine oxide present in fish muscle.[1] Under anaerobic conditions trimethylamine oxide acts as a terminal electron acceptor and the bacteria that perform this reduction have not been shown to metabolize trimethylamine further.[1] Trimethylamine can also be produced from choline,[1] betaine,[1] and ergothioneine[4] by intestinal microorganisms. In mammals, trimethylamine is produced in the gut primarily from ingested trimethylamine oxide in fish or from ingested choline.[1] Trimethylamine is oxidized to trimethylamine oxide by mammalian liver enzymes[5,6] and avian renal enzymes[7] and both trimethylamine and trimethylamine oxide are excreted in the urine.[8] As trimethylamine oxide was found in the urine of our patients and not in their discharges, one could speculate that a reduction process occurs in the human vagina.

We strongly suspect that microorganisms in the flora of patients who have bacterial vaginosis and a fishy odor are involved in the production of trimethylamine but at this time we have not demonstrated its immediate biosynthetic precursor or the origin of this precursor. Using the thin-layer chromatographic procedure of Sarada and Ramasastri, we were unable to detect choline in any of the vaginal discharges; therefore, it is an unlikely vaginal precursor.[9]

Even though the identified amines[2] and possibly certain sulfur compounds[10] contribute to the malodorous nature of the discharge, we believe that trimethylamine is a major compound producing the fishy odor. Regardless, the intriguing possibility is that bacterial vaginosis and the associated recurrent odorous discharge may be due occasionally to an underlying metabolic problem and not always secondary to a sexually transmitted disease as has been perceived. If this could be demonstrated, it would have major

clinical implications. Further work is being conducted in our laboratory to elucidate this problem.

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Individiual Variation in the Major Alarm Pheromone Components of Two *Crematogaster* Species

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Abstract—Variation in the ratio of the two major alarm pheromone components. 3-Octanone and 3-octanol, in individuals of two species of ants in the genus *Crematogaster* has been analysed by gas chromatography. The mean values for each species differ but certain individuals from each species either can have very similar ratios or very different ratios. This variability between individuals seems likely to rule out the possibility of these two substances conveying subtlety of information, such as species recognition or nest mate recognition.

Introduction

The compositional variability of insect pheromone blends, and the behavioural responses to such blends, has attracted considerable interest [1 and references therein]. The earlier studies on this topic often focused attention on the chemotaxonomic value of the data, but the emphasis on compositional variability in the more recent literature has highlighted recognition and behavioural responses. This is due, in part, to the use of pooled samples in earlier studies because of analytical constraints. For example, the chemotaxonomic value of 3-octanone and 3-octanol, produced as major constituents of the alarm pheromone blend of ants in the genus *Crematogaster* [2], and the genus *Myrmica* [3] has been illustrated usually from pooled samples.

The development of high efficiency capillary columns for gas chromatography and the ability to inject microlitre volumes into these columns now allows analyses of extracts of individual insects or glands, even when the amount of material present is in the low to sub-nanogram range. Such results allow a greater scope for interpretation.

Two local species of ant, *C. castanea* and *C. liengmei,* produce 3-octanone and 3-octanol as major volatile components in the heads [4], as do various other species in this genus [5]. The 3-octanol of both of these species is exclusively

the *S*-(+)-enantiomer [4] and it has been suggested that these two related compounds may be able to convey gross information [6]. This suggestion was based on the finding that a substantial difference in the liquid phase ratio of certain binary mixtures may produce only a slight change in the gas phase ratio. One of the binary mixtures investigated was 3-octanone: 3-octanol and changes in composition such as those reported for species of *Crematogaster* [2] and *Myrmica* [3, 7] result in only slight changes in the gas phase composition.

It was these various findings that prompted an investigation of the individual variation of the 3-octanone:3-octanol in the two local species of *Crematogaster, C. castanea* and *C. liengmei.* Gas chromatographic analyses of solvent extracts of individual heads of worker ants illustrated that a considerable variation in the 3-octanone:3-octanol ratio exists within each species.

Results and Discussion

The extraction of a single head containing only nanogram amounts of 3-octanone and 3-octanol in 30 µl hexane ensures complete extraction of these substances. In addition, the ability to analyse each extract in duplicate leads to more reliable estimates of liquid ratios. Employing a 5 µl injection volume allows the detection of low to sub-nanogram amounts of substances in a 30 µl extract without the need for any concentration

TABLE 1. RATIO OF 3-OCTANONE TO 3-OCTANOL IN THE HEADS OF INDIVIDUAL WORKERS OF *C. CASTANEA* AND *C. LIENGMEI*

C. castanea 3-octanone:3-octanol	*C. liengmei* 3-octanone:3-octanol
88:12	69:31
86:14	68:32
85:15	61:39
81:19	61:39
81:19	60:40
81:19	56:44
78:22	55:45
78:22	55:45
77:23	54:46
76:24	51:49
76:24	Mean 59:41
75:25	
73:27	
73:27	
70:30	
70:30	
Mean 78:22	

Each ratio is the average of duplicate analyses on an individual head extract on the BP-20 capillary column.

step. The concentration step and solute focusing is done in the packed bed connected to the capillary column.

Analyses of sixteen worker heads of *C. castanea* and ten worker heads of *C. liengmei* gave the ratios of 3-octanone to 3-octanol presented in Table 1. The mean ratio of 3-octanone:3-octanol for *C. castanea* is 78:22 and that for *C. liengmei* is 59:41. From previously published data [6], these liquid ratios correspond to ratios of 95:5 and 91:9 respectively for the ketone and alcohol that would occur in the gas phase in equilibrium with these liquid ratios at 22°. As it is the gas phase that is actually perceived, it is this ratio of components that carries information. The ratio in *C. castanea* varies from 88:12 to 70:30 and the ratio in *C. liengmei* varies from 69:31 to 51:49. These liquid ratios correspond to a gas phase range of ratios of 97:3 to 94:6 and 94:6 to 86:14 respectively [6]

As individuals were chosen randomly from amongst workers of the two colonies, no information is available on their age or physiological condition. Future studies that control these variables, as well as genetic variation due to the possible presence of multiple queens, should be attempted. It seems quite possible that the ratio of components in a blend could change with age and physiological status and have some behavioural meaning.

The gas phase range in composition varies less than the liquid phase ratios. This is the case when a binary mixture of the two pure compounds is analysed [6], and for the purpose of this discussion, the same is assumed to be true for these two major alarm pheromone components in their natural secretion. If this is the case then the similarity of gas phase composition of these two major components in the alarm pheromone blend of certain worker ants supports the contention that these two substances are unlikely to convey subtlety of information such as species recognition [6]. However, the liquid ratio of pooled samples may well have chemotaxonomic value.

Ultimately one needs to be able to analyse the gas phase composition of the alarm pheromone blend released by an individual. Only then will the true individual variation be available for consideration and assessment. As recently stated by Tumlinson and Teal [8], one should first determine what signals an individual or species perceives in order to understand and evaluate its chemical communication processes. They pointed out that a general problem in understanding insect chemical communication has been that we approach it incorrectly.

Experimental

Lactose and Melibiose Metabolism in *Erwinia chrysanthemi*

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A Lac⁺ mutant of *Erwinia chrysanthemi* was isolated from the Lac⁻ wild type on lactose agar. β-Galactosidase was expressed independently of lactose transport in both the mutant and the wild type, and neither strain expressed thiogalactoside transacetylase. Lactose transport and α-galactosidase, constitutive in the Lac⁺ strain, were coordinately induced in the Lac⁻ strain by melibiose and raffinose but not by isopropyl-β-D-thiogalactopyranoside or thiomethyl-β-D-galactopyranoside. Melibiose was a strong inhibitor of both the melibiose- and the raffinose-induced lactose permeases, whereas raffinose was a strong inhibitor of only the raffinose-induced lactose permease.

Many members of the family *Enterobacteriaceae* possess operons that code for enzymes involved in lactose and melibiose metabolism. In *Escherichia coli*, the *lacZ* gene codes for β-galactosidase, the *lacY* gene codes for lactose permease, and the *lacA* gene codes for thiogalactoside transacetylase (36). The *mel* operon of *E. coli* contains the *A* and *B* genes which code for α-galactosidase and melibiose permease, respectively (30). *Klebsiella aerogenes* possesses both a plasmidborne and a chromosomal *lac* operon (1, 21, 22, 24). Both *K. aerogenes* and *Salmonella typhimurium* possess *mel* operons similar to the *E. coli* operon (12, 23).

The substrate specificities of the *lac* and *mel* permeases differ among the various members of the *Enterobacteriaceae*. The *lac* permease of *E. coli* has wide specificity and transports a variety of α- and β-galactosides, including lactose, isopropyl-β-D-thiogalactopyranoside (IPTG), thiomethyl-β-D-galactopyranoside (TMG), melibiose, and galactose (26). The *mel* permeases of *E. coli* and *S. typhimurium* are similar in specificity and can transport TMG, melibiose, and galactose, but not lactose or IPTG (12, 19, 26, 30). The *mel* permease of *K. aerogenes* differs from those of *E. coli* and *S. typhimurium* in being able to transport lactose in addition to TMG, melibiose, and galactose (23, 35).

The *raf* operon is plasmidborne in *E. coli* (18). It codes for the three genes involved in raffinose metabolism: α-galactosidase, raffinose permease, and invertase (29, 32). All three activities are coordinately induced by melibiose and raffinose (29, 32). Lactose transport by the raffinose permease is competitively inhibited by melibiose and raffinose (32).

Erwinia chrysanthemi pv. *zeae*, the causal agent of soft rot in corn, is a member of the *Enterobacteriaceae* (34). Only two reports dealing with lactose metabolism in *E. chrysanthemi* have appeared (6, 8) despite the use of lactose fermentation as a taxonomic marker in the classification of the *Enterobacteriaceae*. The 322 strains of *E. chrysanthemi* studied by Dickey (3) were all able to hydrolyze *o*-nitrophenyl-β-D-galactopyranoside (β-ONPG), although most of them were classified as Lac⁻. Other workers have classified lactose fermentation by *E. chrysanthemi* as being either variable or delayed (5, 11).

Our studies on lactose metabolism by a locally isolated strain of *E. chrysanthemi* showed that it was normally lactose nonutilizing (Lac⁻). However, lactose-utilizing (Lac⁺) mutants are readily isolated from the parent strain by extended incubation on lactose agar (6). The Lac⁻ and Lac⁺ strains were found to be physiologically and pathogenically similar except for their growth on lactose (6). Since the activity of the β-galactosidase from the Lac⁻ and Lac⁺ strains was similar on both lactose and β-ONPG, we suggested that the Lac⁻ strain differed from the Lac⁺ strain by the absence of a lactose permease (6). The present study confirms that the Lac⁻ strain does differ from the Lac⁺ strain by the absence of a lactose permease coordinately induced with β-galactosidase. Evidence is presented that *E. chrysanthemi* possesses two permeases involved in lactose transport. One permease appears to be associated with the *mel* operon and the other with the *raf* operon. It is concluded that the Lac⁺ phenotype is due to a mutation allowing constitutive expression of the *mel* operon.

MATERIALS AND METHODS

Chemicals. D-[*glucose*-1-¹⁴C]lactose (specific activity, 57.7 mCi mmol⁻¹) was purchased from the Radiochemical Centre (Amersham, England). [*methyl*-¹⁴C]TMG ([¹⁴C]TMG) (specific activity, 35 mCi mmol⁻¹) was obtained from Schwarz/Mann (Orangeburg, N.Y.). [*isopropyl*-¹⁴C]IPTG ([¹⁴C]IPTG) (specific activity, 28 mCi mmol⁻¹) was purchased from Service des Molecules Marquées, Gif-Sur-Yvette, France. β-ONPG and α-ONPG, nonradioactive IPTG and TMG, and all carbohydrates were purchased from the Sigma Chemical Co. (St. Louis, Mo.). Dithiothreitol was obtained from Boehringer GmbH (Mannheim, Federal Republic of Germany). Sodium dodecyl sulfate was purchased from the Pierce Chemical Co. (Rockford, Ill.).

Media. The yeast-salts medium described by Mildenhall et al. (14) was adjusted to pH 7.3 before autoclaving (15 lb/in², 121°C, 20 min). The cooled medium was supplemented with 5 g of filter-sterilized (0.22-μm-pore-size Millex filter; Millipore Corp., Bedford, Mass.) carbon source liter⁻¹. This medium was solidified by the addition of Difco agar (15 g liter⁻¹). Cells for transport studies were grown in M63 medium (15) supplemented with 50 mM NaCl as described by Robbie and Wilson (25) and adjusted to pH 7.0 before autoclaving. Sterile medium received the following sterile supplements: 1 mM MgSO₄, 0.9 g of yeast extract liter⁻¹,

and 4 g of glycerol liter^{-1}. When the effects of different sugars on enzyme production were measured, they were added to the medium to a final concentration of 4 g liter^{-1}. Filter-sterilized IPTG and TMG (0.22-μm-pore-size Millex filter) were used at a final concentration of 0.5 mM.

Organisms. A strain of *E. chrysanthemi* (FH1) isolated locally from corn (13) was used in these studies and has been deposited, as strain SR260, in the collection of Arthur Kelman (Department of Plant Pathology, University of Wisconsin, Madison). This strain, which is Lac$^-$, has been designated EC-C. A Lac$^+$ mutant, designated EC-S, was isolated from the wild type by growth on yeast-salts-lactose agar (6). Both strains were maintained on Difco nutrient agar at 20 to 22°C and subcultured monthly.

Enzyme assays. β-Galactosidase was assayed by the method of Miller (15). The cells were lysed by a modification of the lysis method of Putnam and Koch (20) involving replacement of the sodium dodecyl sulfate with sodium deoxycholate.

α-Galactosidase was assayed in toluene-permeabilized cells, since the lysis mixture completely inhibited enzyme activity. The details of the assay were similar to those described by Schmitt and Rotman (31). The effects of 5 mM dithiothreitol and 5 mM Mn^{2+}, reported by Schmitt and Rotman (31) and Burstein and Kepes (2) to stabilize α-galactosidase, were tested. In the presence of these compounds, the reaction was stopped by the addition of 1 ml of 1 M Na$_2$CO$_3$–10 mM EDTA, since the colored complex formed between dithiothreitol and Mn^{2+} in alkaline solution interferes with the assay (31).

A molar extinction coefficient of 21,300 M^{-1} cm^{-1} at 420 nm and pH 10.3 for *o*-nitrophenol (9) was used to calculate enzyme activity, 1 unit of which is defined as the hydrolysis of 1 nmol of α- or β-ONPG min^{-1}.

Thiogalactoside transacetylase was assayed as described by Miller (15). One unit of thiogalactoside transacetylase activity is defined as an increase in A_{412} of 0.01 unit h^{-1}.

Preparation of cells for transport studies. Overnight cultures were grown either in nutrient broth (Difco Laboratories, Detroit, Mich.) or in M63 medium supplemented with the appropriate carbon source on an orbital shaker (30°C, 200 rpm) and then diluted (1:50) into M63 medium supplemented with 4 g of glycerol per liter^{-1} or various sugars or both as described in the text. After growth on the orbital shaker at 30°C for 8 h (late log or early stationary phase), the cells were sedimented by centrifugation (10,000 × g, 4°C, 10 min), suspended to the original culture volume in M63 medium without glycerol or yeast extract but containing chloramphenicol (0.15 mg ml^{-1}), and recentrifuged. The wash was repeated, and the cells were suspended to an A_{600} of about 4.0 and stored on ice.

Transport of ^{14}C-labeled IPTG, TMG, and lactose. All assays were performed in M63 medium without glycerol or yeast extract in a final volume of 1 ml in tubes (16 by 100 mm). After equilibration at the experimental temperature for 5 min, uptake was initiated by the addition of 100 μM [^{14}C]IPTG (0.25 mCi mmol^{-1}), [^{14}C]TMG (0.5 mCi mmol^{-1}), or [^{14}C]lactose (2.5 or 0.125 mCi mmol^{-1}). Samples (0.5 ml) were removed after 10 min, filtered through a 0.45-μm-pore-size HAWP filter (Millipore Corp.) on a Millipore filtration manifold, and washed with five 0.5-ml aliquots of suspension medium at room temperature (20 to 22°C). The filtration and wash process was completed within 15 s. The filters were counted in either 10 ml of Beckman EP scintillation fluid (Beckman Instruments International S.A., Geneva, Switzerland) or 10 ml of Packard Filtercount (Packard Instruments

TABLE 1. β-Galactosidase activity of strains EC-C and EC-S grown on different carbon sources

Carbon source[a]	β-Galactosidase activity[b] (U/10^9 cells)	
	EC-C (Lac$^-$)	EC-S (Lac$^+$)
Glycerol	6.0	6.1
Glycerol + raffinose	5.8	3.7
Glycerol + melibiose	9.4	7.3
Glycerol + galactose	11.9	8.7
Glycerol + lactose	13.9	66.0
Glycerol + IPTG	66.5	72.3
Glycerol + TMG	64.8	73.8

[a] Strains EC-C and EC-S were grown to the late exponential phase in yeast-salts-glycerol medium. Galactose, lactose, melibiose, and raffinose were added at 4 g liter^{-1}, whereas IPTG and TMG were present at 0.5 mM.

[b] The assay mixture contained 0.1 ml of cells, 0.9 ml of Z buffer, and 25 μl of lysis mixture. After vortexing for 10 s, the assay mixture was equilibrated at 30°C for 5 min, and the reaction was started by the addition of 0.2 ml of β-ONPG (13.2 mM in H$_2$O). The reaction was stopped 10 to 15 min later by the addition of 1.0 ml of 1 M Na$_2$CO$_3$, and after dilution of the mixture with 2 ml H$_2$O, the A_{420} and A_{550} were determined. A correction for turbidity was made by subtracting 1.711 × A_{550} from the A_{420}. Each value represents the mean of three determinations. Variation in all instances was less than 5%.

International S.A., Zurich, Switzerland) in a Beckman model 8100 liquid scintillation counter.

The effect of different sugars on substrate uptake was determined by adding them, together with the substrate, to the assay mixture. The effects of 4 mM 2,4-dinitrophenol and 20 mM sodium azide on substrate uptake were determined after preincubation of the cells with these compounds for 30 min. The effect of 70 mM formaldehyde on substrate uptake was determined by adding it, together with the substrate, to the assay mixture (10).

RESULTS

Induction of β-galactosidase in EC-C (Lac$^-$) and EC-S (Lac$^+$) by different carbon sources. β-Galactosidase was produced by both EC-C and EC-S strains when they were grown on glycerol as a carbon source (Table 1). The levels were increased 10-fold by growth in the presence of 0.5 mM IPTG or TMG, but lactose was effective as an inducer only with strain EC-S. Melibiose, raffinose, and galactose induced β-galactosidase activity weakly or not at all in either strain.

Thiogalactoside transacetylase activity in EC-C and EC-S. No thiogalactoside transacetylase activity was detected in either strain EC-C or EC-S after induction by 1 mM IPTG even though high levels of β-galactosidase were present (Table 2). Neither was thiogalactoside transacetylase activity detected in unheated samples of either EC-C and EC-S, although a similar increase in the nonspecific breakdown of acetyl coenzyme A was observed in both samples. It is unlikely that the failure to detect thiogalactoside transacetylase was a result of a defective assay procedure since, under similar conditions, the enzyme could be detected in induced *E. coli*. Therefore, it was concluded that thiogalactoside transacetylase activity was absent in both strains of *E. chrysanthemi*.

Uptake of ^{14}C-labeled lactose, IPTG, and TMG by EC-C and EC-S cells grown on different carbon sources. Lactose transport was constitutive in EC-S and induced by melibiose and raffinose in EC-C (Table 3). Galactose was a poor inducer of lactose transport in EC-C, and neither IPTG, TMG, nor lactose induced a lactose transport system in EC-C.

TABLE 2. Thiogalactoside transacetylase and β-galactosidase activity in EC-C, EC-S, and Lac⁺ and Lac⁻ strains of *E. coli*

Strain	Thiogalactoside transacetylase (U ml⁻¹)		β-Galactosidase (U ml⁻¹)	
	Induced[a]	Uninduced	Induced[a]	Uninduced
EC-C (Lac⁻)	<0.4	<0.5	610.0	63.1
EC-S (Lac⁺)	<0.4	<0.5	537.7	40.8
E. coli Lac⁺	33.7	<0.5	2,042.8	13.1
E. coli Lac⁻	1.5	<0.4	1,006.9	56.5

[a] Overnight cultures of EC-C, EC-S, and both a Lac⁺ and a Lac⁻ strain of *E. coli* K-12 were grown in M63 medium supplemented with 4 g of glycerol liter⁻¹ and 0.9 g of yeast extract liter⁻¹ at 30°C on an orbital shaker. After dilution (1:25) into fresh medium containing either 1 mM or no IPTG, the cultures were grown to an A_{600} of about 0.6 and harvested by centrifugation (10,000 × *g*, 4°C, 10 min). The cells were suspended in 0.1 of the original culture volume of 0.05 M Tris–0.01 M EDTA (pH 7.9) and sonicated at 4°C. The samples were divided in two. One sample was heated at 70°C for 5 min, centrifuged, and assayed for thiogalactoside transacetylase. The other sample was not heated before centrifugation and assay for β-galactosidase.

Neither EC-C nor EC-S was able to transport IPTG irrespective of the nature of the inducer in the growth medium (data not shown). Measurement of [¹⁴C]TMG accumulation by EC-C and EC-S produced inconclusive results, because similar levels were found in both formaldehyde-treated and untreated cells (data not shown).

The following carbohydrates at a concentration of 2 mM inhibited lactose uptake by EC-S cells grown on glycerol plus lactose (10-min assay, 30°C; percent inhibition compared with the control given in parentheses): TMG (26%), IPTG (87%), thiodigalactoside (91%). Similar values were obtained in the case of EC-C cells grown on glycerol plus melibiose. Under the same experimental conditions, 2-deoxy-D-glucose, galactose, and glucose at 1 mM inhibited lactose transport by EC-S cells grown on glycerol plus lactose by 22, 44, and 56%, respectively. Little or no inhibition of lactose uptake was observed by D-(+)-arabinose, L-(−)-arabinose, L-(−)-fucose, L-rhamnose, mannitol, or D-mannose (data not shown).

Lactose transport by glycerol-grown EC-S cells did not deviate greatly from linearity over the first 2 min when assayed at room temperature (22°C) (Fig. 1). Similar curves were obtained for melibiose-grown EC-C cells. Therefore, the average rate of transport over this period was used as an approximation of the initial rate of transport in competition experiments, the results of which are presented in Table 4. Raffinose was a potent inhibitor of lactose transport by strain

TABLE 3. Uptake of [¹⁴C]lactose by strains EC-C and EC-S grown on different carbon sources

Carbon source[a]	Lactose uptake (nmol 10 min⁻¹ 10⁹ cells⁻¹)[b]	
	EC-C	EC-S
Glycerol	0.17	10.0
Glycerol + IPTG	0.43	16.7
Glycerol + TMG	0.29	16.3
Glycerol + lactose	0.17	10.7
Glycerol + melibiose	6.6	6.9
Glycerol + raffinose	2.13	8.1
Glycerol + galactose	0.96	8.1

[a] Cultures were grown in M63 medium with various carbon sources (4 g liter⁻¹) and assayed for lactose transport as described in Materials and Methods.
[b] Ten-minute assays performed at 30°C. Each point is the mean of duplicate determinations.
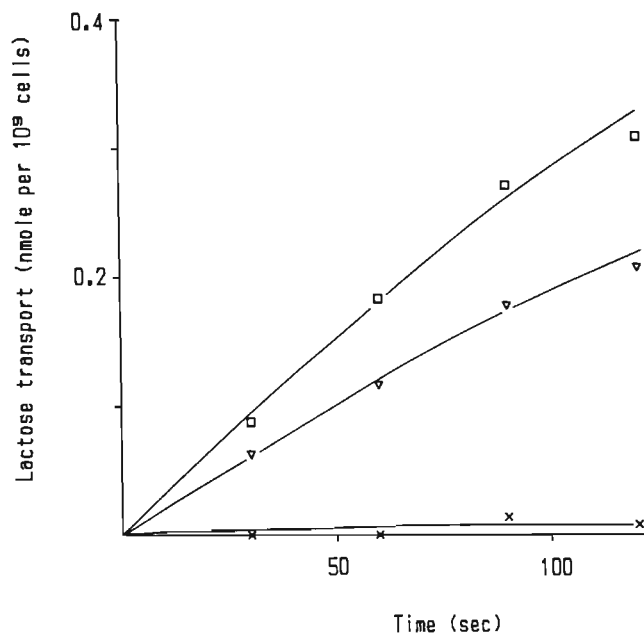
FIG. 1. Inhibition by melibiose and raffinose of lactose transport by the constitutive lactose transport system of strain EC-S. Strain EC-S was grown to the late exponential phase in M63 medium supplemented with 0.9 g of yeast extract liter⁻¹ and 4 g of glycerol liter⁻¹. The cells were washed and assayed for lactose uptake in the absence (□) or presence (×) of 2 mM melibiose or raffinose (∇).

EC-C grown on raffinose, whereas it was a relatively poor inhibitor of lactose transport by EC-S grown on glycerol. Melibiose, irrespective of the inducer present during growth of the cells, strongly inhibited lactose transport by both EC-C and EC-S cells.

Formaldehyde, 2,4-dinitrophenol, and sodium azide all inhibited lactose transport by greater than 90%, indicating the presence of a carrier involved in active transport.

α-Galactosidase activity in permeabilized EC-C and EC-S cells. The lysis mixture used in the assay of β-galactosidase destroyed α-galactosidase activity in EC-S cells. Consequently, the effects of toluene, dithiothreitol, and Mn²⁺ on α-galactosidase activity were investigated in glycerol-grown EC-S cells. Toluene permeabilization increased α-galactosidase activity by about 30%, with little further increase by the addition of dithiothreitol or Mn²⁺, either singly or in combination (data not shown). Therefore, cells were merely tolueneized in all subsequent α-galactosidase assays.

High constitutive levels of α-galactosidase were found in EC-S cells regardless of the carbon source, whereas the enzyme was induced by melibiose and, to a lesser extent, by galactose and raffinose in strain EC-C (Table 5).

DISCUSSION

The strains of *E. chrysanthemi* used in this study (EC-C and EC-S) do not have a *lac* operon analogous to that found in *E. coli*. In *E. coli*, the *lacZ*, *lacY*, and *lacA* genes are expressed coordinately (36). In contrast, β-galactosidase in *E. chrysanthemi* can be induced in EC-C without coordinate induction of either a lactose permease or a thiogalactoside transacetylase. Furthermore, in EC-S, a lactose permease is expressed constitutively, whereas the β-galactosidase remains inducible. Last, melibiose, an inducer of the *lac* operon in *E. coli* (16), does not induce β-galactosidase in *E.*

TABLE 4. Inhibition of lactose transport in strains EC-C and EC-S by melibiose and raffinose

Competing sugar[a]	EC-C		EC-S	
	Lactose uptake (nmol min^{-1} 10^9 cells^{-1})[b]	% Inhibition	Lactose uptake (nmol min^{-1} 10^9 cells^{-1})[b]	% Inhibition
Glycerol-grown cells				
None			0.308 ± 0.004	0
Melibiose			0.008 ± 0.008	97
Raffinose			0.206 ± 0.012	33
Melibiose-grown cells				
None	0.162 ± 0.024	0	ND[c]	
Melibiose	0.032 ± 0.014	80	ND	
Raffinose	0.192 ± 0.025	0	ND	
Raffinose-grown cells				
None	0.139 ± 0.040	0	ND	
Melibiose	0.032 ± 0.019	77	ND	
Raffinose	0.032 ± 0.019	77	ND	

[a] Growth conditions were similar to those reported in Table 3.
[b] Average ± standard error of the mean (three determinations).
[c] ND, Not determined.

chrysanthemi although it does induce a permease able to transport lactose. Thus, lactose transport and hydrolysis are not coordinately regulated in EC-C and EC-S.

Melibiose and raffinose both induced a lactose transport system in strain EC-C. The raffinose-induced lactose transport system in EC-C differs from the constitutively expressed lactose transport system in EC-S by its increased sensitivity to inhibition by raffinose. Therefore, E. chrysanthemi probably possesses two independently regulated permeases capable of lactose transport, one associated with the mel operon and the other with the raf operon.

The results presented in this paper strongly suggest that the mel operon is expressed constitutively in EC-S. High levels of α-galactosidase and lactose transport are coordinately expressed in EC-S, whereas, relative to lactose transport, only low levels of α-galactosidase are induced by raffinose in strain EC-C, i.e., the ratio of lactose permease to α-galactosidase activity is lowest in the case of glycerol-grown EC-S, intermediate in melibiose-induced EC-C, and highest in raffinose-induced EC-C. In addition, raffinose does not inhibit lactose transport in glycerol-grown EC-S to the same extent as found for raffinose-grown EC-C.

Taken together, these findings imply that lactose transport can occur via an inducible mel permease and an inducible raf permease in E. chrysanthemi and that the Lac$^+$ phenotype of EC-S is due to a regulatory mutation allowing constitutive expression of the mel operon. These data confirm and extend the observation by Hugouvieux-Cotte-Pattat and Robert-Baudouy (8) that melibiose and raffinose induce a permease capable of lactose transport in E. chrysanthemi. It is possible that this permease, produced constitutively by what they called the lmrT gene, is the mel rather than the raf permease reported here.

Although the E. chrysanthemi mel permease resembles those of E. coli, S. typhimurium, and K. aerogenes in failing to transport IPTG (data not shown), it also differs in not being able to transport TMG (12, 19, 26, 30, 35). However, the E. chrysanthemi mel permease resembles the K. aerogenes mel permease in its ability to transport lactose (23, 35). In addition, the transport of lactose by the raffinose-induced permease in EC-C and its inhibition by melibiose and raffinose resembles the behavior of the raf permease in E. coli (29).

Although IPTG is not transported by E. chrysanthemi, it is nevertheless a potent inhibitor of lactose transport by this organism. This illustrates the danger of assuming that competition studies provide indisputable evidence that the competitor itself is transported.

The whole question of galactosidase transport in E. coli is complex, with at least four permeases involved (26). The problem is further complicated by the multiple common inducers and substrates of these permeases (26). The unravelling of galactoside transport in E. coli required the isolation of the appropriate mutants (26, 30), and it is apparent that a similar approach is required in E. chrysanthemi.

Substrates of the phosphoenolpyruvate:sugar phosphotransferase system such as glucose, 2-deoxyglucose, and mannose have been found to inhibit both the lac and mel permeases in E. coli (4, 27). The inhibition is thought to involve an allosteric interaction between components of the phosphotransferase:sugar transport system with the lac and mel permeases (28). A similar mechanism may be involved in the inhibition of lactose transport by both glucose and 2-deoxyglucose in strain EC-S.

TABLE 5. α-Galactosidase activity of EC-C and EC-S grown on different carbon sources

Carbon source[a]	α-Galactosidase activity (U/10^9 cells)[b]	
	EC-C	EC-S
Glycerol	0.34	33.8
Glycerol + IPTG	0.51	31.5
Glycerol + lactose	0.17	30.0
Glycerol + melibiose	17.7	17.7
Glycerol + raffinose	3.1	16.8
Glycerol + galactose	1.9	20.2

[a] Growth conditions were similar to those reported in Table 1.
[b] The reaction mixture contained 0.1 ml of bacterial cells, 0.5 ml of 100 mM Tris-hydrochloride buffer (pH 7.5), 0.3 ml of H$_2$O, and 0.025 ml of toluene. After vortexing for 10 s and preincubation of the cells for 5 min at 30°C, the reaction was started by the addition of 0.1 ml of the substrate (20 mM α-ONPG in H$_2$O) and stopped after 10 to 15 min by the addition of 1 ml of 1 M Na$_2$CO$_3$. After the addition of 2 ml of H$_2$O, the A_{420} and A_{550} were determined. The A_{420} was corrected for turbidity as described in Table 1, footnote b.

We cannot explain why growth on melibiose results in significantly lower α-galactosidase and permease activity in EC-S (Tables 3 and 5), but catabolite repression might be one cause. Similarly, it is not clear why IPTG and TMG in the growth medium should enhance lactose transport in strain EC-S (Table 3). One possibility is that the high levels of β-galactosidase induced by these compounds in *E. chrysanthemi* play a role by hydrolyzing the intracellular lactose. This would reduce the lactose concentration gradient across the membrane against which the transport system must work.

The resistance of the α-galactosidase from *E. chrysanthemi* to toluene is similar to that of the *raf* α-galactosidase from *E. coli* (29) but is different from the *mel* α-galactosidases of *E. coli* and *S. typhimurium*, both of which are sensitive to toluene inactivation (2, 7, 12, 31, 33). Furthermore, neither Mn^{2+} nor dithiothreitol, both of which stabilize α-galactosidase activity in cell extracts of *E. coli* (2, 31), activated the *Erwinia* enzyme. The *Erwinia* α-galactosidase also differs from the *E. coli* enzyme in that a cofactor such as NAD^+ is not required for activity (2).

Many members of the *Enterobacteriaceae* have been found to acquire new metabolic functions by mutations that allow the enzymes of different pathways to be used for new functions (17). Two types of mutations have been discovered. First, regulatory mutations may occur that allow enzymes not inducible by their substrates to be expressed constitutively, thereby permitting metabolism of the substrates (17). A second type of mutation may change the substrate specificity of an enzyme and lead to the metabolism of a compound that is not normally a substrate of the enzyme (17).

The β-galactosidases in strains EC-C and EC-S have been shown to have similar activity on β-ONPG and lactose (6). Therefore, the difference in lactose metabolism between EC-C and EC-S cannot be explained by an altered specificity of β-galactosidase for lactose. Our results suggest that EC-S becomes phenotypically Lac⁺ owing to a mutation allowing constitutive expression of the *mel* operon, i.e., a regulatory mutation.

Lactose metabolism of *E. chrysanthemi* has not been studied in detail, and it is not known whether a *lac* operon exists in any other strains of this organism. However, the data presented in this paper may explain why many strains of *E. chrysanthemi* are reported to have either a variable or a delayed ability to ferment lactose (3, 5, 11).

ACKNOWLEDGMENTS

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SYNTHESIS OF ENANTIOMERICALLY ENRICHED 2-HEPTANOL AND 3-OCTANOL BY MICROBIAL REDUCTASES OF *Curvularia falcata* AND *Mucor* SPECIES

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Abstract—Certain insects produce 2-heptanol or 3-octanol in various glandular secretions and recent studies have shown that the 3-octanol of two different genera of ants (*Crematogaster* and *Myrmica*) can be either the (S)-(+) or mainly the (R)-(−) enantiomer, respectively. Synthesis of each of these alcohols can be achieved in relatively high enantiomeric purity by certain microbial reductases. The corresponding ketone of each alcohol is reduced by *Curvularia falcata*, giving an alcohol which is about 90% the (S)-(+) enantiomer, and two *Mucor* species give as much as 80% the (R)−(−) enantiomer. The synthesis of certain chiral alcohols from their corresponding ketones by microbial reductases can offer a simple procedure for obtaining sufficient amounts of these substances for certain behavioral studies.

Key Words—Chiral alcohols, 2-heptanol, 3-octanol, microbial reductases, *Curvularia falcata*, *Mucor* species.

INTRODUCTION

The mandibular glands of three species of *Myrmica* ants contain 3-octanol as a component of their alarm pheromone blend, and this alcohol is >90% the (R)-(−) enantiomer (Attygalle et al., 1983). Bioassays of the pheromonal activity of the separate enantiomers of 3-octanol for three species of these ants indicated that they respond only to the (R)-(−) enantiomer; the (S)-(+) enantiomer is

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inactive (Cammaerts et al., 1985). The separate enantiomers of 2-octanol, which are commercially available, gave little or no response with these *Myrmica* ants. In contrast, two species of *Crematogaster* ants produce exclusively (*S*)-(+)-3-octanol in their mandibular glands (Brand, 1985), but no bioassays have been conducted with the separate enantiomers on these species.

The results of Cammaerts et al. (1985) illustrate the need for behavioral studies on the separate enantiomers of such simple alcohols as 3-octanol in insect species that produce them. However, the separate enantiomers generally are not available to the biologist wishing to bioassay them, and a chemist might not be persuaded easily to synthesize the enantiomers of such mundane alcohols. For their bioassays, Cammaerts et al. (1985) isolated (*S*)-(+)-3-octanol by preparative gas chromatography from oil of Japanese peppermint (*Mentha japonica*), and (*R*)-(−)-3-octanol from heads of worker ants of *Myrmica ruginodis*. However, in many cases, the preparation of microliter quantities of a compound from the insects themselves may not be feasible.

The two alcohols, 2-heptanol and 3-octanol, occur in various insect secretions (Blum, 1981). While the separate enantiomers of 2-heptanol are available from Norse Laboratories, Newbury Park, California 91320, the separate enantiomers of 3-octanol are not available commercially. The enantiomers of each of these simple alcohols could be produced by the action of an appropriate dehydrogenase on the corresponding ketone, a reaction obviously taking place in vivo. Reduction of ketones by microbial reductases, using whole cells, often with high yields of only one enantiomer, is quite common, and this procedure can be used to obtain appreciable amounts of product (MacLeod et al., 1964; Sariaslani and Rosazza, 1984, and references therein; Wong and Drueckhammer, 1985, and references therein).

We have synthesized each enantiomer of 2-heptanol and 3-octanol from the corresponding ketone in 80–90% purity with microbial reductases. The chirality of the alcohol obtained was determined by gas chromatography of the (*R*)-(+)-*trans*-chrysanthemoyl esters as described previously (Attygalle et al., 1983; Brand, 1985). *Curvularia falcata* gives approximately 90% (*S*)-(+)-2-heptanol and (*S*)-(+)-3-octanol, and two *Mucor* species produce 70–80% (*R*)-(−)-2-heptanol and (*R*)-(−)-3-octanol. These studies have been done using whole cells in their culture medium and the procedure can be scaled up to produce sufficient quantities of material for behavioral work.

METHODS AND MATERIALS

All fungal strains were obtained from the personal collection of J.P.N. Rosazza, University of Iowa. The successful strains used were *Curvularia falcata* QM-72 D, *Mucor recurvatus* UI-36, and *M. mucedo* 20094 P. The organisms were grown and maintained on soybean meal–glucose medium consisting of (grams per liter distilled water): glucose 20 g, yeast extract 5 g, soybean

meal 5 g, NaCl 5 g, and K_2HPO_4 5 g. The pH of the medium was adjusted to 7.0 before autoclaving.

Cultures were inoculated from an actively growing culture (5% inoculum), and grown with vigorous shaking in 100 ml medium in 250 ml Erlenmeyer flasks or 1 liter medium in Fernbach flasks. After 1 to 3 days of growth, the cultures were placed in an anaerobic chamber (Coy Mfg. Co.; gas phase: 85% N_2, 10% H_2, 5% CO_2) and allowed to become anaerobic for several hours before addition of either 2-heptanone or 3-octanone (200 μl for 100 ml medium or 2 ml for 1 liter medium). Preliminary studies showed that yields of the alcohol were higher with cultures kept anaerobic rather than aerobic. The period of exposure to either ketone varied from 6 hr to 8 days.

After exposure to either ketone, all the cultures were extracted twice with ether and the ether dried over anhydrous Na_2SO_4. *C. falcata* cultures turn black with time, and it is only when the organism is black that it is extracted easily with ether. Extraction of the earlier yellowish-brown stage results in an emulsion that is extremely difficult to break. *Mucor* cultures did not give any extraction problems.

Gas chromatographic determination of the ketone–alcohol ratio was done at 70°C using a Supelcowax 10 fused silica capillary column (15 m). Derivatization of the alcohols was done on the ketone–alcohol mixture with (R)-(+)-*trans*-chrysanthemic acid as described previously (Brand, 1985). Separation of the 2-heptanyl esters was achieved at 115°C and the 3-octanyl esters at 130°C on the Supelcowax 10 column. GC-MS of the standard 2-heptanyl and 3-octanyl esters and of the same esters from microbially produced alcohols was carried out on a 5% phenyl methyl silicone fused silica capillary column (25 m) at 140°C, interfaced to a Finnigan 1015 quadrupole mass spectrometer.

RESULTS

Gas chromatography of the ether extracts from *C. falcata* and *Mucor* cultures indicated that the added ketone and its corresponding alcohol were the only major volatile compounds in the extract. The formation of the (R)-(+)-*trans*-chrysanthemates was performed on the ketone–alcohol mixture without any further purification. Confirmation of the structures of the (R)-(+)-*trans*-chrysanthemoyl esters was obtained by comparison of the retention times with standards on the Supelcowax 10 column, and by GC-MS on the 5% phenyl methyl silicone column interfaced to a Finnigan 1015 quadrupole mass spectrometer. Equivalent mass spectra were obtained from the esters of standard 2-heptanol and 3-octanol and the microbially produced alcohols. The characteristic base peak at *m/e* 123 was obtained in all cases with a molecular ion at *m/e* 266 for the 2-heptanyl ester and at *m/e* 280 for the 3-octanyl ester.

2-Heptanone. *C. falcata* gave 2-heptanol that was 87–90% the (S)-(+) enantiomer, *M. recurvatus* gave about 76% the (R)-(−) enantiomer, and *M.*

mucedo gave 70–80% the (*R*)-(−) enantiomer. The recovery of the ketone plus alcohol as a percentage of the initial ketone added was as high as 70%.

3-Octanone. *C. falcata* gave 3-octanol that was 86–90% the (*S*)-(+) enantiomer, *M. recurvatus* gave about 76% the (*R*)-(−) enantiomer, and *M. mucedo* gave 75–80% the (*R*)-(−) enantiomer. The recovery of the ketone plus alcohol as a percentage of the initial ketone added was as high as 90%. In one experiment with 3 liters of *C. falcata* treated with 3-octanone and kept anaerobic for eight days, the ratio of ketone to alcohol recovered was 20:80, with the 3-octanol being 90% the (*S*)-(+) enantiomer. In another experiment, 3 liters of *M. mucedo*, kept anaerobic for 6 days, gave 10:90 ketone to alcohol but the 3-octanol was only 61% the (*R*)-(−) enantiomer. In each of these latter two experiments 0.3–0.4 ml of the alcohol was obtained.

DISCUSSION

Cammaerts et al. (1985) conducted satisfactory bioassays on *Myrmica* ants using 3-octanol that is approximately 90% the (*R*)-(−) enantiomer. This illustrates that certain behavioral experiments are possible with an alcohol that is highly enriched in only one enantiomer and that absolute purity of the enantiomer is not necessary for some studies. The (*S*)-(+)-2-heptanol and the (*S*)-(+)-3-octanol of about 90% purity produced by *C. falcata* and the (*R*)-(−) enantiomer of 75–80% purity produced by *M. mucedo* might be adequate for similar behavioral studies on some insects that produce these alcohols. However, the lack of activity of one enantiomer, as is the case with *Myrmica* (Cammaerts et al., 1985), may be an important criterion in the interpretation of bioassay data on alcohols of the above purity. An extensive literature documents the inhibitory effects of small percentages of unnatural enantiomers on the response of certain insects, and a cautionary note is offered on this point when bioassaying enantiomers that are only highly enriched and are not absolutely pure.

Neither the *C. falcata* nor the *Mucor* species reduced all the ketone to the alcohol. The best conversions obtained were a 20:80 ratio of 3-octanone to 3-octanol with *C. falcata* after anaerobic exposure to the ketone for eight days, and a 10:90 ratio with *M. mucedo* after six days. Sih and Chen (1984) have pointed out that a major complication in using intact cells for the reduction of ketones is that the process may be only partially enantioselective. They conclude that this usually arises from the combined action of competing enzymes of opposite chirality in intact cells. A ratio of 9:1 of the *S*:*R* enantiomers was obtained with *C. falcata* whether the exposure time was 6 hr or eight days, and in contrast, the *M. mucedo* usually gave about 80% the (*R*)-(−) enantiomer with an 18-hr exposure, but only 61% after six days. We have not attempted to optimize conditions for these organisms and do not know whether competing enzymes are present. Certain additional microorganisms tested, e.g., *C. pallescens* ATCC 12018 and bakers' yeast, did not produce any alcohol under the

usual conditions employed. However, it seems certain that suitable organisms and conditions could be found that would give better than 90% of either enantiomer in good yield.

As only one enantiomer of the alcohol was a major product, we wondered whether these organisms would selectively oxidize the same enantiomer if given the racemic alcohol under aerobic conditions. Our attempts to do this failed completely, and the reduction of the ketone seems irreversible with these organisms. This irreversibility of reduction has been mentioned by MacLeod et al. (1964) and by Sih and Chen (1984) and is understandable under anaerobic fermentative conditions. We have no explanation as to why our organisms failed to reoxidize any alcohol under aerobic conditions. In previous experiments we have found a fungus that would oxidize racemic *trans*-verbenol to verbenone quantitatively under aerobic conditions (Brand et al., 1976). A different approach employing the enantioselective hydrolysis of certain racemic acyclic alcohol acetates by microbial esterases, to yield chiral alcohols such as (S)-$(+)$-3-octanol, has been described by Oritani and Yamashita (1980).

As the reduction of the ketone is incomplete, solvent extracts of the culture medium contain variable amounts of the added ketone. Separation of the alcohol from the ketone could be achieved by adsorption column chromatography and preparative gas chromatography.

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A Coherent Approach to the Svedberg Equation

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Introduction
Sedimentation velocity has largely been superseded by simpler and cheaper methods for determining molecular weights of macromolecules. However, as a section on the Svedberg equation is considered important enough to be included in most modern textbooks of physical biochemistry,[1,2] students are bound to encounter the equation at some stage. The simple mechanical derivation of the Svedberg equation, which is the one likely to appeal to most students, has been in existence for years. Therefore it is surprising that the logic used in this derivation in many textbooks, including that of Svedberg and Pedersen,[3] is incorrect as it is inconsistent with the definition of force in physics. We propose a logical and physically consistent derivation, based mainly on published experimental findings, and arrive at a similar equation containing an extra term. It turns out that this extra term is numerically very small and can be ignored, but we feel the logic of the presentation should not be ignored, particularly when teaching undergraduates.

Textbook approach
In the usual textbook approach a particle, of mass m, is said to sediment under the action of a centrifugal force of magnitude $m\omega^2 r$. Opposing sedimentation are the forces of buoyancy and viscous drag with magnitudes $m\omega^2 r \bar{v} \rho$ and $f(dr/dt)$ respectively. These various forces are claimed (we believe incorrectly) to cancel each other giving

$$m\omega^2 r(1 - \bar{v}\rho) - f(dr/dt) = 0 \qquad (1)$$

which upon manipulation yields the Svedberg equation

$$M = \frac{RTs}{D(1 - \bar{v}\rho)} \qquad (2)$$

In support of Eqn (1) some authors[4–6] go so far as to say that, following an initial period of acceleration, sedimentation proceeds at constant velocity. However, rearrangement of Eqn (1) clearly reveals that the sedimentation velocity, dr/dt, is not constant but, like the centrifugal field, increases with r. Furthermore, experiment shows that it is log r and not r itself, that increases linearly with time during centrifugation.[5] This result proves the particle accelerates and suggests that Eqn (1) cannot represent the (radial) forces involved. Provision should be made for a net force responsible for the observed acceleration of a particle during centrifugation and for the acceleration actually implied by Eqn (1). Consequently Eqn (1) should be rewritten

$$m\omega^2 r(1 - \bar{v}\rho) - f(dr/dt) = m(d^2r/dt^2) \qquad (3)$$

where the right hand side is the net force (tacitly assumed to be zero[1–6]).

Additional term
The question now is: do equations (1) and (3) lead to similar molecular weights? The answer is to be found by solving Eqn (3) for m and comparing the result with Eqn (2). Experimental data[5] show that log r is linearly related to t, consequently we shall assume that a solution of Eqn (3) is

$$r = c_1 e^{c_2 t} \qquad (4)$$

where c_1 and c_2 are constants. It follows that

$$d\log r/dt = 1/r \cdot dr/dt = \text{a constant} \qquad (5)$$

and, therefore, at constant angular velocity,

$$1/\omega^2 r \cdot dr/dt = \text{a constant} = s \qquad (6)$$

where s is the sedimentation coefficient. Differentiating, we obtain

$$d^2r/dt^2 = s^2\omega^4 r \qquad (7)$$

which, together with Eqn (3), leads to

$$M = \frac{RTs}{D(1 - \bar{v}\rho - s^2\omega^2)} \qquad (8)$$

Equations (2) and (8) will give the same result provided $(1 - \bar{v}\rho) \gg s^2\omega^2$.

In the following comparison attention is confined to proteins but a similar line of reasoning can be adopted with other chemical species. For most proteins $s \leq 50$ S and $\bar{v} \leq 0.75$ cm^3 g^{-1}. Assuming ρ and ω to have magnitudes of 1.0 g cm^{-3} and 7900 radians s^{-1} (75,000 rpm) respectively, the following numerical estimates are obtained: $(1 - \bar{v}\rho) = 0.25$, and $s^2\omega^2 = 1.6 \times 10^{-15}$. The rather extreme values chosen above deliberately minimise the difference between the two terms. For all practical purposes the term $s^2\omega^2$ can be ignored and its exclusion from the Svedberg equation does not affect the outcome of molecular weight determinations. However, our derivation of the Svedberg equation has the advantage of a logical coherency, that should enhance its presentation to undergraduates.

Conclusion
In conclusion it is worth pointing out that, according to Eqn (7), the acceleration depends on r and, theoretically, could be made as large as one pleases simply by increasing the size of the centrifuge! This makes no difference to the molecular weight calculation because both dr/dt and d^2r/dt^2 are proportional to r and, consequently, r actually

cancels in Eqn (3). Therefore as far as the calculation of molecular weights is concerned, it is not necessary to assume that the net force on a sedimenting particle in a centrifuge is zero or even close to zero.

Acknowledgement

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Filmstrip: Monoclonal Antibodies

This is a set of 20 frames accompanied by a booklet containing a few lines describing each slide. Seven of the frames are colour photographs of various laboratory operations, understandable to anyone familiar with the techniques, but possibly not so clear to the uninitiated. There are a few schematic diagrams to illustrate the steps in producing antibodies as well as their possible applications (including curing cancer!). The rest are micrographs. The style of the text is too superficial for a university course. For schools or technical colleges it would have been beneficial to supplement the laboratory photographs with diagrams showing the essential features of the equipment being used. Some actual, rather than hypothetical, illustrations of the uses of monoclonal antibodies could also have been offered. Overall, I felt that a teacher with £15 to spend on a teaching aid might find this something of a disappointment.

John Kenny

Enzyme Nomenclature — Textbook Errors

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The books by Stryer[1] and Martin *et al*[2] contain several mistakes in the names of enzymes. The commonest mistake is in the use of 'synthetase' and 'synthase'. According to Rules 23 and 29 framed by the Commission on Biochemical Nomenclature,[3] 'synthetase' is not to be used for enzymes other than the ligases (Main Class 6). Section A of Table 1 shows these errors, the pages where they appear in the book and the correct names with EC numbers. Another very common mistake is the direct attachment of '-ase' to the name of substrate for enzymes other than the hydrolases (Main Class 3). In column 2, section B, Table 1 are listed the enzymes which are not hydrolases and accordingly, should not be named as substrate–plus–'ase' (Rule 10). The correct names are

given in column 4 of the same section. Hydroxylating enzymes were earlier placed in sub-group 1.99.1. After a better understanding of their mechanism, these are now called oxygenases and are placed in sub-classes 1.13 and 1.14. Section C of Table 1 lists such enzymes with the correct recommended names. Section D is a list of enzymes with miscellaneous errors in their names along with the correct names. In this section, several of the names used in the book are really not the names of individual enzymes; these indicate the kind of reaction catalysed.

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Table 1 Enzyme nomenclature mistakes with corrections

Name of enzyme	Page	Correct Name	EC Number
Section A			
Thymidylate synthetase	527[1]	Thymidylate synthase	2.1.1.45
	353[2]		
Triacylglycerol synthetase complex	458[1]	(i) Glycerophosphate acyltransferase	2.3.1.15
		(ii) Diacylglycerol acyltransferase	2.3.1.20
δ-aminolaevulinate synthetase	506[1]	δ-aminolaevulinate synthase	2.3.1.37
Glycogen synthetase	365[1]	Glycogen (starch) synthase	2.4.1.11
Lactose synthetase	377[1]	Lactose synthase	2.4.1.22
Geranyl-pyrophosphate synthetase	241[2]	Dimethylallyltransferase	2.5.1.1
Squalene synthetase	241[2]	Farnesyltransferase	2.5.1.21
Phosphoribosyl-1-pyrophosphate synthetase	518[1]	Ribosephosphate pyrophosphokinase	2.7.6.1
	356[2]		
ATP synthetase	135[2]	Adenosinetriphosphatase	3.6.1.3
Hydroxymethyl glutaryl-CoA synthetase	393[1]	Hydroxymethyl glutaryl-CoA synthase	4.1.3.5
Citrate synthetase	285[1]	Citrate(si)-synthase	4.1.3.7

Peroxidases and other enzymes in maize guttation fluid

The presence of peroxidase (EC 1.11.1.7) and catalase (EC 1.11.1.6) in maize guttation fluid was reported several years ago[1] but, to our knowledge, no subsequent publications dealing with either these or other guttation fluid enzymes have appeared. In this communication, we present a rationalisation for the occurrence of the peroxidase in maize guttation fluid and list several other enzymes found in this exudate.

Materials and methods

Growth of plants and collection of guttation fluid. Zea mays (strain PNR 6428) was grown from seed in vermiculite in a Fisons Fi-totron 600 H growth cabinet. The temperature during the day was 30°C with a relative humidity of 70%. The day-length was 14 h, light being provided by both neon and tungsten sources with a combined irradiance of about 95 μE m^{-2} s^{-1} at the vermiculite surface. During the last third of the light period, plants were watered liberally with a 1 g l^{-1} solution of Chemicult (Fedmis), a hydroponic nutrient powder. The cabinet was switched off during the 10-h period of darkness to halt air circulation and consequent evaporation of the guttation droplets. While the cabinet was switched off, temperature and relative humidity were not controlled. At the end of the dark period guttation fluid was collected by micropipette from the leaves and whorl and stored frozen. Collection was halted after the seedlings had reached a height of about 40 cm (from the base of the stem to the tip of the tallest leaf).

Enzyme assays. All assays were performed at 30°C. Peroxidase activity was measured by the time taken for the absorption at 470 nm, A_{470}, to increase from zero to 0,05 with guaiacol as oxidisable substrate.[2] The 1,5 ml reaction mixture contained 145 μmol sodium phosphate, 10,2 μmol guaiacol and 0,07 μmol H_2O_2 at a pH of 6,5.

Malate dehydrogenase activity was measured by the decrease in A_{340} in a 2,5 ml reaction mixture containing 115 μmol sodium phosphate, 0,35 μmol NADH and 1,7 μmol oxaloacetate at a pH of 7,4.

The formation of a precipitate from coniferyl alcohol was measured by the increase in A_{620}[3] in a 1,3 ml reaction mixture containing 60 μmol sodium phosphate, 1,1 μmol coniferyl alcohol (originally in 20 μl methanol) and 0,7 μmol H_2O_2 at a pH of 6,5.

Molecular exclusion chromatography. Guttation fluid (3 ml) was chromatographed at about 20°C on a column (1,6 × 91 cm) of Bio-Gel P-100, equilibrated in 0,05 M sodium phosphate buffer, pH 6,0. Fractions of 3,4 ml were collected at a flow rate of 8 ml h^{-1}.

Electrofocusing. Analytical electrofocusing was performed in 375-μm-thick 5% polyacrylamide slabs (Isolab) over the pH range 3 to 10 at 6°C. Before application to the gel, the guttation fluid was concentrated 10-fold by freeze-drying. Peroxidase activity was detected *in situ*[4] by soaking the slab in a solution containing 5 mM H_2O_2, 5 mM guaiacol, and 200 mM potassium phosphate at a pH of 6,4. The pI values of the peroxidases were estimated from the positions of pI markers (FMC Corp.) focused on the same gel as the peroxidases, but visualised with Coomassie Blue.

Results and discussion

Maize guttation fluid contains peroxidase activity (9,1 nkat ml^{-1}) and malate dehydrogenase (EC 1.1.1.37) activity (0,18 nkat ml^{-1}) and is also able to catalyse the formation of a precipitate in the presence of coniferyl alcohol and H_2O_2 ($\triangle A_{620}$ = 0,046 min^{-1} ml^{-1}). No catalase activity (at pH 7) was detected even in freshly collected guttation fluid. API ZYM tests indicated the additional presence of acid phosphatase

(EC 3.1.3.2), phosphoamidase (EC 3.9.1.1) and β-galactosidase (EC 3.2.1.23).

The peroxidase activity was resolved by electrofocusing into four major components, which appeared on the gel as two pairs of isozymes. One pair had pI values around 4,7 and the other around 5,2. In addition, some peroxidase activity focused at about pH 8.5

Chromatography of guttation fluid on Bio-Gel P-100 gave two major peaks of peroxidase activity corresponding to molecular masses of 38 000 and 24 000. The pH optimum of the peroxidase activity was about 6,5.

For reasons made clear below, it seems likely that the peroxidase in guttation fluid is, or was, involved in the lignification of xylem. In this context, two additional facts may be significant. First, maize xylem fluid contains relatively large amounts of malate[5] and, second, maize guttation fluid is able to catalyse a cyanide-sensitive oxidation of NADH in the absence of any other added substrate (unpublished results). If it is assumed that the endogenous NADH oxidation is a manifestation of peroxidase-catalysed H_2O_2 production, then maize guttation fluid contains all the enzyme activities required for the terminal stages of lignin synthesis. Thus malate dehydrogenase is able to catalyse the formation of NADH from malate and NAD$^+$. The peroxidases, in turn, catalyse the oxidation of this NADH, producing H_2O_2 and subsequently use the H_2O_2 as a co-substrate for the polymerization of various phenolic precursors to lignin.[3,6-8] The anionic nature of the major peroxidase components in guttation fluid strongly suggests that these enzymes were once bound to the cell wall.[9,10] It is known also that both the malate dehydrogenase and the peroxidases involved in lignin synthesis are normally bound to the cell wall.[3,6-8] We propose that these enzymes become detached from the wall during differentiation of the vessels and are consequently found free in the guttation fluid. The origins and functions of the enzymes revealed by the API ZYM test remain obscure.

Guttation fluid is probably less multifarious than most plant homogenates. It may therefore prove to be a convenient starting material for the isolation and further study of selected plant enzymes, particularly those involved in the final stages of lignin synthesis.

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388

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Volatile constituents of a Southern African ant, *Polyrhachis schistacea* (Gerstaecker)

The formicine ant genus, *Polyrhachis*, occurs widely in Africa, the Middle East, Asia, and Australia.[1-4] *P. schistacea* (Gerstaecker), a ground-dwelling weaver ant found on the coast of Natal, Transkei and the eastern Cape, forms the subject of the present investigation. Like many other formicine species, it is aroused readily and vigorously defends its nest entrance when disturbed. Worker ants, if prodded with a finger, will bite and squirt formic acid from the poison gland, together with various hydrocarbons from the Dufour's gland. More than 74 alkanes and alkenes have been identified in defensive secretions of ant species, and the Dufour's gland of formicines is the principal source of these compounds. *n*-Undecane, *n*-tridecane and *n*-pentadecane are quantitatively the most important.[5] We report here on the chemical composition of the volatile substances in the head (mandibular gland) and in the gaster (Dufour's gland) of workers of *P. schistacea*.

Worker ants were collected near Margate (30°51′S, 30°22′E) on the southern coast of Natal, South Africa, and hexane extracts of heads and of gasters were made. Representative specimens were identified by Dr A.J. Prins of the South African Museum, Cape Town. Gas chromatograms were obtained on an HP Ultra-1 (50 m) and a DB-225 (30 m) capillary column, with temperature rise programmed from 50°C to 250°C at 5°C per min. These chromatograms indicated the presence of volatile compounds in both extracts. The head extract had a major (91%) and a minor (9%) peak, whereas the gaster extract gave a complex chromatogram with more than 20 identifiable peaks. Mass spectra of eluting peaks were obtained with a Hewlett Packard 5890 GC and 5970 MSD.

Head extract. The two peaks in the chromatograms of this extract were identified from their mass spectra as 4-heptanone and the corresponding alcohol, 4-heptanol. 4-Heptanone, 6-methyl-5-heptene-2-one, and 6-methyl-5-heptene-2-ol have been found in the mandibular gland of *P. simplex* from Israel.[1] One Australian *Polyrhachis* species has no volatile low-molecular weight compounds in the head extract,[2] whereas another, *P. ?doddi*, has large mandibular glands with numerous volatile compounds present.[3] 4-Heptanol has not been reported before from

Table 1. Volatile compounds present in the Dufour's gland of workers of *Polyrhachis schistacea* (Ultra-1 column).

Peak no.	Retention time	E.C.L.*	Area %	Compound name	Identification method
1	12.81	11.00	6.0	*n*-undecane	GC, MS
2	14.84	11.71	0.9	3-methyl undecane	MS
3	15.54		0.15	dodecene	MS
4	15.67	12.00	6.3	*n*-dodecane	GC, MS
5	16.83		0.8	decenal?	MS
6	17.51	12.61	0.1	2-methyl dodecane	MS
7	17.71	12.68	0.2	3-methyl dodecane	MS
8	18.07		0.3	tridecene	MS
9	18.27		3.2	tridecene	MS
10	18.69	13.00	52.6	*n*-tridecane	GC, MS
11	19.74		0.4	undecenal?	MS
12	20.04	13.52	0.75	5-methyl tridecane	MS
13	20.53	13.71	2.9	3-methyl tridecane	MS
14	20.80		0.1	tetradecene	MS
15	20.90		0.25	tetradecene	MS
16	20.99		0.3	tetradecene	MS
17	21.29	14.00	2.9	*n*-tetradecane	GC, MS
18	23.48		1.7	pentadecene	MS
19	23.98	15.00	10.9	*n*-pentadecane	GC, MS
20	25.77	15.72	0.1	3-methyl pentadecane	MS
21	25.96		0.5	2-ketone *m/z* 58	MS
22	26.48	16.00	0.6	*n*-hexadecane	GC, MS
23	26.87		0.6	unknown	
24	27.92		0.2	unknown	
25	28.13		0.25	heptadecadiene?	
26	28.34		0.7	heptadecene	MS
27	28.44		0.15	2-ketone *m/e* 58 base	MS
28	28.89	17.00	4.4	*n*-heptadecane	GC, MS
29	33.76		1.6	primary alcohol?	MS

*Equivalent Chain Length

this genus, and the absolute configuration of this alcohol has not been determined. Both 4-heptanone and 4-heptanol occur in the mandibular glands of the myrmicine ant *Zacryptocerus varians*.[6]

Gaster extract. The composition of the gaster extract of *P. schistacea* comprises mainly *n*-alkanes, alkenes and methyl-branched alkanes from C-11 to C-17 (Table 1). Chromatograms obtained from excised Dufour's glands of *P. schistacea* establish that these compound all occur in this gland. The gaster extract is very similar in composition to that of the Australian *Polyrhachis* species reported previously[2,3] but differs from *P. lamellidens*[7] in that the latter contains no methyl-branched alkanes, and *n*-undecane is the major component, whereas in *P. schistacea n*-tridecane is the main component.

The structure of the normal alkanes was confirmed by comparison of the retention times with those of standard *n*-alkanes and by comparison of their mass spectra with those of the authentic standards. On a non-polar Ultra-1 column, alkenes elute before the corresponding alkane. Only standard 1-alkenes were available for comparative purposes, and it is not possible to identify the double bond position unequivocally in various alkenes from their mass spectra alone. Therefore, while 1-alkene standards were used to confirm that various compounds are indeed alkenes, no double bond positions can be assigned. The 3- and 5-methyl branched alkanes were identified from a comparison of their mass spectra with published spectra.[8,9] 2-Methyl dodecane has been identified on the basis of a comparison of the spectra of 2- and 4-methyl dodecane.[8] For the 2-methyl branched compound the ratio of *m/z* 57 to *m/z* 43 is approximately 1:1, whereas for the 4-methyl branched compound the ratio of *m/z* 57 to *m/z* 71 is approximately 1:1.[8] In addition, the Equivalent Chain Length presented

in Table 1 compares favourably with results given by Howard et al.[10]

Mass spectra of minor components in some extracts of *P. schistacea* have indicated the likely presence of trace amounts of α-farnesene, 2-ketones and a long-chain primary alcohol. Not all extracts contain these trace components and they have not been unequivocally identified. The presence of α-farnesene may be related to age, as it was detected only in worker ants that had been kept in the laboratory for more than six months.

As the Dufour's gland contents are released during attack, it is concluded that this grandular secretion is used for defence as in the case of many other formicine ant species. No role has been determined for the mandibular gland secretion.

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A first report of *Prochloron* from South Africa

The purpose of this communication is to report the discovery of *Prochloron* in South African coastal waters. *Prochloron* is an algal symbiont in some colonial ascidians and its presence in local waters extends the known distribution of the alga into the south-western Indian Ocean.

Prochloron is a prokaryotic, unicellular green alga which is the sole representative of the Division Prochlorophyta.[1,2] It is placed in the Kingdom Monera along with the blue-green algae (Division Cyanophyta) and bacteria because of its prokaryotic structure. It is unique among the algae, however, because it is the only prokaryote known to contain chlorophylls *a* and *b*. These two pigments are usually restricted to the eukaryotic green algae (Division Chlorophyta, Kingdom Protista) and all divisions of higher plants (Kingdom Plantae).

The discovery of *Prochloron* just over a decade ago has stimulated a lot of interest among evolutionary biologists, who have raised many questions regarding the origin of the alga. Did *Prochloron* evolve independently of the Cyanophyta or are these prokaryotes on the same phylogenetic line? If they are monophyletic, which evolved first? Did *Prochloron* evolve from cyanophytes with the loss of phycobiloproteins and acquisition of chlorophyll *b*, or did the cyanophytes evolve from a *Prochloron*-like ancestor?

this would have been accompanied by the appearance of phycobiloproteins. These questions are still unanswered.

The biochemical similarities between photosynthetic prokaryotes and green chloroplasts have led scientists to speculate that chloroplasts have their origin in a symbiotic prokaryote. The symbiogenesis theory proposes that an early photosynthetic prokaryote was incorporated into a primitive heterotroph to form the first eukaryotic plant cell.[3-7] Whether the endosymbiont was a cyanophyte or a *Prochloron*-like cell will be debated for some time to come. The problem will probably be resolved at the molecular level by complete analysis of the nucleic acids of green chloroplasts, cyanophytes and *Prochloron*.

Prochloron is widely distributed in warm tropical and subtropical waters and has been found in the Caribbean, the eastern and western Pacific Ocean, Hawaii, the north-eastern Indian Ocean and the Red Sea. It occurs as a symbiont in a number of genera of colonial didemnid ascidians, namely, *Didemnum*, *Diplosoma*, *Echinoclinum*, *Lissoclonium*, and *Trididemnum* (Protochordata, Ascidiacea).[8-10] The alga has also been found on the surface of the sea cucumber *Synaptula* (Echinodermata, Holothuria).[11] The strength of the symbiotic relationship between *Prochloron* and its didemnid ascidian hosts is demonstrated by the fact that the alga cannot be grown away from its host for any appreciable length of time. Limited success has been obtained by providing isolated cells with tryptophan.[12]

The writer first collected *Prochloron* from a small colony of *Diplosoma* at Rocky Bay (just south of Scottburgh on the Natal south coast) on 1985-08-21. The ascidian host has been identified as *Diplosoma virens* (by Dr F. Monniot, pers. comm.*). The ascidian was approximately 4 cm in diameter and was conspicuous on the shore as a distinctly grey-green patch. The colony was growing in the lower intertidal region at a position coinciding with the uppermost limit of distribution of another ascidian, *Pyura stolonifera* (red bait).

On a subsequent visit to Rocky Bay (1989-01-09), extensive subtidal colonies of *Diplosoma*, containing *Prochloron*, were discovered while skin-diving. The colonies were up to 0.5 m² in area and were common on rocks at a depth of 1 – 2 m below the spring low tide level.

In the laboratory, cells of *Prochloron* (expressed and *in hospite*) were fixed for light microscopy, and scanning and transmission electron microscopy. Under the light microscope very little detail was seen except that the cells had a distinct cell wall, a clear central area, and a pigmented peripheral cytoplasm (Fig. 1). Scanning electron micrographs of *Diplosoma* colonies revealed that *Prochloron* was abundant in the cloacal chambers surrounding individual zooids. The cloacal chambers formed an anastomosing network leading to a common cloacal pore. The ultrastructure of the Rocky Bay *Prochloron* (Fig. 2) is typical of Group II *Prochloron* cells.[9,13] The central cytoplasm, referred to as the centroplasm, is usually devoid of cell inclusions and is surrounded by a peripheral band of cytoplasm containing thylakoids. The latter region is called the chromoplasm. The cell wall is multi-layered (Fig. 3).

Cell size measurements of 100 expressed *Prochloron* cells from several colonies showed that the mean diameter of the algal cells was 16.2 μm (range 12.3 – 25.0 μm). These measurements correspond closely with those reported for *Prochloron* from other ascidian hosts in other parts of the world.[9,14]

Approximately 7% of the cells (estimated from a sample of 600 cells) were in various stages of cell division, which occurs through binary fission with a cross wall developing by centripetal wall growth (see Fig. 1). The percentage of dividing cells also corresponds with that reported for *Prochloron* in *Diplosoma* collected in the Palau Archipelago (western Pacific). Lewin *et al.*[15]

Are Ant Alarm-Defense Secretions only for Alarm Defense?

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There is a remarkable chemical similarity between inhibitors of seed germination isolated from the plant, *Amaranthus palmeri,* and certain ant alarm-defense secretions. We suggest that these ant secretions could possess the additional property of inhibiting seed germination in the nests of granivorous species.

Bradow and Connick [1 – 4] established that various 2-ketones and alcohols in the range C_4 to C_{11} from *A. palmeri* significantly inhibit germination of onion, carrot, tomato, and *A. palmeri* itself. 2-Octanone, 2-heptanone, and 2-heptanol are particularly effective inhibitors. Coincidentally, many of the arthropod defensive compounds listed in [5] are ketones and related alcohols containing 7 or 8 carbon atoms and are particularly widespread in ants. For example, 2-heptanone and 2-heptanol are commonly found in either the mandibular gland of worker ants of *Atta* and *Crematogaster* or in the anal gland of worker ants of *Iridomyrmex, Conomyrma, Azteca,* and *Monacis.* 2-Octanone has been found as a minor constituent in the mandibular gland of *Pseudomyrmex* workers.

These correlations led us to conduct seed-germination experiments on tomato and on the grass, *Eragrostis curvula,* similar to those described in [3, 4]. Since it is myrmicine ants that are the most markedly granivorous [6] we selected, as potential inhibitors, the following compounds which occur among the myrmicine genera *Pogonomyrmex, Crematogaster, Myrmica,* and *Myrmicaria:* 4-methyl-3-heptanone, 3-octanone, d-2-octanol, l-2-octanol, and the monoterpenes (+)-α-pinene, (–)-α-pinene, and dl-limonene [5]. Varying degrees of inhibition were observed ranging from as little as 30 % to as much as 85 %.

It is significant that 4-methyl-3-heptanone, which caused an 80 % inhibition of the germination of *E. curvula,* is a mandibular gland component of *Pogonomyrmex* species. These ants depend largely on seeds for their main dietary staple and at certain seasons of the year store a large surplus of seeds in the nest [6].

The unrestricted growth of the roots and shoots of etiolated seedlings within the galleries and confines of a granivorous ant nest would cause a chaotic situation. Similar chaos would reign in the nests of nongranivorous ants if seeds entered the nest fortuitously. Clearly seed germination in the nests of many ants species is controlled. The above results lead us to propose that some ant alarm-defense secretions play a part in this control.

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Honeybees Can be Recruited by a Mechanical Model of a Dancing Bee

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"We may be able to tell our bees (to) fertilize those apple trees five minutes fly to the south-east. To do this we should presumably need a model bee to make the right movements, and perhaps the right noise and smell. It would probably not be a paying proposition, but there is no reason to regard it as an impossible one."

J. B. S. Haldane: The Future of Biology (1927)

Honeybee (*Apis mellifera*) workers can inform their nestmates of the distance, direction, and profitability of food sources by means of wagging dances performed on the vertical combs in the hive [1,2]. The direction of the wagging run (in which the dancer wags her body from side to side and emits sounds) indicates the direction of the food, such that upward on the comb signifies the azimuth of the sun in the field. Several parameters of the dance are correlated with the distance to the food source, but it is not known which of these parameters serves as the distance signal. It is also not known how dance followers detect the dancer's movements in the darkness of the hive, although evidence suggests that airborne sounds produced

Stereospecific Hydroxylation of Indan by *Escherichia coli* Containing the Cloned Toluene Dioxygenase Genes from *Pseudomonas putida* F1

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Escherichia coli JM109(pDTG601), containing the *todC1C2BA* genes encoding toluene dioxygenase from *Pseudomonas putida* F1, oxidizes indan to (−)-(1*R*)-indanol (83% *R*) and *trans*-1,3-indandiol. Under similar conditions, *P. putida* F39/D oxidizes indan to (−)-(1*R*)-indanol (96% *R*), 1-indanone, and *trans*-1,3-indandiol. The differences in the enantiomeric composition of the 1-indanols formed by the two organisms are due to the presence of a 1-indanol dehydrogenase in *P. putida* F39/D that preferentially oxidizes (+)-(1*S*)-indanol.

Pseudomonas putida F1 initiates the degradation of toluene by incorporating both atoms of oxygen into the aromatic nucleus to form (+)-*cis*-(1*R*,2*S*)-dihydroxy-3-methylcyclohexa-3,5-diene (*cis*-toluene dihydrodiol [5, 6]). The enzyme catalyzing this reaction, toluene dioxygenase, also catalyzes the benzylic oxidation of indan and indene to (−)-(1*R*)-indanol and (+)-(1*S*)-indenol, respectively (9). We have now reinvestigated the stereospecificity of indan oxidation by the toluene dioxygenase present in *P. putida* F39/D, a mutant strain of *P. putida* F1 that lacks the enzyme *cis*-toluene dihydrodiol dehydrogenase (5), and here we compare the results with those for *Escherichia coli* JM109(pDTG601), a recombinant organism that contains the structural genes (*todC1C2BA*) for toluene dioxygenase cloned in the vector pKK223-3 (10). The results show that differences in the enantiomeric composition of the (−)-(1*R*)-indanol formed by the two organisms are due to the presence of a dehydrogenase in *P. putida* F39/D which preferentially oxidizes (+)-(1*S*)-indanol to 1-indanone.

Cells of *E. coli* JM109(pDTG601) were grown with shaking (200 rpm) at 37°C on mineral salts basal medium (8) supplemented with 20 mM glucose, 1 mM thiamine, and ampicillin (100 µg/ml). When the culture reached a turbidity of 0.5 at 600 nm, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to give a final concentration of 1 mM. After 1 h, cells were harvested and suspended to a turbidity of 2.0 at 600 nm in 50 mM phosphate buffer, pH 7.2, containing 20 mM glucose. Cell suspensions, 50 ml in 250-ml Erlenmeyer flasks, were incubated at 30°C on a rotary shaker (200 rpm) in the presence of indan. After 6 h, the contents of each flask were extracted three times with equal volumes of ethyl acetate. The organic extracts were dried over anhydrous sodium sulfate and concentrated under vacuum at 30°C. The products formed were separated on a 25-m Hewlett Packard Ultra-1 nonpolar fused silica capillary column programmed from 80 to 150°C at 2°C/min and from 150 to 280°C at

20°C/min. The carrier gas was helium (25 cm s^{-1}), and detection was by a Hewlett Packard 5970 mass selective detector. All products were identified by their retention times and by showing that their mass spectra were identical to the spectra of authentic compounds. The major product formed from indan was 1-indanol (99%). A minor product (~1%) was identified as *trans*-1,3-indandiol. 1-Indanone was not detected. In control experiments, IPTG-treated cells of *E. coli* JM109(pKK223-3) did not oxidize indan. When the experiments described above were conducted with toluene-induced cells of *P. putida* F39/D, the products formed from indan were 1-indanol (88%), 1-indanone (11%), and *trans*-1,3-indandiol (~1%).

The enantiomeric composition of the 1-indanol formed from indan by *E. coli* JM109(pDTG601) and *P. putida* F39/D was determined by gas chromatography of its isopropyl urethane derivative (4, 7). Samples were dissolved in CH$_2$Cl$_2$, and the enantiomeric isopropyl urethanes were separated on a 50-m XE-60-(*S*)-valine-(*S*)-phenylethylamide fused silica column (7) at 170°C with helium as the carrier gas (25 cm s^{-1}). Under these conditions, the retention times for the (−)-(1*R*)-indanol and (+)-(1*S*)-indanol derivatives were 21.91 and 22.31 min, respectively. The results obtained showed that *E. coli* JM109(pDTG601) oxidized indan to (−)-(1*R*)-indanol (83% *R*) in contrast to the (−)-(1*R*)-indanol (96% *R*) formed by *P. putida* F39/D. The formation of almost enantiomerically pure 1-indanol by *P. putida* F39/D indicated that the 1-indanol dehydrogenase previously detected in this organism (9) preferentially oxidizes (+)-(1*S*)-indanol. This observation was confirmed by incubating toluene-induced cells of *P. putida* F39/D for 6 h with authentic (−)-(1*R*)-indanol (>97% *R*). Analysis of the reaction mixture revealed the presence of 1-indanol (85%), 1-indanone (11%), and *trans*-1,3-indandiol (4%). Gas chromatography of the isopropyl urethane derivative of the remaining (−)-(1*R*)-indanol showed that it consisted of more than 97% of the *R* enantiomer. In contrast, the same cell suspension oxidized (+)-(1*S*)-indanol completely to 1-indanone in 6 h. Analogous experiments with *E. coli* JM109(pDTG601) showed that *trans*-1,3-indandiol (10%) and a trace of 1-indanone (<1%) were the only products formed from (−)-(1*R*)-indanol, and approximately 90% of the added 1-indanol was recovered

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TABLE 1. Indanol dehydrogenase activity in cell
extracts of *P. putida* F39/D[a]

Substrate	pH	Sp act[b]
(+)-(1*S*)-Indanol	7.2	0.052
(−)-(1*R*)-Indanol	7.2	<0.001
(+)-(1*S*)-Indanol	8.0	0.069
(−)-(1*R*)-Indanol	8.0	0.002

[a] Enzyme activities were determined by monitoring the increase in A_{340} due
to the reduction of NAD. Substrates were present in saturating amounts, and
activity was linear with respect to protein concentration. Reactions were
initiated by the addition of either (+)-(1*S*)- or (−)-(1*R*)-indanol to the reaction
mixture.
[b] Micromoles of NADH formed per minute per milligram of protein.

from the reaction mixture. The recombinant *E. coli* did not
oxidize (+)-(1*S*)-indanol (>98% *S*).

Cell extracts, prepared from toluene-induced cells of *P.
putida* F39/D, were examined for their ability to oxidize
(−)-(1*R*)- and (+)-(1*S*)-indanol in the presence of NAD$^+$ and
NADP$^+$. The results obtained with NAD$^+$ as the electron
acceptor are shown in Table 1. NADP$^+$ was less effective,
giving rates three times lower than those obtained with
NAD$^+$. The rate of oxidation of (−)-(1*R*)-indanol was negli-
gible at pH 7.0, and at pH 8.0 it was 35 times lower than the
rate observed with (+)-(1*S*)-indanol.

The results of the present study show that the differences
in the enantiomeric composition of the 1-indanol formed
from indan by *E. coli* JM109(pDTG601) and *P. putida* F39/D
are due to the preferential oxidation of (+)-(1*S*)-indanol by a
dehydrogenase present in the latter organism. The partial
resolution of racemic 1-indanol into the *R* enantiomer by a
different strain of *P. putida* (strain UV4) has been reported
and is also attributed to the preferential oxidation of (+)-
(1*S*)-indanol (2). In a previous report, we showed that *P.
putida* F39/D oxidizes indan to (−)-(1*R*)-indanol (88 to 92%
R). The reaction times in these experiments were limited to
1 h in order to minimize the effect of an inducible indanol
dehydrogenase on the chirality of the 1-indanol formed from
indan (9). However, in light of the present studies, it now
appears that the enantiomeric composition of the 1-indanol
observed previously was due to either partial resolution by
the dehydrogenase and incomplete recovery of 1-indanone
from large-scale reaction mixtures or the loss of (+)-(1*S*)-
indanol during isolation, purification, and derivatization
procedures. The in situ formation of the enantiomeric iso-
propyl urethane derivatives of 1-indanol and their resolution
by gas chromatography on a chiral capillary column are
significantly more sensitive and accurate than chiroptical
methods and procedures involving the resolution of di-
astereomers (3). Consequently, the enantiomeric composi-
tion of the 1-indanol (83% *R*) formed by *E. coli* JM109
(pDTG601) reflects the true enantiospecificity of toluene
dioxygenase.

The reaction products formed from indan by toluene
dioxygenase are shown in Fig. 1. The formation of *trans*-1,3-
indandiol has not been observed previously, and its absolute
stereochemistry was not determined in the present study.
However, it seems probable that, starting with (−)-(1*R*)-
indanol, it would be the (1*R*,3*R*) enantiomer.

P. putida F39/D oxidizes indene to (+)-(1*S*)-indenol (63%
S) and (−)-*cis*-(1*S*,2*R*)-indandiol (65% *S*,*R*) (9). These prod-
ucts, which have similar enantiomeric compositions, are also
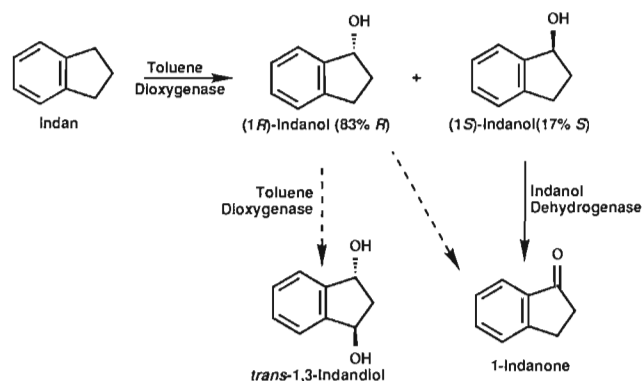formed from indene by the cloned toluene dioxygenase in *E.*

FIG. 1. Proposed reactions in the oxidation of indan to 1-indanol
by toluene dioxygenase present in *E. coli* JM109(pDTG601) and *P.
putida* F39/D. Also shown is the oxidation of (+)-(1*S*)-indanol to
1-indanone by a dehydrogenase present only in *P. putida* F39/D.
The dehydrogenase shows slight activity with (−)-(1*R*)-indanol as
indicated by the dashed line. The oxidation of (−)-(1*R*)-indanol to
trans-1,3-indandiol is a minor reaction catalyzed by toluene dioxy-
genase in both organisms. The absolute stereochemistry of the
indandiol was not determined.

coli (data not shown). Thus, it is of interest to note that
although *P. putida* UV4 oxidizes indene to (−)-*cis*-(1*S*,2*R*)-
indandiol (60% *S*,*R*), the 1-indenol formed by this organism
is the *R* enantiomer (99% *R*) (1). These observations suggest
that there may be subtle but significant differences in the
active sites of the toluene dioxygenases from *P. putida* F1
and *P. putida* UV4.

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GUTTATION FLUID PEROXIDASES FROM *HELIANTHUS ANNUUS*

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Abstract—The isoperoxidases (EC 1.11.1.7) in guttation fluid of *Helianthus annuus* appeared as two bands, G1 (slower moving) and G2 (slightly faster), following disc gel electrophoresis and again as two bands, SG1 (M_r 39 000) and SG2 (M_r 36 000) on SDS gels. These were electrophoretically indistinguishable from the corresponding isoperoxidases R1 and R2 (on non-SDS gels) and SR1 and SR2 (on SDS gels) found in bleed taken from just above the root (root bleed). In addition, what appeared to be SR1 and SR2 were also found in root apoplast washes. The total peroxidase activity of root bleed was very similar to that of bleed taken from higher up the hypocotyl, indicating that no additional peroxidase activity entered the sap above the root. These observations suggest that the root is the main, if not the sole, source of the peroxidases in guttation fluid. R1, but not G1, showed a slight but consistent increase in electrophoretic mobility on non-SDS gels following treatment with commercial acid phosphatase. Similarly the mobility on SDS gels of SR1, but not of SG1, increased after incubation with acid phosphatase. This indicates that R1 and G1 are not identical in all respects and that, after release from the root into the xylem sap, R1 becomes modified *in vivo* before appearing in the guttation fluid as G1.

INTRODUCTION

Peroxidases occur in the guttation fluid and xylem sap from several plants [1–3]. In a recent study [3], it was concluded that the roots were the source of one of the peroxidases found in cucumber guttation fluid. In the present work, we have examined the peroxidases in the guttation fluid and xylem sap of *Helianthus annuus*, and have found support for the idea that guttation fluid peroxidases originate in the roots.

RESULTS AND DISCUSSION

Disc gel electrophoresis revealed the presence of two closely spaced isoperoxidases, G1 (smaller mobility) and G2 (slightly greater mobility), in *Helianthus annuus* guttation fluid, both bands staining with equal intensity. Following non-denaturing SDS gel electrophoresis, two bands (SG1 and SG2) were again observed, at positions corresponding to M_r values of 39 000 and 36 000, respectively.

Attempts were made to isolate G1 and G2 from organ homogenates in order to establish which organ(s) was responsible for the production of these enzymes. However, no readily detected peroxidase bands corresponding to those from guttation fluid were obtained following disc gel electrophoresis of root, hypocotyl or cotyledon homogenates.

As peroxidases are adsorbed by disrupted plant tissue [4], it is possible that the guttation fluid enzymes were present in the homogenates but remained undetected.

Even if G1 and G2 had been found in organ homogenates, we would not have been able to decide whether the enzymes originally resided within the cells or in the xylem sap. Showing that an enzyme exists in an organ homogenate is, therefore, no better than showing the enzyme to be present in the xylem sap of that organ. Since xylem sap in the form of bleed is readily obtainable and, further, since bleed comes into contact with very little disrupted tissue, we considered it advantageous to switch our search for guttation fluid peroxidases from homogenates to bleeds.

Hypocotyls were cut transversely at various heights above the root and the bleed collected and subjected to analysis by disc gel electrophoresis. Isoperoxidases R1 and R2, corresponding to G1 and G2, were found in all bleeds regardless of the position of the cut. These were the sole peroxidase components in bleeds from the upper half of the hypocotyl. Bleeds taken from the lower half of the hypocotyl contained, in addition to these two peroxidases (R1 and R2), a fast moving streak of peroxidase activity and a fast moving band, R3. Both the streak and R3 were most prominent in bleeds taken from just above the root (root bleed).

Despite the presence of the streak and R3 on non-SDS gels, only two peroxidase bands, SR1 and SR2, were obtained following SDS electrophoresis of root bleed. SR1 and SR2 were electrophoretically indistinguishable from the corresponding enzymes in guttation fluid, SG1 and SG2. In addition, root apoplast washes contained a pair of peroxidase bands electrophoretically identical to SR1 and SR2, and therefore also identical to SG1 and

SG2. Thus it was established that root bleed, which is taken to reflect root xylem sap in composition, contained peroxidases electrophoretically identical to those in guttation fluid, suggesting that the roots are the source of guttation fluid peroxidases. To address the question of whether the roots are the sole source, the total peroxidase activity of root bleed was compared with that from bleed taken from higher up the hypocotyl. The two activities were very similar, suggesting that no additional peroxidases were entering the xylem sap from higher up the hypocotyl. We conclude that roots are the main, if not the sole, supplier of peroxidases to the xylem sap and, hence, to the guttation fluid.

An attempt was made to modify both root bleed (R) and guttation fluid (G) peroxidases by commercial enzymes, arguing that if R and G enzymes were subject to similar modification, this would constitute further evidence of their identity. Commercial trypsin and proteinase K had no apparent effect on the peroxidases of either root bleed or guttation fluid as judged by disc gel electrophoresis. In contrast a small, but consistently observed, increase in the mobility of R1 was observed following incubation of root bleed with commercial acid phosphatase, while G1 was unaffected. A similar effect was noted on SDS gels where the mobility of SR1, but not SG1, increased as a result of acid phosphatase treatment.

It would be premature to ascribe the modification of a root bleed peroxidase by commercial acid phosphatase to phosphatase action *per se*. We do not know how pure the commercial enzyme is and, besides, it is difficult to reconcile the observed increase in anodic character of the modified peroxidase with the loss of a negatively charged phosphoryl group. Most plant peroxidases are glycoproteins [5] and we consider it more likely that the glycan of R1 is the region affected, although we cannot comment further on this. Regardless of what causes the modification, G1 and R1 cannot be identical in all respects.

It appears that the roots are the source of guttation fluid peroxidases in *H. annuus*, but that at least one of the peroxidases, R1, after release by the root, is subtly modified on its journey up the sap to the surface of the cotyledon.

The fate of the streak of peroxidase and band R3, present in root bleed only, is puzzling. It seems unlikely that these isozymes become adsorbed in the lower part of the hypocotyl, since there is no significant change in total peroxidase activity in bleed taken from higher up. The possibility of their conversion to either R1 or R2, or both, cannot be ruled out. The increase in intensity of the R1 and R2 bands on the gel resulting from such a conversion might have passed unnoticed as it is difficult to judge intensity accurately by eye. It is noteworthy that no extra bands, ascribable to the streak or band R3 appeared on SDS gels following electrophoresis of root bleed.

EXPERIMENTAL

Plant material and growth conditions. Seeds of *Helianthus annuus* L. cv S0 210 (Agricol Seed Co., Brackenfell, South Africa) were grown in vermiculite in a Fisons Fitotron 600 H growth cabinet. The growth conditions were as described before [2] except for the light intensity. At the vermiculite surface, it was 70 μE sec^{-1} m^{-2} for plants homogenized to obtain peroxidase and 168 μE sec^{-1} m^{-2} for all other experiments.

Chemicals. Acid phosphatase was obtained from Boehringer Mannheim. Rainbow markers for M_r determination by SDS gel electrophoresis were purchased from Amersham.

Extraction of plant material. Fresh plant material, collected 12 to 14 days after planting, was washed in H_2O at 2°, briefly patted dry with absorbent paper, and homogenized in a pre-chilled pestle and mortar with one vol. of cold 50 mM Tris–HCl buffer, pH 8. Homogenates were centrifuged at 18 000 g for 20 min and the supernatant fraction retained for electrophoresis.

Guttation fluid and bleeds. Guttation droplets were collected from the cotyledons just before the commencement of the light period, using a pipette with a plastic tip. Bleeds were collected, in similar fashion, in the early afternoon, over a period of 30 min from hypocotyls cut transversely at various heights above the root surface.

Root apoplast washes. The method resembled that of ref. [6]. Roots were excised, washed as described above, covered with H_2O at 2° and vacuum infiltrated for 3 min. Infiltrated roots were patted dry and centrifuged at 1000 g for 5 min at room temp. in tubes fitted near the bottom with perforated teflon grids. The grid acted as a filter holding back the roots but allowing the apoplast wash through. The wash, thus freed of solids, was collected from beneath the grid.

Concentration, dialysis and storage. Concn was by freeze drying. Samples for dialysis were transferred to Spectra/Por 1 dialysis tubing and dialysed overnight against H_2O at 4°. Storage of samples was at −70°.

Assay of peroxidase. The assay mixture contained, in 1.5 ml, 50 to 100 μl of enzyme, 78 μmol of K-P$_i$ and 11.2 μmol of guaiacol. The pH was 6.5. After a short preincubation at 30°, 10 μl of 10 mM H_2O_2 was added to start the reaction. The enzyme prepn was diluted until the time taken for the A at 470 nm to reach 0.05 was between 15 and 30 sec.

Incubation with acid phosphatase. Bleed (concd 20-fold) and guttation fluid (concd 10-fold) were separately incubated for 16 hr at *ca* 25° in a total vol of 115 μl, 4.4 mM with respect to Na succinate buffer, pH 5, and containing 34 μg MgSO$_4$ and 44 μg of acid phosphatase (1.5 nkat). Toluene (2 μl) was added to retard microbial contamination.

Electrophoresis. Between 3 and 20 μl of sample was subjected to electrophoresis, in an anodic system, on discontinuous 7.5% (running gel) vertical slabs (10 × 8 × 0.075) cm at 4° and a constant current of 12 mA. Buffers and gel formulation were as for system 1 of ref. [7]. Peroxidases were visualized by soaking the slabs in 100 mM Na succinate buffer, pH 4, for 5 min and thereafter in a soln containing 5.3 mM H_2O_2, 5.5 mM guaiacol and 200 mM K-P$_i$, pH 6.5.

SDS gel electrophoresis was performed at 4° according to the method of ref. [8], expect that gelatin was omitted from the gel. M_r was estimated from the relative mobili-

ties of sample and Rainbow markers, run on the same gel. The distance migrated by a solute zone on the gel was, in all cases, taken as the distance from the origin to the centre of the zone. After washing the gels for 5 min in 2.5% Triton X-100, peroxidases were visualized in the same way as in non-SDS gel electrophoresis. Peroxidases retained their activity during this mild SDS procedure.

Plant extracts were dialysed and concd 10-fold before electrophoresis. Apoplast washes were concd 10-fold but were not dialysed before electrophoresis. Other samples were adjusted to ensure that the final concn of the guttation fluid or bleed was 10 to 20 times greater than the concns in the plant and were subjected to electrophoresis without prior dialysis.

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DUFOUR'S GLAND AND POISON GLAND CHEMISTRY
OF THE MYRMICINE ANT, *Messor capensis* (Mayr)

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Abstract—The Cape harvester ant, *Messor capensis* (Mayr), is widespread
in the more arid regions of southern Africa, where it forms trails many meters
long and harvests considerable quantities of seeds. The poison gland contains
primarily the alkaloid, anabaseine, with minor amounts of the related alkaloid,
anabasine, and an unidentified compound. The Dufour's gland contains pre-
dominantly alkanes and alkenes of carbon chain length 12–23. *n*-Pentadecane
is the major component, with lesser amounts of *n*-pentadecene, *n*-tridecane,
n-heptadecane, *n*-tetradecane, *n*-heneicosene, and *n*-tricosene. The dienes,
n-heneicosadiene and *n*-tricosadiene are rather unusual components of the
Dufour's gland of ants.

INTRODUCTION

Analyses of the Dufour's gland and poison gland volatiles of many myrmicine
ants have shown that the Dufour's gland usually contains a series of hydrocar-
bons, and other relatively nonpolar compounds, of a carbon chain length of
11–21 (Blum, 1981). In contrast, the poison gland produces a variety of inter-
esting behavioral substances, many of which are nitrogen-containing (Blum,
1981; Wheeler and Duffield, 1988).

For example, the 2,6-dialkylpiperidines (MacConnell et al., 1971; Brand
et al., 1972) and the 3,5-dialkylpyrrolidines and pyrrolines (Pedder et al., 1976;
Jones et al., 1979) of *Solenopsis* species, and the 2,5-dialkylpyrrolidines of
Megalomyrmex species (Jones et al., 1991) are generally considered to be used

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in defense. Trail following is elicited by 3-butyl-5-methylindolizidine in *Monomorium pharaonis* (Ritter et al., 1973), by methyl 4-methylpyrrole-2-carboxylate in *Atta texana* (Tumlinson et al., 1971), and various alkylpyrazines in *Tetramorium* (Attygalle and Morgan, 1983), *Pheidole pallidula* and possibly *Acromyrmex* species (Evershed and Morgan, 1983), and *Messor bouvieri* (Jackson et al., 1991).

The alkaloid anabaseine (3,4,5,6-tetrahydro-2,3'-bipyridine) occurs in the poison gland of two *Aphaenogaster* species (Wheeler et al., 1981), and the related alkaloid, anabasine, is the major volatile product in the poison gland of *Messor ebeninus* (Forel) (Coll et al., 1987); these substances may be part of the alarm–defense system of these ants. Anabasine is a well-known alkaloid produced by tobacco plants, and anabaseine has been isolated from certain marine hoplonemertine species (Kem, 1971; Kem et al., 1971).

The Cape harvester ant, *Messor capensis*, is widespread in the drier areas of southern Africa, and these soil dwelling ants follow long distinct trails, harvesting large quantities of seeds. They are fairly slow and deliberate in their movements and only show signs of excitement when severely provoked. Their main defensive behavior seems limited to biting and wiping the poison gland contents on an offending object such as forceps or stalk of grass.

Analysis of the volatiles from single Dufour's glands and poison glands, by solventless sample introduction, has established the presence of a variety of normal alkanes and alkenes from C_{12} to C_{23} in the former gland and anabaseine, anabasine, and a third compound, which is possibly a derivative of octadecenoic acid in the latter gland. While anabaseine is the major component of the poison gland, these two alkaloids have not been reported to occur together in either *Aphaenogaster* species (Wheeler et al., 1981) or *M. ebeninus* (Coll et al., 1987).

METHODS AND MATERIALS

Worker ants were collected from colonies on the University of Fort Hare farm (26°50'E, 32°47'S), from the Alice golf course, and from Adelaide, all in the eastern Cape region of southern Africa. Individual Dufour's glands and poison glands were removed and placed in the cup of a glass capillary as described by Burger et al. (1990) for solventless introduction in a gas chromatograph. The main modification to the method comprised the placement of an on/off toggle valve in the carrier gas line immediately before the injection port. This valve was closed for a period of 2–3 sec while the capillary tube containing the sample was introduced, after which a purge off time of 20 sec was set. GC-MS analyses were run on a Hewlett Packard 5890 GC and 5970 MSD. A HP Ultra 1 column (50 m) was temperature programmed from 50°C to 260°C at 5°C/min, and held at 260°C. Helium was used as carrier gas at 25 cm/sec. A portion of the contents

of the poison glands was reduced with $NaBH_4$ and $NaBD_4$ in ethanol, followed by the addition of water and extraction into *n*-hexane.

RESULTS

Poison Gland. Chromatograms of the poison gland indicated the presence of one major and two minor compounds. The first of the three peaks (approximately 5 area %) gave a mass spectrum with a molecular ion at *m/z* 162 (40%), a base peak at *m/z* 84 (100%), and characteristic fragment ions at *m/z* 105 (60%) and 133 (45%). Both the retention time and the mass spectrum were identical to those obtained for an authentic sample of anabasine (Sigma Chemical Co., St. Louis, Missouri).

The second and major peak (approximately 92 area %) gave a molecular ion and base peak at *m/z* 160 (100%), with significant fragment ions at *m/z* 159 (70%), 131 (44%), 104 (41%), and 145 (26%). Reduction of a poison gland extract with $NaBH_4$ led to an appreciable reduction in this second peak together with an increase in the anabasine peak. Reduction of a similar extract with $NaBD_4$ gave a mass spectrum for the anabasine containing one atom of deuterium, and in addition, the mass spectrum of this major peak was identical to the published spectrum of anabaseine (Kem, 1971) (Scheme 1).

The third peak (approximately 3 area %) gave a mass spectrum with a typical alkene fragmentation pattern in the low mass region. Significant ions at *m/z* 264 and 265 (40%), however, are suggestive of an octadecenoic acid derivative. A possible molecular ion at *m/z* 324 (20%) was apparent, but no structure has been assigned to this compound.

Dufour's Gland. Chromatograms of single Dufour's glands, introduced by solventless injection, illustrated the presence of numerous compounds. The identities of these compounds are presented in Table 1. The majority of peaks were readily identified as *n*-alkanes and *n*-alkenes. Standards of all the identified

Anabaseine   Anabasine
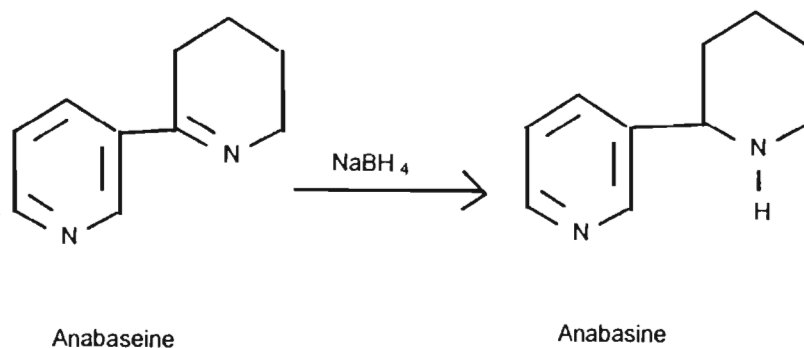
SCHEME 1.

TABLE 1. DUFOUR'S GLAND VOLATILES OF ANT, *M. capensis*[a]

Peak	Retention time (min)	Area (%)	Identity		Evidence
1	21.99	0.1	*n*-Dodecane	*n*-C12	GC, MS
2	24.68	0.1	*n*-Tridec-?-ene	*n*-C13:1	MS
3	25.06	6.4	*n*-Tridecane	*n*-C13	GC, MS
4	27.34	0.2	*n*-Tetradec-?-ene	*n*-C14:1	MS
5	27.56	0.5	*n*-Tetradec-?-ene	*n*-C14:1	MS
6	27.88	3.4	*n*-Tetradecane	*n*-C14	GC, MS
7	29.80	0.3?			
8	30.12	9.4	*n*-Pentadec-?-ene	*n*-C15:1	MS
9	30.39	12.7	*n*-Pentadec-?-ene	*n*-C15:1	MS
10	30.80	42.3	*n*-Pentadecane	*n*-C15	GC, MS
11	31.95	0.1	5-Methylpentadecane	5-Me-C15	MS
12	32.42	0.2	3-Methylpentadecane	3-Me-C15	MS
13	32.60	0.2	*n*-Hexadec-?-ene	*n*-C16:1	MS
14	33.11	0.2	*n*-Hexadecane	*n*-C16	GC, MS
15	34.79	0.4	*n*-Heptadeca-?, ?-diene	*n*-C17:2	MS
16	35.03	1.8	*n*-Heptadec-?-ene	*n*-C17:1	MS
17	35.55	3.9	*n*-Heptadecane	*n*-C17	GC, MS
18	37.32	0.3	*n*-Octadec-?-ene	*n*-C18:1	MS
19	39.27	0.3	*n*-Nonadeca-?, ?-diene	*n*-C19:2	MS
20	39.44	0.5	*n*-Nonadec-?-ene	*n*-C19:1	MS
21	39.53	1.0	*n*-Nonadec-?-ene	*n*-C19:1	MS
22	39.68	0.1	2-Ketone (*m/z* 58 base peak)		
23	39.98	0.6	*n*-Nonadecane	*n*-C19	GC, MS
24	41.62	0.2	*n*-Eicos-?-ene	*n*-C20:1	MS
25	43.43	0.3	*n*-Heneicosa-?, ?-diene	*n*-C21:2	MS
26	43.59	1.2	*n*-Heneicos-?-ene	*n*-C21:1	MS
27	43.72	2.9	*n*-Heneicos-?-ene	*n*-C21:1	MS
28	45.82	0.4	*n*-Docos-?-ene	*n*-C22:1	MS
29	48.20	2.3	*n*-Tricosa-?, ?-diene	*n*-C23:2	MS
30	48.46	7.5	*n*-Tricos-?-ene	*n*-C23:1	MS
31	48.57	0.4	*n*-Tricos-?-ene	*n*-C23:1	MS

[a]Typical results obtained from a single gland.

n-alkanes were available for comparison of retention time and mass spectrum. All other compounds were identified by interpretation of the mass spectrum obtained, as well as by comparison with those in the NBS library of mass spectra available in the data base of the Hewlett Packard 5970 Mass Selective Detector. Standard 1-alkenes from C_{13} to C_{19} were available for comparison of retention times and mass spectra of the identified *n*-alkenes. The trace components, 5-methylpentadecane and 3-methylpentadecane, were identified from the pres-

ence of increased intensities of m/z 168 and 169 and m/z 196 and 197, respectively.

DISCUSSION

Harvester ants are an important component of ecosystems, and the genus *Messor* consists of more than 100 species worldwide (Robertson, South African Museum, Cape Town, personal communication, 1992). *M. capensis* (Mayr) is a granivorous ant that collects, utilizes, and, to some extent, distributes seeds. As it occurs in semiarid regions, some concern has been expressed about its influence on the seed bank and an investigation of the nest population-density, diaspore collection and utilization, and foraging strategy of *M. capensis* has been conducted (Vorster, 1989).

The success of solventless introduction of small-scale samples from insects for gas chromatographic analysis on capillary columns has been adequately demonstrated by Morgan (1990) and Burger et al. (1990). Our results show that about 92% of the detectable volatiles in the *M. capensis* poison gland is anabaseine, with anabasine being a minor component (approximately 5%). This result is similar to that obtained on *Aphaenogaster* species (Wheeler et al., 1981), where anabaseine comprised approximately 90% of the volatiles of the poison gland. In contrast, Coll et al. (1987), using packed column GC-MS, found that anabasine constituted over 90% of the volatiles in the poison gland secretion of *M. ebeninus* collected in Israel and is the major component of the poison gland of the Mediterranean harvester ant, *Messor bouvieri* (Jackson et al., 1989). The fact that two Mediterranean species of *Messor* have anabasine as a major component of this gland and a southern African species of *Messor* and a North American species of *Aphaenogaster* have anabaseine as a major component may have significant chemotaxonomic value.

The Dufour's gland of *M. capensis* contains n-pentadecane (42%) as the major component, as does the secretion of this gland of *M. ebeninus* (Coll et al., 1987). Table 1 lists the various compounds identified in the glandular secretion of *M. capensis* and the area percent given is typical of proportions found in a number of analyses of single glands. Most of the identified compounds are commonly found in Dufour's gland secretions of ants (Blum, 1981). 8,11-Nonadecadiene has been identified in the Dufour's gland secretion of several Attini (Evershed and Morgan, 1981; Salzemann et al., 1992), and this secretion of *M. bouvieri* (Jackson et al., 1989) contains principally 6,9-heptadecadiene, with small amounts of pentadecadiene and nonadecadiene. The secretion of *M. capensis* contains four dienes, C17:2, C19:2, C21:2, and C23:2. The double bond positions in the monoenes and dienes has not been determined.

When individual workers of *M. capensis* are carefully held and the tip of

the abdomen touched, they release a visible amount of secretion. Touching the tip of the abdomen with the capillary used for the solventless introduction of samples resulted in some of the secretion entering the cup of the capillary. Chromatographing this secretion established that only the poison gland contents were discharged, as there was no evidence of any Dufour's gland components in the chromatograms obtained. The solventless sample introduction of individual glands in this study produced extremely good chromatograms and trace components are easily detectable. However, no alkylpyrazines responsible for trail following, as found in *M. bouvieri* (Jackson et al., 1991), have been detected. Certain behavioral studies are in progress but no definitive data are available.

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A reinvestigation of the chemistry of the Dufours gland of the formicine ant, *Anoplolepis custodiens*

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Abstract. The components of individual Dufours glands excised from *Anoplolepis custodiens* workers were analysed by GC-MS. In addition to the *n*-alkanes and *n*-alkenes previously reported[2] in these glands, primary alcohols (C_{19}–C_{22}), secondary alcohols (C_{20}–C_{23}), 2-ketones (C_{20}–C_{23}) and possibly carboxylate ethyl esters (C_{19} and C_{21}) were identified as components of these glands. It seems possible that these high-boiling compounds are used by the workers in laying trails on the hot sandy surfaces of their characteristic habitat and in lining of the inner walls of nests, but no standard compounds have been available to us for any behavioral studies.
Key words. Anoplolepis custodiens; Dufours gland; *n*-alkanes; *n*-alkenes; 1-alcohols; 2-alcohols; 2-ketones.

A positive correlation between the presence of the formicine ant, *Anoplolepis custodiens*, in citrus fields and the incidence of red scale has long been observed[1]. This ant is thus of considerable economic importance in citrus farming in South Africa and its control could solve much of the problem of red scale in citrus. Methods for the biological control of this ant remain to be developed and it is hoped that control measures using pheromones might be possible if certain behavioral compounds of this pugnacious ant are characterised and tested.

The alarm-defense system of this ant has been investigated previously and it was reported that the Dufours gland contains only hydrocarbons[2]. In these early investigations, packed GC columns were used and eluting compounds were trapped on graphite for MS analysis. The possibility that additional components exist in this gland was considered and the increased sensitivity, resolution, and high temperature capability of present day capillary GC columns and the ready availability of GC-MS, prompted our reinvestigation of the chemistry of this glandular secretion. In addition, the solventless injection procedure used in this work minimised the physical and chemical processing of the samples prior to GC or GC-MS analyses. This is an important point since the components of the Dufours glands are present in nanogram quantities only.

Worker ants were collected on the University of Fort Hare farm (26°50′E, 332°47′S) and kept in the laboratory at room temperature and fed dead insects and sucrose solution. Dissected Dufours glands were placed in the cup of a glass capillary for solventless introduction in a gas chromatograph[3]. For NaBH$_4$ reduction, a Dufours gland was sealed in a glass capillary containing finely powdered NaBH$_4$ (1 mg or less) and the tube crushed in the GC inlet as described by Morgan[4] for solventless sample introduction. For BSTFA derivatisa-

tion, a Dufours gland was placed in the cup of the capillary tube containing 1 µl of BSTFA (Regis Chemical Company, IL, USA) and introduced into the GC inlet[3]. GC-MS analyses were run on a Hewlett Packard 5890 GC and 5970 MSD. A splitless injection with purge off time of 0.5 min was used with a HP-Ultra 1 column (50 m × 0.2 mm i.d. × 0.3 µm film thickness) held at 35 °C for 3 min, then temperature programmed to 300 °C at 5 °C min^{-1}, and finally held at 300 °C for 10 min.

Helium (25 cm s^{-1}) was used as the carrier gas. The mass selective detector monitored m/z 26 to 450 using 70 eV ionisation and the power supply to the electron multiplier was 2000 volts.

Chromatograms of single Dufours glands of workers indicated the presence of at least 39 components. The identities and evidence for the identities of these components are summarised in the table. The majority of the components were readily identified as *n*-alkanes and *n*-alkenes. Standards of *n*-alkanes were available for comparison of retention time and mass spectrum, whereas standard 1-alkenes only were available. The identified alkanes and alkenes confirmed previously reported results[2] except the additional presence of two dodecenes and three tridecenes are reported here. The difference between the two dodecenes (and the three tridecenes) is in the position of double bond within the molecule. An alkene with the unsaturation point closer to the centre of the molecule elutes earlier than the one with the unsaturation towards either end of the molecule. However, the position of the double bond in each alkene has not been determined.

The primary alcohols, 2-alcohols, 2-ketones and the components tentatively identified as carboxylate ethyl esters have not been reported as components of this gland before. Together, these components contribute an average of 26% to the total volatiles of this gland. Each

Identities and evidence for the identifications of components of single Dufours glands from *A. custodiens* workers

Peak No.	Kovats index	Area %	Component identity	Evidence for identity*
1	1000	2.57	*n*-Decane	GC, MS
2	1087	0.96	*n*-Undec-?-ene	MS
3	1100	27.88	*n*-Undecane	GC, MS
4	1173	0.08	3-methyl undecane	MS
5	1191	0.06	*n*-Dodoc-?-ene	MS
6	1195	0.09	*n*-Dodec-?-ene	MS
7	1200	0.63	*n*-Dodecane	GC, MS
8	1284	0.75	*n*-Tridec-?-ene	MS
9	1286	0.29	*n*-Tridec-?-ene	MS
10	1294	3.67	*n*-Tridec-?-ene	MS
11	1300	4.59	*n*-Tridecane	GC, MS
12	1400	0.05	*n*-Tetradecane	GC, MS
13	1488	tr	*n*-Pentadec-?-ene	MS
14	1500	0.41	*n*-Pentadecane	GC, MS
15	1600	tr	*n*-Hexadecane	GC, MS
16	1678	tr	*n*-Heptadec-?-ene	MS
17	1700	0.10	*n*-Heptadecane	GC, MS
18	1790	tr	*n*-Octadec-?-ene	MS
19	1800	1.00	*n*-Octadecane	GC, MS
20	1881	0.50	*n*-Nonadec-?-ene	MS
21	1900	14.06	*n*-Nonadecane	GC, MS
22	1986	0.12	*n*-Eicos-?-ene	MS
23	2000	3.16	*n*-Eicosane	MS
24	2086	0.78	*n*-Heneicos-?-ene	MS
25	2100	10.16	*n*-Heneicosane	MS
26	2168	0.35	Nonadecan-1-ol	MS, BSTFA
27	2188	0.26	Eicosan-2-one	MS, NaBH$_4$
28	2197	0.46	Eicosan-2-ol	MS, NaBH$_4$, BSTFA
29	2269	0.48	Eicosan-1-ol	MS, BSTFA
30	2287	5.52	Heneicosan-2-one	MS, NaBH$_4$
31	2295	5.77	Heneicosan-2-ol	MS, NaBH$_4$, BSTFA
32	2370	1.96	Heneicosan-1-ol	MS, BSTFA
33	2387	0.33	Docosan-2-one	MS, NaBH$_4$
34	2393	0.14	Docosan-2-ol	MS, NaBH$_4$, BSTFA
35	2408	2.39	Nonadecanoate ethyl ester?	
36	2481	0.34	Docosan-1-ol	MS, BSTFA
37	2487	2.80	Tricosan-2-one	MS, NaBH$_4$
38	2491	0.74	Tricosan-2-ol	MS, NaBH$_4$, BSTFA
39	2509	4.19	Heneicosanoate ethyl ester?	MS

*BSTFA, identification was based on the mass spectrum of the BSTFA derivative.
NaBH$_4$, evidence for the identity was obtained from increase/decrease in peak size after incubation of the gland with sodium borohydride.

of these compounds was identified by interpretation of the mass spectrum obtained, as well as by comparison with those in the NBS library of mass spectra available in the data base of the Hewlett Packard 5970 MSD. Support for the identities of 2-ketones and 2-alcohols was derived from the observation that peaks tentatively identified as those of 2-ketones were reduced in size, and those tentatively identified as the corresponding 2-alcohols, were increased in size when glands were treated with NaBH$_4$.

Further evidence for the identities of 1-alcohols and 2-alcohols was obtained from the mass spectra of the products of bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) reaction with these compounds. Peaks of m/z 75, 103 and M-15 (base peak) were quite discernible in the mass spectra of the primary alcohols, whereas for the 2-alcohols, peaks of m/z 73 and 75 were equally abundant (ca 33% of base peak). The base peak in each

mass spectrum of the 2-alcohols was m/z 117 and the M-15 peak contributed about 15% of the base peak in each mass spectrum.

The mass spectra of components 35 and 39 were each characterised by a base peak of m/z 43, an abundant M-45 peak (about 45% of base peak) and a rather uncommon m/z 88 peak. The M-45 and m/z 88 ions are indicative of ethyl esters[5] and components 35 and 39 are thus tentatively identified as nonadecanoate ethyl ester and heneicosanoate ethyl ester, respectively.

These more polar compounds of rather low volatility (1-alcohols, 2-alcohols, 2-ketones and possible carboxylate ethyl esters) were detected readily in glands of all workers analysed. Alate females, in contrast, showed evidence only on some chromatograms of possible trace amounts of certain of these compounds (no functional queens were available for analysis). As workers of *A. custodiens* follow long trails on bare ground, which is

often extremely hot, one of us (JMB) has long been of the opinion that the non-polar compounds reported previously[2] are not the most likely compounds responsible for trail marking. Alate females are not expected to lay trails and it is considered possible that these more polar compounds reported here may be employed in trail marking by workers, and even in lining the inner walls of nests.

It has recently been reported that pseudomyrmicine ants living in tropical environments have higher melting substances in their Dufours glands than ants living in colder climates[6] and relatively long-chain hydrocarbons have been identified as components of Dufours glands from *Atta laevigata* where they were also identified on foraging trails in the field as components of the territorial odour[7].

As most of these long chain compounds occurring in the Dufours gland of *A. custodiens* are not commercially available (to our knowledge), we appeal to anyone who could supply us with standards for behavioral studies to communicate with us.

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Structure of an extracellular polysaccharide produced by *Erwinia chrysanthemi*

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ABSTRACT

Erwinia chrysanthemi pv zeae strain SR260, a phytopathogen of corn, produced from lactose an acidic extracellular polysaccharide which was purified and found to consist of L-rhamnose, D-mannose, D-glucose, and D-glucuronic acid in the ratio of 3:1:1:1. A combination of chemical (carboxyl-group reduction, methylation analysis, periodate oxidation, Smith degradation, and lithium–ethylenediamine degradation) and physical (1 and 2D NMR spectroscopy) methods revealed that the polysaccharide is composed of a hexasaccharide repeating unit **1**:

$$
\begin{array}{ccc}
\text{III} & \text{II} & \text{I} \\
\end{array}
$$

$$\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1 \rightarrow 4)\text{-}\alpha\text{-D-Man}p\text{-}(1 \rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1 \rightarrow$$
$$3$$
$$\uparrow$$
$$1$$
$$\alpha\text{-L-Rha}p\text{-}(1 \rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1 \rightarrow 4)\text{-}\alpha\text{-D-Glc}p\text{A}$$

$$
\begin{array}{ccc}
\text{C} & \text{B} & \text{A} \\
\end{array}
$$

$$\mathbf{1}$$

INTRODUCTION

Erwinia chrysanthemi is a Gram-negative bacterial phytopathogen that causes soft-rot in a number of tropical and sub-tropical plants[1-8]. *E. chrysanthemi* produces copious amounts of an extracellular polysaccharide (EPS) when grown on lactose. At present, little is known about the polysaccharides produced by phytopathogens. Neither the factors that distinguish the surface carbohydrates of

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virulent and avirulent cells nor their involvement in pathogenicity are known. The structure of the extracellular polysaccharide (EPS, 1) produced by *E. chrysanthemi pv. zeae* strain SR260, a strain pathogenic to corn, is reported here.

EXPERIMENTAL

Production of polysaccharide. —*E. chrysanthemi pv zeae* strain SR260 was grown on a modified Scott's medium[9] solidified with 1.5% Difco agar (Difco Laboratories, Detroit, MI). Before use, the yeast extract was ultrafiltered (Millipore PSAC 1000, 1000 molecular weight cutoff, Millipore Corporation, Bedford, MA) to remove high-molecular-weight polysaccharides, and the ultrafiltrate was used to make up the medium.

The surface of the medium, which was allowed to dry for 48 h at room temperature, was densely streaked in a cross-pattern with bacterial cells and the plates were incubated first for 24 h at 30°C, and for a further 4 days at room temperature (20–22°C). The dense, mucoid growth was carefully scraped from the plates with a bent glass "hockey stick", diluted with water to decrease the viscosity, and centrifuged (10 000g, 90 min, 4°C) to remove the bacterial cells. The supernatant was decanted and the bacterial cells were suspended in water and pelleted again by centrifugation. The supernatants were combined and the crude polysaccharide was recovered by lyophilization. The yield of crude polysaccharide was ~ 0.67 g per L of medium.

Purification of polysaccharide. —Crude polysaccharide in water, 5 mg mL^{-1}, pH 8.5, was precipitated by the slow addition of 3 vol of 95% (w/v) EtOH. The EtOH precipitation step was repeated twice more.

Residual water was removed from the polysaccharide by solvent exchange, first with abs EtOH, and finally with anhyd Et$_2$O. Recovery of the EPS ranged from 36 to 50% of the crude polysaccharide, depending on the batch.

The polysaccharide was converted to the free acid either by electrodialysis or by passage over a cation-exchange resin (Bio-Rad AG50-X8, H$^+$) which had previously been extensively washed with deionized water.

Analytical methods. —Descending paper chromatography was carried out on Whatman No. 1 chromatography paper using 12:5:4 EtOAc–pyridine–H$_2$O as solvent. Thin-layer chromatography was carried out on 0.1-mm cellulose-coated plates (Merck, Darmstadt, Germany) using 15:30:40:15 HCO$_2$H–butanone–*tert*-butanol–H$_2$O as solvent. Standards containing D-glucose, D-mannose, D-glucuronic acid, and L-rhamnose were chromatographed together with hydrolyzates of the EPS on these media. Sugars were visualized with the alkaline silver reagent[10]. Total carbohydrate was determined by the phenol–H$_2$SO$_4$ procedure[11]. Uronic acid was measured either by a modified carbazole reaction[12] or by the biphenyl procedure[13]. Kdo was determined as described by Karkhanis et al.[14]. Protein assays were carried out by the Coomassie Blue method as described by Bradford[15] using a commercial kit (Bio-Rad Protein Assay Kit, Bio-Rad Laboratories, Richmond,

CA) and by absorbance at 280 nm. Solutions of the EPS were also scanned in the UV to detect nucleic acids. A Beckman (Palo Alto, CA) model 121MB amino acid analyzer using a standard protein hydrolyzate protocol was used to analyze for amino sugars; galactosamine, glucosamine, and mannosamine were used as standards.

All GLC analyses were performed on either a Hewlett–Packard 5890 (Hewlett–Packard, Avondale, PA) or a Varian 3700 (Walnut Creek, CA) gas chromatograph equipped with a FID detector. Helium was used as a carrier gas (20 cm s^{-1}) in all the analyses. GLC–MS was performed on a Hewlett–Packard 5890 GC coupled to a Hewlett–Packard 5970 mass selective detector (MSD).

Monosaccharides were analyzed, as their alditol acetates, by GLC after hydrolysis (2 M CF$_3$CO$_2$H, 121°C, 1 h) and derivatization, essentially as described by York et al.[16]. The alditol acetates were analyzed isothermally at 220°C on a J & W DB 225 fused silica capillary column (30 m × 0.25 mm, J & W Scientific, Folsom, CA).

Methylation was performed as described by York et al.[16] and the resulting per-*O*-methylated alditol acetates were analyzed as already described for the alditol acetates except that a temperature program (180°C for 3 min increased to 220°C at 2°C min^{-1}, and held at 220°C for 37 min) was used. GLC–MS was used to confirm the identity of the partially methylated alditol acetates using conditions similar to those just described. Standards prepared by the methylation of glycogen and dextran (NRRL *β*-1355 Fraction S) were used to confirm the identities of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol, respectively.

Quantitative monosaccharide analysis was also performed using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) on a Dionex BioLC (Sunnyvale, CA) fitted with a Carbopak PA1 anion-exchange column. The eluents used were as follows: A = 100 mM NaOH; B = 100 mM NaOH–1000 mM NaOAc; C = deionized water. The following elution program was employed to detect both the mono- and oligo-saccharides (up to a glucan 40-mer): T_0, 20% A–80% C (20 mM NaOH); T_{20}, 20% A–80% C; T_{25}, 100% A (100 mM NaOH); T_{55}, 50% A–50% B (100 mM NaOH–500 mM NaOAc); T_{65}, 50% A–50% B. This gradient program allowed the separation of a variety of mono- and oligo-saccharides in a single 65-min run.

The absolute configuration of the monosaccharides was determined by gas chromatography of the trimethylsilyl (Me$_3$Si) derivatives of their R-(−)-butan-2-ol glycosides as described by Gerwig et al.[17]. After butanolysis, the mixture was treated as described by Chaplin[18] and not neutralized with AgNO$_3$ as described by Gerwig et al.[17]. The Me$_3$Si derivatives of the R-(−)-butan-2-ol glycosides were prepared according to Sweeley et al.[19] and separated by gas chromatography on a HP OV-101 (Hewlett–Packard) fused silica column (25 m × 0.25 mm) using the following temperature program; 180°C for 3 min increased to 220°C at 2°C min^{-1} and held at this temperature for 20 min. Standard Me$_3$Si derivatives were prepared by butanolysis of pure D sugars (except for L-rhamnose) with R-(−)-butan-2-

ol or *RS*-($\pm$)-butan-2-ol to obtain the retention times corresponding to the L sugars (or D-rhamnose).

Ultracentrifugal analysis of the EPS was performed on a Beckman Model E analytical centrifuge. The EPS (1 mg mL^{-1} in phosphate buffered saline, pH 7.3) was analyzed by ultracentrifugation at 54 800 rpm in an AN-H head on a Beckman Model E Analytical Ultracentrifuge fitted with Schlieren optics.

Reduction of glycosyluronic residues. —Reduction of the D-glycosyluronic residues in the EPS was carried out by reaction with a water-soluble carbodiimide [1-ethyl-3(3-dimethylaminopropyl)-carbodiimide, EDC] as described by Taylor and Conrad[20]. The extent of reduction, which was repeated if necessary, was examined by uronic acid analysis or by methanolysis and analysis of the Me$_3$Si ethers as described above.

Low-pressure gel-permeation and anion-exchange chromatography. —The following gel-permeation chromatography columns and elution conditions were used in this study: Bio-Gel P-2 (-400 mesh, 1.6×80 cm) eluted with water at a flow rate of 18 mL h^{-1}; Bio-Gel A-1.5m (200–400 mesh, 1.6×80 cm) eluted with water at a flow rate of 20 mL h^{-1}; Sepharose CL6 (200–400 mesh, 1.5×90 cm) eluted with water at a flow rate of 18 mL h^{-1}; Spectra/Gel^{TM} (200–400 mesh, 1.5×88 cm) eluted with water at a flow rate of 20 mL h^{-1}.

All columns were operated at room temperature and were calibrated with a series of maltodextrins (maltose to maltoheptose obtained from Boehringer Mannheim) or a series of dextrans obtained from Sigma (St. Louis, MO).

Anion-exchange chromatography of the EPS was performed on a column (2.5×33 cm) of DEAE-cellulose, which was equilibrated in 10 mM KCl. The column was eluted initially with two bed-volumes of 10 mM KCl followed by a linear gradient of 10–500 mM KCl (500 ml). Column fractions were analyzed for neutral carbohydrate and uronic acid.

Lithium–ethylenediamine degradation. —Degradation of the EPS by Li–ethylenediamine was performed by the method of Lau et al.[21] and the products fractionated by chromatography on Bio-Gel P-2. Fractions across the low molecular-mass peak were analyzed by HPAE-PAD chromatography and fractions containing a single component were pooled for NMR analysis.

Periodate oxidation and Smith degradation. —Initial Smith degradations of the native- (**1**, Scheme 1) and carboxyl-reduced EPS (**2**) were performed as described by Smith and Montgomery[22]. Briefly, the deionized EPS was oxidized for 200 h in 0.05 M NaIO$_4$ (calculated so that there was a five-fold excess of periodate) at 4°C in the dark. The excess periodate was decomposed by the addition of a five-fold excess of ethylene glycol and the solution was kept at room temperature for 30 min. Thereafter, the sample was extensively dialyzed against deionized water and freeze-dried. The polyaldehyde was taken up in water, and reduced with a five-fold excess of NaBH$_4$ for 16 h at room temperature. The excess NaBH$_4$ was decomposed by the addition of Bio-Rad AG50-X8 (H$^+$) ion-exchange resin during which time the pH was not allowed to fall below 4.5. The resin was filtered through a
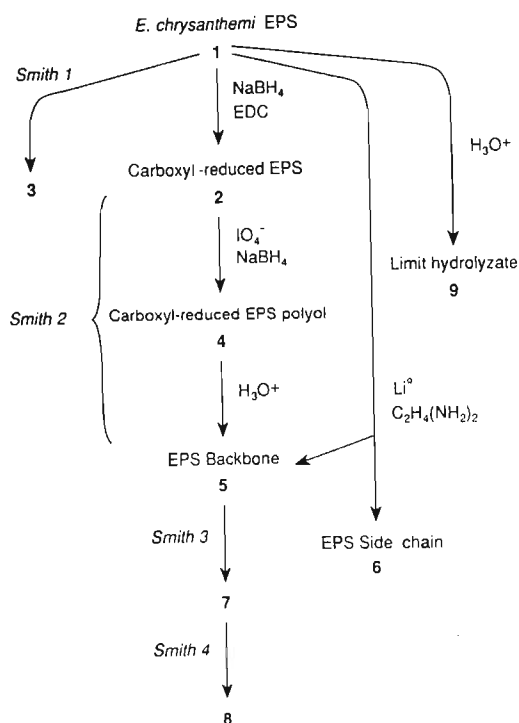
Scheme 1. Chemical degradations of *E. chrysanthemi* extracellular polysaccharide (**1**).

glass wool-plugged funnel and the polyalcohol was freeze-dried. Borate was removed from the lyophilized material by repeated evaporation with MeOH at diminished pressure and temperature (< 40°C) and the residue taken up in 1 M CF_3CO_2H and maintained at room temperature for 24 h. After diluting the sample two-fold, the CF_3CO_2H was removed by lyophilization. The products were then fractionated by chromatography on Bio-Gel P-2 yielding a high-molecular-weight fragment, **3**.

Smith degradations on **1** and **2** were also performed in the presence of 0.5 M NaCl as described by Aalmo et al.[23], and in the presence of $NaClO_4$ as described by Dudman[24]. Briefly, deionized EPS (1 mg mL^{-1}) in 0.05 M $NaIO_4$–0.2 M $NaClO_4$ was oxidized in the dark for 96 h at 4°C. The sample was quenched by the addition of ethylene glycol (1 mL) followed 30 min later by $NaBH_4$ (0.5 g). Reduction was allowed to proceed overnight (16 h) after which the excess borohydride was destroyed by the addition of glacial AcOH. The pH of the solution was not allowed to fall below 4.5. After extensive dialysis of the solution against distilled water and lyophilization, the oxidation and reduction was repeated. The resulting polyalcohol (**4**), a portion of which (100 μg) was subjected to methylation analysis, was hydrolyzed by 1 M CF_3CO_2H for 24 h at room temperature and after two-fold dilution, the CF_3CO_2H was removed by lyophilization. The resulting products were purified by chromatography on Bio-Gel P-2 (−400 mesh). The

fraction eluting in the void volume (**5** in the case of the Smith degradation of **2**) of the Bio-Gel P-2 column was further analyzed by chromatography on Spectra/Gel™ HW-75F (Spectrum Industries, Inc., Los Angeles, CA) (200–400 mesh) and found to elute as a single peak. The high-molecular-mass polymer was subjected to a second Smith degradation, and the products purified by chromatography on Bio-Gel P-2. Fractions containing the low-molecular-mass fragments were examined by HPAE-PAD chromatography, and those exhibiting a single component (**7**) were pooled for NMR spectroscopic analysis.

The low-molecular-weight fragment (**7**) from the EPS backbone (derived by Smith degradation of either the carboxyl-reduced EPS or the Li–ethylenediamine degradation of the native EPS) was subjected to a third Smith degradation as just described, except that periodate oxidation was carried out in the absence of $NaClO_4$ for 24 h at room temperature. The excess $NaBH_4$ was decomposed by the addition of Bio-Rad AG50-X8 (H^+) and the dialysis step was omitted. After removal of the excess borate by repeated evaporation from 1% AcOH in MeOH (4 times) and MeOH (4 times), the fragments (**8**) were hydrolyzed in 2 M CF_3CO_2H (121°C, 1 h) and the monosaccharide composition determined by GLC of the alditol acetates.

Production of an aldobiouronic acid (**9**).—Electrodialyzed EPS (175 mg) was hydrolyzed (1 M CF_3CO_2H, 100°C, 6 h), and the CF_3CO_2H evaporated under diminished pressure. The residual syrup was chromatographed on a column (1 × 11 cm) of Bio-Rad AG1-X8 (acetate form) with a linear gradient of 0 to 3 M AcOH. The uronic acid containing peak was pooled as four fractions, and freeze dried. HPAE-PAD analysis of the pooled fractions showed that the first three fractions (**9**) were > 95% pure, and were used for subsequent NMR spectroscopy.

^1H NMR analysis.—Samples for NMR analysis (2–5 mg) were exchanged three times D_2O (Aldrich, Milwaukee, WI) by lyophilization, before being dissolved in D_2O (0.5 mL), containing a trace of acetone as internal standard whose resonance was set equal to 2.225 ppm. All spectra were recorded on a Bruker AMX 600 spectrometer. Standard programs, supplied by Bruker, were used to record the COSY-45 and NOESY-45 spectra.

RESULTS AND DISCUSSION

Production of EPS by E. chrysanthemi.—The Lac^+ strain of *E. chrysanthemi* used in this study produced an extracellular polysaccharide from glucose, galactose, lactose, fructose, and sucrose. The structure of the polysaccharide (**1**) produced on lactose is reported here.

Purity of the EPS.—The polysaccharide (**1**), after purification by ethanol precipitation, migrated as a single component when the EPS was either analyzed by analytical ultracentrifugation or chromatographed over DEAE-cellulose, Bio-Gel A 1.5, Sepharose CL6, or Spectra/Gel™ HW-75F. Qualitative analysis of the monosaccharide composition of fractions across the single peak of polysaccharide
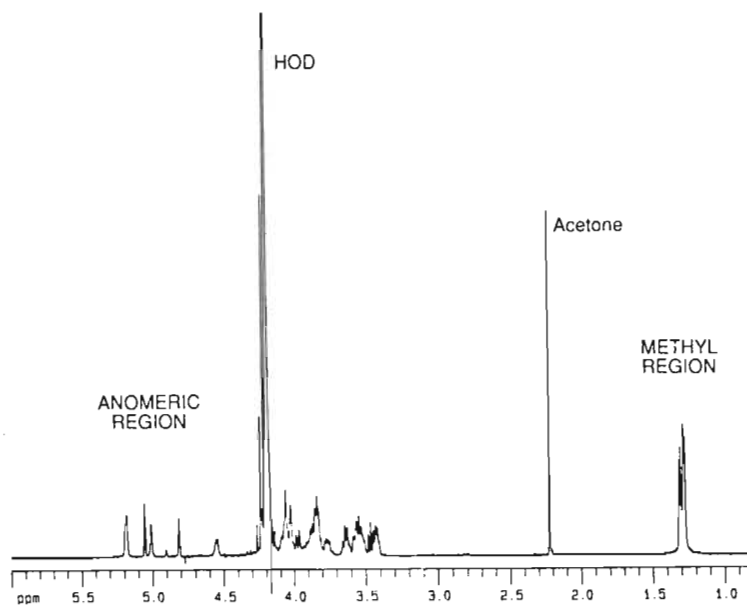
Fig. 1. ¹H NMR spectrum of EPS (1) obtained at 600 MHz in D₂O at 350 K.

that eluted from all of these columns, revealed a similar pattern, thus supporting the conclusion that the EPS was homogeneous.

Initial examination of **1** by high-field proton NMR (Fig. 1) revealed four well-resolved and two overlapped 1-proton resonances in the anomeric region and three 3-proton doublets in the methyl group region. Thus **1** could be considered tentatively a polymer of a repeating unit of six monosaccharide residues, probably three of which were 6-deoxyhexoses. The complex and extensively overlapped ring-proton region (3.4–4.3 ppm), however, precluded the extraction of any further structural information from the polysaccharide **1**, even using two-dimensional NMR analysis. The structure of **1** was then determined through a combination of physical and classical chemical methods (Scheme 1).

Composition. —Quantitative data could not be obtained initially because of the difficulty in completely hydrolyzing the glucosyluronic linkages of the EPS. After the GlcA residues of **1** had been reduced to form the carboxyl-reduced EPS (**2**), however, analysis was carried out by three different methods, which yielded quantitative data for all monosaccharide components (Table I). EPS did not contain GlcN, GalN, ManN, Fuc, Xyl, Ara, Fru, pyruvate, acetate, succinate and propanoate, and Kdo, or protein and nucleic acid. After the stereochemistry of the component monosaccharides had been established by GLC of the Me₃Si derivatives of their R-(−)-butan-2-ol glycosides, it was concluded that the composition of EPS (**1**) was 3 : 1 : 1 : 1 L-Rha : D-Man : D-Glc : D-GlcA.

Linkage analysis. —Methylation analysis of the native **1** (Table II) revealed the presence of terminal nonreducing and 1,3-linked rhamnose residues in a ratio of

TABLE I

Monosaccharide composition of carboxyl-reduced EPS (2)

Method	Derivative	Apparent molar ratio [a]				
		Rha	Man	Glc	(Glc + GlcA) [b]	GlcA
GC	Alditol acetates	3.00	0.97		1.95	
GC [c]	Partially methylated					
	Alditol acetates	3.00	0.91	1.07		1.07 [d]
HPAE-PAD	None	3.00	1.02		2.29	

[a] Results relative to Rha = 3. [b] Glucose derived from both glucose and reduced GlcA residues. [c] Data obtained by summing the values for each partially methylated alditol acetate from the cleavage of the fully methylated EPS followed by reduction and acetylation (see Experimental section). [d] Value obtained for 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-glucitol, which arises from the carboxyl-reduced GlcA.

~ 1:2. The value for the 1,5-di-O-acetyl-2,3,4-tri-O-methylrhamnitol was lower than expected, probably because of the volatility of its methylated methyl glycoside obtained from the methanolysis of the methylated EPS. The amount of 1,3,4,5-tetra-O-acetyl-2,6-di-O-methylmannitol recovered was lower than expected, and is probably due to the resistance of the uronic acid linkage to acid hydrolysis. The incompletely hydrolyzed per-O-methylated aldobiuronic acid is not detected by GLC of its alditol acetate derivative.

Methylation analysis of 2 (Table II) confirmed the presence of the partially methylated alditol acetates detected in the native EPS (1) and, in addition, revealed the presence of 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylglucitol, which arises from the reduction of glucuronic acid.

The molar ratios of the partially methylated alditol acetates suggests that 1 has a repeating unit of six monosaccharides in which only the terminal rhamnose residue and the glucuronic acid residue (or the glucose residue derived from the glucuronic acid) contain unmodified vicinal hydroxyl groups and are therefore susceptible to periodate oxidation.

TABLE II

Methylation analysis of E. chrysanthemi EPS and its derivatives

Me sugar [b]	EPS or derivative [a]					
	1 (Mol%)	2	3	4	5	5 [c]
2,3,4 Me$_3$Rha	16.8	15.3	27.0	< 1.0	3.0	4.2
2,4 Me$_2$Rha	41.2	34.2	36.2	48.8	30.4	29.8
2,4,6 Me$_3$Glc	24.2	17.8	39.6	27.5	33.5	30.4
2,3,6 Me$_3$Glc		17.8		< 1.5		4.6
2,3,6-Me$_3$Man			12.7		30.7	26.4
2,6 Me$_2$Man	17.9	15.0	22.2	23.7	2.4	4.6

[a] See Scheme 1. [b] 2,3,4 Me$_3$Rha = 1,5-di-O-acetyl-2,3,4-tri-O-methyl rhamnitol, etc. [c] Derived by Li–ethylenediamine degradation of 1.
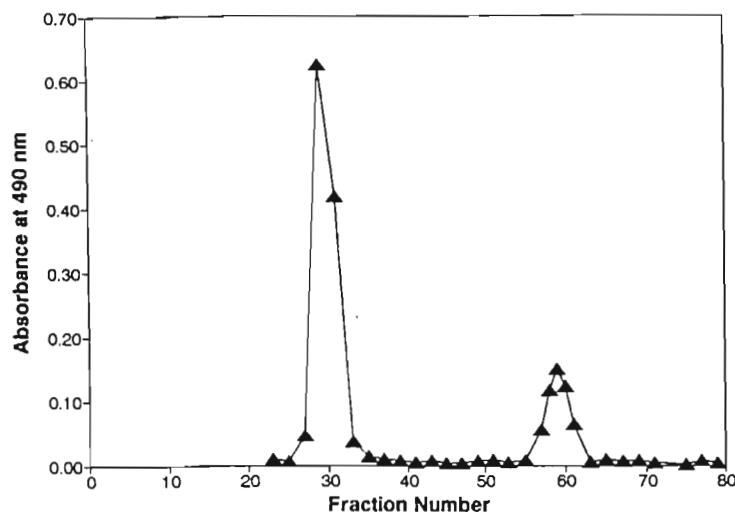
Fig. 2. Chromatography of the products from Smith degradation of the carboxyl-reduced EPS (**2**) on Bio-Gel P-2.

Sequence analysis. — In order to elucidate the sequence of the sugar residues, and to further characterize the branching pattern, Smith degradations were performed on **1** and its derivatives as summarized in Scheme 1. The native EPS (**1**) proved difficult to oxidize completely (Smith-1), even in the presence of sodium chloride or sodium perchlorate, which have been found to facilitate the oxidation of acidic polysaccharides[23,25]. Different polysaccharides are oxidized by periodate at varying rates[23] and this is particularly noticeable in the case of the acidic polysaccharides, where ionic repulsion[25] and the facile formation of inter- and intra-residue hemiacetal linkages[26,27] hinder the completeness of oxidation. Methylation analysis of the Smith-degraded native EPS (**3**), as shown in Table II, revealed that only ~ 30% of the side-chain had been removed.

Periodate oxidation of the carboxyl-reduced EP2 (**2**) and chromatography of the product on Bio-Gel P-2 revealed two components (Fig. 2), one of high (**4**) and one of low molecular weight. Methylation analysis of the high-molecular-weight polyalcohol, both before (**4**) and after (**5**) mild acid hydrolysis, demonstrated complete oxidation (Table II).

Side-chain sequences. — The observation that a high molecular polysaccharide (**5**) remained after complete Smith degradation indicated that the two periodate-susceptible residues of **2** must be in the side chain. This finding was confirmed when the results of the methylation analysis were considered. As observed in Table II, there is the loss of the terminal nonreducing Rha and the 4-linked Glc residues in the Smith degradation of **2** to **4**. In addition, there is the loss of a third residue (3-linked Rha) after the mild acid hydrolysis of **4** to form **5**. Since a 3-linked Rha residue cannot be oxidized by periodate, its loss indicates that it is present *between* the nonreducing terminal Rha and the 4-linked Glc residues of the side chain.

TABLE III

^{1}H NMR data ^{a} and residue assignments for oligosaccharides derived from **1** (see also Fig. 5).

Cmpd ^{b}	Residue		Chemical shifts (ppm) and coupling constants (Hz)					
	Symbol ^{c}	Assignment ^{d}	H-1($J_{1,2}$)	H-2($J_{2,3}$)	H-3($J_{3,4}$)	H-4($J_{4,5}$)	H-5($J_{5,6}$)	H-6($J_{6,6'}$)
5	I	α-L-Rha$p$ ^{e}	5.175 (< 2.0)	4.277 (< 2.0)	3.885 (9.7)	3.535 (9.5)	4.058 (6.2)	1.269 ^{f} (< 0.1)
	II	α-D-Man$p$ ^{e}	5.043 (< 2.0)	4.058 (3.0)	4.009 (9.4)	3.893 (9.6)	3.506 (4.9) (2.4)	3.840 (− 13.5) 4.027
	III	β-D-Glc$p$	4.537 (8.3)	3.434 (8.8)	3.621 (8.9)	3.478 (9.3)	3.524 (5.5) (< 2.0)	3.737 (− 11.5) 3.938
6	B′	L-Rhamnitol	3.642 (7.2) 3.823 (3.6) ($J_{1,1'} = − 11.7$)	3.892 (5.6)	3.946 (2.0)	3.606 (8.3)	3.736 (6.3)	1.273 ^{f} (< 0.1)
	C	α-L-Rha$p$ ^{e}	4.952 (1.6)	4.029 (3.3)	3.805 (9.6)	3.464 (9.6)	3.861 (6.3)	1.287 ^{f} (< 0.1)
7	I	α-L-Rha$p$ ^{e}	5.140 (1.7)	4.051 (3.5)	3.788 (9.8)	3.457 (9.7)	4.016 (6.3)	1.252 ^{f} (< 0.1)
	II′	Erythritol	3.763 (5.3) 3.849 (2.9) ($J_{1,1'} = − 12.0$)	3.815 (5.6)	3.796 (6.5) (3.5)	3.672 3.862 ($J_{4,4'} = − 12.3$)		
	III	β-D-Glc$p$	4.567 (8.1)	3.430 (9.2)	3.606 (9.1)	3.472 (10.8)	3.470 (4.1) (1.3)	3.724 (− 12.2) 3.909
9	II	α-D-Man$p$ ^{g}	5.148 (1.9)	4.056 (3.2)	3.939 (9.0)	3.858 (11.0)	3.764 (6.1) (6.1)	3.858 (< 0.1) 3.858
		β-D-Man$p$ ^{g}	4.906 (< 0.1)	4.070 (2.5)	3.775 (11.1)	3.764 (9.7)	3.420 (6.3) (2.1)	3.737 (− 12.3) 3.903
	A	α-D-Glc$p$A	5.286 (3.9)	3.619 (9.7)	3.817 (9.6)	3.570 (9.8)	4.528	

^{a} Obtained at 600 MHz in D$_2$O at 298 K. ^{b} See Scheme 1. ^{c} Symbols as used in Fig. 6. Residue sequence designators were chosen to be consistent throughout the entire series of derivatives. ^{d} Assigned according to procedures of Koerner et al.[28] using 600-MHz 2D COSY-45 spectra and integrated 1D spectra for each compound. ^{e} Anomeric form assigned from 2D NOESY data presented in Table IV. ^{f} Three-proton singlet. ^{g} Based on the integration of well-resolved resonances the equilibrium anomeric composition was 72% α anomer and 28% β anomer; no furanose forms were observed.

TABLE IV

Cross-peaks [a] observed for anomeric protons in the NOESY spectra of oligosaccharides derived from **1**

Compound [b]	Anomeric signal (ppm)	Upfield signal (ppm)	Interpretation [c]
5	5.175	4.277	I-1 → I-2 (1,2-*eq / eq*)
	5.043	4.277	II-1 → I-2 (glycosidic)
	5.043	4.058	II-1 → II-2 (1,2-*eq / eq*)
6	4.952	4.029	C-1 → C-2 (1,2-*eq / eq*)
7	5.140	4.051	I-1 → I-2 (1,2 *eq / eq*)
		3.606	I-1 → III-3 (glycosidic)
	4.567	3.470	III-1 → III-5 (1,5-*ax / ax*)
9	5.148	4.056	II-1 → II-2 (1,2 *eq / eq*)
	5.286	3.619	A-1 → A-2 (1,2-*eq / ax*)

[a] Obtained at 600 MHz; mixing time 185 ms. [b] See Scheme 1. [c] Symbols as used in Fig. 6. Abbreviations used: *eq*, equatorial; *ax*, axial. Assigned according to the procedure of Koerner et al.[28]

Finally, the observation that mild acid hydrolysis of **4** results in the exposure of the 3 hydroxyl group of the mannose residue in **5** to methylation shows that the side chain is linked to the 3 position of the branch point residue and that the side chain is composed of no more than the three residues already noted.

Addressing the structure of the branch point, the NMR spectrum of aldobiouronic acid (**9**), composed of equimolar Glc*p*A and Man*p* (Table III), the small vicinal coupling constant ($J_{1,2}$ 3.9 Hz) manifest by the anomeric proton of the Glc*p*A residue (A), as well as its single 1,2 equatorial–axial and lack of 1,3 or 1,5 diaxial cross peaks (Table IV), indicated that this proton was equatorial and that the acid moiety was α-D-Glc*p*A. Although there are no *trans*-glycosidic cross-peaks detected in the 2D NOESY spectrum of **9**, the sequence of this disaccharide can be deduced from its 1D spectrum. The Man*p* residue is observed to be in an anomeric equilibrium (Table III), so that the Man*p* residue must be at the reducing end of the disaccharide and the sequence of **9** must be A → II (the residue symbolism is shown in Fig. 6).

The degradation of the native EPS (**1**) by Li–ethylenediamine, as described by Lau et al.[21], produced two fractions, which were separated by chromatography on Bio-Gel P-2 (Fig. 3, panel A). Monosaccharide and methylation analysis of the high-molecular-weight fragment gave data (Table II) identical to that obtained for the backbone (**5**) generated by the Smith degradation of the carboxyl-reduced EPS (**2**). Rechromatography of fractions from the overlapping region of the low-molecular-weight peak on Bio-Gel P-2 gave rise to two incompletely resolved peaks of these components (Fig. 3, panel B). The major component (**6**), which constituted more than 80% of the material, eluted in the leading edge of the peak and was composed of rhamnose and rhamnitol; the minor component (< 20%) eluted in the trailing edge of the peak was composed of only rhamnose. The production of a mixture of an aldose and an alditol was a similar finding to that obtained by Lau et
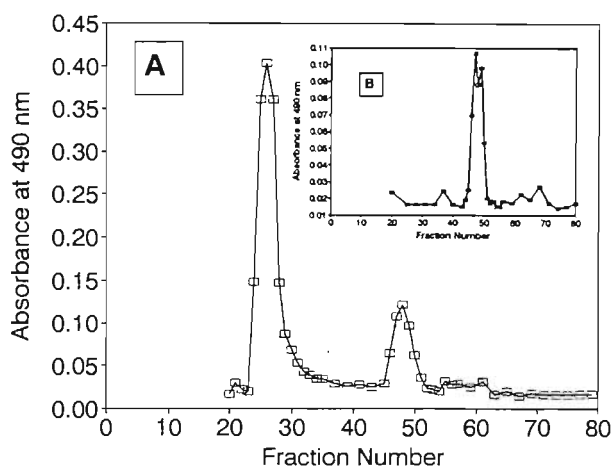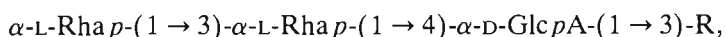
Fig. 3. (A) Chromatography of the products produced by degradation of the native EPS (1) by Li–ethylenediamine on Bio-Gel P-2. (B) Rechromatography of the trailing edge of the low-molecular peak in chromatogram A (fractions 49 to 51) on Bio-Gel P-2.

al.[21] in their studies on the application of the Li–ethylenediamine degradation of uronic acid-containing polysaccharides.

Examination of **6** by NMR (Table III) showed that the anomeric proton at the Rha *p* residue (C-1) manifests a 1,2-equatorial–equatorial, but no 1,3 or 1,5 diaxial cross-peaks, indicating its α-L-linkage. No *trans*-glycosidic cross-peaks were detected between the rhamnitol (B′) and Rha *p* (C) residues of **6**, possibly due to the conformational flexibility of the rhamnitol residue. However, it may be deduced from the 1D data (Table III) that the rhamnosyl residue (C) must be the terminal and nonreducing residue and the sequence of the last two residues of the side chain must be C → B. The anomeric form of the 3-linked Rha residue (B) in the side chain could not be assigned from the data available. However, in light of the findings that Rha residues I and C were α-L-linked and that L-Rha residues are usually found to be α-L-linked in bacterial polysaccharides[30], it is reasonable to assume that residues B is α-L-linked. Thus it can be concluded that the structure of the side chain of the carboxyl-reduced **2** is:

α-L-Rha *p*-(1 → 3)-α-L-Rha *p*-(1 → 4)-α-D-Glc *p*-(1 → 3)-R
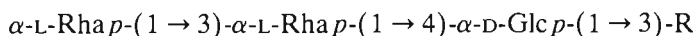
and that of the parent structure **1** is:

α-L-Rha *p*-(1 → 3)-α-L-Rha *p*-(1 → 4)-α-D-Glc *p*A-(1 → 3)-R,

where R is the Man in the backbone of the polysaccharide.

Backbone sequence. —Monosaccharide analysis of the high-molecular-weight fragment (**5**), obtained either by Smith degradation (No. 2) or by reductive cleavage, revealed that it was composed of glucose, rhamnose, and mannose in a 1:1:1 ratio. From the methylation analysis of **5** (Table II, column 5) the backbone polysaccharide **5** was composed of three residues: 3-linked Rha, 3-linked Glc, and 4-linked Man. For such a composition there are theoretically six possible trisaccha-
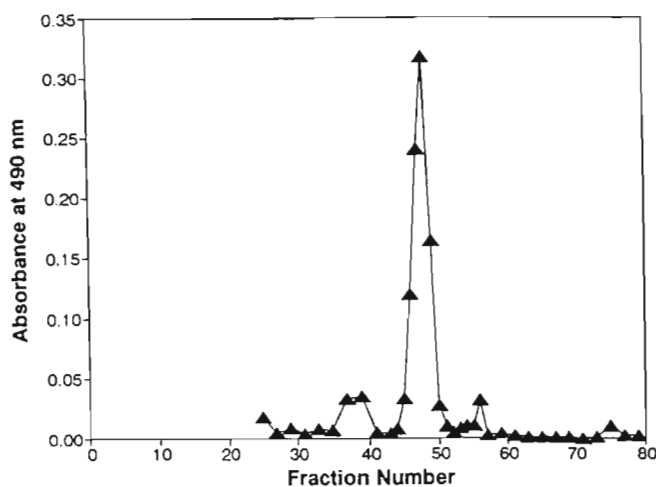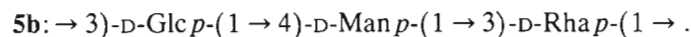
Fig. 4. Chromatography of the products from Smith degradation of the EPS backbone (5) on Bio-Gel P-2.

ride sequences for the structure of the backbone; however, the degeneracy resulting from polymer formation allows for only two unique sequences for the repeating unit of **5**. These two possible sequences are:

5a: → 3)-L-Rha$p$-(1 → 4)-D-Man$p$-(1 → 3)-D-Glc$p$-(1 →

and

5b: → 3)-D-Glc$p$-(1 → 4)-D-Man$p$-(1 → 3)-D-Rha$p$-(1 → .

The resolution of these two structures was achieved by carrying out two more Smith degradations (No. 3 and 4, Scheme 1), first on **5** to form **7** (Fig. 4), then on **7** to form **8**. Since only Glc was detected in **8** the two possibilities are reduced to **5b**.

Analysis of the proton NMR spectra of **5** confirmed its composition of equimolar Glc$p$, Man$p$, and Rha$p$. Assignment of a β-D-anomeric form to the Glc$p$ residue follows from the large vicinal coupling of its H-1 proton ($J_{1,2}$ 8.3 Hz). However, the small vicinal coupling of the H-1 resonances of Man$p$ and Rha$p$ residues, a characteristic of the *manno* configuration of these residues, shed no light on their anomeric configuration, which was however resolved in the 2D NOESY spectra of **5** (Table IV)[28]. In this spectrum, the H-1 resonance of β-D-Man$p$ and β-L-Rha$p$ residues would have been expected to manifest intense cross-peaks due to strong 1,3 and 1,5 diaxial interactions, as well as strong 1,2 axial–equatorial interaction; whereas, the H-1 resonances of the α-linked anomers of these residues would have been expected to manifest a cross peak due to only a strong 1,2 equatorial–equatorial interaction. In fact, only 1,2 cross-peaks are observed for the H-1 resonances of these residues. Thus, the D-Man$p$ and L-Rha$p$ residues of **5** must both be α-linked. Also observed in the NOESY spectra of **5** was a strong cross-peak arising from a *trans*-glycosidic interaction between the Man H-1 (II-1) and Rha H-2 (I-2). This finding was useful in confirming that the

sequence of these two residues is II → I but was apparently contradictory with the methylation data (Table II, **5**), since the cross-peak indicated that the glycosidic linkage site on the backbone was Rha O-2; the methylation data indicated that the linkage site on the residue was O-3. The resolution of this dilemma is the realization that NOESY cross-peaks can occasionally occur preferentially to adjacent protons rather than linkage-site protons[29]. In the case of the backbone Rha
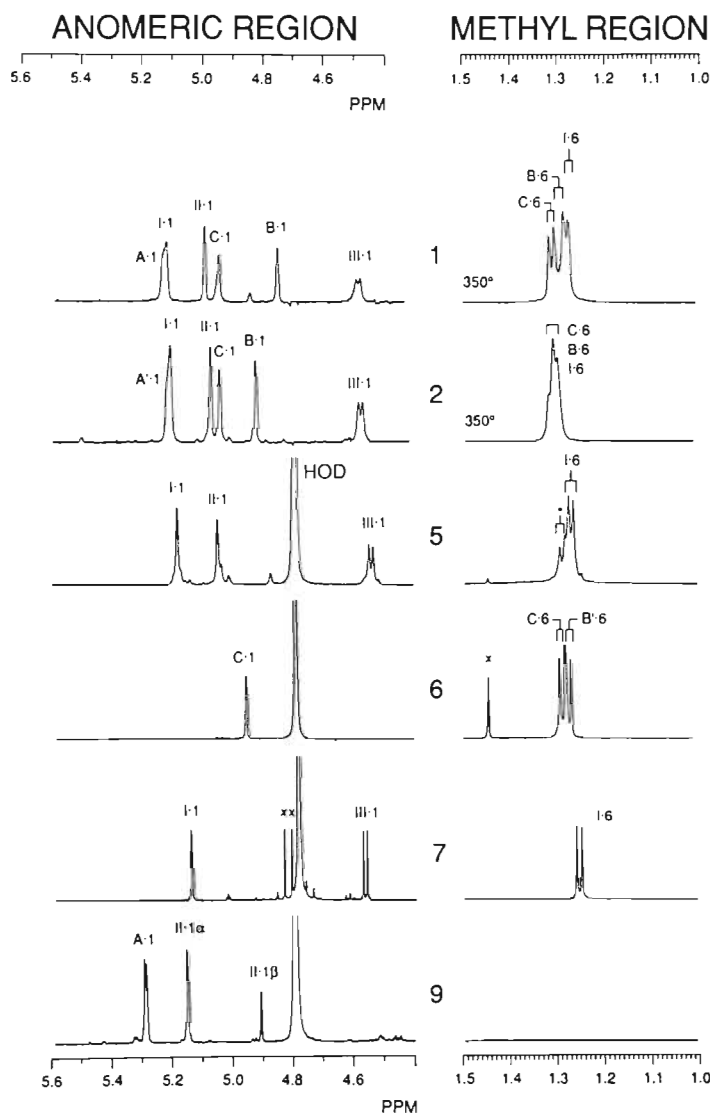

Fig. 5. The anomeric and methyl regions observed in the ¹H NMR spectra of EPS (**1**) and its derivatives (**5–7** and **9**), obtained at 600 MHz in D₂O at 298 K except where noted. Resonance assignments are as defined in Fig. 6. Other symbols are: X, nonsaccharide resonances and *, resonances from trace amounts of side chain rhamnose residues remaining attached to **5**.

(I) residue, this is certainly possible, since both H-2 and H-3 of this residue are on the same side of the pyranose ring.

Analysis of the NMR spectra of **7** presented another opportunity to study the polysaccharide backbone as well as to observe directly the effects of Smith degradation on **5**. As may be seen in Table III, compound **7** was composed of equimolar Rha$p$, Glc$p$, and erythritol. The survival of these two residues confirms the decomposition of the Man$p$ residue (II) by the Smith degradation (No. 2). The observation of equimolar erythritol confirms the periodate cleaved between the II-2 and II-3 carbons. As observed for **5**, the large vicinal coupling displayed by the H-1 proton of D-Glc$p$ ($J_{1,2}$ 8.1 Hz) indicates that this residue (III) is β-linked. Inspection of the NOESY spectrum of **7** reveals an intense cross peak for H-1 of Rha$p$ due to a 1,2 equatorial–equatorial interaction, but no evidence of 1,3- or 1,5-diaxial interaction, which confirms the α-linkage at this residue (I). Also noted
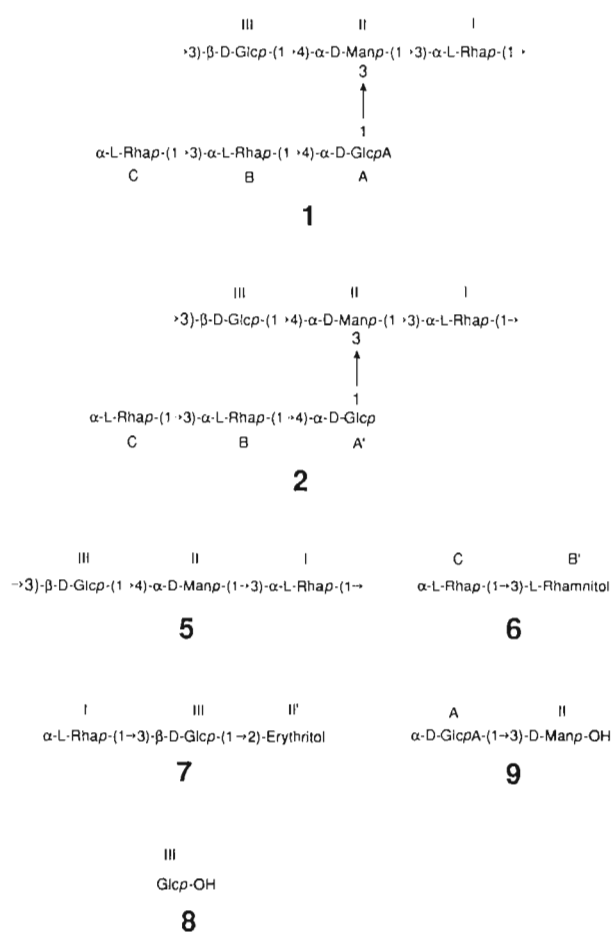

Fig. 6. The structure of EPS (**1**) and its derivatives.

is a cross-peak due to a 1,5 diaxial interaction for the D-Glc p residue (III), thus confirming again its β-linkage. Lastly and most importantly, an intense cross-peak is seen between H-1 of Rha p and H-3 of Glc p due to a *trans*-glycosidic interaction (I-1 → III-3). This observation confirmed both the I → III sequence required by the repeating trisaccharide backbone and the O-3 linkage site on the Glc p residue.

Finally, the issue of the nature of the repeating unit of the backbone of the polysaccharide, for example, an $(XYZ)_n$ versus $(XXXYYYZZZ)_m$ structures was further resolved by observing how many microenvironments there are in which a particular sugar residue exists. For the former pattern, the answer would be *one* for each sugar; whereas, for the latter pattern there are *three* for each. A multiplicity of microenvironments would result in a broadening of the resonances for each proton in each sugar. The 1D and 2D proton studies of the polymer backbone (**5** and **7**), as well as the side chain (**6**) and backbone (**9**) fragments, all manifest only sharp and unique resonances for residue protons, when the multiplicity of anomerization in **9** is discounted. Particularly striking in this regard is the finding that Smith degradation (No. 3) of **5** results in a magnetically homogenous product (**7**), as is best seen in Fig. 5. Thus, we can conclude that there is only a single hexasaccharide repeating unit.

Taking all of the chemical and NMR data together, we conclude that the structure of the EPS (**1**) and its derivatives are as shown in Fig. 6.

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Hexadecanol and hexadecyl formate in the venom gland of formicine ants

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SUMMARY

The venom apparatus of workers of five representative species of formicine ant, *Anoplolepis custodiens*, *Camponotus vagus*, *Formica polyctena*, *Lasius niger* and *Polyrhachis gagates*, have all been shown to contain a mixture of hexadecanol, its formate and acetate, unaccompanied by homologous compounds. These compounds are not found in venom or in the venom reservoir but in the convoluted gland. It is suggested that the gland is lined with hexadecanol and its esters to protect the tissue from the corrosive venom.

1. INTRODUCTION

Wray (1670) first described the venom of formicine ants when he distilled wood ants and obtained a new acid which he called formic. Today it is recognized that all formicine ants have a venom consisting of a strong aqueous solution of formic acid, of concentration up to 60% (Stumper 1951, 1952; Osman & Brander 1961). Cavill & Clark (1971) reported that formic acid is the only volatile compound present in formicine venom, but the presence of peptides and free amino acids have also been reported in the venoms of *Formica polyctena* (Osman & Brander 1961) and *Campo-notus pennsylvanicus* (Hermann & Blum 1968). Acetic acid is a minor component in at least some cases, as A. B. Attygalle (personal communication) has identified it in the venom of *Camponotus floridanus* and we have identified it in the venom of *Anoplolepis custodiens* (unpublished results). In the present work we have sometimes identified both formic and acetic acids.

Formic acid is also used as a defensive secretion of some other arthropods, including millipeds (Polydesmidae), Coleoptera and certain moth larvae (Blum & Hermann 1978). In the case of a carabid beetle producing a mixture of formic acid and alkyl esters as a defensive secretion, Attygalle *et al.* (1992) have again shown that 1% of the secretion is acetic acid.

The venom apparatus of ants consists essentially of three parts, the tubular filaments, attached at one end to the convoluted gland and otherwise floating in the haemolymph, the convoluted gland, which Billen (1990) has shown for *Solenopsis*, has the same cellular structure as the filaments but is coiled into a compact ball and attached to the third part, the reservoir, in which the venom is stored. Hefetz & Blum (1978) have shown that the formic acid of formicine ants is

biosynthesized in the venom gland from a C_1 source with the aid of folic acid. It is presumed that synthesis occurs in the filaments and convoluted part of the gland. Hefetz & Blum (1978) refer to intact and homogenized glands but do not say if the reservoir was included or not.

In the course of examination of some formicine ants we found that either extracts of venom glands or whole venom apparatuses contained a small amount of hexadecyl formate accompanied by hexadecanol and hexadecyl acetate. Closer examination has shown that these compounds are not in the venom or its reservoir but are located in the convoluted gland. We have demonstrated their presence in five representative genera of the subfamily Formicinae.

2. MATERIALS AND METHODS

Insects were obtained from colonies of *Anoplolepis custodiens* and *Polyrhachis gagates* collected in South Africa and sent live to Keele. *Camponotus vagus* and *Lasius niger* collected in southern France and *Formica polyctena* from Belgium were sent live to Keele where they are maintained in artificial nests until dissection.

Individual venom glands and parts of glands were dissected under water with the aid of a binocular dissecting microscope and venom collected from the reservoir in glass capillaries as described by Morgan (1990). The dissected materials were freed of moisture and either solvent extracted in a small tissue grinder with hexane (100 µl) or sealed in glass capillaries for gas chromatography, using the solvent-less solid sampling method of Morgan & Wadhams (1972).

Gas chromatography-mass spectrometry was done with a Hewlett Packard 5890 gas chromatograph coupled to a 5970B Mass Selective Detector, which was controlled with a HP 59970C Chemstation. Chromatography was done on a fused silica capillary

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177

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Table 1. *Average amounts of hexadecanol, its formate and acetate and their average proportions, in the venom glands of some formicine species*

(Results are averages of five determinations.)

species	amount µg			proportion %		
	$C_{16}OH$	C_{16} formate	C_{16} acetate	$C_{16}OH$	C_{16} formate	C_{16} acetate
Anaplolepis custodiens	0.12	0.90	0.1	11	82	9
Camponotus vagus	1.8	0.27	0.2	79	12	9
Formica polyctena	0.08	1.84	—	4	96	—
Lasius niger	0.7	1.1	0.03	38	60	2
Polyrhachis gagates	1.1	2.2	trace	33	67	—

column (12 m × 0.22 mm i.d.) coated with immobilized polydimethylsiloxane. The injection port temperature was 250°C. The sample capillary was held in the injection port for 3 min before crushing it. The column was initially at 30°C for 2 min after crushing, then raised to 250°C at 3°C min^{-1}.

The identification of compounds was confirmed by comparison of their mass spectra and retention times with those of commercial or synthesized materials.

Hexadecyl formate and hexadecyl acetate were prepared by heating hexadecanol (5 mg), formic acid (2 µl) and acetic acid (2 µl) together with a drop of concentrated sulphuric acid in a Keele Microreactor (Attygalle & Morgan 1986) for 12 h at 100°C. The reaction mixture was neutralized with $NaHCO_3$, extracted with hexane (200 µl) and a portion (1 µl) was injected (splitless) onto the chromatography column.

3. RESULTS

Three compounds identified as hexadecanol, hexadecyl formate and hexadecyl acetate were first observed in solvent extracts of *A. custodiens* and *P. gagates*. Three other species of formicine ant were then selected at random to represent other genera, i.e. *C. vagus, F. polyctena* and *L. niger*, and in each the same substances were identified. Hexadecanol is not an unusual substance in hymenopteran exocrine glands (Wheeler & Duffield 1988; Blum 1981), nor are various of its simple esters, but these are normally accompanied by homologous compounds (tetradecanol, octadecanol, etc.), but in these venom glands we found no evidence of homologues at all.

The proportions of the three substances varied with sample and species. Some values are given in table 1, together with the amounts of these substances per individual gland. The amounts did not vary greatly; the largest amount was 4.7 µg of hexadecyl formate in a sample of *F. polyctena*. The smallest were two samples of *A. custodiens* which contained 0.4 µg of hexadecyl formate, the major compound. The mean proportions hide a wide variation in the actual proportions found. For example, three out of four samples of *C. vagus* contained no hexadecyl formate. Hexadecyl acetate was sometimes absent, frequently an unquantifiable trace component, but in one sample of *A. custodiens* and one of *C. vagus* it was over 20%.
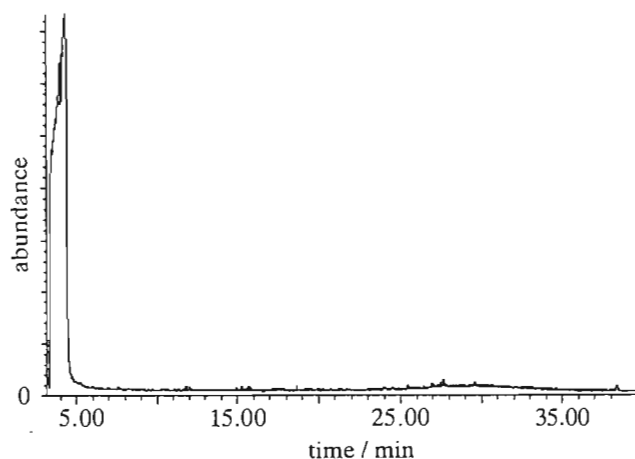

Figure 1. Total ion chromatogram of the secretion from a single venom reservoir of a worker of *Formica polyctena*. The only substance visible is formic acid (large peak at 4 min).

Frequently very small amounts of undecane and other hydrocarbons were present. These from their pattern were easily recognized as Dufour gland contaminants and indicate contamination with a very small proportion of the Dufour gland contents.

To investigate the location of the hexadecyl formate further, the venom was removed from the reservoir of
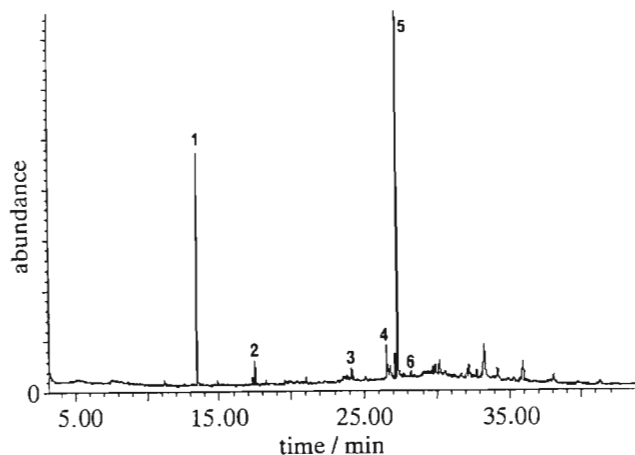

Figure 2. Total ion chromatogram of the empty venom apparatus of a single worker of *Formica polyctena* after the removal of the venom. Numbered peaks are: 1, undecane; 2, tridecane; 3, heptadecane (all contaminants from the Dufour gland secretion); 4, 1-hexadecanol; 5, hexadecyl formate; 6, hexadecyl acetate. Other peaks are produced by silicone products from the stationary phase.
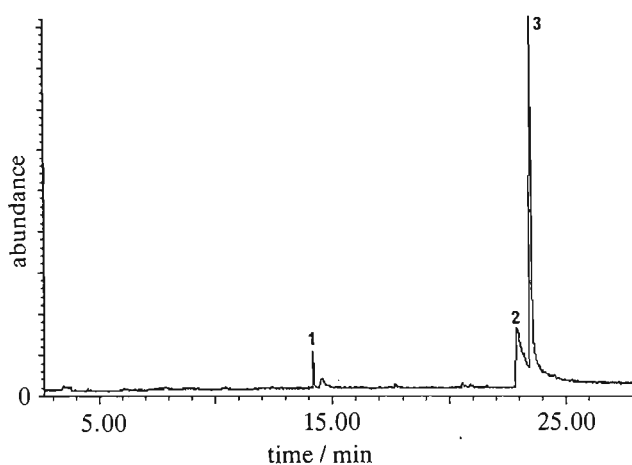
Figure 3. Total ion chromatogram from a single convoluted gland portion of the venom apparatus of a worker of *P. gagates*. Numbered peaks are: 1, tridecane; 2, hexadecanol; 3, hexadecyl formate.

some glands of *F. polyctena* and *P. gagates*, and the venom and the empty glands were chromatographed separately. The venom surprisingly did not contain the alcohol and esters (figure 1), but they were in the gland itself (figure 2). Further dissection of the gland located the compounds in the convoluted gland portion (figure 3), although it was absent from the reservoir and the filaments.

4. DISCUSSION

The venom glands of a random selection of five species of formicine ants have all been shown to contain microgram quantities of hexadecanol, hexadecyl formate and hexadecyl acetate in variable proportions. One of us (J.M.B.) has also identified hexadecyl formate in two further species, *Camponotus arminius* and *Polyrhachis schistacea*. These compounds are not located in the venom itself but are found in the convoluted gland. We did not find them in the filament part of the gland, but the filaments are much smaller than the convoluted section, and that makes a firm statement about the filaments more difficult. It was also surprising to find the hexadecanol and its esters unaccompanied by C_{14} or C_{18} homologues, as is usually found in nature for long-chain alcohols or carboxylic acids.

From our experiments, no explanation for the presence of these compounds was forthcoming. Water-insoluble long-chain compounds with an apolar chain and a polar end group, such as hexadecanol, form ordered monolayers on aqueous solutions. These monolayers of close-packed oriented molecules are known as Langmuir-Blodgett films. The films have unusual properties, for example, they inhibit evaporation and act as insulators. Although the venom reservoir has a thick cuticular lining, it would appear from Billen (1990) that the convoluted gland has no such protection. It is tempting to suggest (but difficult to envisage a technique for testing) that hexadecanol forms a monolayer film on the secretory ducts to protect cells from the corrosive venom. Contact of hexadecanol with the venom would convert some of it

to formate and acetate. In some cases both these acids could be observed in the venom by their mass spectra. Langmuir-Blodgett films are best prepared from compounds with homogeneous chain length. This would be consistent with finding only C_{16} chains. The polar hydroxyl groups would be directed towards the venom. If these became esterified with polar formate and acetate groups they would alter the monolayer very little.

We have examined the venom glands of a large number of other ants, chiefly myrmicines, both those with alkaloidal and protein venoms and those such as attines with only vestigal stings, but have not encountered the present compounds in them. Hexadecanol and its formate and acetate in the venom gland have no correlation with the presence of esters or alcohols in Dufour gland secretions. Only hydrocarbons have been identified in the Dufour glands of *A. custodiens* (Schreuder & Brand 1972) and *F. polyctena* (Francke *et al.* 1980). Those of *C. vagus* (B. D. Jackson, unpublished results) and *L. niger* (Attygalle *et al.* 1987) contain hexadecanol and its acetate, but there, as in some other formicines, e.g. *Formica rufa* (Francke *et al.* 1985), a whole range of homologous alcohols and esters are found. *P. gagates* and *P. schistacea* Dufour glands contain primarily a series of hydrocarbons (L. C. Lopez, E. D. Morgan & J. M. Brand, unpublished results).

The mass spectra of alcohols, alkenes and formates are all very similar (Bagnères *et al.* 1991); the formates are best recognized by their retention times in gas chromatography and the very weak but characteristic mass spectral ions at m/z 47, corresponding to protonated formic acid.

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Oxidation of Polychlorinated Biphenyls by *Pseudomonas* sp. Strain LB400 and *Pseudomonas pseudoalcaligenes* KF707

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Biphenyl-grown cells and cell extracts prepared from biphenyl-grown cells of *Pseudomonas* sp. strain LB400 oxidize a much wider range of chlorinated biphenyls than do analogous preparations from *Pseudomonas pseudoalcaligenes* KF707. These results are attributed to differences in the substrate specificity of the biphenyl 2,3-dioxygenases from both organisms.

Current interest in the microbial degradation of polychlorinated biphenyls (PCBs) stems from the fact that many members of this class of compounds are persistent environmental pollutants (14). Most studies on the degradation of PCBs by aerobic bacteria have been conducted with biphenyl-grown cells. For example, biphenyl-grown cells of *Pseudomonas* sp. strain LB400 oxidize a wide range of PCBs, including congeners that contain four to six chlorine substituents (4, 5). The same PCBs are oxidized by *Alcaligenes eutrophus* H850 (2–4). The genes (*bph*) encoding the enzymes responsible for the oxidation of biphenyl to benzoate in LB400 have been cloned (12) and shown to hybridize strongly to the genome of H850 but not to DNA from four other bacterial strains known to degrade PCBs (17). These observations led to the suggestion that DNA probes containing the LB400 *bph* genes can be used to determine the distribution and fate of LB400 and H850 *bph* genes in the environment and also to facilitate the quantitation and isolation of new strains containing related genes with potentially greater degradative activity (12, 17).

The initial reaction in the degradation of biphenyl by bacteria, including LB400, involves the enzymatic incorporation of both atoms of molecular oxygen into the aromatic nucleus to form *cis*-(2*R*, 3*S*)-dihydroxy-1-phenylcyclohexa-4,6-diene (*cis*-biphenyl dihydrodiol) (8, 9, 18). The enzyme that catalyzes this reaction, biphenyl 2,3-dioxygenase, has not been studied in detail (9). However, the nucleotide sequences of the *bph* genes encoding biphenyl 2,3-dioxygenase in LB400 (6) and a different pseudomonad, *Pseudomonas pseudoalcaligenes* KF707 (16), have been determined. The sequences from both organisms show significant homology (52 to 66% identity at the predicted amino acid level) to the structural genes (*todC1C2BA*) encoding the multicomponent toluene dioxygenase from *Pseudomonas putida* F1 (20). These data suggest that the biphenyl 2,3-dioxygenases in LB400 and KF707 are organized as shown in Fig. 1. Thus, it is not surprising to find that the *todC1C2BADE* genes from F1 hybridize to the isofunctional genes in LB400 (19). However, the relationship does not

extend to enzyme activity. The F1 toluene dioxygenase cannot oxidize chlorinated biphenyls containing more than two chlorine substituents (19), and toluene is not a substrate for the biphenyl 2,3-dioxygenase in LB400 cell extracts. There is considerably greater homology, at the nucleotide and predicted amino acid levels, between the individual components of the biphenyl 2,3-dioxygenases from LB400 and KF707 (Table 1). Nevertheless, the results presented below indicate that there are wide differences between the substrate specificities of the biphenyl 2,3-dioxygenases of the two organisms.

Cultures of *Pseudomonas* sp. strain LB400 (5) and *P. pseudoalcaligenes* KF707 (7) were provided by Herman L. Finkbeiner, Research and Development Center, General Electric Company, Schenectady, N.Y., and Kensuke Furukawa, Department of Agricultural Chemistry, Kyushu University, Fukuoka, Japan, respectively. Both organisms were grown in a mineral salts medium (15) containing 0.005% (wt/vol) yeast extract and 0.3% (wt/vol) biphenyl. Cells were harvested in the logarithmic phase of growth, washed twice with 50 mM sodium phosphate buffer (pH 7.5), and resuspended in the same buffer to a turbidity of 1.0 at 600 nm. The transformation procedures with defined PCB congener mixes 1B and 2B were essentially those of Bedard et al. (4). PCB congeners were separated on a 25-m (0.2-mm inside diameter) nonpolar fused silica capillary column at 210°C with inlet and electron capture detector temperatures set at 250°C. The carrier gas was helium (0.7 ml/min), and injections were split 100:1. The makeup gas for the electron capture detector was nitrogen (24 ml/min). The reported depletion percentages were calculated by normalization to the recovery of 2,4,6,2',4'-pentachlorobiphenyl, a nondegradable internal standard, from experimental and perchloric acid-treated controls.

Table 2 shows the results obtained when biphenyl-grown cells of LB400 and KF707 are incubated for 24 h with congener mixtures 1B and 2B. The congeners oxidized by LB400 are the same as those reported previously for this organism (4, 5), with minor differences in the extent of degradation observed with 4,4'-dichlorobiphenyl and some of the more highly chlorinated molecules. KF707 has been shown to oxidize 4-, 3,4-, 2,4,5-, 2,3-, 2,4'-, and 2,4,4'-chlorobiphenyls (7). The first three substrates, 4-, 3,4-, and 2,4,5-chlorobiphenyls, are not components of congener mixes 1B and 2B and were not investigated in this study. However, we were able to confirm the oxidation of 2,3-,
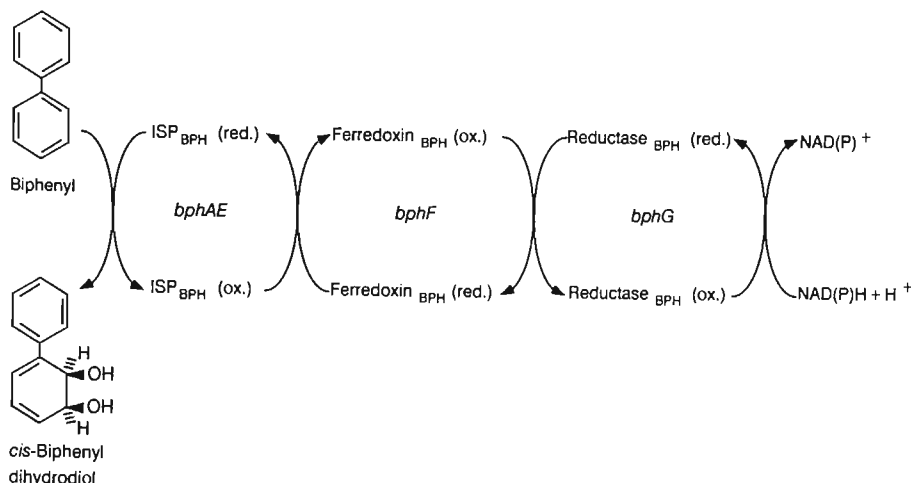
FIG. 1. Proposed organization of the biphenyl 2,3-dioxygenase complex in *Pseudomonas* sp. strain LB400 and *P. pseudoalcaligenes* KF707. The flow of electrons from NAD(P)H through ferredoxin$_{BPH}$ reductase (Reductase$_{BPH}$) and a [2Fe-2S] ferredoxin (Ferredoxin$_{BPH}$) to the terminal oxygenase component (ISP$_{BPH}$) is based on homology of the *bph* genes with the *todC1C2BA* genes that encode the individual components of toluene dioxygenase (6, 16, 20). The *bph* genes in LB400 have been identified as *bphA* (ISP$_{BPH}$, large subunit), *bphE* (ISP$_{BPH}$, small subunit), *bphF* (ferredoxin$_{BPH}$), and *bphG* (ferredoxin$_{BPH}$ reductase). The isofunctional *bph* genes in KF707 have been designated *bphA1A2A3A4* (16). Abbreviations: ox., oxidized; red., reduced.

2,4'-, and 2,4,4'-chlorobiphenyls by KF707. A comparison of the results presented in Table 2 reveals that LB400 and KF707 show similar substrate specificities with respect to the oxidation of 2,3-, 2,4'-, 2,4,4'-, 2,5,4'-, 2,3,2',3'-, and 2,4,3',4'-chlorobiphenyls. However, LB400 also oxidizes 2,2'- and 2,5,2'-chlorobiphenyls and congeners containing chlorine substituents at the 2,5 positions on one ring and two or three chlorine substituents at the positions on the second ring shown in Table 2. With the exception of 2,5,4'-trichlorobiphenyl, KF707 shows little or no oxidation of these substrates. The greater versatility of LB400 with respect to PCB degradation is also seen in the oxidation of 2,4,2',4'-, 2,4,5,2',3'-, and 2,4,5,2',4',5'-chlorobiphenyls, which are not oxidized by KF707. However, KF707 is superior to LB400 with respect to the degradation of 4,4'-dichlorobiphe-

nyl. It has been noted previously for LB400 that the presence of chlorine at the 2 position enhances the degradation of 2,4,4'-trichlorobiphenyl relative to 4,4'-dichlorobiphenyl (4).

We recently described the preparation of LB400 cell extracts that oxidize biphenyl at the 2,3 position (9). This procedure, used to prepare cell extracts from biphenyl-grown cells of KF707 and LB400, enabled us to examine the possibility that the differences in congener specificity between LB400 and KF707 are due to differences in the efficiency of transport of the various chlorinated biphenyls. Transformations were performed in duplicate as described above with incubation times of 0.5 and 2 h. Reaction mixtures contained cell extract (LB400, 1.1 mg of protein; KF707, 0.94 mg of protein), NADH (1.0 mM), and ferrous ammonium sulfate (0.4 mM) in 1.0 ml of 50 mM 2-(*N*-

TABLE 1. Comparison of the nucleotide and predicted amino acid sequences of the *bph* genes encoding the individual components of biphenyl 2,3-dioxygenase from *Pseudomonas* sp. strain LB400 and *P. pseudoalcaligenes* KF707[a]

Function	Sequence						Sequence difference	
	LB400			KF707				
	Gene	Region[b]	Size[b]	Gene	Region	Size	Nucleotides	Amino acids
Large subunit	*bphA*	1439–2818	1,380 (459)[c]	*bphA1*	500–1876	1,377 (458)	46 (3)[d]	19 (1)[e]
Small subunit	*bphE*	2936–3502	567 (188)	*bphA2*[f]	1994–2560	567 (188)	2	1
Unknown	ORF1[g]	3540–3959	420 (139)	ORF3	2598–3017	420 (139)	1	1
Ferredoxin	*bphF*	4034–4363	330 (109)	*bphA3*	3092–3421	330 (109)	2	1
Reductase	*bphG*	4360–5586	1,227 (408)	*bphA4*	3418–4644	1,227 (408)	2	1

[a] Nucleotide and amino acid sequences were compared by an alignment of the respective sequences by the GAP program of the University of Wisconsin Genetics Computer Group. Differences between the sequences were noted by visual inspection. Sequences were obtained from GenBank, using accession numbers M83673 and M86348 for KF707 and LB400, respectively.

[b] In nucleotides.

[c] The number of amino acids is given in parentheses.

[d] The nucleotide sequences of the *bphA* and *bphA1* genes, encoding the large subunits of the terminal oxygenases from LB400 and KF707, respectively, show 46 bases that are mismatched. In addition, three adjacent bases in *bphA* are not present in *bphA1*.

[e] The amino acid sequences of the large subunits show 19 amino acids that are mismatched and that the LB400 large subunit contains one amino acid more than the KF707 large subunit.

[f] Open reading frame analysis identified nucleotide 1919 as the starting point for the KF707 *bphA2* gene (16). However, comparison with the highly homologous *todC2* gene, for which the starting point is known (20), indicates that *bphA2* begins at position 1994. This is also the position reported for the isofunctional *bphE* gene in LB400 (6). Thus, for the purpose of comparison, we have used position 1994 as the starting nucleotide for the KF707 *bphA2* gene.

[g] LB400 and KF707 both have an open reading frame (ORF) with an unknown function between *bphE* (*bphA2*) and *bphF* (*bphA3*).

TABLE 2. Oxidation of PCB congeners by biphenyl-grown cells and cell extracts of LB400 and KF707

Congener	% Depletion[a]			
	LB400 cells	LB400 extracts[b]	KF707 cells	KF707 extracts[b]
2,3-	100	100	100	100
2,4'-	100	100	100	100
2,2'-	100	100	18	0
2,5,2'-	100	100	10	0
2,5,4'-	98	100	100	54
2,3,2',3'-	100	100	81	50
2,3,2',5'-	100	100	9	0
2,5,3',4'-	100	100	18	9
2,5,2',5'-	100	100	9	0
2,4,5,2',5'-	100	98	0	0
2,3,4,2',5'-	97	96	0	0
2,4,5,2',3'-	50	53	0	0
4,4'-	25	11	100	71
2,4,4'-	89	54	99	94
2,4,2',4'-	81	86	0	0
2,4,3',4'-	43	34	31	24
3,4,3',4'-	6	11	0	0
2,4,5,2',4',5'-	41	61	0	0
2,4,6,2',4'- (IS)[c]	0	0	0	0

[a] Combined data obtained from the incubation of intact cells and cell extracts with congener mixtures 1B and 2B. Congener depletion differences between LB400 and KF707 (for both intact cells and cell extracts) are boxed. Values for LB400 extracts were the same at 0.5 and 2 h. Values for KF707 extracts were determined at 0.5 and 2 h. The 2-h values are shown.

[b] The specific activities of biphenyl 2,3-dioxygenase in cell extracts of LB400 and KF707 were 2.5 and 1.4 nmol of cis-biphenyl dihydrodiol formed per min per mg of protein, respectively (9).

[c] (IS), internal standard.

morpholino)ethanesulfonic acid (MES) buffer (pH 6.0). The KF707 mixtures also contained 1.0 μM flavin adenine dinucleotide. The congener specificities of LB400 and KF707 cell extracts are significantly different and parallel the results obtained with intact cells (Table 2). Thus, differences in congener transport are not responsible for the differences in PCB oxidation observed with LB400 and KF707.

The defined congener depletion assay used in this study provides valuable comparative information on the substrate specificity of the enzyme(s) responsible for the initial reaction in the degradation of a wide range of PCB congeners. Care must be taken when one compares the extents of degradation of different congeners, since Bedard et al. (4) have shown that the results can be influenced by other congeners present in the reaction mixture. Nevertheless, the patterns of congener depletion observed with LB400 cells and extracts are similar to each other and clearly different from the results of analogous experiments conducted with KF707.

The wide range of PCB congeners oxidized by LB400 may be due to the presence of more than one enzyme capable of initiating PCB degradation or to the ability of biphenyl 2,3-dioxygenase to catalyze hydroxylation at other positions on the biphenyl molecule (1, 13). Although the existence in LB400 of more than one enzyme capable of initiating the oxidation of PCBs cannot be ruled out at this time, it seems unlikely for the following reasons. First, the only biphenyl dioxygenase that we have been able to detect in LB400 cell extracts is the multicomponent 2,3-dioxygenase system predicted from sequence data (6, 9). Second, homogeneous preparations of the oxygenase component of the LB400 biphenyl 2,3-dioxygenase system oxidize 2,5,2',5'-tetrachlo-

robiphenyl at the 3,4 position (8a). Third, all attempts to demonstrate, by mutagenesis and subcloning, the presence of more than one oxygenase in LB400 have been unsuccessful (12).

The differences in congener specificity between LB400 and KF707 probably reflect differences in the active sites of the oxygenase components of the biphenyl 2,3-dioxygenase present in both organisms. It has been suggested that the small subunit of the oxygenase component of toluate 1,2-dioxygenase may play a role in substrate recognition (10). This seems unlikely for biphenyl 2,3-dioxygenase, as the small subunits of the oxygenase components of LB400 and KF707 differ by only a single amino acid (6, 16). There are 20 amino acid differences between the predicted sequences for the large subunits of the terminal dioxygenases from LB400 and KF707, but most of them can be regarded as conservative. Histidine and tyrosine residues are probable ligands for binding iron at the active site in other multicomponent systems similar to biphenyl 2,3-dioxygenase (11). Thus, the change of histidine and phenylalanine at positions 255 and 277 in the KF707 large subunit to glutamine and tyrosine in the LB400 large subunit may play a role in determining the substrate specificity of each enzyme. We are currently purifying the biphenyl 2,3-dioxygenases from LB400 and KF707 to determine the factors responsible for substrate specificity.

Finally, the results suggest that DNA-DNA hybridization experiments may produce unreliable results when they are used to determine the presence and biodegradation potential of bacteria in environments contaminated with PCBs.

ADDENDUM IN PROOF

Similar results to those presented here for intact cells of LB400 and KF707 were reported in a poster by F. J. Morello and B. D. Erickson at a conference, The Role of Reducing Toxicity and Exposure to Environmental Pollutants, held at the National Institute of Environmental Health Sciences, 26 and 27 April 1993.

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Regiospecific and Stereoselective Hydroxylation of 1-Indanone and 2-Indanone by Naphthalene Dioxygenase and Toluene Dioxygenase

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The biotransformation of 1-indanone and 2-indanone to hydroxyindanones was examined with bacterial strains expressing naphthalene dioxygenase (NDO) and toluene dioxygenase (TDO) as well as with purified enzyme components. *Pseudomonas* sp. strain 9816/11 cells, expressing NDO, oxidized 1-indanone to a mixture of 3-hydroxy-1-indanone (91%) and 2-hydroxy-1-indanone (9%). The (*R*)-3-hydroxy-1-indanone was formed in 62% enantiomeric excess (ee) (*R:S*, 81:19), while the 2-hydroxy-1-indanone was racemic. The same cells also formed 2-hydroxy-1-indanone from 2-indanone. Purified NDO components oxidized 1-indanone and 2-indanone to the same products produced by strain 9816/11. *P. putida* F39/D cells, expressing TDO, oxidized 2-indanone to (*S*)-2-hydroxy-1-indanone of 76% ee (*R:S*, 12:88) but did not oxidize 1-indanone efficiently. Purified TDO components also oxidized 2-indanone to (*S*)-2-hydroxy-1-indanone of 90% ee (*R:S*, 5:95) and failed to oxidize 1-indanone. Oxidation of 1- and 2-indanone in the presence of [^{18}O]oxygen indicated that the hydroxyindanones were formed by the incorporation of a single atom of molecular oxygen (monooxygenation) rather than by the dioxygenation of enol tautomers of the ketone substrates. As alternatives to chemical synthesis, these biotransformations represent direct routes to 3-hydroxy-1-indanone and 2-hydroxy-1-indanone as the major products from 1-indanone and 2-indanone, respectively.

Toluene dioxygenase (TDO) and naphthalene dioxygenase (NDO) are multicomponent enzyme systems which catalyze the enantiospecific incorporation of dioxygen into toluene and naphthalene to form (+)-*cis*-(1*S*,2*R*)-dihydroxy-3-methylcyclohexa-3,5-diene (*cis*-toluene dihydrodiol [9, 15]) and (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene (*cis*-naphthalene dihydrodiol [14]), respectively. The absolute relationship of the hydroxyl groups is the same in each dihydrodiol, and the configurational differences are due to the priorities established by the Prelog-Cahn-Ingold sequence rules. Interest in the reactions catalyzed by TDO, NDO, and other bacterial dioxygenases is related to the use of arene-*cis*-diols as chiral synthons in the preparation of a wide range of compounds not easily obtained by chemical synthesis. Examples include inositols, conduritols, acyclic sugars, and a wide range of biologically active natural products (6, 7, 21).

The reactions catalyzed by TDO and NDO are not limited to *cis*-hydroxylation of the aromatic nucleus. For example, TDOs from *Pseudomonas putida* F1 and *P. putida* UV4 catalyze the oxidation of indene (1, 27) and indan (4, 27). In addition, F1 oxidizes indole (8), and UV4 oxidizes related benzocyclic (4) and heterocyclic (2, 3) aromatic compounds. The substrate specificity of NDO from *Pseudomonas* sp. strain NCIB 9816 has not been examined in detail. However, strain 9816/11, a mutant that lacks a functional *cis*-naphthalene dihydrodiol dehydrogenase, oxidizes indan to (*S*)-1-indanol (96% enantiomeric excess [ee]), whereas *P. putida* F39/D and UV4, which

lack a functional *cis*-toluene dihydrodiol dehydrogenase, oxidize indan to (*R*)-1-indanol (80 and 98% ee, respectively) (4, 27).

Previous reports on the microbial transformation of indan have made no reference to the formation of hydroxyindanones. Typical microbial transformation products identified from indan include 1-indanol, 1-indanone, and *trans*-1,3-indandiol (4, 5, 27). In contrast, it has been reported that when male rats were treated with indan, the major urinary metabolites formed were 2-hydroxy-1-indanone and 3-hydroxy-1-indanone in a ratio of approximately 2:1 (20, 28).

We recently detected the formation of 1-indanone and two hydroxyindanones as minor metabolites when salicylate-induced cells of strain 9816/11 were incubated with (±)-1-indanol for 24 h. This observation, coupled with the fact that chemical syntheses of 2-hydroxy-1-indanone (17, 26) and 3-hydroxy-1-indanone (16) require several steps, often with air-sensitive reagents, prompted the present study of the regiospecific oxidation of 1- and 2-indanone and the enantiomeric composition of the hydroxyindanone products formed by NDO and TDO. The results presented provide the first detailed description of the bacterial oxidation products formed from 1- and 2-indanone.

MATERIALS AND METHODS

Growth of *Pseudomonas* strains and transformation of substrates. *Pseudomonas* sp. strain 9816/11 is a spontaneous mutant which oxidizes naphthalene to *cis*-naphthalene-1,2-dihydrodiol and lacks *cis*-naphthalene dihydrodiol dehydrogenase activity (8a). *P. putida* F39/D is a mutant which oxidizes toluene quantitatively to *cis*-toluene dihydrodiol (9). Strains 9816/11 and F39/D and the wild-type strains NCIB 9816-4 and F1 were grown in mineral salts medium (MSB) (22) and

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induced during growth with 0.05% (wt/vol) salicylate and toluene vapor, respectively. Growth in MSB containing 0.05% (wt/vol) 1- and 2-indanone as the sole carbon source was also determined. Resting cell incubations were carried out using washed cells suspended (turbidity, 2.0 to 2.5 at 600 nm) in 50 or 500 ml of 50 mM sodium-potassium phosphate buffer (pH 7.2), in 250-ml Erlenmeyer or 2.8-liter Fernbach flasks, respectively, containing 0.05% of either 1- or 2-indanone and 0.2% (wt/vol) pyruvate. Racemic (synthetic) 2- and 3-hydroxy-1-indanones were provided at 0.01% under identical conditions. After 24 h of incubation (30°C, 220 rpm), cells were removed by centrifugation and the supernatants were extracted three times with sodium hydroxide-washed ethyl acetate. Solvent was dried over anhydrous sodium sulfate (Na_2SO_4) and concentrated under reduced pressure (30°C) prior to analysis of transformation products.

[^{18}O]Oxygen studies with purified NDO and TDO. The terminal oxygenase (ISP_{NAP}) of NDO was purified from *Escherichia coli* JM109(DE3)(pDTG121) as previously described by Suen and Gibson (24). $Reductase_{NAP}$ and $ferredoxin_{NAP}$ were purified from recombinant *E. coli* strains expressing individual or multiple NDO components (23). The ISP_{TOL} and $reductase_{TOL}$ components of TDO were purified from isopropyl-β-D-thiogalactoside (IPTG)-induced cells of *E. coli* JM109 (pDTG601A) which express the cloned *todC1C2BA* genes (29). $Ferredoxin_{TOL}$ was purified from JM109(pDTG614) (29). Specific details of these purification procedures will be published elsewhere. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis showed that all components of TDO and NDO were homogeneous. When used alone or together, the purified reductase and ferredoxin components showed no activity on the substrates used in this study.

Transformation reactions were carried out in 1-dram (3.7-ml) glass vials fitted with Teflon-lined self-sealing septa and screw-on open-top caps (Altech Associates, Inc., Deerfield, Ill.). The reaction mixtures contained the following in a final volume of 1.0 ml: 20 μg of $reductase_{NAP}$, 35 μg of $ferredoxin_{NAP}$, 50 μg of ISP_{NAP}, 0.75 mM NADH, and 0.25 mM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ in 50 mM 2-(4-morpholino)ethanesulfonic acid buffer (pH 6.8). Concentrations of $reductase_{TOL}$, $ferredoxin_{TOL}$, and ISP_{TOL} were 20, 35, and 50 μg, respectively. After flushing the headspace of each vial with dry nitrogen gas (3 min), 2.0 ml of the nitrogen headspace was removed with a gas-tight syringe and replaced with 2.5 ml of 98 atom% [^{18}O]oxygen (Icon Isotopes, Summit, N.J.). The oxygen composition of the headspace of each reaction vial was analyzed at the beginning and end of the experiment. Reactions were initiated by addition of 0.5 mM 1-indanone or 2-indanone dissolved in 10 μl of methanol. The reaction vials were incubated horizontally at 24°C with gentle agitation (60 rpm) for 2 h. The reactions were terminated by the addition of 2.0 ml of ethyl acetate through the Teflon-lined septa. The vials were gently shaken, the ethyl acetate was removed, and the aqueous phase was extracted two more times. The combined extracts were dried over Na_2SO_4 and concentrated (35°C under dry nitrogen) to approximately 100 μl prior to analysis (see below).

Analysis, purification, and identification of metabolites. Thin-layer chromatography (TLC) of extracts was performed on silica gel 60 F_{254} sheets (E. Merck, no. 5735) with chloroform-acetone (80:20) as the developing solvent. Compounds were visualized by observing quenching of fluorescence under UV light (254 nm) and by exposure to iodine vapor. Hydroxyindanones were purified by radial-dispersion chromatography (RDC) using a Chromatotron (Harrison Research, Palo Alto, Calif.). Ethyl acetate extracts were applied to 2.0-mm-thick silica plates and eluted with a chloroform-acetone step gradi-

ent (0 to 30% acetone; 10% steps over 1 h) at a flow rate of 7 ml/min. Fractions (ca. 8 ml) were analyzed by TLC prior to further characterization of the different hydroxyindanones. Hydroxyindanones were isolated from enzymic reaction mixtures by preparative-layer chromatography (PLC) (0.5-mm silica thickness; Merck) using multiple elution (three developments) with chloroform-acetone (95:5).

N,O-bis-(Trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA plus 1% TMSCl; Pierce Chemical, Rockford, Ill.) was used to generate trimethylsilyl (TMS)-ether derivatives. The (S)-(+)- and (R)-(−)-methoxyphenylacetate (O-methylmandelate) esters of 2-hydroxyindanone were synthesized essentially as described by Trost et al. (25). For example, 2-hydroxy-1-indanone (compound II; 9 mg, 0.061 mmol), (S)-(+)- and (R)-(−)-methoxyphenylacetic acids (11 mg, 0.067 mmol, 1.1 eq), N,N-dicyclohexylcarbodiimide (13 mg, 0.061 mmol, 1.0 eq), and 4-dimethylaminopyridine (1 mg, 0.008 mmol, 0.1 eq) were dissolved in 4 ml of methylene chloride, and the mixture was stirred at 22°C for 72 h. The esters of 3-hydroxy-1-indanone were prepared by treatment of an anhydrous tetrahydrofuran solution of 3-hydroxy-1-indanone (compound I; 20 mg, 0.14 mmol) at 0°C under argon with n-butyl lithium (8 mg, 0.126 mmol, 0.9 eq), and then the methoxyphenylacetyl chlorides (30 mg, 0.16 mmol, 1.2 eq) were added. Reaction mixtures were filtered through Celite 545 (Fisher Scientific, Fair Lawn, N.J.) with hexane elution, and the methoxyphenylacetyl esters (R_f value of 0.6 by TLC; chloroform-acetone, 8:2) were isolated by PLC (1.0-mm silica) using chloroform-acetone (95:5) as the developing solvent.

Gas chromatography-mass spectrometry (GC-MS) was performed on a Hewlett-Packard model 5890 gas chromatograph equipped with a Hewlett-Packard Ultra-1 capillary column (inside diameter, 0.2 mm by 25 m; film thickness, 0.33 μm). The column temperature was programmed from 70 to 240°C at 10°C/min with a helium flow of 25 cm/s. Temperatures of the injection port and transfer line were 220 and 280°C, respectively. Samples (1 μl) were injected at a split ratio of 50:1, and mass spectra were obtained using a Hewlett-Packard model 5970 mass selective detector with electron impact ionization (70 eV). Headspace atmosphere (for [^{18}O]oxygen incorporation studies) was similarly analyzed, except that the column temperature was 150°C.

Chiral stationary-phase high-performance liquid chromatography (CSP-HPLC) was performed with a Waters Associates 510 solvent pump, a U-6K injector, a model 490E multiwavelength detector, and Maxima 820 workstation software (for acquisition of data and peak integration). Enantiomers were separated on a Chiralcel OB column (25 by 4.6 cm; Chiral Technologies, Exton, Pa.) using a mobile phase of hexane and 2-propanol (9:1) at a flow rate of 0.5 ml/min. The detector monitored wavelengths at 210, 254, 270, and 280 nm. Under these conditions, the (R)- and (S)-enantiomers of 3-hydroxy-1-indanone eluted with retention volumes (R_v) of 11.9 and 14.3 ml, respectively. The (R)- and (S)-2-hydroxy-1-indanone enantiomers eluted in the same order, at 12.6 and 17.1 ml, respectively.

Proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance (NMR) spectra were recorded on a Bruker WM-360 spectrometer at 360.14 and 90.56 MHz, respectively. Chemical shifts (δ) are reported in parts per million with respect to tetramethylsilane, and coupling constants (J values) are given in hertz. Abbreviations for ^{1}H NMR signals are as follows: s, singlet; d, doublet; t, triplet; dd, doublet of doublets; and m, multiplet. Optical rotations at 25°C were determined using a Perkin-Elmer model 141 polarimeter. Absorption spectra were re-
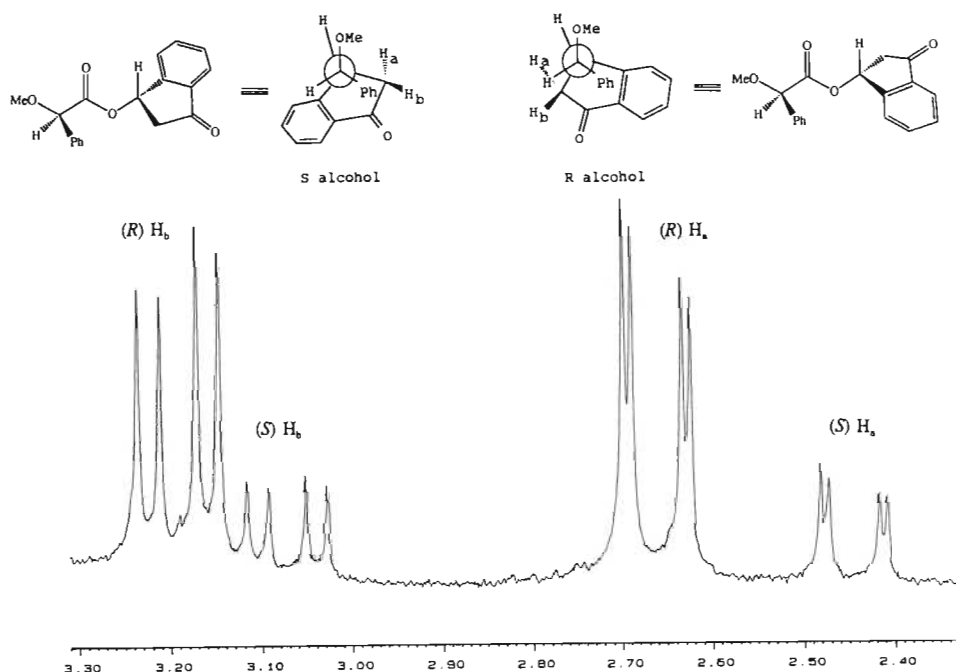
FIG. 1. Portion of the ^{1}H NMR spectrum (CDCl$_3$) of the (R)-(−)-methoxyphenylacetate ester of 3-hydroxy-1-indanone formed by strain 9816/11. The signals corresponding to the diastereomeric esters of (R)-3-hydroxy-1-indanone (dd, 2.65 and 3.20 ppm) and (S)-3-hydroxy-1-indanone (dd, 2.45 and 3.08 ppm) are indicated. Extended Newman projections have the intervening ester linkage omitted to make a more convenient view of the esters. See text for discussion of the method and results.

corded over the 200- to 350-nm range using a Beckman DU-70 spectrophotometer.

Syntheses of 3- and 2-hydroxy-1-indanone. (±)-3-Hydroxy-1-indanone was prepared from 1,3-indandione in glacial acetic acid by treatment with zinc dust as described by Lacy et al. (16). (±)-2-Hydroxy-1-indanone was synthesized by treating the TMS-enol ether of 1-indanone with m-chloroperoxybenzoic acid (19). The enol ether was prepared as previously described (13) by treatment of a tetrahydrofuran solution of 1-indanone at −78°C under argon with lithium diisopropylamide and then by quenching with TMSCl-triethylamine.

RESULTS

Growth studies and transformations by wild-type and mutant strains. *Pseudomonas* sp. strains 9816-4, 9816/11, F1, and F39/D grew well on 2-indanone but not on 1-indanone (data not shown). Transformation of 1- and 2-indanone by induced and uninduced cells of strains 9816-4 and F1 gave the same hydroxyindanone products observed with strains 9816/11 and F39/D; however, various additional products were detected. For this reason, mutant strains 9816/11 and F39/D, as well as purified NDO and TDO components, were used to focus on the initial hydroxylation reactions.

Transformations catalyzed by *Pseudomonas* sp. strain 9816/11. Incubation of salicylate-induced cells (500 ml) of *Pseudomonas* sp. strain 9816/11 with 1-indanone led to the accumulation of two polar products which showed R_f values of 0.4 (compound I) and 0.5 (compound II) by TLC in chloroform-acetone (8:2). GC and ^{1}H NMR analysis showed that compounds I and II were formed in a ratio of approximately 9:1. Compound I (52 mg) was isolated by RDC and had the following characteristics: GC-MS, retention time (R_t) of 12.8 min, molecular ion [M$^+$ (% relative intensity)] at m/z 148 (100) with major fragment ions at m/z 131 (15), 119 (37), 105 (53),

103 (24), 91 (46), 77 (46), 65 (13), 51 (35), and 50 (32); ^{1}H NMR (CDCl$_3$) δ 2.6 (dd, J = 18.9, 2.4 Hz, 1H), 3.11 (dd, J = 18.9, 6.8 Hz, 1H), 5.41 (dd, J = 6.5, 2.3 Hz, 1H), 7.48 (t, J = 6.9 Hz, 1H), 7.65–7.73 (m, 3H); ^{13}C NMR (CDCl$_3$) δ 46.96 (CH$_2$), 67.86 (CH), 122.5 (CH), 126.4 (CH), 129.0 (CH), 135.0 (CH), 136.6 (C), 156.8 (C), 203.0 (C=O); UV spectrum (MeOH) λ_{max}, 206, 243, 280 nm; [α]$_D$ −87.5° (c 2.0, CHCl$_3$). These data (with the exception of optical rotation) are identical to those of synthetic (±)-3-hydroxy-1-indanone, and our mass spectral data are in agreement with those previously reported (28). During GC-MS analysis, up to 16% of the total ion current area consisted of a compound with an R_t of 8.7 min and an M$^+$ at m/z 130. This compound was identified as 1-indenone and was shown to result from dehydration of compound I in the GC inlet. When the injection port was lowered to 140°C (from 220°C), less than 1% 1-indenone was produced.

The absolute configuration and enantiomeric composition of compound I formed by strain 9816/11 were determined by ^{1}H NMR analysis of its (R)- and (S)-methoxyphenylacetyl esters (25). Trost et al. have shown that the methoxyphenylacetyl esters of secondary alcohols assume a conformation in which the oxygen atoms are in alignment (see Fig. 1), most likely because of electronic effects (25). When this occurs, the phenyl group will eclipse substituents on either side of the alcohol, depending on the stereochemistry of the alcohol. The substituent which is eclipsed by the phenyl ring is then shifted upfield as a result of the shielding it experiences by the phenyl ring.

The ^{1}H NMR spectrum (CDCl$_3$) of the ester formed with (R)-methoxyphenylacetic acid (Fig. 1) revealed the presence of two sets of signals corresponding to the diastereomeric esters of (R)-3-hydroxy-1-indanone (δ 2.65 [dd, J = 19.0, 3.0 Hz, 1H], 3.20 [dd, J = 19.0, 7.3 Hz, 1H], 3.38 [s, 3H], 4.75 [s, 1H], 6.26 [dd, J = 7.0, 2.9 Hz, 1H], 7.07–7.65 [m, 9H]) and (S)-3-hydroxy-1-indanone (δ 2.45 [dd, J = 19.0, 3.0 Hz, 1H], 3.08 [dd, J = 19.0, 7.3 Hz, 1H], 3.35 [s, 1H], 4.72 [s, 3H], 6.30 [dd, J = 7.0, 2.9

TABLE 1. Products formed from 1-indanone and 2-indanone by induced whole cells of strains 9816/11 and F39/D

Strain	Substrate	Product	Relative yield (%)[a]	Enantiomeric composition (%)[b]
9816/11	1-Indanone	3-Hydroxy-1-indanone	91	81 (R)-3-hydroxy-1-indanone
		2-Hydroxy-1-indanone	9	51 (R)-2-hydroxy-1-indanone
9816/11	2-Indanone	2-Hydroxy-1-indanone	99	53 (S)-2-hydroxy-1-indanone
		2-Hydroxy-2-inden-1-one	1	NA[c]
F39/D	2-Indanone	2-Hydroxy-1-indanone	72–96	88 (S)-2-hydroxy-1-indanone
		2-Hydroxy-2-inden-1-one	4–28	NA

[a] Relative product yields were determined from peak area integrations of total ion current chromatograms.
[b] The enantiomeric compositions of isolated hydroxyindanones were determined by integrations of CSP-HPLC peak areas of the individual enantiomers. The individual enantiomers were collected and shown to have superposable UV spectra.
[c] NA, not applicable.

Hz, 1H], 7.07–7.65 [m, 9H]). The downshifted signals (dd at 2.65 and 3.20 ppm; Fig. 1) for the (R)-methoxyphenylacetyl ester indicated that the hydroxyl group at carbon position C-3 was predominantly of the (R)-configuration. For the (S)-methoxyphenylacetyl ester, the magnitude of the integrals of the same signals were reversed relative to those of the (R)-ester (data not shown). The enantiomeric composition of the product, determined by integration of the proton signals arising from the two diastereomers, was 80% (R)-3-hydroxy-1-indanone. CSP-HPLC was also used to determine the enantiomeric purity of compound I. On the basis of the integration of peak areas, the enantiomeric composition was shown to be 81% (R)-3-hydroxy-1-indanone (62% ee) (Table 1).

Compound II (5 mg) was a minor product formed from 1-indanone by strain 9816/11. The product was isolated by PLC and characterized by GC-MS, ^{1}H and ^{13}C NMR, and UV spectral analyses: GC-MS, R_t of 11.6 min and M$^+$ (% relative intensity) at m/z 148 (100) with major fragment ions at m/z 131 (14), 119 (60), 105 (29), 91 (87), 77 (16), 65 (34), 51 (21), and 50 (24); ^{1}H NMR (CDCl$_3$), δ 3.03 (dd, J = 16.5, 5.1 Hz, 1H), 3.58 (dd, J = 16.5, 7.9 Hz, 1H), 4.54 (dd, J = 7.9, 5.1 Hz, 1H), 7.42 (t, J = 7.5 Hz, 1H), 7.47 (d, J = 7.7 Hz, 1H), 7.64 (t, J = 7.5 Hz, 1H), 7.75 (d, J = 7.6 Hz, 1H); ^{13}C NMR (CDCl$_3$) δ 35.1 (CH$_2$), 74.1 (CH), 124.3 (CH), 126.7 (CH), 127.9 (CH), 134.0 (C), 135.8 (CH), 150.9 (C), 206.8 (C=O); UV spectrum (MeOH) λ$_{max}$, 207, 246, and 292 nm. The above data were identical with those of synthetic 2-hydroxy-1-indanone. In contrast to compound I, compound II did not dehydrate under the GC conditions used. CSP-HPLC separated the enantiomers of compound II and showed that compound II formed by strain 9816/11 from 1-indanone was essentially racemic (51:49; Table 1).

When salicylate-induced cells (500 ml) of strain 9816/11 were incubated with 2-indanone, a single product was observed by TLC, with an R_f value of 0.5. The product (65 mg) was isolated by RDC, and analysis by GC-MS, ^{1}H NMR, and ^{13}C NMR showed that this compound was identical to compound II. CSP-HPLC resolved two enantiomers and showed that (S)-2-hydroxy-1-indanone was in slight excess (6% ee) (Table 1). GC-MS analysis of ethyl acetate extracts also detected a minor product (compound III) in trace amounts (Table 1). This compound was identified in reactions with F39/D (see below).

Salicylate-induced cells of strain 9816/11 were also incubated with synthetic (±)-3-hydroxy-1-indanone and (±)-2-hydroxy-1-indanone to determine if a dehydrogenase activity influenced the enantiomeric compositions of these products. After 24 h of incubation, CSP-HPLC analysis showed that the composition of the 3-hydroxy-1-indanone was still racemic while (S)-2-hydroxy-1-indanone was found in slight excess (54:46) (data not shown).

Transformations catalyzed by *P. putida* F39/D. Toluene-induced cells (50 ml) of strain F39/D showed extremely poor transformation of 1-indanone to compound I (4% yield by GC-MS at 24 h). However, the same cells (500 ml) catalyzed efficient conversion of 2-indanone to a product identified (GC-MS, ^{1}H NMR, and ^{13}C NMR) as compound II (72 to 96% yield; Table 1). Compound II (73 mg, [α]$_D$ +21° [c 1.4, CHCl$_3$]) was isolated by RDC and derivatized with both methoxyphenylacetyl chlorides. ^{1}H NMR analysis of (R)-(−)- and (S)-(+)-methoxyphenylacetyl esters of compound II showed two sets of signals corresponding to the diastereomeric esters of (R)-2-hydroxy-1-indanone (CDCl$_3$ δ 3.05 [dd, J = 16.9, 4.4 Hz, 1H], 3.50–3.78 [m, 2H], 4.91 [s, 3H], 5.47 [dd, J = 8.1, 4.9 Hz, 1H], 7.28–7.81 [m, 9H]) and (S)-2-hydroxy-1-indanone (CDCl$_3$ δ 2.77 [dd, J = 16.9, 4.4 Hz, 1H], 3.50–3.78 [m, 2H], 4.93 [s, 3H], 5.53 [dd, J = 8.1, 4.9 Hz, 1H], 7.28–7.81 [m, 9H]). Integration of the signals (dd at 2.77 and 3.05 ppm) indicated that (S)-2-hydroxy-1-indanone was formed in ee by F39/D (data not shown). CSP-HPLC analysis confirmed the enantiomeric composition of compound II as 88% (S)-2-hydroxy-1-indanone (76% ee; Table 1). In a separate experiment, toluene-induced cells of PpF39/D were incubated for 24 h with synthetic (±)-2-hydroxy-1-indanone. CSP-HPLC analysis of the recovered substrate showed its composition to be (S)-2-hydroxy-1-indanone of 54% ee (data not shown).

A minor product (compound III) formed from 2-indanone by both F39/D and 9816/11 cells was detected by GC-MS (R_t of 12.6 min) and showed a M$^+$ (% relative intensity) at m/z 146 (13) with fragment ions at m/z 118 (74), 90 (100), 89 (55), 74 (4), 63 (34), 51 (11), 39 (15), and 27 (6). Compound III formed a TMS derivative which showed a M$^+$ (% relative intensity) at m/z 218 (19) and fragment ions at m/z 205 (40), 147 (61), 133 (20), 117 (33), 103 (27), 73 (100), 59 (12), and 45 (22). The formation of a TMS derivative indicated the presence of an exchangeable hydroxyl proton and supports the identification of compound III as 2-hydroxy-2-inden-1-one.

Oxidation of 1- and 2-indanone by purified NDO and TDO components in the presence of [^{18}O]oxygen. 1-Indanone and 2-indanone were incubated with purified NDO and TDO components in the presence of an atmosphere containing ≥90% [^{18}O]oxygen. Product distribution was determined by GC-MS of reaction extracts. Enantiomeric compositions of the PLC-isolated products were determined by CSP-HPLC. The reaction products formed and their normalized ^{18}O-labeled isotopic enrichment are summarized in Table 2. Mass spectra of all hydroxyindanones formed in the presence of [^{18}O]oxygen showed M$^+$ + 2 at m/z 150, indicating the incorporation of a single atom of molecular dioxygen. The lack of any M$^+$ + 4 ion indicated that the possibility of product formation through dioxygenation of the enol tautomer of either substrate was unlikely. Purified NDO oxidized 1-indanone to 78% (R)-3-

TABLE 2. Incorporation of [^{18}O]oxygen into 1-indanone and 2-indanone by purified NDO and TDO components and enantiomeric purity of the products

Enzyme	Substrate	Product (enantiomeric composition [%])[a]	Relative yield (%)	Isotopic [^{18}O]oxygen enrichment (%)	Product [^{18}O]oxygen enrichment (%)[b]	Normalized enrichment (%)[c]
NDO	1-Indanone	(R)-3-Hydroxy-1-indanone (78)	87	90	92	100
		(R)-2-Hydroxy-1-indanone (61)	13	90	88	98
NDO	2-Indanone	(S)-2-Hydroxy-1-indanone (88)	100	92	77	84
TDO	2-Indanone	(S)-2-Hydroxy-1-indanone (95)	100	91	60	66

[a] Product identification based on GC-MS characteristics with relative product yields determined from peak area integrations of total ion current chromatograms. No products were detected in 2-h reaction mixtures containing 1-indanone and TDO components. Enantiomeric composition was determined from integrations of CSP-HPLC resolved peaks corresponding to the enantiomers of the isolated products.
[b] Determined by the intensities of the m/z at $(M^+ + 2)/[M^+ + 2)] \times 100$.
[c] Defined as $([^{18}O]oxygen$ enrichment of product/$[^{18}O]oxygen$ enrichment of headspace$) \times 100$.

hydroxy-1-indanone (87% yield, 56% ee) and 61% (R)-2-hydroxy-1-indanone (13% yield, 22% ee). Purified TDO did not oxidize 1-indanone. Both TDO and NDO oxidized 2-indanone to (S)-2-hydroxy-1-indanone (100% yield); however, the enantiomeric purity of the product formed by TDO (90% ee) was higher than that from NDO (76% ee) (Table 2). 2-Hydroxy-2-inden-1-one (compound III) was not formed by purified NDO and TDO components.

DISCUSSION

TDO and NDO have relaxed substrate specificities and are capable of catalyzing dioxygenation and monooxygenation reactions with different substrates. Thus, TDO oxidizes indan to (R)-1-indanol, while NDO forms the (S)-enantiomer (27). NDO seems to be more versatile than TDO, since it has the ability to catalyze the desaturation of indan to indene (12) and phenetole to ethenyloxybenzene (18). In addition, NDO catalyzes O-dealkylation reactions with anisole and phenetole, while TDO does not show desaturase or O-dealkylation activity with phenetole (18).

The present study provides further examples of monooxygenation reactions catalyzed by NDO and TDO. Salicylate-induced cells of strain 9816/11 oxidized 1-indanone to (R)-3-hydroxy-1-indanone and 2-hydroxy-1-indanone as the major and minor products, respectively (Table 1). Purified NDO formed the same products (Table 2) (Fig. 2A). In contrast, toluene-induced cells of F39/D and purified TDO did not oxidize 1-indanone efficiently. This illustrates further differences in the substrate specificities of NDO and TDO.

Induced cells of strain 9816/11 and F39/D oxidized 2-indanone almost exclusively to (S)-2-hydroxy-1-indanone. A minor product formed by both organisms was identified as 2-hydroxy-2-inden-1-one (Table 1). The latter product may result from dehydrogenation or desaturation of the 2-hydroxy-1-indanone. However, on the basis of the observed product, it is difficult to determine whether desaturation or dehydrogenation is occurring, since 1,2-indandione and 2-hydroxy-2-inden-1-one are merely keto-enol tautomers and thus offer little insight into the mechanism of the oxidation. Purified NDO and TDO both oxidized 2-indanone to (S)-2-hydroxy-1-indanone as the only detectable product (Table 2) (Fig. 2B).

The 3-hydroxy-1-indanone formed by strain 9816/11 and NDO was the (R)-enantiomer (ca. 60% ee). In contrast, induced cells of strain 9816/11 and purified NDO showed less enantiospecificity in the formation of (R)-2-hydroxy-1-indanone (Tables 1 and 2).

The 2-hydroxy-1-indanone formed from 2-indanone by induced cells of strains 9816/11 and F39/D was the (S)-enantiomer, with F39/D showing a higher enantiospecificity than strain 9816/11 (Table 1). The same pattern was observed with

purified NDO and TDO, although NDO produced (S)-2-hydroxy-1-indanone in 76% ee compared with the 6% ee for induced cells of strain 9816/11. The reason for this difference was not pursued further in the present study.

Experiments with [^{18}O]oxygen showed that for 1-indanone, the single atom of oxygen incorporated into (R)-3-hydroxy-1-indanone and (R)-2-hydroxy-1-indanone by purified NDO was derived exclusively from dioxygen (Table 2). This suggests that the hydroxylation reaction is tightly coupled. In contrast, a significant percentage of the oxygen in the (S)-2-hydroxy-1-indanone formed from 2-indanone by NDO and TDO was not from molecular oxygen and was presumed to be derived from water. This suggests that an iron-bound oxygen species in the oxygenase components of NDO and TDO may be the site of exchange of oxygen with water. A similar exchange reaction may also be involved in the oxidation of indan to (R)-1-indanol by TDO (27).

The formation of 3-hydroxy-1-indanone and 2-hydroxy-1-indanone as the major bacterial oxidation products from 1- and 2-indanone, respectively, offers a biocatalytic alternative to multistep chemical syntheses of these compounds. In addition, the characterization of these oxidation products may aid in identification of metabolites in the bacterial catabolic pathways of larger indeno-aromatic hydrocarbons. In fact, an unidentified metabolite (compound IV) with an M^+ at m/z 148 formed from fluorene by an *Arthrobacter* species (strain F101) (10) has
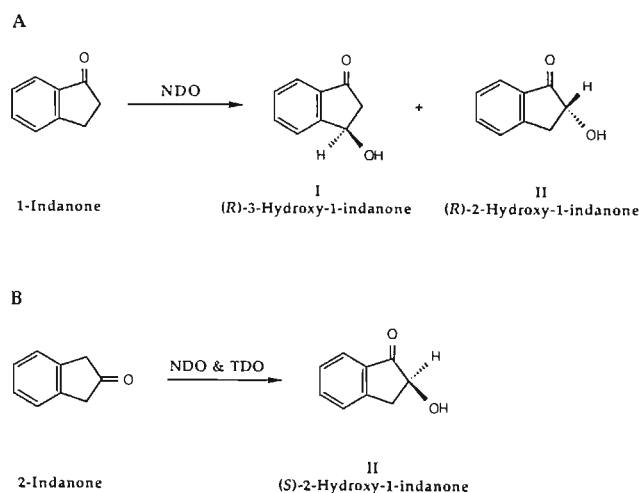
A

1-Indanone   →(NDO)→   I
(R)-3-Hydroxy-1-indanone   +   II
(R)-2-Hydroxy-1-indanone

B

2-Indanone   →(NDO & TDO)→   II
(S)-2-Hydroxy-1-indanone

FIG. 2. (A) Products formed from 1-indanone by NDO; (B) oxidation of 2-indanone to (S)-2-hydroxy-1-indanone by NDO and TDO. The enantiomeric compositions of compounds I and II are given in Table 2.

spectral data (^{1}H NMR, MS, and UV data) which are essentially identical to those reported for 3-hydroxy-1-indanone in the present study. Also, 1-indanone was recently identified as a metabolite in the degradation of fluorene by *Pseudomonas cepacia* F297 (11). Hence, the 3-hydroxy-1-indanone formed by strain F101 could conceivably result via hydroxylation of 1-indanone in the manner demonstrated for NDO.

ACKNOWLEDGMENTS

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Desaturation, Dioxygenation, and Monooxygenation Reactions Catalyzed by Naphthalene Dioxygenase from *Pseudomonas* sp. Strain 9816-4

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The stereospecific oxidation of indan and indene was examined with mutant and recombinant strains expressing naphthalene dioxygenase of *Pseudomonas* sp. strain 9816-4. *Pseudomonas* sp. strain 9816/11 and *Escherichia coli* JM109(DE3)[pDTG141] oxidized indan to (+)-(1*S*)-indanol, (+)-*cis*-(1*R*,2*S*)-indandiol, (+)-(1*S*)-indenol, and 1-indanone. The same strains oxidized indene to (+)-*cis*-(1*R*,2*S*)-indandiol and (+)-(1*S*)-indenol. Purified naphthalene dioxygenase oxidized indan to the same four products formed by strains 9816/11 and JM109(DE3)[pDTG141]. In addition, indene was identified as an intermediate in indan oxidation. The major products formed from indene by purified naphthalene dioxygenase were (+)-(1*S*)-indenol and (+)-(1*R*,2*S*)-indandiol. The results show that naphthalene dioxygenase catalyzes the enantiospecific monooxygenation of indan to (+)-(1*S*)-indanol and the desaturation of indan to indene, which then serves as a substrate for the formation of (+)-(1*R*,2*S*)-indandiol and (+)-(1*S*)-indenol. The relationship of the desaturase, monooxygenase, and dioxygenase activities of naphthalene dioxygenase is discussed with reference to reactions catalyzed by toluene dioxygenase, plant desaturases, cytochrome P-450, methane monooxygenase, and other bacterial monooxygenases.

Pseudomonas sp. strain 9816-4 grows with naphthalene as the sole source of carbon and energy (9). The initial reaction is catalyzed by a multicomponent enzyme system designated naphthalene dioxygenase (NDO) (11, 12, 23, 24). NDO catalyzes the NAD(P)H-dependent enantiospecific incorporation of dioxygen into naphthalene to form (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene (*cis*-naphthalene dihydrodiol) (26, 27) (Fig. 1). An analogous reaction is catalyzed by toluene dioxygenase (TDO) from *Pseudomonas putida* F1, where enantiomerically pure (+)-*cis*-(1*S*,2*R*)-dihydroxy-3-methylcyclohexa-3,5-diene (*cis*-toluene dihydrodiol) is the first detectable oxidation product (17, 31, 60). TDO also catalyzes the enantiospecific oxidation of naphthalene to (+)-*cis*-naphthalene dihydrodiol (18, 39).

In addition to the enantiospecific oxidation of naphthalene and toluene, NDO and TDO from the above strains oxidize many related aromatic compounds to optically active dihydrodiols (10, 18, 28, 30). Other bacterial dioxygenases show similar properties, and more than 130 chiral arene *cis*-dihydrodiols have been produced from a small number of strains (7, 35, 48). The high enantiomeric purity of these compounds has led to their use as chiral synthons in the enantiospecific synthesis of a wide variety of biologically active natural products (7, 8, 46,

57). The present studies focus on another facet of this interesting group of dioxygenases, that is, their ability to catalyze reactions other than the formation of arene *cis*-dihydrodiols. For example, the TDO expressed by *P. putida* F39/D oxidizes indan to (1*R*)-indanol and oxidizes indene to *cis*-(1*S*,2*R*)-indandiol and (1*S*)-indenol (55). Similar reactions have been reported for TDO from *P. putida* UV4, although the 1-indenol produced by this strain is the (1*R*)-enantiomer (3, 5).

We now report the identification and absolute stereochemistry of the products formed from indan and indene by NDO from *Pseudomonas* sp. strain 9816-4 and confirm earlier observations on the desaturation of indan to indene by NDO (22).

MATERIALS AND METHODS

Organisms. *Pseudomonas* sp. strain 9816/11 is a mutant which oxidizes naphthalene stoichiometrically to (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene (40). This organism is a derivative of *Pseudomonas* sp. strain 9816-4 (9, 59), which harbors the genes for naphthalene catabolism on an 83-kb NAH plasmid designated pDTG1 (45). *Pseudomonas* sp. strain 9816/C84, a cured strain, was used as a control in experiments with strain 9816/11. *Escherichia coli* strain JM109 (DE3)[pDTG141] contains the structural genes (*nahAaAbAcAd*) for NDO in plasmid pT7-5 (50). Expression of NDO in this strain is inducible by the addition of isopropylthiogalactopyranoside (IPTG). *E. coli* JM109(DE3)[pT7-5] was used as a control in experiments with strain JM109(DE3)[pDTG141].

Biotransformation experiments. Strain 9816/11 was grown at 30°C in mineral salts basal medium (MSB) (49) with 0.2% (wt/vol) pyruvate as a carbon source in the presence of 0.05% (wt/vol) salicylate or anthranilate. These aromatic acids induce the synthesis of naphthalene catabolic enzymes in strain 9816 (2). Transformations of indan and indene, except where noted otherwise, were conducted with washed cell suspensions (turbidity, 2.0 to 2.5 at 600 nm) in 200 or 800 ml of 50 mM sodium-potassium phosphate buffer (pH 7.2) in 1.0-liter Erlenmeyer or 2.8-liter Fernbach flasks, respectively. Pyruvate (0.2%, wt/vol) was provided as an energy source, and indan or indene was added at a concentration of 0.05% (vol/vol). The cell suspensions were incubated on a rotary shaker (220 rpm) at 30°C, and at designated times, cells were removed by centrifugation, and the supernatant solutions were extracted three times with sodium hydroxide-washed ethyl acetate. The organic extract was dried over anhydrous Na$_2$SO$_4$ and concentrated under reduced pressure (30°C) prior to analysis of transformation products.

E. coli JM109(DE3)[pDTG141] was grown in a 5.0-liter Bioflow II Fermentor

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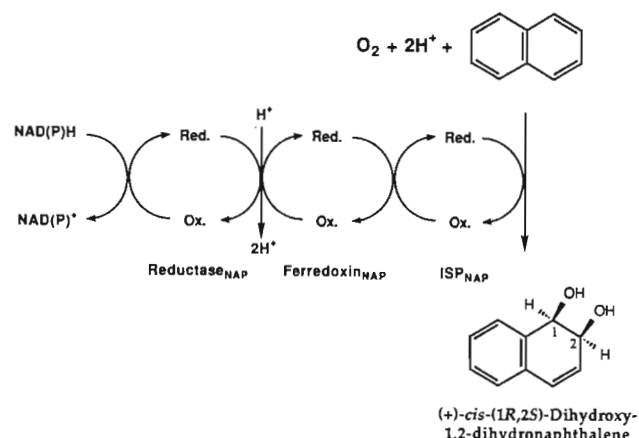
FIG. 1. Sequence of electron transfer from NAD(P)H to the oxygenase component (ISP_{NAP}) of NDO, resulting in the formation of (+)-cis-(1R,2S)-dihydroxy-1,2-dihydronaphthalene. The individual components reductase$_{NAP}$, ferredoxin$_{NAP}$, and ISP$_{NAP}$ have been purified (11, 23, 24), and their structural genes have been cloned and sequenced (38, 47). The redox state of each protein is indicated as reduced (Red.) and oxidized (Ox.).
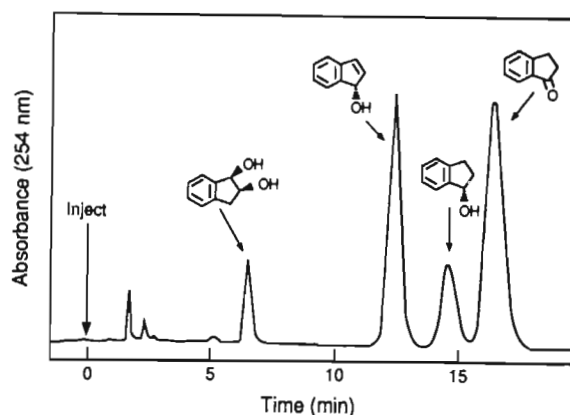

FIG. 2. HPLC separation of products formed from indan by strain 9816/11. Cells were grown as described in Materials and Methods. Products were separated by reverse-phase HPLC on a radial-compression 10-μm C-18 column. The solvent used was methanol-water (36:64) at a flow rate of 1.9 ml/min.

(New Brunswick, Inc.) as previously described (42). IPTG-induced cells were incubated with indan and indene as described above except that glucose (0.2%, wt/vol) was provided as the energy source and indan and indene were provided at 0.1 and 0.025% (vol/vol), respectively. Unused cells were stored at −70°C.

Oxidation of indan to indene by purified NDO. The individual components of NDO (reductase$_{NAP}$, ferredoxin$_{NAP}$, and ISP$_{NAP}$) were purified to homogeneity as described previously (11, 23, 24). Reactions were carried out in 2.34 ml of 50 mM Tris-HCl buffer, pH 7.5, in 15-ml conical glass tubes. Reaction mixtures contained reductase$_{NAP}$ (73 μg of protein), ferredoxin$_{NAP}$ (48 μg of protein), ISP$_{NAP}$ (860 μg of protein), NADH (5.0 μmol), flavin adenine dinucleotide (5.0 nmol), and indan (3.38 μmol). Reactions were initiated by the addition of indan and were conducted at room temperature for 10 min. At this time, reaction mixtures were extracted twice with diethyl ether. The organic extract was dried over anhydrous sodium sulfate and concentrated to a small volume prior to analysis by high-pressure liquid chromatography (HPLC) and gas chromatography/mass spectrometry (GC/MS). Reverse-phase HPLC was conducted with a Beckman Ultrasphere 5-μm C-18 column, and separation of products was achieved with a programmed linear gradient of methanol-water (50 to 95% methanol) at a flow rate of 1.0 ml/min (see Fig. 3).

Oxidation of indan, indene, and (1S)-indanol by purified NDO. The experiments described in Table 3 were conducted with ISP$_{NAP}$ purified from *E. coli* JM109(DE3)[pDTG121] as recently described by Suen and Gibson (52). Reductase$_{NAP}$ and ferredoxin$_{NAP}$ were purified from *E. coli* JM109(DE3)[pDTG141] (32). Transformation reactions were carried out in 15-ml conical polyethylene tubes. Reaction mixtures contained, in 2.0 ml of 50 mM 2-(4-morpholino)-ethanesulfonic acid (MES) buffer (pH 6.8), reductase$_{NAP}$ (20 μg of protein), ferredoxin$_{NAP}$ (70 μg of protein), ISP$_{NAP}$ (50 μg of protein), ferrous ammonium sulfate (0.05 μmol), NADH (0.25 μmol), and indan, indene, or (1S)-indanol (0.5 μmol in 20 μl of methanol). Reactions were initiated by addition of substrate, and each tube was capped and incubated horizontally at 23°C with gentle agitation (~60 rpm) for 1 h. Oxidation products were extracted with ethyl acetate as described above prior to analysis by GC/MS.

Analytical procedures. Thin-layer chromatography (TLC) and preparative-layer chromatography (1.0- or 2.0-mm silica thickness: Merck) were conducted as described previously (43). Radial-dispersion chromatography was performed on 2.0-mm-thickness silica plates, and products were eluted with a chloroform-acetone step gradient (0 to 40% acetone; 20% steps over 1 h) at a flow rate of 7.0 ml/min. All fractions were analyzed by TLC (solvent: chloroform-acetone, 80:20, vol/vol), and those containing products were combined for further characterization. Open-column silica gel chromatography was used where indicated in the text. GC-MS was conducted as described previously (43). Relative yields of products were determined from the integration of their total ion current peak areas. Proton ^{1}H nuclear magnetic resonance (^{1}H NMR) spectra, absorption spectra, and optical rotation values were obtained as described previously (43). Reported $[\alpha]_D$ values were determined in chloroform at 25°C.

Chiral stationary-phase HPLC was conducted as described previously (43). Enantiomers of cis-1,2-indandiol were separated on a Chiralcel OJ column (25 cm by 4.6 mm; Chiral Technologies, Exton, Pa.) with a mobile phase of hexane and 2-propanol (9:1) at a flow rate of 0.5 ml/min. Under these conditions, the (+)-cis-(1R,2S)- and (−)-cis-(1S,2R)-enantiomers of indandiol eluted with retention times of 18.5 and 23.6 min, respectively. 1-Indenol enantiomers were separated on a Chiralcel OB-H column (25 cm by 4.6 mm) under identical conditions, with (−)-(1R)-indenol and (+)-(1S)-indenol eluting at 15.1 and 25.3

min, respectively. The Chiralcel OB-H column also resolved (−)-(1R)-indanol and (+)-(1S)-indanol, which eluted at 11.8 and 17.4 min, respectively.

Chemicals. Indan, indene, (+)-(1S)-indanol, (−)-(1R)-indanol, 1-indanone, 1,3-indandione, indole, indoline, and 2,3-dihydrobenzofuran were obtained from Aldrich Chemical Company, Milwaukee, Wis. Indan, free from contaminating indene, was obtained by reverse-phase HPLC on a semipreparative Waters C-18 column. The solvent was 70% methanol, and the flow rate was 2.5 ml/min. Under these conditions, indene elutes at 15 min and indan elutes at 22 to 23 min. The middle fractions containing pure indan were pooled to give a stock indan solution (13 mM) for enzyme experiments. Racemic cis-1,2-indandiol was prepared by treatment of indene with osmium tetroxide. trans-1,3-Indandiol was prepared by reducing 1,3-indandione with sodium borohydride. (−)-cis-(1S,2R)-Indandiol was prepared by reacting (±)-cis-indandiol with (−)-menthoxyacetyl chloride. The crude diesters were purified by silica gel chromatography and recrystallized from methanol. Direct hydrolysis of the first crystals obtained gave (−)-cis-(1S,2R)-indandiol (mp, 105 to 107°C; $[\alpha]_D$, 50.4°) (25).

RESULTS

Oxidation of indan by strains 9816/11 and JM109(DE3) [pDTG141]. When a suspension of anthranilate-induced cells of 9816/11 was incubated overnight with indan, several oxidation products were detected by TLC. The major product was identified as 1-indanone based on its R_f value, HPLC retention time, absorption spectrum, and the properties of its semicarbazone derivative. Minor products, tentatively identified by TLC and HPLC, were 1-indanol, 1-indenol, and 1,2-indandiol. In addition, several unidentified minor polar peaks were observed.

In order to identify primary indan oxidation products, the experiment was repeated, and product formation was determined after 1 h. Four major metabolites were detected by HPLC (Fig. 2) and identified as cis-1,2-indandiol, 1-indenol, 1-indanol, and 1-indanone by comparing their HPLC retention times and absorption spectra with those of authentic compounds. Sufficient material for further structural studies was obtained by incubating a 2.4-liter suspension of induced cells with indan for 1 h. Cells were removed by centrifugation, and ethyl acetate extraction of the clear supernatant solution followed by silica gel column chromatography gave crude preparations of cis-1,2-indandiol, 1-indanone, and a mixture of 1-indanol and 1-indenol. The monols were separated by HPLC. All four indan metabolites were crystallized and shown to have ^{1}H NMR and mass spectra identical to those given by authentic compounds (55). The specific rotations, $[\alpha]_D$, for cis-1,2-indandiol, 1-indanol, and 1-indenol were +53°, +32°, and +128°, respectively.

The formation of cis-1,2-indandiol and 1-indenol was unex-

TABLE 1. Yields, enantiomeric composition, and absolute configuration of hydroxylated products formed from indan by strains expressing NDO[a]

Strain[b]	1-Indanol			cis-1,2-Indandiol			1-Indenol		
	Yield (%)	Enantiomeric composition[c] (%)	Absolute configuration[d]	Yield (%)	Enantiomeric composition (%)	Absolute configuration	Yield (%)	Enantiomeric composition (%)	Absolute configuration
9816/11	64	79	(+)-(1S)-	7	86	(+)-(1R,2S)-	8	69	(+)-(1S)-
JM109(DE3)[pDTG141]	54	93	(+)-(1S)-	18	91	(+)-(1R,2S)-	19	83	(+)-(1S)-

[a] Products were identified by GC/MS. Yields were determined by integration of total ion current peak areas under the conditions described in Materials and Methods.

[b] Other products formed by strain 9816/11 were 1-indanone (18%) and 2-hydroxy-1-indanone (~1%). Approximately 1% indan remained at the end of the experiment. The only other product formed by strain JM109(DE3)[pDTG141] was 1-indanone (3%). Approximately 6% indan remained at the end of the experiment.

[c] Determined by integration of peak areas of enantiomers separated by chiral stationary-phase HPLC under conditions described in Materials and Methods.

[d] Absolute configuration of the major enantiomer.

pected, since previous studies showed that TDOs from *P. putida* F39/D (55) and *P. putida* UV4 (5) oxidize indan to (−)-(1R)-indanol and 1-indanone as the major products. In a second experiment, salicylate-induced cells of strain 9816/11 and IPTG-induced cells of JM109(DE3)[pDTG141] were incubated with indan for 5 and 24 h, respectively. Products were separated by preparative-layer chromatography (*cis*-1,2-indandiol, R_f 0.2, and indanol-indenol mixture, R_f 0.6) and identified by GC/MS. Absolute configurations were determined by chiral stationary-phase HPLC and are shown in Table 1. A significant amount of indanone (16%) was produced by strain 9816/11. We have noticed in other experiments that 1-indanone formation increases with the time of incubation of indan with strain 9816/11 and appears to be correlated with a decrease in the enantiomeric purity of (+)-(1S)-indanol recovered (data not shown). Indan oxidation was not observed in control experiments with strains 9816/C84 and JM109(DE3)[pT7-5].

Oxidation of indene by strains 9816/11 and JM109(DE3)[pDTG141]. Experiments analogous to those described above for indan were conducted with indene. Salicylate-induced cells of strain 9816/11 oxidized indene to two major products, which were isolated by radial-dispersion chromatography and identified as (+)-*cis*-(1R,2S)-indandiol ([α]$_D$, +40°) and (+)-(1S)-indenol ([α]$_D$, +128°). The same diol ([α]$_D$, +34°) and monol ([α]$_D$, +137°) products were formed by JM109(DE3)[pDTG141]. Both organisms formed small amounts of 1-indanone (Table 2).

The formation of (+)-*cis*-(1R,2S)-indandiol and (+)-(1S)-indenol from indan (Table 1) can be explained by the desaturation (dehydrogenation) of indan or the dehydration of 1-indanol to yield indene, which would then serve as a substrate to give the products shown in Table 2. However, indene was never detected as an intermediate in the metabolism of indan or 1-indanol by strains 9816/11 and JM109(DE3)[pDTG141].

Only minor amounts of (1S)-indenol were formed from (1S)-indanol by both strains, thus eliminating the desaturation of (1S)-indanol as a major source of (1S)-indenol.

Oxidation of indan to indene by purified NDO. In order to unequivocally establish the role of NDO in the observed dioxygenation, monooxygenation, and desaturation reactions, purified NDO components (reductase$_{NAP}$, ferredoxin$_{NAP}$, and ISP$_{NAP}$) were incubated with indan in the presence of NADH. Reaction products were extracted with ether, separated by HPLC, and identified by their retention times and absorption spectra (Fig. 3). Indan oxidation was not observed when any one of the NDO components or NADH was omitted from the reaction mixture. The identity of indene was confirmed by analyzing the ether extract by GC/MS. The reaction product had a retention time of 6.98 min, and its mass spectrum gave a molecular ion M$^+$ at *m/z* 116 (100%) and major fragment ions at *m/z* 115 (91%), 89 (10%), 63 (12%), and 58 (32%). These properties are identical to those given by authentic indene. When indene was oxidized by purified NDO, the only products detected were (1S)-indenol and *cis*-(1R,2S)-indandiol (Table 3). In contrast, the major products formed from indan were (1S)-indanol and (1S)-indenol. Smaller amounts of indene, (1R,2S)-indandiol, and 1-indanone were also detected. (1S)-Indanol was a poor substrate for NDO. The major oxidation product detected was a diol with the same retention time as *trans*-1,3-indandiol. Minor amounts of (1S)-indenol and 1-indanone were also produced.

Oxidation of indoline and dihydrobenzofuran by strain 9816/11. Previous studies have shown that NDO and TDO expressed by 9816/11 and *P. putida* F39/D, respectively, oxidize indole to indigo (13). The proposed reaction sequence involves *cis*-dihydroxylation of the heterocyclic ring followed by nonenzymatic dehydration to yield indoxyl, which autooxidizes to indigo. Salicylate-induced cells of 9816/11 oxidized indoline to

TABLE 2. Yields, enantiomeric composition, and absolute configuration of the hydroxylated products formed from indene by strains expressing NDO[a]

Strain[b]	cis-1,2-Indandiol			1-Indenol		
	Yield (%)	Enantiomeric composition[c] (%)	Absolute configuration[d]	Yield (%)	Enantiomeric composition (%)	Absolute configuration
9816/11	53	90	(+)-(1R,2S)-	43	94	(+)-(1S)-
JM109(DE3)[pDTG141]	56	86	(+)-(1R,2S)-	42	81	(+)-(1S)-

[a] Products were identified by ^{1}H NMR and GC/MS. Yields were determined by integration of total ion current peak areas under the conditions described in Materials and Methods.

[b] 1-Indanone (~4%) was the only other product formed by 9816-11. 1-Indanone (~2%) was the only other product formed by JM109(DE3)[pDTG141].

[c] Determined by integration of peak areas of enantiomers separated by chiral stationary-phase HPLC under conditions described in Materials and Methods.

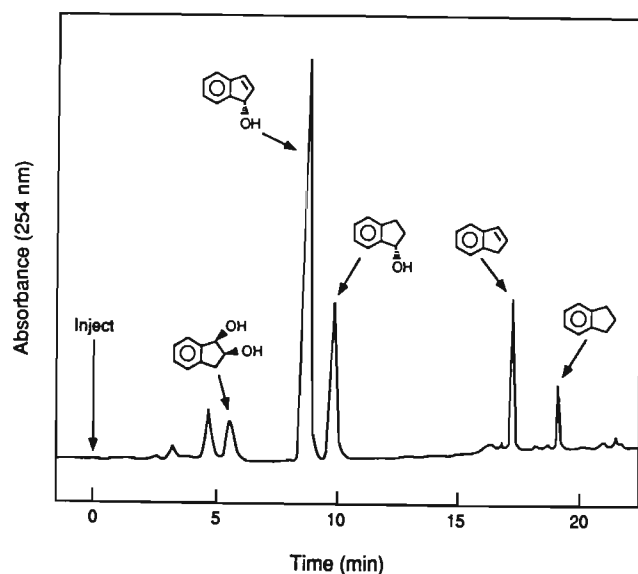[d] Absolute configuration of the major enantiomer.

FIG. 3. HPLC separation of the products formed from indan by purified NDO. The experimental conditions and procedures used for the identification of products are described in Materials and Methods.

TABLE 3. Yields, molecular weights, and retention times of products formed from indan, indene, and (1S)-indanol by purified NDO[a]

Product	M[+]	Retention time (min)	% of product formed from:		
			Indan	Indene	(1S)-Indanol
Indene	116	6.98	2.4	12.5	—[b]
(1S)-Indenol	132	9.45	20.1	57.9	0.8
(1S)-Indanol	134	9.76	67.1	—	85.5
1-Indanone	132	10.4	3.5	—	2.2
(1R,2S)-Indandiol	150	12.35	7.0	29.6	—
trans-1,3-Indandiol	150	12.53	—	—	11.5

[a] Reactions were carried out for 1 h, and products were separated and identified by GC/MS as described in Materials and Methods. The absolute configurations given are based on results with intact cells (Tables 1 and 2).
[b] —, not detected.

a blue compound, which was extracted with hot chloroform and shown to have TLC properties, absorption, and mass spectra identical to those given by authentic indigo. In contrast, toluene-induced cells of P. putida F39/D did not oxidize indoline to indigo.

The structural similarity of 2,3-dihydrobenzofuran to indoline suggested that it may serve as a substrate for the desaturase activity of NDO. Several products were formed from 2,3-dihydrobenzofuran by 9816/11. One of these was identified by GC/MS as 2,3-benzofuran. The identities of the other products were not determined in the present study.

DISCUSSION

The results presented show that NDO catalyzes dioxygenation, monooxygenation, and desaturation reactions with indan. The formation of (+)-(1S)-indenol and (+)-cis-(1R,2S)-indandiol from this substrate was of interest, since previous studies with TDO from P. putida F39/D (6, 55) and UV4 (5) showed that the major products formed from indan are (−)-(1R)-indanol and 1-indanone. The variable enantiomeric composition of the (1R)-indanol formed by these strains is due to the presence of a dehydrogenase which preferentially oxidizes (1S)-indanol to 1-indanone (5, 6). A similar dehydrogenase is probably responsible for the lower enantiomeric composition of the (1S)-indanol formed from indan by 9816/11. This conclusion is based on the higher enantiomeric composition of the (1S)-indanol formed by JM109(DE3)[pDTG141] and the larger amount of 1-indanone formed by 9816/11 after 5 h (Table 1). In addition to the compounds listed in Table 1, 9816/11 also produced 2-hydroxy-1-indanone as a minor product. Subsequent studies showed that NDO oxidizes 1-indanone to racemic 2-hydroxy- and (3R)-hydroxy-1-indanone (~80% R-enantiomer). In contrast, 1-indanone is not oxidized by TDO from strain F39/D (43).

Table 2 shows that NDO oxidizes indene to (+)-cis-(1R,2S)-indandiol and (+)-(1S)-indenol. The former product is of higher enantiomeric composition and of the opposite configuration than the (−)-cis-(1S,2R)- indandiol formed by the TDO-containing strains F39/D (55) and UV4 (5). In addition, the

(+)-(1S)-indenol formed by NDO is of higher enantiomeric purity than the same product formed by F39/D (55) and of opposite configuration to the (−)-(1R)-indenol formed in high enantiomeric purity by UV4 (5).

The formation of (+)-cis-(1R,2S)-indandiol in high enantiomeric purity by NDO in strains 9816/11 and JM109(DE3)[pDTG141] provides a direct route to this enantiomer. Recent studies by Boyd and his associates have shown that naphthalene-grown cells of P. putida NCIMB 8859 oxidize indene to 1,2-indandiol, with an excess of the (1R,2S)-enantiomer. The concentration of indene used in these experiments (0.5 to 1.0 mg ml^{-1}) is critical, since the same NCIMB 8859 cells catalyze the enantiospecific removal of (1R,2S)-indandiol from a racemic mixture of cis-1,2-indandiol provided at lower concentrations (0.2 to 0.4 mg ml^{-1}) (1). These observations, those described in this paper, and studies currently in progress (40) indicate that NDO may provide a family of chiral synthons that differ in configuration from those formed by TDO. The explanation for these differences awaits detailed studies on the structure and mechanism of action of both dioxygenases.

The oxidation of indan and indene to (1S)-indenol and cis-(1R,2S)-indandiol by NDO suggests that the enzyme catalyzes the desaturation of indan or the dehydration of 1-indanol to yield indene, which then serves as a substrate for the monooxygenase and dioxygenase activities of the enzyme. Attempts to detect indene formation from indan during whole-cell experiments with strains 9816/11 and JM109(DE3)[pDTG141] were unsuccessful. Indene was finally detected by HPLC analysis of the products formed from indan by purified NDO (Fig. 3) and identified by showing that its retention time, absorption spectrum, and mass spectrum were identical to those of authentic indene. Additional evidence for the desaturase activity of NDO was provided by showing that strain 9816/11 oxidizes indoline (1,2-dihydroindole) to indigo. The oxidation of indole to indigo by NDO and other oxygenases, including TDO, has been reported previously (4, 13). Thus, it was of interest that the TDO expressed by strain F39/D did not oxidize indoline to indigo, a characteristic consistent with its inability to desaturate indan to indene. Other desaturation reactions catalyzed by NDO include the formation of benzofuran from dihydrobenzofuran (this study) and ethenyloxybenzene from phenetole (41).

Strains 9816/11 and JM109(DE3)[pDTG141] and purified NDO did not form detectable amounts of indene from (1S)-indanol, and only very low yields of 1-indenol were formed from this substrate by purified NDO (Table 3). The enzyme did, however, oxidize (1S)-indanol to a compound tentatively identified as trans-1,3-indandiol. Thus, all of the available ev-
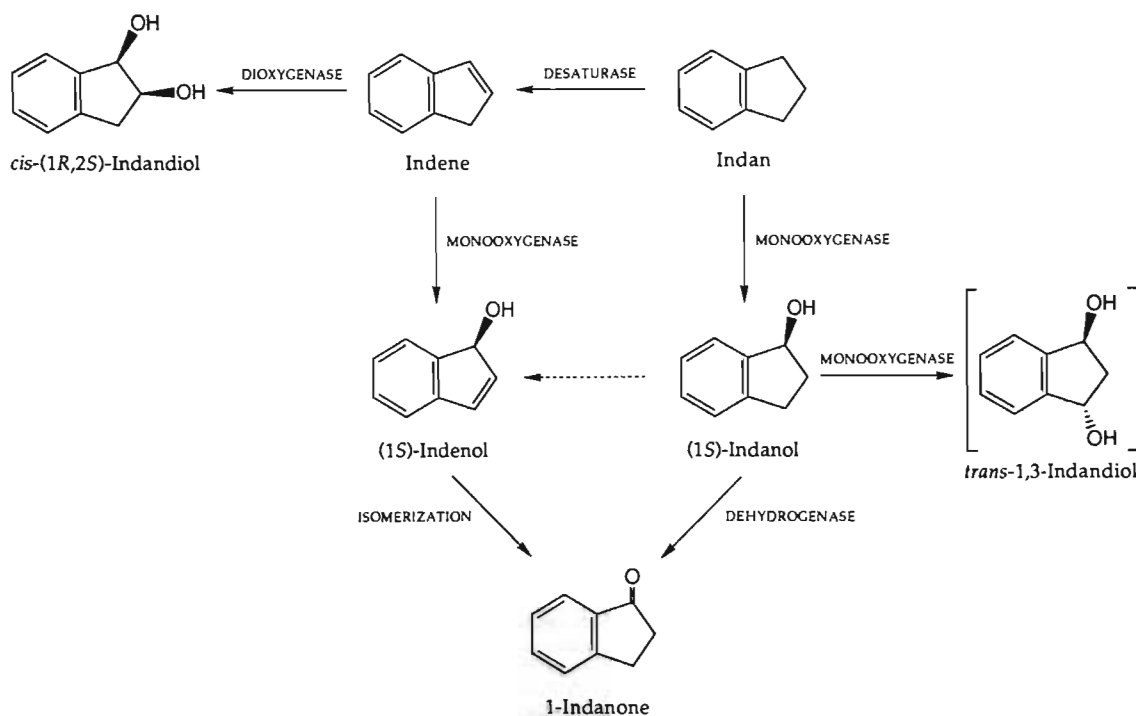
FIG. 4. Desaturase, monooxygenase, and dioxygenase reactions catalyzed by NDO. The formation of 1-indanone from (1S)-indenol probably occurs by nonenzymatic isomerization. The oxidation of (1S)-indanol to 1-indanone is catalyzed by a dehydrogenase present in cells of strain 9816/11. The desaturation of (1S)-indanol to (1S)-indenol is a minor reaction, indicated by ---->.

idence indicates that NDO catalyzes the desaturation of indan to indene, which then serves as a substrate for the formation of cis-(1R,2S)-indandiol and (1S)-indenol, as shown in Fig. 4. The formation of 1-indanone from (1S)-indenol probably occurs by nonenzymatic isomerization (16), whereas 1-indanone formation from (1S)-indanol appears to be catalyzed by a dehydrogenase, as discussed above.

The desaturation reaction catalyzed by NDO and the structural organization of the enzyme (Fig. 1) both have features in common with plant stearoyl-acyl carrier protein (stearoyl-ACP) Δ^9 desaturase. This enzyme forms oleoyl-ACP in a reaction that requires NAD(P)H, oxygen, ferredoxin oxidoreductase, ferredoxin, and a terminal desaturase component which contains non-heme iron (34, 36). Recent studies with the cloned desaturase from the castor bean plant have shown that the iron is in the form of diiron-oxo clusters, which are involved in the generation of a high-valency iron-oxo species responsible for the desaturation reaction (14).

The desaturation and monooxygenation reactions catalyzed by NDO are also analogous to reactions catalyzed by cytochrome P-450. For example, the initial abstraction of a hydrogen atom from indan by an $(FeO)^{3+}$ species followed by oxygen rebound (hydroxylation) or removal of the β-hydrogen atom (desaturation) would account for the observed products (20, 21). In this context, it is of interest that NDO can catalyze O-dealkylation (41), N-dealkylation, and sulfoxidation reactions (32), which are also typical cytochrome P-450 reactions. Other non-heme iron oxygenases that catalyze reactions similar to those catalyzed by cytochrome P-450 are the soluble forms of methane monooxygenase (19, 33) and 4-methoxybenzoate monooxygenase (56). Ammonia monooxygenase appears to be responsible for the cytochrome P-450-type reactions catalyzed by Nitrosomonas europaea (29, 54). The iron at the active site of methane monooxygenase (15,

44) and plant stearoyl-ACP Δ^9 desaturase (14) is in the form of diiron clusters. In addition, deduced amino acid sequence analyses (14) show that the conserved motif proposed for the iron-binding sites in all known diiron-oxo proteins are also present in the putative oxygenase components of toluene 4-monooxygenase (58) and phenol hydroxylase (37). Lipscomb has proposed that the diiron-oxo clusters in methane monooxygenase are converted during catalysis to an [Fe(IV)·Fe(IV)]=O species, which would be a strong oxidant similar to the Fe(IV)=O species proposed for cytochrome P-450 (33). An iron-peroxo complex $[FeO_2]^+$ has been proposed for the oxygenating species in 4-methoxybenzoate O-demethylase (56).

The unique feature of NDO that sets is apart from the oxygenases described above is its ability to catalyze the enantiospecific incorporation of dioxygen into naphthalene and related compounds. It seems probable that this reaction is catalyzed by a strong oxidizing species generated by the interaction of oxygen with mononuclear iron present in the large (α) subunit of ISP_{NAP} (51). The monooxygenase, desaturase, and other diverse reactions catalyzed by NDO are probably catalyzed at the same site and, like cytochrome P-450, reflect differences in the "fit" of the substrate in the active site, the available oxidizable sites of the different substrates, and the rate-limiting steps in catalysis (20, 21). Current studies are directed towards understanding the role of mononuclear iron in NDO, which may involve Fenton-type reactions. In this context, it is of interest that H_2O_2 and Fe^{2+} in organic solvents can catalyze desaturation, monooxygenation, and dioxygenase reactions (53).

ACKNOWLEDGMENTS

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STEREOSPECIFIC SULFOXIDATION BY TOLUENE AND NAPHTHALENE DIOXYGENASES

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SUMMARY: Studies on the sulfoxidation of aryl alkyl sulfides by purified toluene dioxygenase and naphthalene dioxygenase showed that naphthalene dioxygenase produces sulfoxides of (S) absolute configuration in high enantiomeric purity while those formed by toluene dioxygenase are of variable enantiomeric purity depending on the p-substituents on the benzene ring. Oxygen uptake experiments with naphthalene dioxygenase showed that the reaction rate and degree of oxygen incorporation are affected by both aryl and alkyl substituents. $^{18}O_2$-Incorporation experiments showed that the oxygen atom of methyl phenyl sulfoxide formed by toluene dioxygenase and naphthalene dioxygenase is derived exclusively from O_2. Accompanying studies showed that chloroperoxidase produces single (R)-sulfoxides (>98% enantiomeric excess) from the aryl alkyl sulfides examined in the present study. © 1995 Academic Press, Inc.

Toluene dioxygenase (TDO) from *Pseudomonas putida* F1 (1) and naphthalene dioxygenase (NDO) from *Pseudomonas* sp. NCIB 9816-4 (2) are the initial enzymes involved in the aerobic catabolism of toluene and naphthalene, respectively. In both enzyme systems the oxygenase components are iron-sulfur proteins (ISP_{TOL} and ISP_{NAP}) which have an $\alpha_2\beta_2$ subunit composition (1, 2). The large (α) subunits each contain a Rieske-type [2Fe-2S] center and mononuclear iron (3, H. Jiang and D.T. Gibson, unpublished observations). The exact function of the small (β) subunits are not known. ISP_{TOL} and ISP_{NAP}, in the presence of NAD(P)H and two other electron transport proteins, catalyze the enantiospecific addition of dioxygen (O_2) to their respective substrates to form, in most cases, homochiral *cis*-dihydrodiols. It is now known that both dioxygenases are capable of oxidizing a variety of compounds by reactions involving stereospecific *cis*-dihydroxylation (4, 5) and monohydroxylation (6, 7). Other reactions include desaturation (8, 9), O-dealkylation (8), dechlorination (10, 11), N-dealkylation, and O_2-dependent alcohol oxidation (K. Lee and D.T. Gibson, unpublished observations).

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Abbreviations: TDO, toluene dioxygenase; NDO, naphthalene dioxygenase; HPO, horseradish peroxidase; CPO, chloroperoxidase; GC-MS, gas chromatography-mass spectrometry; CSP-HPLC, chiral-stationary phase high-performance liquid chromatography; e.e., enantiomeric excess.

We now report the stereospecific oxidation of aryl alkyl sulfides (thioanisole and its derivatives) to sulfoxides by purified TDO and NDO and compare the results obtained when the same substrates are oxidized by horseradish peroxidase (HPO) and chloroperoxidase (CPO). The highly selective formation of (S)-aryl alkyl sulfoxides by NDO contrasts with previous reports that racemic or (R)-aryl alkyl sulfoxides are produced by oxygenases (for a review see 12). Asymmetric sulfoxidations by intact cells of different bacteria expressing TDO and NDO have been recently reported (13). However, the present study with purified enzymes provides definitive proof that the oxygenase components of TDO and NDO can function as sulfoxidases.

MATERIALS AND METHODS

Materials. Aryl alkyl sulfides were obtained from Aldrich Chemical Co., Milwaukee, WI. [^{18}O]-O_2 (98 atom %) was from Icon Isotopes, Summit, NJ. Chloroperoxidase (CPO) from *Caldariomyses fumago* (RZ: approx. 0.6) and horseradish peroxidase (HPO) type II (RZ: 1.5-2.0) were from Sigma Chemical Company, St. Louis, MO. TDO and NDO components were purified to homogeneity from recombinant *E. coli* strains (14, 15). Details of the purification procedures will be published elsewhere.

Enzymatic oxidation procedures. Transformations of aryl alkyl sulfides were conducted in capped 15-ml Corning polyethylene tubes. Reaction mixtures for CPO and HPO contained in 2.0 ml of 50 mM sodium citrate buffer (pH 5.0), 1.0 mM H_2O_2 and CPO (72 µg of protein) or HPO (1 mg of protein). Reaction mixtures for TDO and NDO contained in 2 ml of 50 mM sodium 2-(N-morpholino)ethane sulfonate (MES) buffer (pH 6.8), 1.0 mM NADH, 0.25 mM Fe(NH$_4$)$_2$(SO$_4$)$_2$·6H$_2$O and the respective reductase (20 µg of protein), ferredoxin (35 µg of protein) and ISP (50 µg of protein) components of TDO or NDO. Reactions were initiated by the addition of 20 µl of 50 mM substrate in methanol to give a final substrate concentration of 0.5 mM. Tubes were incubated horizontally with agitation (60 rpm) at 23°C for 2 h with the exception of reaction mixtures containing methyl p-nitrophenyl sulfide. Due to lower transformation efficiencies this substrate was incubated with each enzyme for 5 h.

Oxygen uptake studies. Oxygen uptake by NDO was measured with a Clark-type oxygen electrode (Rank Brothers, Cambridge, England) equipped with a temperature-controlled water bath and a magnetic stirrer. Reactions were conducted in air-saturated 50 mM MES buffer (pH 6.8). The agitation rate was approximately 200 rpm at 24°C and the dissolved oxygen concentration was taken as 250 µM (16). Each reaction mixture contained in 0.8 ml of 50 mM MES buffer (pH 6.8), 0.25 mM NADH, reductase$_{NAP}$ (6 µg of protein), ferredoxin$_{NAP}$ (15 µg of protein), and ISP$_{NAP}$ (25 µg of protein). Reactions were initiated by the addition of 4 µl of a 25 mM solution of the substrate in methanol. $^{18}O_2$-Incorporation experiments were conducted as described previously (7).

Identification of metabolites. Reaction mixtures were extracted three times with equal volumes of NaOH-washed ethyl acetate. The organic extracts were combined, dried over anhydrous sodium sulfate and concentrated to approximately 30 µl under nitrogen. All extracts were analyzed by gas chromatography-mass spectrometry (GC-MS) as previously described (7). Under these conditions the retention times of methyl phenyl sulfoxide, ethyl phenyl sulfoxide, methyl p-tolyl sulfoxide, p-methoxyphenyl methyl sulfoxide, and methyl p-nitrophenyl sulfoxide were 10.6, 12.3, 12.7, 14.8, and 16.2 min, respectively. Relative yields of products were determined by integration of total ion current peak areas. For example, a 100% relative yield indicates that no substrate was detected at the end of the reaction period. Derivatization with phenylboronic acid was carried out by mixing equal volumes of reaction products and phenylboronic acid in ethyl acetate at room temperature for five minutes. Products were identified by GC-MS as previously described (7).

Determination of metabolite chirality. The enantiomeric compositions of the sulfoxide reaction products were determined by chiral stationary phase-high performance liquid chromatography (CSP-HPLC, 7) on a Chiralcel OB-H column (4.6 mm x 25 cm, 5 µm particle size, obtained from Chiral Technologies Inc., Exton, PA). Sulfoxides were eluted from the column with hexane and 2-propanol (9:1, vol/vol) at a flow rate of 1.0 ml/min. The column effluent was monitored at 254 nm and the areas under each peak were integrated. Analysis of the enantiomeric composition of methyl p-nitrophenyl sulfoxide was achieved with hexane and 2-

propanol (6:4, vol/vol) at a flow rate of 0.5 ml/min. Under these conditions, the retention volumes of the R and S enantiomers of methyl phenyl sulfoxide, ethyl phenyl sulfoxide, methyl p-tolyl sulfoxide, p-methoxyphenyl methyl sulfoxide, and methyl p-nitrophenyl sulfoxide were 37 and 21.8 ml, 30.8 and 14.3 ml, 39.6 and 15.6 ml, 71.3 and 29.2 ml, and 23.1 and 18.4 ml, respectively.

RESULTS

TDO and NDO, with one exception, preferentially oxidized the sulfur atoms of the aryl alkyl sulfides used in this study (Table 1). The sulfoxides were identified by comparison of their GC-MS properties with the sulfoxides formed by commercial preparations of HPO and CPO. Both hemoproteins are known to catalyze sulfoxidation of aryl alkyl sulfides (17-19). In addition, the use of CSP-HPLC to determine the enantiomeric composition of the sulfoxides formed by HPO and CPO provided a convenient procedure for determining the enantiomeric composition of the sulfoxides formed by TDO and NDO. For example, under the conditions used in the present study, HPO catalyzed the oxidation of the aryl alkyl sulfides to essentially racemic sulfoxides (Table 1). These results are in accord with previous reports (17, 18), with the exception of the stereospecific oxidation by HPO type IV which yielded (S)-sulfoxides in 60-70% e.e. (19). The identity of the sulfoxide enantiomers formed by HPO were confirmed by CSP-HPLC of the sulfoxides formed by CPO. Previous studies have shown that CPO oxidizes aryl alkyl sulfides to

Table 1. Stereospecific sulfoxidation of aryl alkyl sulfides by heme-containing peroxidases and Rieske [2Fe-2S] cluster, mononuclear iron-containing dioxygenases[1]

| | Enantiomeric Excess (% e.e.) (Relative Yield, %) | | | |
Substrate	Toluene Dioxygenase	Naphthalene Dioxygenase	Horseradish Peroxidase	Chloro-peroxidase
Methyl phenyl sulfide (R=H, R'=CH₃)	R > 98 (100)	S > 98 (100)	0 (100)	R > 98 (100)
Ethyl phenyl sulfide (R=H, R'=CH₂CH₃)	R > 98 (100)	S = 93 (100)	R = 16 (100)	R > 98 (100)
Methyl p-tolyl sulfide (R=CH₃, R'=CH₃)	S = 38 (35.3)[2]	S > 98 (100)	S = 6 (100)	R > 98 (100)
p-Methoxyphenyl methyl sulfide (R=OCH₃, R'=CH₃)	S = 32 (17)	S > 98 (100)	S = 6 (72.4)	R > 98 (100)
Methyl p-nitrophenyl sulfide (R=NO₂, R'=CH₃)	S = 86 (3)	S > 98 (100)	R = 10 (2.3)	R > 98 (4.8)

[1] Details of the reaction conditions and product analyses are given under Materials and Methods.
[2] The major product (62%) was identified as methyl p-tolyl sulfide cis-dihydrodiol.

(R)-sulfoxides (39-92% e.e.) (18). However, Table 1 shows that CPO oxidized the listed aryl alkyl sulfides almost exclusively to the (R)-enantiomers (>98% e.e.). The lower enantiomeric purities reported by Colonna et al. (18) may be due to longer incubation times (4 to 10 days), suboptimal incubation temperatures (4°C) or the methods used to determine enantiomeric purity (^{1}H-NMR and optical rotation).

TDO catalyzed the stereospecific oxidation of methyl phenyl sulfide and ethyl phenyl sulfide to enantiomerically pure (R)-sulfoxides (Table 1). In contrast, substituents at the *para*-position reduced not only reaction rate but also stereoselectivity. Thus, the electron withdrawing *p*-nitro substituent gave a low product yield with a high percentage of the (S)-enantiomer. GC-MS analyses of the products formed from methyl *p*-tolyl sulfide showed the presence of three compounds with molecular ions (M$^+$) at *m/z* 154, 154, and 172 in addition to methyl *p*-tolyl sulfoxide (M$^+$, *m/z* 154). The three compounds represented 62% of the total product yield and suggested that the major product was a 2,3-dihydrodiol which had undergone elimination of water, during work up or in the GC injection port, to yield phenols at the 2 and 3 positions. The formation of a *cis*-dihydrodiol was confirmed by the formation of the monobenzene boronate derivative (M$^+$, *m/z* 258) shown in Figure 1. This derivatization procedure led to the disappearance of the three compounds with molecular ions (M$^+$) at *m/z* 154, 154, and 172. These results suggest that the major product formed from methyl *p*-tolyl sulfide is the *cis*-dihydrodiol (*cis*-1,2-dihydroxy-3-methyl-6-methylthiocyclohexa-3,5-diene). The absolute stereochemistry of this reaction product was not determined.

NDO oxidized the aryl alkyl sulfides to the corresponding sulfoxides with an (S) absolute configuration. All of the sulfoxides were obtained in 100% yield and were of high enantiomeric
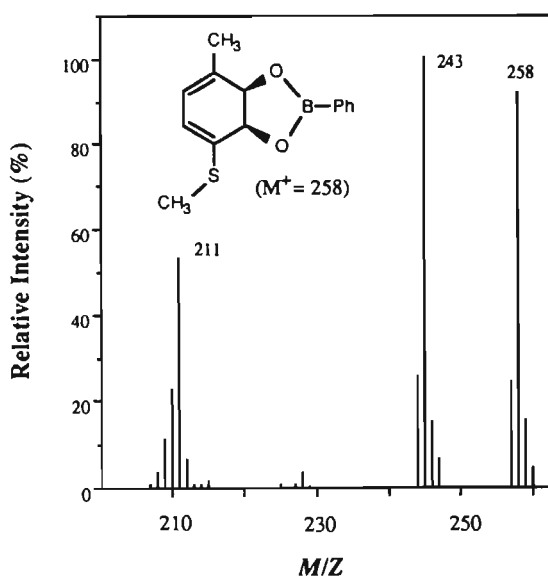

Fig. 1. Mass spectrum of the phenylboronic acid derivative of methyl *p*-tolyl sulfide *cis*-dihydrodiol formed by toluene dioxygenase (absolute stereochemistry is not intended).

purity (Table 1). Studies on the rate of formation of the sulfoxides was determined polarographically (Fig. 2). The initial rates of oxygen consumption in the presence of methyl phenyl sulfide, ethyl phenyl sulfide, and methyl p-tolyl sulfide were 1.7 µmol/min/mg ISP_{NAP}. The same oxidation rate was observed with naphthalene (data not shown). Oxygen uptake rates observed with p-methoxyphenyl methyl sulfide and methyl p-nitrophenyl sulfide were 1.56 and 0.90 µmol/min/mg ISP_{NAP}, respectively. In addition, the ratio of oxygen consumed to substrate added was greater than 1:1 for ethyl phenyl sulfide and methyl p-nitrophenyl sulfide. These observations indicate that substrate oxidation is partially uncoupled from oxygen consumption in the presence of these compounds.

When methyl phenyl sulfide was oxidized by purified TDO and NDO in the presence of $^{18}O_2$ ($^{18}O_2$:$^{16}O_2$ = 91:9) the ratios of the molecular ion ($M^+ + 2$) and (M^+) peaks at m/z 142 and 140 were 92:8 for both enzymes. These results show that the oxygen atom in methyl phenyl sulfoxide is derived exclusively from dioxygen.

DISCUSSION

The sulfoxidation of aryl alkyl sulfides adds to the growing list of reactions catalyzed by NDO and TDO (4-11). Other reactions include N-dealkylation and O_2-dependent alcohol oxidations (K. Lee and D.T. Gibson, unpublished results). The oxygenation of the pro-(R) or pro-(S) lone pair of electrons on the sulfide can be determined by the position of the substrate in the active site (regiospecificity) and the mobility of the substrate within the active site (stereospecificity) (20). Thus the sulfur atoms, rather than the aromatic or alkyl groups of the substrates shown in Table 1, appear to be in close proximity to the reactive oxygen species at the
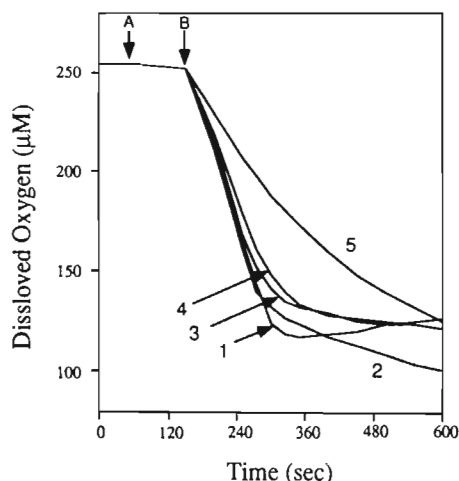
Fig. 2. Oxygen uptake by naphthalene dioxygenase determined in the presence of methyl phenyl sulfide (1), ethyl phenyl sulfide (2), methyl p-tolyl sulfide (3), p-methoxyphenyl methyl sulfide (4), and methyl p-nitrophenyl sulfide (5). Additions of NADH (A) and substrate (B) are indicated by arrows.

active site of both enzymes. The only exception is the formation of a sulfoxide and a *cis*-dihydrodiol from methyl *p*-tolyl sulfide by TDO. These results suggest that this substrate can adopt two orientations with respect to the reactive oxygen species responsible for the oxygenation reaction.

The results obtained with NDO suggest that the aryl alkyl substituents and the physiological substrate naphthalene, may occupy similar positions in the active site of the enzyme. This would account for the consistent formation of homochiral (*S*)-sulfoxides in high yields from the sulfides listed in Table 1. In a recent communication, naphthalene-grown cells of *Pseudomonas putida* strain NCIMB 8859 were reported to form (*S*)-sulfoxides from methyl phenyl sulfide and ethyl phenyl sulfide (13). Thus it is possible that this organism may contain a similar enzyme to the NDO purified from *Pseudomonas* sp. NCIB 9816-4. Oxygen uptake studies with purified NDO (Fig. 2) show that, with the exception of ethyl phenyl sulfide and methyl *p*-nitrophenyl sulfide, the enantiospecific oxygenation reactions are tightly coupled with respect to product formation.

TDO catalyzes the oxidation of methyl phenyl sulfide and ethyl phenyl sulfide to the corresponding (*R*)-sulfoxides (>98% e.e.). High yields of both sulfoxides were obtained (Table 1). Similar results were recently reported for TDO expressed by intact cells of *P. putida* strain UV4 and a recombinant *E. coli* strain (13). In these organisms, increasing the size of the alkyl substituent from methyl to isopropyl greatly reduced the yield of sulfoxides without significantly affecting their enantiomeric purity. The effect of larger alkyl substituents on sulfoxidation by purified TDO and NDO was not addressed in the present study. However, substituents at the *para*-position of the phenyl ring produced a remarkable change in the yields and enantiomeric purity of the sulfoxides formed by TDO. Substrates with electron-withdrawing (NO_2) and electron-donating (OCH_3) groups both yielded low amounts of sulfoxides with an excess of the (*S*)-enantiomer. Thus it appears that steric factors related to the phenyl ring may play a large role in determining the enantiospecificity of TDO. In contrast, *para*-substituents of the phenyl ring did not significantly affect the yield or the enantiomeric purity of the sulfoxides formed by NDO.

Sulfoxidation reactions are catalyzed by a diverse group of oxygenases. These include, cytochrome P-450s (21), heme-containing peroxidases (17-19), and copper-containing dopamine β-hydroxylase (22). All of these enzymes utilize free radical mechanisms to produce their respective sulfoxide products. Many of the reactions catalyzed by cytochrome P-450s including, monohydroxylation, *N*- and *O*-dealkylation, desaturation, O_2-dependent alcohol oxidation and sulfoxidation are also catalyzed by NDO and, to a lesser extent TDO. However, the oxygenase components of NDO and TDO which contain Rieske [2Fe-2S] centers and are thought to contain mononuclear iron at their respective active sites differ from cytochrome P-450s in their reactions with substrates containing π electrons. The dioxygenases produce chiral *cis*-dihydrodiols whereas cytochrome P-450s oxidize many of the same substrates to epoxides or monols. Current studies are directed towards identification of the reactive oxygen species generated by TDO and NDO which will further our understanding of the diverse oxidative reactions catalyzed by these unique dioxygenases.

14

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Desaturation and Oxygenation of 1,2-Dihydronaphthalene by Toluene and Naphthalene Dioxygenase

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Bacterial strains expressing toluene and naphthalene dioxygenase were used to examine the sequence of reactions involved in the oxidation of 1,2-dihydronaphthalene. Toluene dioxygenase of *Pseudomonas putida* F39/D oxidizes 1,2-dihydronaphthalene to (+)-*cis*-(1S,2R)-dihydroxy-1,2,3,4-tetrahydronaphthalene, (+)-(1R)-hydroxy-1,2-dihydronaphthalene, and (+)-*cis*-(1R,2S)-dihydroxy-1,2-dihydronaphthalene. In contrast, naphthalene dioxygenase of *Pseudomonas* sp. strain NCIB 9816/11 oxidizes 1,2-dihydronaphthalene to the opposite enantiomer, (−)-*cis*-(1R,2S)-dihydroxy-1,2,3,4-tetrahydronaphthalene and the identical (+)-*cis*-(1R,2S)-dihydroxy-1,2-dihydronaphthalene. Recombinant *Escherichia coli* strains expressing the structural genes for toluene and naphthalene dioxygenases confirmed the involvement of these enzymes in the reactions catalyzed by strains F39/D and NCIB 9816/11. 1-Hydroxy-1,2-dihydronaphthalene was not formed by strains expressing naphthalene dioxygenase. These results coupled with time course studies and deuterium labelling experiments indicate that, in addition to direct dioxygenation of the olefin, both enzymes have the ability to desaturate (dehydrogenate) 1,2-dihydronaphthalene to naphthalene, which serves as a substrate for *cis* dihydroxylation.

Pseudomonas putida F1 and *Pseudomonas* sp. strain NCIB 9816-4 initiate the oxidation of toluene and naphthalene by the addition of both atoms of molecular oxygen and two hydrogen atoms to the aromatic nucleus to form (+)-*cis*-(1S,2R)-dihydroxy-3-methylcyclohexa-3,5-diene (*cis*-toluene dihydrodiol) (14, 21) and (+)-*cis*-(1R,2S)-dihydroxy-1,2-dihydronaphthalene (*cis*-naphthalene dihydrodiol) (19, 20), respectively. These reactions are catalyzed by multicomponent enzyme systems designated toluene dioxygenase (TDO) (16, 31) and naphthalene dioxygenase (NDO) (13).

Current interest in TDO and NDO stems from the fact that, in addition to the enantiospecific reactions shown above, both enzymes also oxidize a wide range of substrate analogs to optically active products (5, 6, 10, 11, 25). Many of these compounds have been used as chiral synthons to synthesize a number of compounds of interest to the pharmaceutical and specialty chemical industries (references 9, 10, and 17 and references cited therein).

In addition to forming arene *cis*-dihydrodiols, the TDO expressed by strain F39/D catalyzes the monohydroxylation of indan to (1R)-indanol (7, 29). The same reaction is catalyzed by TDO expressed by *P. putida* UV4, a strain studied extensively by Boyd and his colleagues (5, 6). In contrast, NDO from strain NCIB 9816/11 oxidizes indan to (1S)-indanol and also catalyzes the desaturation of indan to indene. The latter compound is then oxidized by the enzyme to (1S)-indenol and *cis*-(1R,2S)-indandiol (15). The TDOs expressed by F39/D and UV4 do not catalyze the desaturation of indan to indene (6, 29). Strain UV4 does, however, oxidize 1,2-dihydronaphthalene (compound I, Fig. 1) to (+)-(1R)-hydroxy-1,2-dihydronaphthalene (compound II), (+)-*cis*-(1S,2R)-dihydroxy-

1,2,3,4-tetrahydronaphthalene (compound III), naphthalene (compound IV), and (+)-*cis*-(1R,2S)-naphthalene dihydrodiol (compound V) (4). Three possible reaction sequences have been proposed to account for the observed results. These are shown in Fig. 1 and are explained as follows: (i) oxidation of compound I to compound II, dehydration of compound II to compound IV, and *cis* hydroxylation of compound IV to compound V (reactions A→B→D→H) and (ii) oxidation of compound I to compound II, *cis* hydroxylation of compound II, and dehydration of the putative triol to compound V (reactions A→B→C→I). Support for sequences i and ii was provided by deuterium isotope experiments (4). Subsequent studies with 1,4-dihydronaphthalene (1) led to the suggestion that compound V could be formed by sequence (iii): oxidation of compound I to a benzyl radical followed by a second hydrogen abstraction to yield compound IV and subsequent *cis* hydroxylation to compound V (reactions A→E→H). We now report the isolation and identification of the products formed from deuterium-labeled compound I by TDO and NDO from strains F39/D and NCIB 9816/11, respectively, and provide evidence for the oxidation of compound I to compound V through desaturation and dioxygenation reactions A→E→H shown in Fig. 1.

MATERIALS AND METHODS

Materials. The following chemicals were obtained from the sources indicated: toluene, Fisher Scientific Co., Fair Lawn, N.J.; 2,3-dichloro-5,6-dicyano-1,4-benzoquinone, naphthalene (compound IV), 1,2-dihydronaphthalene (compound I), (−)-menthoxyacetic acid, 1-tetralone, oxalyl chloride, and sodium borodeuteride, Aldrich Chemical Co., Milwaukee, Wis.; 1,4-epoxy-1,4-dihydronaphthalene, Janssen Chimica, New Brunswick, N.J. Compound I was distilled prior to use. (±)-*cis*-1,2-Dihydroxy-1,2,3,4-tetrahydronaphthalene (compound III) was prepared from compound I by using OsO₄ as described previously (28). (±)-1-Hydroxy-1,2-dihydronaphthalene (compound II) and (±)-*cis*-1,2-dihydroxy-1,2-dihydronaphthalene (compound V) were synthesized as previously described (8, 18). [4-²H]1,2-dihydronaphthalene was prepared from 1-tetralone by reduction with NaBD₄ followed by dehydration (20% H₂SO₄; 16 h). [4-²H]1,2-dihydronaphthalene was then converted into [1-²H]naphthalene by using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone as previously described for naphthalene

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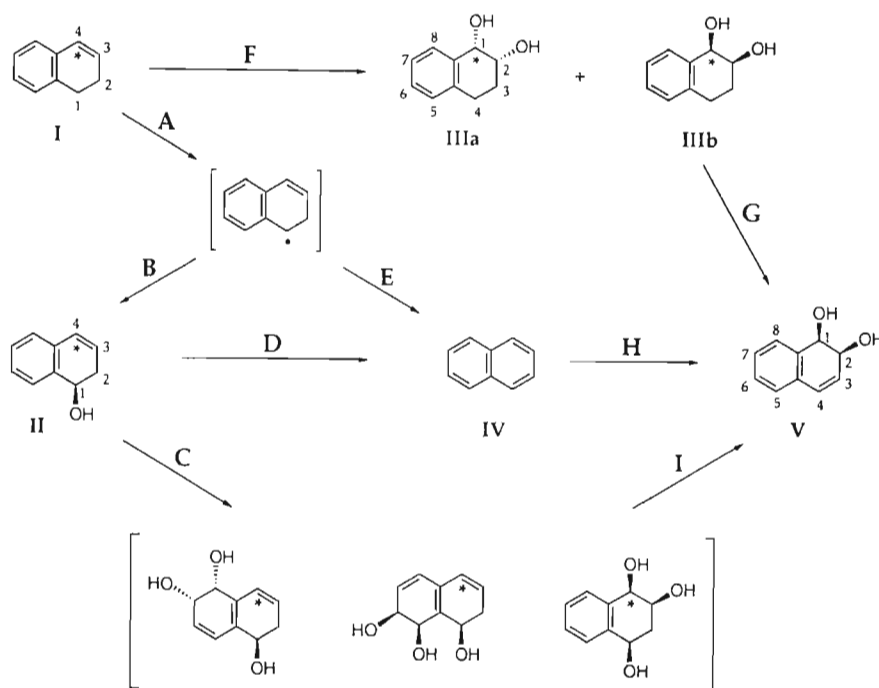
§ Deceased.

FIG. 1. Possible routes for the formation of *cis*-naphthalene dihydrodiol (compound V) from 1,2-dihydronaphthalene (compound I). An asterisk indicates the position of the deuterium label.

(30). Thin-layer chromatography and preparative-layer chromatography plates were obtained from EM Science, Gibbstown, N.J.

Analytical methods. High-performance liquid chromatography (HPLC) analyses were performed on a Waters μ-Porasil column (3.9 by 300 mm). The solvents used to separate the diesters of compounds V and III were ethyl acetate-hexanes at 0 to 10% over 10 min and 0 to 5% over 120 min. respectively. Gas chromatography-mass spectrometry (GC-MS) analyses were conducted as previously described (24). The relative yield of each product was determined by integration of the area under each peak excluding the solvent front. Each compound was expressed as a percentage of the total peak area derived from integration of the total ion chromatogram. In all cases, the compounds identified accounted for more than 95% of the total ion current, and only the area under the solvent front was excluded before relative percentages were computed. Thin-layer chromatography was performed on silica gel 60 F_{254} sheets (0.2 mm thickness). The solvents used were chloroform-acetone (80:20, vol/vol) and hexane-ethyl acetate (90:10, vol/vol). Preparative-layer chromatography was performed on silica gel 60 F_{254} plates (1.0 mm thickness). Optical rotation measurements were determined by using a Perkin-Elmer model 141 polarimeter. Reported $[\alpha]_o$ values were obtained in chloroform at 25°C. Proton (^{1}H), deuterium (^{2}H), and carbon (^{13}C) nuclear magnetic resonance (NMR) spectra were obtained with a Bruker WM-360 spectrometer at 360, 360, and 90.1 MHz, respectively.

Organisms and culture conditions. Mutant and recombinant bacterial strains expressing NDO and TDO were cultivated as described below. *Pseudomonas putida* F39/D is a dihydrodiol dehydrogenase mutant of *P. putida* F1 which oxidizes toluene to (+)-*cis*-(1S,2R)-dihydroxy-3-methylcyclohexa-3,5-diene (14, 21). Strain F39/D was grown at 30°C with rotary shaking (200 rpm) in a mineral salts medium (MSB [26]), pH 7.2, containing 0.2% pyruvate. Toluene was provided in the vapor phase. *Pseudomonas* sp. strain NCIB 9816/11 is a naphthalene dihydrodiol dehydrogenase mutant which oxidizes naphthalene to (+)-*cis*-(1R,2S)-dihydroxy-1,2-dihydronaphthalene (22). Strain NCIB 9816/11 was grown as described above and was induced with 0.05% (wt/vol) salicylate (3). Induced cells were harvested in the late exponential phase of growth by centrifugation (9,000 × *g*; 10 min; 4°C), washed once in MSB, and resuspended in 100 and 500 ml of MSB for kinetic experiments and isolation of metabolites, respectively. Growth of *Escherichia coli* JM109(pDTG141) (27) and JM109(pDTG601A) (32, 33), which express the structural genes encoding naphthalene and toluene dioxygenases, respectively, were grown in a 5.0-liter Bioflo II Fermentor (New Brunswick, Inc.) as previously described (23). Isopropyl-β-D-thiogalactopyranoside (IPTG)-induced cells were used for biotransformation reactions. Whole cells were incubated with 0.05% (vol/vol or wt/vol) substrate in MSB containing 0.1% pyruvate for strains F39/D and NCIB 9816/11 (turbidity, 2.0 to 2.5 at 600 nm) and 0.2% glucose for JM109(pDTG141) and JM109(pDTG601A) (turbidity, 12 to 15 at 600 nm). Flasks were incubated for stated times at 30°C with shaking (250 rpm). At designated intervals, or at 20 h for the isolation of metabolites, the contents of each flask were centrifuged (9,000 × *g*; 10 min; 4°C)

and the supernatant solutions were extracted three times with equal volumes of sodium hydroxide-washed ethyl acetate. The combined organic extracts were dried over anhydrous Na_2SO_4 and concentrated at 30°C under reduced pressure prior to analysis.

Cell extract experiments. Cell extracts were prepared by passage of cell suspensions through a chilled French pressure cell (20,000 lb/in^2); this was followed by removal of particulate material by centrifugation (10,000 × *g*; 10 min; 4°C). Clarified cell extract (1 ml) was used in reaction mixtures which contained a final volume of 4 ml: 0.05% substrate, 0.6 mM NADH, and 0.05 M Tris-HCl (pH 7.5). Flasks (25 ml) were incubated at 30°C with shaking (250 rpm) for stated times and extracted as described above.

Isolation of metabolites. Ethyl acetate extracts containing metabolites produced by strain F39/D were purified by radial dispersion chromatography on silica gel plates (2.0 mm thickness). Products were eluted with a chloroform-acetone step gradient (0 to 30% acetone; 5% steps over 1 h) at a flow rate of 7 ml/min. Under these conditions, compound II (R_f, 0.49) and a mixture of compounds III and V (R_f, 0.21) eluted with 20% acetone. Analytical samples (~20 mg) of compounds III and V were obtained by preparative-layer chromatography (silica thickness, 1.0 mm). Separation was achieved by multiple elution (five times in chloroform-acetone, 94:6). Diol V had the higher R_f of the two diols. Extraction of compounds III and V from the silica gel followed by recrystallization from ether-hexanes provided pure material suitable for analysis. The same procedures were used to isolate compounds II, III, and V formed by strain JM109(pDTG601A) and compounds III and V formed by strains NCIB 9816/11 and JM109(DE3)(pDTG141).

Preparation and isolation of derivatives. The (−)-menthoxyacetyl diester derivatives of diols III and V were synthesized from freshly prepared (−)-menthoxyacetyl chloride as previously described (12). The diastereomers were purified by radial dispersion chromatography (silica thickness, 1 mm) by using an ethyl acetate-hexanes step gradient (0 to 20% ethyl acetate; 4% steps over 1 h) at a flow rate of 4 ml/min. The purified diastereomers were analyzed for purity by ^{13}C NMR and HPLC and compared with authentic samples prepared from racemic diols. The arene hydrate (compound II) was hydrogenated to 1-tetralol as previously described (4) and derivatized with (−)-menthoxyacetyl chloride as described above. The mixture of diastereomers was not resolved by the HPLC conditions used for the diesters, and diastereomeric purity was determined only by ^{13}C NMR.

RESULTS

Oxidation of naphthalene (compound IV) by strains NCIB 9816/11 and F39/D. Induced cells of strains NCIB 9816/11 and F39/D oxidized compound IV to compound V of high enan-
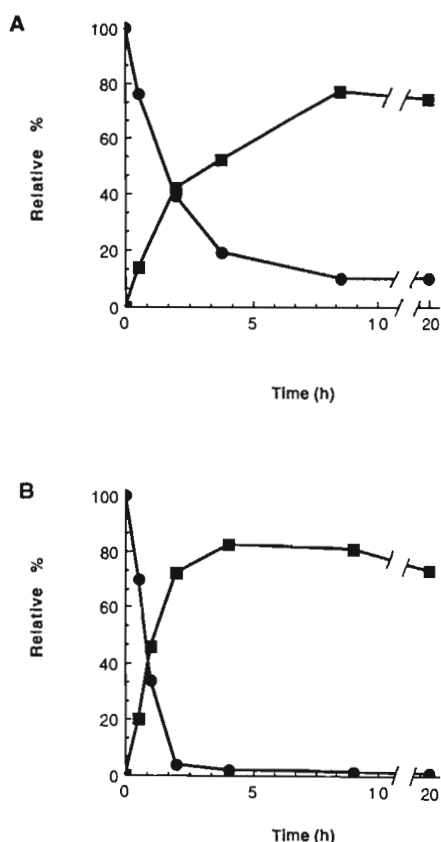
FIG. 2. Oxidation of naphthalene (compound IV) (●) to *cis*-naphthalene dihydrodiol (compound V) (■) by strains F39/D (A) and NCIB 9816/11 (B). Relative yields are given as the percentage of each compound present at the times shown as determined by integration of the total ion current during GC-MS analyses. Experimental details are described in Materials and Methods.

Oxidation of [4-²H]-1,2-dihydronaphthalene by strains NCIB 9816/11 and F39/D. Deuterium NMR analysis of a mixture of compounds III and V formed by strain NCIB 9816/11 showed that the monodeuterated diols were obtained in approximately the same ratio as those determined for the nondeuterated substrate. Proton and deuterium NMR analyses of the purified tetrahydronaphthalene diol (compound III) showed that deuterium was only present at the C-1 position (Fig. 5A), whereas the dihydronaphthalene diol (compound V) was labeled in equal ratios at the C-1, C-4, C-5, and C-8 positions (Fig. 5B). The deuterium NMR spectra of compounds II, III, and V formed by strain F39/D from deuterated compound I are shown in Fig. 6. The deuterated products were obtained in approximately the same ratio as for the nondeuterated substrate. Proton and deuterium NMR analyses of compounds II and III showed that deuterium was located only at the C-4 and C-1 positions, respectively (Fig. 6A and B). In contrast, compound V was labeled equally at the C-1, C-4, C-5, and C-8 positions (Fig. 6C).

Oxidation of 1,2-dihydronaphthalene (compound I) by strains JM109(DE3)(pDTG141) and JM109(pDTG601A). Dihydronaphthalene (compound I) was oxidized by IPTG-induced cells of JM109(DE3)(pDTG141), which express the NDO structural genes (*nahAaAbAcAd*) from NCIB 9816-4, to yield both compounds III and V in a 1.5:1.0 ratio after 20 h. IPTG-induced cells of JM109(pDTG601A), which express the TDO structural genes (*todC1C2BA*), oxidized compound I to compounds II, III, and V in a 2.0:1.0:1.2 ratio after 20 h. All products formed by both recombinant organisms exhibited NMR and mass spectra identical to those exhibited by authentic compounds.

Oxidation of (±)-1-hydroxy-1,2-dihydronaphthalene (compound II) and (±)-*cis*-1,2-dihydroxy-1,2,3,4-tetrahydronaphthalene (compound III) by strains NCIB 9816/11 and F39/D.
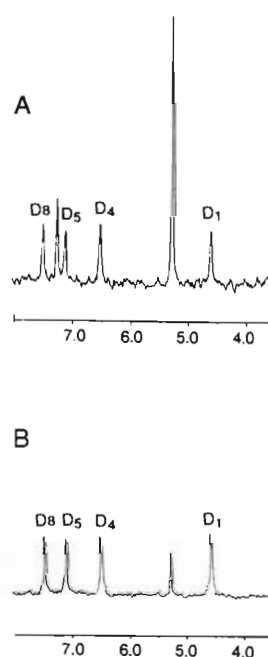
tiomeric purity (>98% enantiomeric excess [e.e.]) as the only detectable product (Fig. 2). High yields (85 to 90%) of the diol were produced by both organisms. The experiment was repeated with [1-²H]-naphthalene, and similar yields of monodeuterated diols were obtained. Analyses of proton and deuterium NMR spectra showed that both organisms produced compound V in which equal ratios of deuterium were located at the C-1, C-4, C-5, and C-8 positions (Fig. 3).

Oxidation of 1,2-dihydronaphthalene (compound I) by strains NCIB 9816/11 and F39/D. Figure 4 shows that compound I was oxidized rapidly by both strains. The major products formed were compounds III and V by strain NCIB 9816/11 and compounds II, III, and V by strain F39/D. Both strains formed small amounts of compound IV (<5%) during the transformation. Each metabolite isolated exhibited NMR and mass spectra identical to those exhibited by authentic compounds. Both diols (compounds III and V) formed by NCIB 9816/11 and F39/D were of high enantiomeric purity (≥98% e.e.), and the tetrahydrodiols III were of opposite configuration (Table 1). Compound II formed by F39/D was chromatographically and spectrally indistinguishable from an authentic sample. The optical rotation of compound II ($[\alpha]_D$ = +53°) was identical to the value previously reported for (+)-(1R)-hydroxy-1,2-dihydronaphthalene (4). In addition, the rotation of the 1-tetralol formed by hydrogenation of compound II, in conjunction with the NMR analysis of the menthoxy acetyl ester derived from 1-tetralol, confirmed that compound II was of 1R configuration and high enantiomeric purity (>98% e.e.) (Table 1).

FIG. 3. Deuterium NMR spectrum of *cis*-naphthalene dihydrodiols (compound V) formed from [1-²H]naphthalene by strains F39/D (A) and NCIB 9816/11 (B). Samples were dissolved in CH₂Cl₂, and chemical shifts are given in parts per million and referenced to CDCl₃ (7.24 ppm) or acetone-d₆ (2.04 ppm) as the internal standard. The signal at 5.28 ppm is due to CD₂Cl₂. The assigned deuterium chemical shifts refer to the numbered structures in Fig. 1.
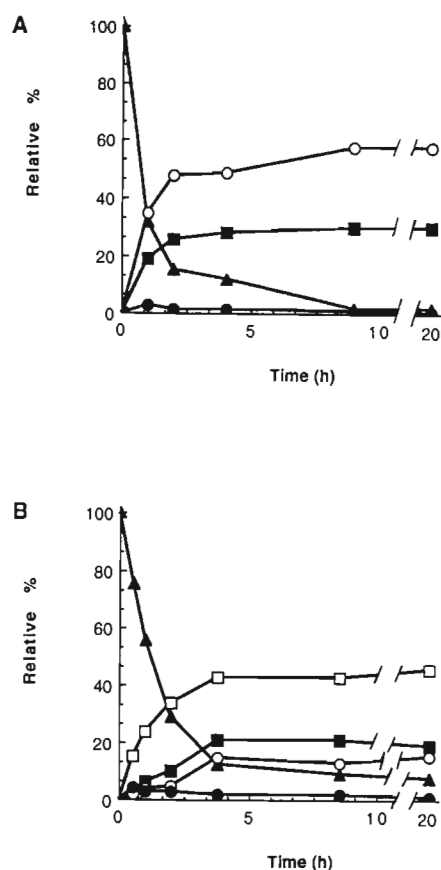
A

B

FIG. 4. (A) Oxidation of compound I (▲) to compounds III (○), IV (●), and V (■) by strain NCIB 9816/11; (B) oxidation of compound I (▲) to compounds II (□), III (○), IV (●), and V (■) by strain F39/D. Relative yields are given as the percentage of each compound present at the times shown as determined by integration of the total ion current during GC-MS analyses. Experimental details are described in Materials and Methods.

A

B

FIG. 5. Deuterium NMR spectra of metabolites formed from [4-^{2}H]1,2-dihydronaphthalene (compound I) by strain NCIB 9816/11. (A) Diol IIIb labeled at the C-1 position; (B) diol V labeled at the C-1, C-4, C-5, and C-8 positions. Chemical shifts are reported in parts per million. The signal at 5.28 ppm is due to CD_2Cl_2. The samples were prepared as described in the legend to Fig. 3.

Racemic compound II was incubated with induced cells of strains NCIB 9816/11 and F39/D. Over a 24-h period, 95% of the starting material was recovered together with approximately 5% 1-naphthol. Similar results were obtained with strain F39/D, for which 85% of the substrate and 15% of 1-naphthol was recovered. In separate experiments, racemic compounds II and III were incubated without cells under the same conditions as those used for the biotransformations. Both compounds were recovered unchanged at the end of the experiment. In control experiments, the same cells oxidized compound IV to compound V.

Racemic compound III was incubated with strains NCIB 9816/11 and F39/D for 24 h as described above for compound II. Both strains formed approximately 5% of diol V, and the majority of the substrate was recovered unchanged.
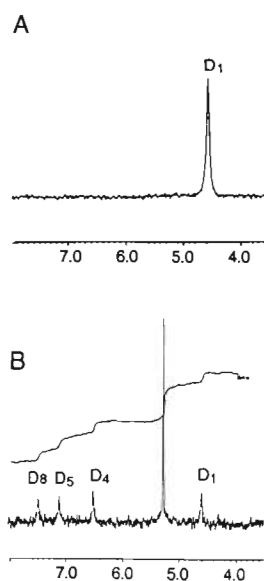
Cell extract experiments. Cell extracts prepared from induced cells of strains NCIB 9816/11 and F39/D oxidized 1,2-dihydronaphthalene (compound I) and naphthalene (compound IV) to the same products and in the same ratios as observed with intact cells. Both cell extracts did not oxidize racemic compounds II, III, and V.

DISCUSSION

The results presented indicate that NDO and TDO catalyze the formation of *cis*-naphthalene dihydrodiol (compound V) by desaturation of 1,2-dihydronaphthalene to naphthalene (compound IV) followed by *cis*-hydroxylation, to yield compound V of high enantiomeric purity (Fig. 1, reactions A→E→H). These conclusions are based on the formation of significant amounts of compound V by induced cells of strains NCIB 9816/11 and F39/D and the detection of low levels of compound IV formed by both organisms. Further support is provided by showing that recombinant *E. coli* strains expressing NDO and TDO activity oxidize compound I to the same products (compounds III and V) in almost the same ratios as those formed by the mutant strains NCIB 9816/11 and F39/D. In addition, the reaction sequence A→E→H may be catalyzed by the TDO expressed by strain UV4 (1), although experiments with deuterated compound I support alternative routes for the oxidation of compound I to compound V by this or-

TABLE 1. Products formed from 1,2-dihydronaphthalene (compound I) by strains NCIB 9816/11 and F39/D[a]

Strain	Ratio[b]			[α]$_D$ of compound:			Configuration (e.e.) of compound:		
	II	III	V	II	III	V	II	III	V
NCIB 9816/11		1.9	1.0		−36.4°	+211°		1R,2S (>98%)	1R,2S (>98%)
F39/D	2.5	1.0	1.2	+53°	+36°	+211°	1R (>98%)	1S,2R (>98%)	1R,2S (>98%)

[a] Both strains were incubated with compound I for 20 h prior to the isolation of products as described in Materials and Methods. All products were identified by comparison of their NMR and GC/MS spectra with those of authentic compounds. Compound numbers refer to the structures in Fig. 1.
[b] Ratios of biotransformation products were determined by integration of the total ion current chromatograms obtained during GC/MS analyses.
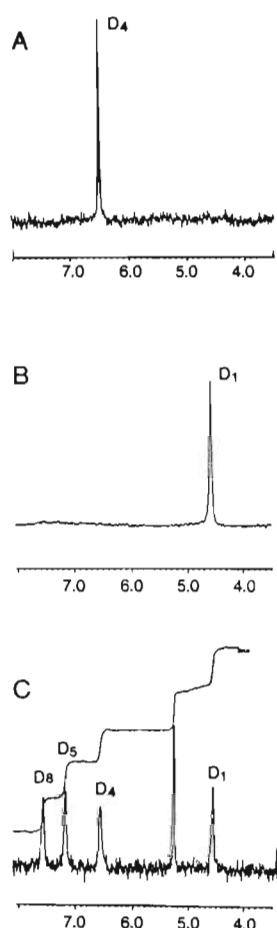
FIG. 6. Deuterium NMR spectra of metabolites formed from [4-²H]1,2-di-hydronaphthalene (compound I) by strain F39/D. (A) Alcohol II labeled at the C-4 position; (B) diol IIIa labeled at the C-1 position; (C) diol V labeled at the C-1, C-4, C-5, and C-8 positions. Chemical shifts are reported in parts per million. The signal at 5.28 ppm is due to CD₂Cl₂. Samples were prepared for NMR analyses as described in the legend to Fig. 3.

the oxidation of compound I to compound V by strains NCIB 9816/11 and F39/D. However, when both strains (or induced cell extracts) were incubated with racemic compound II for 20 h, most of the substrate was recovered unchanged and only a minor amount of 1-naphthol was detected as a reaction product. The reaction sequence A→B→D→H involving the formation of compound V through the arene hydrate intermediate compound II can therefore be eliminated.

The two remaining reaction sequences that could account for the oxidation of compound I to compound V are F→G and A→E→H (Fig. 1). The former sequence (F→G) could possibly explain the difference in the absolute configuration of the tetrahydronaphthalene diols formed by strains NCIB 9816/11 and F39/D. For example, strain F39/D might form a racemic mixture of tetrahydronaphthalene diols (compounds IIIa and IIIb; Fig. 1). If the organism possesses an enantiospecific tetrahydronaphthalene-1,2-diol 3,4-dehydrogenase, the (1R,2S)-tetrahydronaphthalene diol IIIb would be converted to compound V while leaving compound IIIa unchanged. This route was eliminated by showing that long-term (20-h) incubations of strains NCIB 9816/11 and F39/D with racemic compound III yielded only small amounts of compound V (<5%), and the remaining substrate was recovered unchanged at the end of the experiment. In addition, the oxidation of deuterated [4-²H]1,2-dihydronaphthalene through sequence F→G (Fig. 1) would yield compound V containing deuterium at only the C-1 position; the finding that dihydrodiol V contained equal amounts of deuterium label at the C-1, C-4, C-5, and C-8 positions (Figs. 5B and 6C) is consistent with the reaction sequence A→E→H.

The results show that TDO and NDO have different specificities when dihydronaphthalene (compound I) is used as a substrate, even though both dioxygenases oxidize naphthalene to (+)-cis-(1R,2S)-dihydroxy-1,2-dihydronaphthalene (Fig. 7A). The formation of enantiomerically pure (−)-cis-(1R,2S)-dihydroxy-1,2,3,4-tetrahydronaphthalene by NDO and (+)-cis-(1S,2R)-dihydroxy-1,2,3,4-tetrahydronaphthalene by TDO (Table 1) indicate that the direct dioxygenation of compound I by NDO must occur on the opposite face attacked by TDO (Fig. 7B and C). It would seem most plausible to explain these results in terms of the spatial differences between naphthalene and dihydronaphthalene. Naphthalene is a planar molecule, whereas dihydronaphthalene is twisted out of the plane be-

ganism (4). These are shown in Fig. 1 and require the oxidation of compound I to the arene hydrate compound II which can undergo (i) dehydration to compound IV followed by cis hydroxylation to compound V (reactions A→B→D→H) or (ii) cis-hydroxylation to the putative triols shown in Fig. 1 followed by dehydration to compound V (reactions A→B→C→I). Dehydration of compound II occurs rapidly under mild acidic conditions (1). However, no dehydration of compound II was observed under the experimental conditions used in the present study. Deuterated compound I was used to determine if NDO and TDO expressed by strains NCIB 9816/11 and F39/D also use the arene hydrate compound II as a precursor of compound V by the sequences described above for the TDO expressed by strain UV4. If dehydration occurs after the oxidation of compound II to the putative triols shown in Fig. 1, the diol V would show deuterium enrichment at either the C-1, C-5, or C-8 position based on the formation of the three triols shown. Figures 5 and 6 show that dihydrodiol V formed by strains NCIB 9816/11 and F39/D contains equal amounts of deuterium at the C-1, C-4, C-5, and C-8 positions. These results eliminate the reaction sequence A→B→C→I for the formation of compound V.

The equal distribution of deuterium in compound V (Fig. 5B and 6C) supports the sequence A→B→D→H or A→E→H for
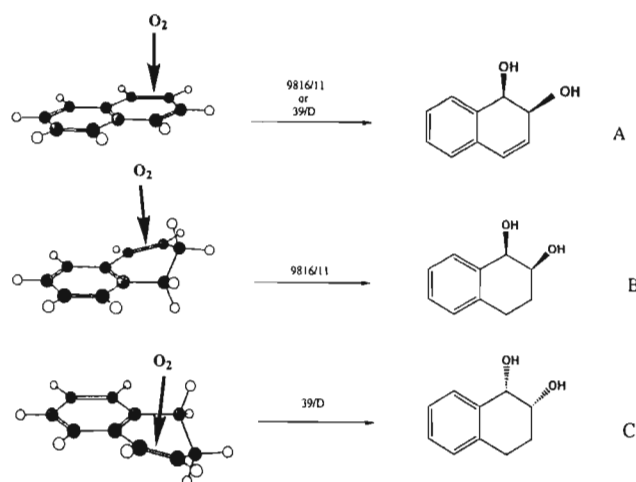

FIG. 7. Dioxygenation of naphthalene (A) and 1,2-dihydronaphthalene by strains NCIB 9816/11 (B) and F39/D (C) which express NDO and TDO, respectively.

cause of the presence of the two sp³ carbon atoms. This twist, combined with the two additional hydrogens, causes dihydronaphthalene to possess a larger "quadrant" than naphthalene (Fig. 7). These differences could force dihydronaphthalene to assume a 180°-different orientation in the active site of TDO compared with NDO (Fig. 7B and C). It is of interest that the enantiospecificities observed in the direct dioxygenation of compound I by NDO and TDO are consistent with those reported for the diols formed from indene. In the case of indene, NDO produced a (+)-(1R,2S)-diol of higher purity (>70% e.e.) than the (−)-(1S,2R)-diol formed by TDO (4, 15, 29).

In conclusion, the results support the reaction sequence A→E→H for the oxidation of compound I to compound V by TDO and NDO. A desaturase reaction via a benzyl radical is proposed for the oxidation of compound I to compound IV, since we have recently shown that the oxidation of indan to indene by NDO shares many similarities with desaturase systems from higher organisms. These include a requirement for an electron donor [NAD(P)H], oxygen, and a desaturase component which contains non-heme iron (15). It appears that naphthalene (compound IV) is released from the active sites of NDO and TDO prior to dioxygenation to compound V. This is supported by the detection of low concentrations of naphthalene during the oxidation of compound I to compound V and also by the formation of compound V with equal amounts of deuterium present at the C-1, C-4, C-5, and C-8 positions when [4-²H]1,2-dihydronaphthalene is used as the substrate. This can only occur if naphthalene is free to rotate in any orientation after its formation.

Recent studies by Boyd and his colleagues have shown that naphthalene-grown cells of *P. putida* NCIMB 8859 oxidize 1,2-dihydronaphthalene to the (−)-(1R,2S)-enantiomer IIIb. The *cis*-naphthalene dihydrodiol dehydrogenase in this strain selectively dehydrogenates this enantiomer and thus oxidizes a racemic mixture of compound III to the (+)-(1S,2R)-enantiomer (91% e.e.), which is the same enantiomer formed from compound I by strains UV4 and F39/D (2).

It is clear that there are many similarities between the TDOs expressed by strains F39/D and UV4 and also between the NDOs expressed by strains NCIB 9816/11 and NCIMB 8859. The observed differences (i.e., the formation of compound V from compound I through the reaction sequence A→B→C→I [4]) may reflect differences in experimental protocols which affect reaction rates and thus lead to differences in yields and purities of the isolated reaction products. The results of the present study are consistent with more recent studies with UV4 (1) and indicate that both TDO and NDO catalyze the oxidation of compound I to compound V by reactions involving the initial desaturation of compound I to compound IV, which then serves as a substrate for dioxygenation to yield (+)-(1R,2S)-compound V (sequence A→E→H, Fig. 1).

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ADDENDUM IN PROOF

A study by D. R. Boyd, N. D. Sharma, R. Agarwal, N. A. Kerley, R. A. S. McMordie, G. N. Sheldrake, P. Williams, and H. Dalton (J. Chem. Soc. Perkin I, in press) reports that strain UV4, unlike strains F39/D and NCIB 9816, oxidizes compound I to compound V through compound II and enantiopure 1R, 7S, 8R-triol (reactions A→B→C→I in Fig. 1).

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