MEMBRANE CHANGES AND LIPID PEROXIDATION DURING AGEING IN SEEDS OF *Lactuca sativa* L.

by

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PREFACE

The work described in this thesis was carried out in the Biology Department, University of Natal from January 1976 to 1986, under the supervision of Professor T.A. Villiers and Dr. G.K. Campbell.

These studies represent original work by the author and have not been submitted in any form to another university.

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ABSTRACT

Ultrastructural and biochemical studies were undertaken in an attempt to identify possible lesions which lead to loss of viability in seeds of Lactuca sativa cv. Great Lakes.

Electron microscopy revealed cotyledonary necrosis ('red heart') in seedlings as a symptom of ageing manifested by delayed mobilization and localized cell death. Ultrastructural studies on root tip cells during imbibition revealed discontinuities in the plasmalemma and fusion of lipid bodies to be one of the earliest observable changes during ageing. Since anhydrous fixation techniques revealed that the membranes of dry seeds were continuous and lamellar, it is suggested that plasmalemma lesions are the result of altered membrane properties which became exacerbated on imbibition rather than the result of an inability of non-lamellar membranes to become re-organised into a lamellar bilayer. Additional ageing related changes seen included damage to the membrane systems of the mitochondria, ER, and plastids, clumping of the nuclear material and dispersal of the ground cytoplasm. γ-irradiated dry seeds were seen to possess plasmalemma abnormalities on imbibition as well as the other ultrastructural lesions described above for aged seeds. However the presence of cytolysome-like structures was uniquely associated with the irradiation treatment.

These changes are critically discussed in relation to methodology, a limited biochemical literature, and the proposal that membrane damage may be an important contributory element to seed ageing.

Biochemical studies revealed a distinct increase in total lipid hydroperoxides with duration and severity of ageing regime, which was not accompanied by losses in total lipid unsaturation. Analysis of membrane phospholipids by HPLC nevertheless revealed changes in the relative percentages of phosphatidylcholine, phosphatidylethanolamine and two unknown products to be associated

with ageing. Analysis of the volatile products in the headspace of dry-heated seeds, and of the volatiles produced in the headspace of seeds during the course of imbibition, revealed a relationship between seed quality and the particular volatile spectrum. Amongst the volatiles identified by GC-MS as possible indicators of seed quality were pentane and ethanethiol; short chain alcohols and aldehydes (butanol, pentanol, hexanol, pentanal and hexanal). The origin of these compounds as likely breakdown products of lipid jhydroperoxides is considered. The status of the free radical theory of seed ageing is discussed and a model is presented to show how free radical action and altered enzyme activity may lead to a state of metabolic imbalance, and also to the evolution of the volatile compounds identified.

CONTENTS

CHAPTER 1

INTRODUCTION

Undoubtedly the holding of seed stocks for planting must be regarded as man's earliest and most important reason for storing seeds. That the lifespan of stored seed is finite has been known for a considerable time (Theophrastus 285 B.C., cited in Evenari, 1980/81). One of the first writers to advocate the protection of seeds from heat, moisture and oxygen to ensure vitality was probably de Candolle (cited in Justice & Bass, 1978). Attempts to assess and measure seed deterioration have been fraught with problems of definition and interpretation. The most widely accepted and useful index of seed deterioration is the reduction in viability (i.e. the ability to germinate). While the measurement of germination by a quiescent seed, when given favourable conditions, would appear to be an easy laboratory measure of seed quality, this is not the case. Germination may be defined as the emergence of the radicle, but at the other extreme it can refer to the establishment of a seedling. However, the establishment of a seedling under ideal, non-stressed laboratory conditions is a far cry from those encountered in the field. Indeed, as Perry (1973) has pointed out, it is largely the absence of a consistent relationship between germination in the laboratory and emergence in the field which has been responsible for the development of the concept of seed vigour. Heydecker (1977) has defined vigour as a "cover name" given to that "complex" of interrelated properties which render seeds versatile enough to germinate and flourish under a wide range of conditions. It should be clear that the above definition creates considerable difficulties, both in terms of conceptualization and measurement, although it does lay emphasis on the more changeable conditions likely to be encountered in the environment. However, vigour has also been used in a more specific sense to provide a physiological perspective by measuring such parameters as the rate of germination, rate of

seedling elongation, or fresh weight increase in test conditions. These measured properties may or may not have any relevance as regards predicting field potential (Halmer & Bewley, 1984).

Although there are difficulties associated with the evaluation of seed viability, it is generally held that loss of viability in a seed population (as measured by germination percentage) provides a reliable index of deterioration in the population as a whole (Roberts, 1972).

Attempts to determine why seeds lose viability are confounded by the fact that the critical events leading to radicle protrusion are poorly understood. As Jann & Amen (1969) have suggested the gap between morphogenesis and biochemistry is still wide. Except for seeds with some kind of after-ripening requirement, it is generally held that after the final drying stage of maturation the seed is in a condition of maximum vigour, and from this point gradually loses vigour and eventually dies (loses viability). In biochemical terms it has been suggested that this reflects a progressive impairment of a seed's potential to synthesize lipids, proteins and all classes of ribonucleic acids (Osborne, 1982). In addition the enzymes responsible for repair to DNA molecules are thought to become impaired with time, leading to morphological abnormalities and chromosomal aberrations. Woodstock (1973) has suggested that in cellular terms there is no sharp distinction between life and death in seeds. Death is viewed as a gradual and cumulative process where more and more cells die until certain critical parts of the seed become unable to perform their essential function. The loss of germination is seen as the last relevant indication of the loss of vigour (Delouche, 1969; Heydecker, 1972), but one which is preceded by many much more subtle changes. Thus vigour and viability may be seen to be on the same continuum of deterioration.

A number of distinct, but interacting components are known to influence seed vigour and viability (Heydecker, 1972). These include:

1. Genetic factors (both species specific and varietal)

2. Pathological factors (seed and soil borne)
3. Pre-harvest and maturational effects
4. Mechanical factors (at harvest and in handling)
5. The storage environment (principally temperature and moisture)
6. Intrinsic factors (including accumulation of toxic metabolites, denaturation of macromolecules and depletion of essential metabolites)

In this thesis the storage environment has been the major concern as a determining factor in seed ageing, but the other factors are of sufficient importance to warrant some discussion; these above six factors will be discussed in turn.

1. GENETIC FACTORS

In considering examples which claim to show genetic factors operating between species and among cultivars as regards longevity it is well to bear in mind that variable environmental conditions, storage and harvest conditions could make genetic differences seem greater than is actually the case; alternatively genetic differences could be masked by the above variables. Nevertheless, different cultivars and harvests of particular species show different viability characteristics under identical storage conditions (Bewley & Black, 1982). On the other hand Roberts (1972) has reported a case of 6 widely differing cultivars of rice which were thought to show marked differences in viability. Careful re-examination under identical storage conditons showed that all cultivars yielded identical survival curves.

Studies with maize have yielded the clearest evidence that longevity has a genetic component in inbred lines (Lindstrom, 1942). However, the relationship was not simple. Certain **luteus** genes on chromosome 10 have been implicated in, although by no means proven to reduce longevity (Weiss & Wentz, 1937). More recently, Scott (1981) has used rapid ageing procedures to select for longevity in maize populations, and believes this may be a feasible approach for the

selection of this trait. In a study under controlled storage conditions it was shown (Harrison, 1966) that differences were apparent in the storability of cultivars of lettuce which amounted to a threefold difference in the most extreme case.

Many reports concerning germination ability of seed after long periods of storage are generally unplanned and often subject to uncontrolled conditions of storage. Many of the seeds with authenticated records for longevity of 100 years or more appear to have hard and impermeable seed coats in common, such as the genera Cassia, Albizia, Trifolium, Canna and Nelumbo. Thus the resistance of coats to water and gases may contribute in some way to longevity in addition to possible species-specific genetic determinants (Harrington, 1972). Flood & Sinclair (1981) have argued that this may be so for Trifolium, while Ohlrogge & Kiernan (1982) have suggested that oxygen may produce an additive effect in ageing, but not be the primary determinant per se. It has been well-documented that removal of hulls from cultivated, economically important grasses reduces storability either by rendering seed tissues liable to infection or by possibly removing mould inhibiting sustances present in the surrounding tissue (Justice & Bass, 1978). Haferkamp et al. (1953) have classified economically important plants into three broad categories. Those with a short storage life, including lettuce, onion, parsnip and peanut; intermediate, such as maize and wheat; those capable of long-term storage such as barley and oats. To-date there has been no attempt at comparative studies which seek possible reasons for such longevity.

2. PATHOLOGICAL FACTORS

Of the microflora which may infect or be harboured within seeds, the fungi are probably the most important and thoroughly investigated. The seeds of grasses, in particular, continually face the risk of infection as a consequence of their open fruit structure. At the other end of the spectrum, seeds of fleshy fruits such as tomatoes and melons, may be free from microflora at maturity, assuming the fruits themselves were reasonably sound. However, soundness should by no means be taken to indicate that tissues are sterile (Pugh, 1973).

Christensen (1972) has classified fungi into two broad groups, namely field and storage fungi. This distinction is based on the conditions that permit the fungi to invade seeds.

Field Fungi

Some fungi invade the seeds while they are maturing on the plants in the field, or they may invade harvested material before drying when the moisture content is still high. These field fungi may be particularly troublesome and abundant during unseasonally prolonged wet conditions prevailing at harvest. Rampant growth by, inter alia, species of the genera Alternaria, Fusarium and Helminthosporium will lead to death of ovules or embryos and shrivelling of seeds, as well as discolouration. Toxic fungal products detrimental to man may also be produced. These fungi remain dormant during storage and in the case of grass seeds have been shown to prevent growth of storage fungi during the initial stages of storage.

Storage Fungi

Certain fungi are not isolatable at harvest but are capable of growing under conditions of limited moisture content. It has been shown that during over-winter storage of cereal grains, field fungi gradually

decline while the storage fungi increase in abundance. The genera Aspergillus and Penicillium constitute the major members of the storage fungi, although Sporodonema, Candida and Pullularia spp may also be present under certain conditions (Christensen, 1972).

Each species of fungus is considered to have its own lower limit of seed moisture content, below which it will not grow. However, this distinction is confounded by the fact that different seed parts will differ in moisture content (Christensen, 1972).

The rate at which storage fungi develop is determined by the extent of invasion, seed damage, the presence of insect vectors and bulk storage effects. The effects of these fungi upon seeds are thought to be, in order of appearance:

1. Decrease in germinability
2. Discolouration
3. Production of mycotoxins
4. Heating
5. Development of mustiness and caking
6. Total decay.

Although there can be little doubt that storage fungi can accelerate viability loss, Roberts (1972) presents evidence which suggests that fungi cannot be solely responsible for loss of viability. Seeds shown to be sterile nevertheless deteriorate, while the presence of known storage fungi reduces mean viability by only 18 - 23%.

3. PRE-HARVEST AND MATURATIONAL EFFECTS

It has been documented (Austin, 1972) that the environmental factors of temperature, rainfall, soil and atmospheric moisture, photoperiod and soil mineral status infuence not only storage reserves of the developing seed, but also vigour and viability. These are seen in the development of abnormal morphological structures and the internal disruption of biochemical mechanisms associated with seed

germination and development of the seedling. It is perhaps easy to understand how environmental influences could influence the seed in the critical final desiccation stage of development, especially since current thinking on seed formation favours the idea of developmental switching with water status (Walbot, 1978; Dure, 1977; Dasgupta & Bewley, 1982).

Persistent wet weather, which led to slower drying rates in soybean seeds, was shown by Howell et al. (1959) to lead to seed weight loss. This was attributed to high respiratory rates over a longer than normal period, leading to loss of substrates.

Many studies have shown that heavier or larger seeds are superior to smaller seeds as regards germination and vigour although no studies have been undertaken to show that this may be extended to include storage for longer periods (Justice & Bass, 1978). Not all seeds mature synchronously on the parent plant and consequently variations in the developmental stage and size of seeds can be expected. The harvest of a seed crop can be considered a compromise between logistical and environmental factors in addition to the maturity of the crop. It was Borthwick (1932) who drew attention to the later- flowering, higher order umbels of carrot, a fact which would be expected to lead to some seeds being harvested in a less than fully mature state.

Austin (1972) presented data to show that germination and field emergence were poorer in seed of carrot harvested earlier, a fact which could be related to the size and weight of seeds. A well-documented, though not necessarily fully understood harvest disorder is "hollow heart" in peas. Seeds are usually normal in outward appearance but have sunken or cracked areas in the centre of the adaxial cotyledon face. While the seeds may germinate well on filter paper in the laboratory, their field performance is poor (Neergaard, 1977), the tissue lining the cavity being prone to fungal attack. Perry & Harrison (1973) showed experimentally that high ambient temperatures during maturation, or the drying of immature seeds could produce this disorder. Field moisture, stress and temperatures of 32°C and above were reported to produce necrotic

spots in the cotyledons of maturing lettuce seeds (Neergaard, 1977).

4. MECHANICAL FACTORS

The use of mechanical harvesting and threshing machinery must inevitably lead to some seeds being damaged although the advantages gained would clearly outweigh disadvantages in commercial practice. The extent of the damage would be clearly influenced by the structure of the fruit and the difficulty in removing such structures from the "seeds". (This latter term is used in the loosest possible sense for that structure which is commercially marketed as a seed, without reference to botanical terminology).

Environmental conditions at maturation can exert an influence by making seed tissues more liable to cracks, bruises and abrasions on threshing, as can excessive dryness. Coincidentally such damage would permit invasion by fungi. Moore (1973) has suggested that ageing of adjacent tissues is accelerated by embryo injuries, although this conclusion is based on interpretation of patterns of tetrazolium staining.

Large seeds such as soya, beans and peanuts are generally more susceptible to injury, while smaller seeds, particularly if they are round, are less liable to damage. Onion and sorghum seeds on the other hand, although small are liable to radicle damage because these project from the bulk of the endosperm. Barley and rice are well-protected by a husk and are considered immune to normal mechanical abuse (Roberts, 1972).

5. THE STORAGE ENVIRONMENT

Seed storage has been seen in its broadest context as beginning at harvest and ending at planting (Harrington, 1973). In his review, Harrington emphasizes the importance of proper drying and packing in moisture-proof containers to minimize the deterioration, and stresses the importance of seed moisture content and temperature as the most important factors affecting longevity. Reported effects of the gaseous storage environment are regarded as variable and in some instances contradictory (Justice & Bass, 1978).

Seeds have been divided into two categories as far as their viability characteristics are concerned (Roberts, 1973). Most species, including all crops and probably all the weeds of arable land, are regarded as orthodox and may be dried to low moisture contents of 5% or less without damage. The other group are described as recalcitrant, lose viability rapidly, and are killed by drying and temperatures well above 0°C (King & Roberts, 1980). An understanding of why viability loss is so rapid may well provide useful comparison or contrasts with orthodox seeds, but will not be considered further.

Living systems obey the laws of thermodynamics but the high order which they display is achieved at the expense of energy. On the other hand, in a dry seed where metabolic activity is considered minimal, the laws of thermodynamics demand that the free energy decreases and disorder increases. The stored seed becomes progressively more liable to lethal stochastic events (Osborne, 1980), including hits by ionizing radiation and autoxidation of lipids by free-radical mechanisms. The seed may thus be seen to accumulate these lesions with time until some potentially lethal event is reached. Background radiation is not considered an important component involved in deterioration during normal storage (Roberts, 1972).

The concept of accumulating damage in dry storage becoming manifest

on imbibition appears to have been first introduced by Villiers (1973). Osborne (1980) has used this concept also, although adding the qualification "long before that happens (lethal stochastic event) .... degradative biochemical changes take place that pre-empt stochastic events as the causative lesions leading to seed senescence".

In the light of what has been said above about deterioration in biochemical terms, it should come as no surprise that a large number of empirical studies have clearly shown that as a general rule, the lower the temperature and moisture content of seeds in storage, the longer the viability. In order to design seed storage systems rationally it is essential to understand the quantitative relationship between environmental factors and longevity. It is possible, knowing the rate of deterioration in a germ plasm sample, to reduce the testing interval and so conserve potentially valuable genetic resources (Roberts, 1983). Two storage "rules of thumb" have been proposed by Harrington (1972) regarding seed storage:

1. Seed life is halved for each 5°C increase in temperature, and
2. Seed life is halved for each 1% increase in seed moisture content.

This, however, only applies when the two storage factors are operating singly. There is an additive effect when the two operate together, and the proposal is intended only as a rough guide. Roberts (1972) has developed formulae which could be used to predict the viability of a seed population over a selected range of storage temperatures and seed moisture contents. Improved formulae were subsequently developed which will accommodate seed lot differences, and a wider range of storage conditions (Ellis & Roberts, 1981a; Roberts & Ellis, 1982). It is further possible to apply the later equations to problems of seed drying and viability (Roberts, 1981).

While mathematical treatments do not explain the fundamental cause of viability loss, Roberts (1982) believes that they can exclude two proposed explanations of seed viability loss as being universally

applicable.

In the first case a relationship between viability loss and moisture content can be shown to be such that the possible role of seed storage fungi can be excluded. For instance, if barley seeds are held at 90°C and 15% moisture content, the mean viability period is less than 2 minutes. Under such conditions microbial activity cannot be invoked in viability loss.

The second case concerns attempts to relate loss of viability to respiratory activity. Viability loss has a Q_{10} value which is very different from the Q_{10} value for respiratory activity and these vary with temperature, being 3 at 10°C, 6 at 45°C, 9 at 65°C, and 12 at 80°C. This suggests that factors seem to operate in temperature dependent ageing other than direct respiratory oxidative effects.

While viability studies have been undertaken on a number of seeds, many remain to be tested. The effects of oxygen and its interrelationship with moisture content and temperature still require detailed analysis.

6. INTRINSIC FACTORS

Many biochemical changes have been found in deteriorating seeds but at the present time it is impossible to identify which are primary or secondary events. This may be due to the limited number of studies carried out which makes it difficult to decide on a common pattern of events. Many workers have studied different facets of deterioration, using different techniques at various stages of deterioration in a wide range of seeds. This makes comparisons difficult, even questionable (Bewley & Black, 1982). While it may seem profitable to undertake studies in which both biochemical and detectable physiological changes are examined on a time-course basis (Abdul Baki & Anderson, 1972), this ideal appears far from realized. Among the many changes which have been identified are the

following:

1. Chromosome aberrations and damage to the DNA
2. Changes in RNA and protein synthesis
3. Changes in enzymes and food reserves
4. Defective respiratory activity and ATP production
5. Membrane changes and leakage

In the light of what has been said above, it should not be assumed that theories without recent support are in any way less acceptable than those currently in vogue, and which command a greater proportion of research effort. In addition, none of the separate categories should necessarily be viewed as being mutually exclusive. In this study the storage environment and membrane changes have been the major concern, but the other factors are of sufficient importance to warrant some discussion

1. Chromosome aberrations and damage to the DNA

Historically this theory is the oldest, having its origins in the chromosome studies of Navashin (1933) and others. A detailed account of this earlier work has been given by Roos (1982).

A good correlation exists between loss of viability and chromosome damage over a fairly wide range of temperatures and moisture contents (Roberts, 1972; Villiers, 1974). These studies have been conducted using squash preparations of germinating root tip cells at anaphase, and involve scoring the number of abnormal mitotic figures. These abnormalities represent gross damage to the DNA, and only when a presumed critical number of aberrant dividing cells is reached does root growth cease, and seedling death ultimately ensue.

Nichols (1941) showed that although 10% aberrations were present in onion root tips at 2 - 5mm growth, these became less with further growth such that at 80 - 100mm length only 1.7% aberrant figures could be noted. This could have been a reflection of selection or indicate repair to the chromosomes. It has been noted that there appears to be a characteristic species-specific level of chromosome damage at which loss of viability ensued (Bewley & Black, 1982). This concept is in agreement with a theoretical model by Roberts et

al. (1967), which considers the possibility of "key cell" numbers in relation to survival curves. Murata et al. (1981) have shown for barley seeds stored under a range of conditions that:

$$\text{Aberrant anaphases} = 5.435 - 0.042X$$

(where X is percentage germination).

Such a relationship was observed to have a correlation co-efficient of 0.937. It is possible that any deviations from the above proposals may reflect the influence of other non-nuclear components such as plastids and mitochondria.

As already noted, chromosomal aberrations give an underestimate of actual damage to the DNA since they measure gross, visible chromosomal damage. Similarly, cells which fail to divide because of the severity of damage would naturally be excluded from the estimates, and their contribution is ignored in such studies. It has however been observed that under severe ageing conditions the correlation between chromosome aberrations and viability is poor. Such observations have been noted by Roberts (1972) for peas, beans and barley, and by Harrison (1966) for lettuce.

While aberrant non-surviving cells, may be eliminated during root growth, minor genetic damage in the form of recessive mutations may occur. These may only become manifest later as pollen abortion or chlorophyll mutants in the progeny. This has potentially serious implications for germplasm work, since they would be undetectable by current techniques, and presumably have no measurable effect on germination.

Another category of abnormality is found in seedlings and plants of aged seeds. These abnormalities have been identified in peas and broad beans as anomalous leaf shapes, chlorotic spots and abnormal branching (Roberts, 1972). Harrison (1966) has reported dwarf and chlorotic plants from aged lettuce seeds. The causes of these conditions are not clear, but it has been suggested by Roberts that these might not be due to genetic changes but rather reflect cytoplasmic or physiological changes which are not heritable traits.

If such gross damage to the genome in the form of chromosomal aberrations can be correlated with viability, it would seem reasonable to expect correlations between viability and lower order levels of molecular dysfunction other than the chromosome. This has indeed been shown to be so in pioneering work by Osborne and co-workers. Initial observations indicated that a filtrate from the DNA of non-viable rye embryos contained more soluble nucleotides and less intact DNA than viable embryos (Roberts & Osborne, 1973). DNA from viable and non-viable embryos was distinguishable by alkaline density-gradient separation, non-viable embryos being found to have a lower mean molecular weight. It was further shown in studies using electrophoretic separation that DNA from the nuclei of non-viable embryos displayed a dispersely-migrating, low molecular weight material throughout the entire gel. This was interpreted as random cleavage of DNA as a result of DNase activity in the dry seed. Evidence was also obtained to suggest that inactivation of a DNase inhibitor occurred with prolonged storage and this was identified as a major reason for seed deterioration. It is noteworthy also that single-stranded DNA breaks may occur in viable seed samples (Osborne, 1980), possibly as a result of strand breaks within the dry and highly condensed chromatin. DNA repair is viewed as one of the early events in imbibition which occurs many hours before the DNA replication phase (Osborne, 1983). Thus restitution of minor single strand breaks may be made without visible chromosome damage, although there may be a lag in germination. Since the repair enzymes themselves are considered to deteriorate in storage, the quality of the repair will deteriorate also. This could lead to the production of "nonsense" information or DNA molecules with impaired functions and hence impair synthetic function. Eventually damage reaches a level at which repair becomes impossible, and gross chromosomal damage becomes evident; still later cell death occurs.

In addition to the above it has also been noted that cytoplasmic changes might influence repair of the genome. The leakage of solutes and ions by the membranes of cells in the early stages of imbibition of aged seeds could bring about changes in intracellular levels of ions and co-factors essential for proper functioning of DNA

repair enzymes (Osborne, 1983), and presumably enzymes in general. This latter point is especially significant when it is realized that "unscheduled" DNA synthesis must also be taking place at the time of presumed repair to membrane systems. This serves to emphasize the interlocking relationships between various aspects of metabolism, and the difficulties in trying to unravel cause and effect.

2. Changes in RNA and Protein Synthesis

Physiological expressions of seed deterioration such as reduced germinability and seedling growth suggest low rates of synthesis. Attention has therefore been directed at the possible relationships and functioning of the RNA and protein synthetic machinery in deterioration. This approach assumed particular significance since the early studies by Marcus (1969) showed that all the necessary components for protein synthesis were present and became activated 30 minutes after imbibition in viable wheat embryos. In addition exposure of germinating embryos to inhibitors of protein synthesis prevents elongation (Walton & Soofi, 1969; Klein et al., 1971) and leads not unreasonably to the assumption that protein synthesis is a pre-requisite for germination.

It is well-established that DNA replication is a relatively late event following imbibition and relatively unimportant in early synthetic reactions.

Studies have thus been undertaken to investigate which species of RNA or component of the protein synthetic machinery deteriorates with ageing. From these studies it is apparent that differential stability exists between the different categories of RNA.

Ribosomal RNA

Although electrophoretic separation of total RNA from dry, non-viable embryos of rye has shown that the 18 S component suffers

progressive degradation and loss of fragments with ageing (Roberts et al., 1973) there is no change in density between viable and non-viable ribosome populations. Further, the in vitro capacity of ribosomes from embryos of different viabilities to bind ^{14}C-phenylalanine-tRNA to ribosomes and to synthesize polyphenylalanine showed only a slight impairment (60% of control values) with ageing. The actual incorporation of ^{14}C amino acid into TCA-insoluble products fell off markedly with ageing. The greater lesion appeared to be in the elongation factor 1 & 2 of the post-ribosomal supernatant fraction. In dry, non-viable embryos elongation factor 1 was almost without activity, whilst the activity of elongation factor 2 was seen to fall by half. Whilst this pattern of deterioration is apparently typical of rye (Roberts & Osborne, 1973) and wheat (Dell'Aquila, Zocchi, Lanzanne & De Leo, 1976) it would seem to apply to dicotyledons also. Elongation factor 1 has been shown to be the site of the lesion in pea (Bray & Chow, 1976).

It has been noted that elongation factor activity lags behind protein synthesis, which might suggest other supernatant components might also be important (Bewley & Black, 1982). With progressive imbibition lesions become amplified which is either the result of intrinsic properties of the molecule or because exogenous components, such as nucleases becoming more active (Osborne, 1983).

Transfer RNA

Studies by Roberts & Osborne (1973) suggest that tRNA from non-viable rye embryos is readily aminoacylated indicating that this RNA fraction is particularly resistant to deterioration.

Messenger RNA

Studies with the inhibitors of RNA synthesis showed that seeds still have the capacity for protein synthesis. This led to the suggestion that RNA produced during embryogenesis was retained during drying and became functional on imbibition (Dure, 1977).

This long-lived poly-A rich RNA has been shown in cotton to constitute $\frac{1}{2}$ - 2/3 of the total RNA at 24 hours imbibition. If this value can be considered in any way representative of seeds in general, it is apparent that DNA-directed RNA synthesis is an event of significance only much later in the germination process.

An examination of the poly A-rich RNA fraction of dry rye embryos during viability loss showed that template activity in non-viable seed was 61% of that of viable seed (90% germination). The situation was worse at 24 hours imbibition (Osborne, 1980). Hybridization studies and base composition analyses further suggested that loss of bases occurred at the non-adenylated ends of the RNA. This shortening presumably influenced the in vitro efficiency of the templates (Osborne, unpublished data, cited in Osborne, 1980). However, since the non-viable embryos studied above do not synthesize any proteins, the inability of the seeds to germinate cannot be attributed to a lack of function of long-lived mRNA. This being so, it seems reasonable to consider the position of DNA-directed RNA synthesis in early germination.

Consistent with the decline in protein synthesis with declining viability there is a lesser, but parallel decline in all classes of newly synthesized RNA (Sen & Osborne, 1977). This is to be expected and is in keeping with studies discussed earlier (Section 1 - Chromosome aberrations and damage to DNA).

At present it is difficult to assess the relative contributions of protein synthesis directed by stored mRNA reserves and newly synthesized RNA species to seed viability.

As was noted at the outset of this review, the precise criterion used to decide germination is of considerable importance to viability studies.

For instance it has been stated that "seeds germinated in the presence of RNA synthesis inhibitors invariably die within a few days, the tissues destined to divide becoming necrotic first ..... thus before the embryo can be considered to have developed into a seedling, all its tissues become dependent on mRNA made during germination" (Dure, 1977). Such a viewpoint would satisfy the agronomist and seed technologist, laying emphasis as it does on seedling establishment.

On the other hand if germination is defined only in terms of radicle emergence it becomes clear that a seed in which cell extension precedes cell division could be scored as viable and yet never develop into a seedling. In such an example the immediate events which renders the seed non-viable would be those cumulative deteriorative events which lead to a failure in cell expansion, and might exclude many (or all) of the DNA-directed RNA syntheses.

3. Changes in Enzymes and Food Reserves

During the early stages of germination many enzyme systems begin to operate, some requiring imbibition in order to operate, while others appear de novo. While many of these are concerned with reserve mobilization and synthesis, a few may be concerned with repair. Mayer (1977) has drawn attention to the importance of a time scale in the appearance of particular enzymes, in order to evaluate their importance.

As many as 10 enzymes have been assayed in vitro for activity and a number have been identified for which correlations exist between germination and loss of viability (Abdul Baki & Anderson, 1972).

For barley five enzymes have been identified (MacLeod, 1952) whose decline in activity appears to be a post-mortem event. Dehydrogenase activity, as measured by the tetrazolium technique, proved to be a good indicator of viability loss. More recent viability studies have made use of the optical density of the extracted formazan, but unless the method is properly standardized this may be of questionable value. The older view that loss of viability is the result of a depletion of food reserves has long been discounted (Barton, 1961) although a considerable body of information exists regarding changes in the food reserves of seeds used as foodstuff by man. One such change in oily and cereal seeds is the increase in "acidity". These are thought to be due to production of free fatty acids by lipases, hydrolysis of phytin by phosphatases and protein hydrolysis by proteases. Zeleny and Coleman (1938) developed a fat acidity test as a measure of the suitability of grain for use as food. The basis of the test was to neutralize free fatty acids with potassium hydroxide. Some studies have suggested that these changes are the result of fungal lipase activity. Correlations could be obtained between extent of mould growth, moisture content and the acidity value (Milner & Geddes, 1946).

Seeds stored in a nitrogen atmosphere at high moisture content did not show increased acidity, presumably because anaerobiosis inhibited fungal growth (Milner, Christensen & Geddes, 1947). Seed germination was unchanged. While the evidence seems clear that, given appropriate conditions, storage fungi may give rise to increased fatty acid levels, Roberts (1972) cites unpublished work by Abdulla which showed that increased free fatty acid levels were associated with loss of viability in barley and wheat. (12% mc, 30° & 40°C). This was suggested to occur under conditions in which fungi were not active. In addition, data presented by Baker (1957) for soybeans shows a steady progressive increase in the fatty acid value as the moisture content was increased progressively between 6% and 16%. It would perhaps be expected that if fungi were active, the fatty acid levels would rise sharply at the higher moisture contents rather than increase uniformly throughout the range. Later studies by Christensen & Kaufman (1969) suggested that the use of fatty acid values was unreliable as an indicator of fungal activity. This line of investigation appears to have progressed no further since then and appears to have been incompletely resolved. Of possible relevance to the above are the observations by Koostra & Harrington (1969) that there is a decrease in the total neutral and polar lipid fractions of artificially aged cucumber seeds (high temperature and humidity). No such changes were seen when ageing took place over many years storage. This topic is discussed more fully in the section concerned with membrane changes.

As far as carbohydrate reserves are concerned there are conflicting data (Abdul Baki & Anderson, 1972). On face value, changes in such polymeric substances would seem to offer little of benefit in understanding the causes of viability loss. Unexplained features in one of the few studies reported is the decline in reducing sugars in barley and wheat (Abdulla, cited by Roberts, 1972), under conditions of deterioration unlikely to favour fungal growth. Studies by Lynch et al. (1962) showed that non-reducing sugars decreased under both aerobic and anaerobic conditions (regarded, though by no means proven, as inhibitory to fungal growth). On the other hand, reducing sugars did not change in air stored seeds, while under anaerobic conditions increases were observed, possibly because of

enzymic breakdown and inhibition of fungal activity. The lack of change of reducing sugars in air is difficult to explain, unless some steady-state condition between breakdown and utilization by the fungi is postulated.

Studies of a more general nature where properties of total seed proteins were examined suggests that crosslinking (as reflected by solubility and proteolytic digestibility) and some degree of proteolysis (as measured by increases in free amino acids) is possible (Ching & Schoolcraft, 1968). Such studies can be criticised in that post-mortem events may be involved, and the generality is so great as to be meaningless.

The above rather unsatisfactory reports serve to highlight that consistent patterns can be seen between particular enzymes, substrates or reserves and viability loss.

4. Respiratory Activity and ATP

Since germination involves energy-dependent cell growth and division, it is not surprising that many studies have examined respiratory changes in deterioration. Significant positive correlations between O_2 uptake and viability and high respiratory quotients have been reported. The latter is thought to reflect increased CO_2 output or reduced O_2 uptake, or both; for two barley cultivars it has been reported that increased CO_2 production occurs with declining vigour, but oxygen consumption values yielded no clear trend (Abdul Baki & Anderson, 1972). The reasons for the increased CO_2 output have not been explained. The suggestion that it reflects increased anaerobic metabolism or possibly increased activity of decarboxylase enzymes is without support. Isotope studies showed that deteriorated seed released more unlabelled CO_2 from some source other than the supplied ^{14}C glucose, furthermore decarboxylase enzymes appear to be ageing-sensitive (Abdul Baki & Anderson, 1972). This was followed by a suggestion that some block to glucose utilization occurred in the endosperm before entry of acetyl CoA into the Krebs cycle, while the embryo shows little or no change. This could possibly lead to distortion in the pattern of CO_2 output. However, isotope studies of this kind are not without their

problems of interpretation, and altered pool sizes could equally well explain such differences (Bewley & Black, 1984).

More recently, Woodstock & Taylorson (1981b) have suggested that as a possible consequence of membrane deterioration in soybean ageing an imbalance develops between glycolysis and the TCA cycle. This conclusion was principally based on the production of ethanol and acetaldehyde, and the known tendency of legumes to produce ethanol during glycolysis. While it is clear that measurement of respiratory events have given yet another indicator of viability loss, lack of standardization on, amongst others, ageing conditions, method of evaluation and the possible differences between different embryonic parts offer little hope of a clear understanding of respiratory patterns at the present time.

Another area of study, though not without problems of interpretation and methodology, concerns experiments on isolated mitochondria by electron microscopy in which attempts have been made to relate structure to function. It is known from the work of Morohashi et al. (1981) that two patterns of mitochondrial development can be distinguished in viable, germinating legume seeds. The first, as typified by peanut cotyledons, requires protein synthesis for the biogenesis of the mitochondria. In peas, however, pre-existing mitochondria are thought to be activated on imbibition. Succinate and NADH oxidation by mitochondria extracted from dry tissue suggests that an almost immediate restitution of the full functionality (Breidenbach et al., 1966). That this might be generally so in seeds has emerged from studies on wheat (Obendorf & Marcus, 1974), lettuce (Pradet, 1982) and soybean (Rodaway et al., 1979) where adenylate levels rise to normal values within 30 minutes. Pradet (1982) concludes from his work on lettuce, and the studies of others, that mitochondria are able to exhibit coupled respiration soon after imbibition, and cautions that in vitro studies must be carefully evaluated before it is assumed that values obtained are representative of in vivo activity.

Many ultrastructural studies have shown mitochondria with either distorted outer membranes, disorganised or almost absent internal

cristae in non-viable embryo tissues (See later membrane changes and leakage). Non-viable crimson clover seeds contain less than 1% of the ATP levels of viable controls (Ching, 1973), while rapidly-aged, non-viable soybeans possessed 35% of control levels of ATP (Abdul-Baki, 1980). At intermediate deterioration axes from 3 year old soybean seeds showed reduced coupling, consuming 40% more oxygen and possessing a P:O ratio 50% of control. Both seed lots were seen to have the same viability (Abu-Shakra & Ching, 1967). Electron microscopy of a mitochondrial pellet showed distorted organelles with swollen cristae and lacking outer membranes. These results are unfortunately more probably a reflection of the extraction and preparative techniques than in vivo appearance. Hallam et al. (1973) have shown one-sided blebs in the mitochondria of ageing tissues which presumably indicates some kind of outer membrane weakness, which could be readily exacerbated by grinding and extraction.

Although more difficult to evaluate, it would seem that examination of whole tissues by electron microscopy should be undertaken in preference to fractionation studies of the kind mentioned above.

In the root cap cells of maize (Berjak & Villiers, 1972 a, b, c) it was shown that mitochondria were amongst the first organelles to show damage in ageing. After 24 hours imbibition abnormalities were no longer evident, from which it was concluded that repair had been effected. More severely aged material took 48 hours to bring about repair.

It is unfortunate, as Bewley & Black (1982) have pointed out, that there are no studies of ATP synthesis or respiration against which ultrastructural observations can be evaluated to determine which lesion is cause and which effect.

The many studies which have shown a correlation between ATP content and vigour in seeds of lettuce, rape, rye, cauliflower and crimson clover (Ching & Danielson, 1972; Ching, 1973; Lunn & Madsen, 1981) have been confounded by later studies which have shown that ATP levels are not good indicators of vigour (Styer,

Cantliffe & Hall, 1980; Mazor, Negbi & Perl, 1984). Similarly the observation that non-viable embryos which have been rendered non-viable by accelerated ageing still have the capacity to reduce tetrazolium salts may be taken to indicate that loss of viability may occur without loss of dehydrogenase activity in the mitochondria.

5. Membrane Changes

(a) Leakage

There seems to be no reason why the ageing-induced changes which lead to the deterioration of macromolecular components such as proteins and nucleic acids, should not also influence membrane components. It is now well recognized that spatial separation produced by membrane systems is of primary importance to all eukaryotic cells. Apart from limiting the nuclear and mitochondrial elements from the cytomatrix, membranes serve as important sites for the localization of enzyme molecules (e.g. membrane pumps) and sites of protein synthesis (rough endoplasmic reticulum). Perhaps the most important role of the plasmalemma lies in maintaining the intracellular ionic milieu and transmembrane gradients. Along with the cell wall, this is also a site of rapid change on contact with water at hydration (Buttrose, 1973).

It has been known for over a century that the electrical conductivity of solutions in which plant tissues are bathed, shows increases with ageing (Abdul Baki and Anderson, 1972) and it has been postulated that a decline in vigour is associated with, or even due to, a 'weakening' of cell membranes (Heydecker, 1972). The relative ease with which the conductivity test can be done has tended to favour its acceptance as a means of vigour testing among seed technologists. The amount of leakage shows negative correlations with viability in seeds of soybean (Yaklich et al., 1979), pea (Larson, 1968), bean (Matthews and Bradnock, 1968) and peanut (Abdel Samad and Pearce, 1978).

However, the precise conditions under which water uptake occurs, are critically important as regards the extent of leakage and germination (Heydecker, 1977; Mullet and Considine, 1980). In a sense, therefore, steeping seeds with large cotyledons in water may be more of a stress test than a true measure of the physiological state of the membranes. The studies by Duke and Kakefuda(1981) show clearly that differences exist between seeds as regards damage by water, and further investigations to seek reasons for this are clearly needed. The fact that ionic leakage during imbibition in high and low vigour wheat did not differ (Mukhtar and Laidman, 1982) stresses the need for caution in this regard, especially since relatively few cereals or seeds with small cotyledons have been examined. Were all seed organs to age uniformly - and there is certainly no evidence to suggest that this is so - conductivity measurements would have gained greater acceptance. Although potassium is the major cation of plant cells, it may account for only between 25 - 50% of electrolyte conductivity in steep waters (Mullet and Considine, 1980). Ion leakage can be a poor indicator of physiological status, as suggested by the work of Heath & Fong (1981) where visual ratings of ozone damage to cotyledons proved a more sensitive indicator of damage than amino acid or ion leakage.

Solute leakage is, of course, a well documented event during the course of imbibition of viable seeds. In pea seeds high rates of leakage are seen during the first two minutes of imbibition, which decline rapidly to a low, constant rate of leakage after five minutes (Simon and Raja Harun, 1972). Later, Simon (1974,1978) argued by analogy on the basis of a phospholipid model system of Stoekenius (1968), that plant membranes in the dry seed assume a hexagonal phase and in this condition cannot regulate leakage during imbibition until the membrane is reconstituted as a bilayer. This was viewed as being a purely physical process although recent suggestions favour involvement of metabolic activity (Mukhtar and Laidman, 1982; Bewley, 1979).

In earlier studies by Larson (1968) it was suggested that leakage reflected a physical rupture of membranes induced by the rapid inrush of water. This may be a more likely explanation of solute

leakage in viable seeds, especially since the studies on lotus seeds by McKersie and Stinson (1980). Membranes from seeds of lotus were prepared by cell fractionation and examined by X-ray diffraction at different water contents. Membranes remained in the lamellar or bilayer phase at hydration levels as low as 5%.

The importance of potassium to cell function cannot be disputed, and the transfer of nearly all cotyledonary reserves to the axis of germinating peas stresses the importance of this ion to axis growth (Ferguson and Bollard, 1976). Altered potassium gradients and intracellular ion concentrations have been shown in animal cells to inhibit cell growth, differentiation and protein synthesis (Cahn and Lubin, 1978); more recently a good correlation has been shown between protein synthesis and K^+ concentration in pollen grains (Bashe & Mascarenhas, 1984). In terms of the polyelectrolyte theory of Dozou and Maurel (1977) it is suggested that highly organised systems concerned with DNA, RNA and protein synthesis, respond to cations in a similar way to the proposed electrostatic effect produced by polyamines (Bagni, Fracassini & Torrigiani, 1982). Thus too high or too low a level of any particular ion as a consequence of progressive changes in membrane properties, might be detrimental to overall cell function, leading to slower growth rates and finally death.

In addition to ions, a number of other substances have been reported to leach out of aged seeds, including sugars, amino acids and inorganic phosphate (Bewley and Black, 1982). While they are undoubtably indicative of the deterioration process, they offer even less predictive value at the present time. More recently it has been shown that loss of large molecular weight components such as malate dehydrogenase (70,000) and glutamate dehydrogenase (300,000) occur from the cells of viable soybean, pea, bean and peanut seeds during imbibition (Duke and Kakefuda, 1981). This may be a highly significant finding as regards seed ageing and raises questions as regards size exclusion characteristics of membranes, possible resorption of lost enzymes or their re-synthesis after some membrane repair.

(b) Nature and possible causes of changes

Ultrastructural examination of the root tips of a number of monocotyledons (Berjak and Villiers, 1972a,b,c; Hallam et al., 1973;), gymnosperms (Simola, 1974;1976) and dicotyledons (Villiers, 1973; van Staden et al., 1975) confirm that membrane systems of the cell suffer deteriorative changes during imbibition, including:

1. Abnormalities to mitochondrial and plastid membranes;
2. Fusion of lipid droplets to form larger bodies or irregular pools in the cytoplasm;
3. Lobing of the nuclear envelope;
4. Loss or fragmentation of the endoplasmic reticulum and Golgi bodies;
5. Rupture of the protein body or vacuolar membrane;
6. Discontinuities in the plasma membrane and its withdrawal from the cell wall;
7. The occasional appearance of floccular material in the extracellular space between plasmalemma and wall.

Other changes, which reflect alterations to the DNA, RNA and protein synthetic machinery, include:

1. Darkly-staining nucleoli and/or heterochromatin;
2. Loss of polysomes and cytoplasmic ribosomes;
3. Dilution of the cytoplasm.

The above-mentioned changes are presented only in a general way, since accurate comparison between the studies is rendered difficult by widely differing conditions of preparation. In some cases, dry seeds were placed directly into fixative, while others were imbibed for several hours before fixation for microscopy. Furthermore, in some cases seeds are described as being non-viable without

particular reference to the conditions which may have brought this about. It is well-recognized that seeds may show varying degrees of deadness (Maguire, 1977) and it is therefore important that the ultrastructural studies should examine ageing over intervals of time during which viability and vigour decline as a result of controlled conditions. Furthermore, changes on imbibition should preferably be given on a time-course basis in an attempt to detect the primary membrane-associated events. This presupposes that membrane systems would be differentially sensitive.

The studies of Berjak and Villiers (1972 a,b,c), Villiers (1972) and Berjak, Gevers and Dini (1985) fulfil some of the above requirements and implicate membrane changes as being important determinants in viability loss.

Villiers (1973) showed that with increasing time and relative humidity of storage, an increase in aberrant mitotic figures occurred in lettuce seeds. Since 1 mm segments of emergent radicle tips were sampled for these studies, it is clear that nuclear damage, per se, did not prevent the initial stages of cell extension, nor the onset of the first mitotic division. If, however, the plasmalemmas of cells of the radicle tip developed some defect such that they were unable to achieve the necessary turgor to initiate cell extension, the seeds would clearly not germinate. Thus, while nuclear and membrane lesions were coincident, it was argued that the primary lesion which rendered the lettuce seed non-viable was extranuclear, and probably membranal.

Ultrastructural examination of seeds from samples where the germination percentage was low, showed that the cells of the radicle were well organised during the early hours of imbibition. However, by the end of 24 hours, vacuolar membranes appeared to have burst, so releasing hydrolytic enzymes into the cytoplasm. This was then followed by disintegration of mitochondria, plastids and nuclear envelope. The plasmalemma was seen to be dissociated from the cell wall, while lipid droplets became confluent in the cytoplasm. Thus, in a comparable manner to the events proposed by Berjak and Villiers (1972b, c) for maize, lettuce embryos were rendered non-

viable after a critical number of cells lost function.

With extended periods of storage, it seemed that the plasmalemma underwent some change of property such that, on imbibition, it failed to perform its function as a differentially permeable membrane and cell turgor could not develop. The membrane was no longer intimately associated with the cell wall, as was seen earlier, and appeared to rupture at the onset of imbibition. Rapid and substantial subcellular disorganization ensued and the embryonic axis became necrotic.

Progressive membrane deterioration could possibly explain the decline of vigour and eventual loss of embryo viability, especially when occurring in conjunction with nuclear damage.

While it is clear from the above, that membrane damage theories of ageing have considerable support, such visual evidence provides little indication of what underlying changes lead to altered membrane properties.

(c) Free radicals and ageing

It is well-known that oxygen is essential for the life of aerobic organisms, as is the fact that high concentrations can be damaging. Thus the use of oxygen by an organism, for oxidation reactions, presents two immediate problems: how to catalyse the reaction; and how to control the reactivity of the oxygen (Bannister and Hill, 1982).

In the free radical theory of ageing, which has been advocated by Harman since the 1950's, the above-mentioned radicals are seen as the single, basic cause of ageing, their effects being modified by environmental and genetic factors. Thus free radicals may arise as a

result of ionizing radiation, and both non-enzymic and enzymic reactions. This theory enjoys considerable support in when applied to animal systems and can provide a reasonable explanation for many aspects of age-associated events in mammals (Harman, 1981).

Recently, however, these ideas have been increasingly applied to plant systems, especially in leaf and flower senescence (Leshem, 1983). Evidence for free radical involvement in seed ageing is slight, often indirectly assessed or inferred by extrapolation from studies on hydrated systems. This may not be an altogether valid assumption.

Direct assessments of free radicals have been made by Conger and Randolph (1959,1968). While those authors were able to demonstrate free radicals in irradiated seeds, this was not the case for seeds aged in the long-term. Since irradiation of the material was taken to represent a fast form of ageing, the disparity might be explained by supposing that free radicals built up at a rate greater than their dissipation. In slower ageing, however, sufficient time for their removal may be available (Villiers, 1973). This suggestion should be testable, by holding irradiated samples and examining the rate of loss of free radicals, relative to viability loss. A decline in radiation-induced free radicals has been correlated with a loss of viability, possibly by concomitant $OH^{\cdot}$ radical attack on nucleic acids (Haber and Randolph, 1967).

The recent studies by Buchvarov and Gantcheff (1984), however, do not appear to support the above arguments about decay rates. Embryonic axes of seeds subjected to an accelerated ageing regime, and in which viability was lost over 6 days, were compared with slow-aged (4 year stored), non-viable seeds. Relative electron spin resonance (ESR) values, which are a measure of the incidence of free radicals, of these two groups differed by only approximately 10%, but were nearly twice that of the controls.

On the other hand, earlier work by Priestley et al. (1980) failed to show significant differences between either free radical levels or

tocopherol levels in seeds over 8 years storage or subjected to an accelerated ageing regime. In that study, however, the cotyledons only were examined, in contrast to the positive results of Buchvarov and Gantcheff (1984) which were reported for the embryonic axes. The latter found also that no ESR signals were discernible from cotyledonary tissue of any of their seed samples.

Pammenter, Adamson & Berjak (1974) approached the problem of putative free radical involvement by holding maize seeds under an accelerated ageing regime (13.6% m.c., 40°C) on a cathodic plate. Under these conditions, it was argued, free electrons would be available to enter the unfilled orbitals of radicals as they were produced. Viability of seeds was greatly extended and ultrastructurally the treatment appeared to ameliorate deteriorative changes, particularly in the mitochondria (Berjak, 1978). However, because of structural considerations of the seed, or some other factor similar treatment by Leopold of soybean seeds, did not produce any beneficial effects (pers. comm.).

It remained for Koostra and Harrington (1969), using thin layer chromatography (TLC) techniques, specifically to implicate the loss of membrane phospholipids in seed deterioration. Harman and Mattick (1976), arguing that fatty acids with two or three unsaturated double bonds would be more vulnerable to free radical attack, showed (by GLC) a convincing parallel decline in the linoleic and linolenic acid levels with loss of viability in pea seeds, subjected to accelerated ageing (100% RH and 30° C). While their analyses were conducted on entire seeds only, they point out that changes in the axis, which comprises only 1% of the total seed weight, could be more critical to germination. Powell and Matthews, (1981) showed a decline in total phospholipids with loss of vigour in pea seeds aged at 94% RH and 25°C, the phosphatidylcholine fraction declining by some 40%, while viability was unchanged at 88%. Increasing conductivity of steepwater, and decreasing dehydrogenase activity (as measured by tetrazolium staining) was noted over the 9 week ageing regime. Although fatty acid levels were not determined, the results of this study are in agreement with those of Harman and Mattick (1976), in showing that changes in lipids, particularly

phospholipids, can precede loss of viability.

Soybean seeds subjected to accelerated ageing (100% RH and 40°C) showed little change in fatty acid levels (Priestley and Leopold, 1979). Over the 5 days of ageing, by which time viability was lost, the fatty acid levels of whole seeds, their polar lipid fraction or the fatty acid levels of the total lipid fraction, showed no change. Nevertheless, examination of individual phospholipid classes by TLC showed a decline in phosphatidylcholine (as phosphate) of some 25%, with a smaller change in phosphatidylethanolamine. Those authors reported no immobile material at the origin of the TLC plates to suggest the presence of peroxidised lipids, as Koostra and Harrington (1969) had reported and concluded that if changes in fatty acids play any rôle in seed deterioration, they must be manifested at an extremely low level.

In a later study, Priestley and Leopold (1983) examined changes taking place in soybean seeds stored at 4° C and low humidity for 44 months. For variety Wayne, it was noted that a 12% decline in viability was accompanied by a 2.5% decline in the molar percentage of linolenic acid. In the variety, Chippewa, a 60% decline in viability was accompanied by losses of 3.1% in total seed fatty acids, a 1.7% loss in total seed polar lipid fatty acids and an 11.5% loss in total axis fatty acids. Significantly, linolenic acid values were nearly 4 times greater in the axis, thus making the axis liable to potentially greater peroxidative damage. This is in keeping with the findings of Buchvarov and Gantcheff (1984), who showed the presence of free radicals in axis, rather than cotyledonary tissue.

Stewart and Bewley (1980) found for soybean seeds under an even more extreme ageing regime (100% RH and 45° C) than that used by Priestley and Leopold (1979) that linoleic and linolenic acid levels declined with ageing. However, in this study, seeds were non-viable by the second day and while substantial changes may have taken place subsequently, it is perhaps more pertinent to examine changes occurring only over the first two days.

In the first day of ageing, viability declined to 24%, while linolenic

acid levels declined by 21% and linoleic acid by some 2% of the control values. During imbibition, malondialdehyde, a breakdown product of linolenic acid hydroperoxides, was measured. Levels declined rapidly within one hour of imbibition from a basal level of approximately 10 μmol/axis, although by the third day they remained elevated for four hours. With further ageing, malondialdehyde declined, being low both in the dry seed and during imbibition. Although these trends are difficult to interpret, it is noteworthy that even fresh seed apparently contained significant levels of malondialdehyde.

Peanut seeds aged at 90% RH and 38° C, showed a decline in the fatty acid levels of the phospholipid fractions (Pearce and Abdel Samad, 1980). On an area percent basis, the change was small (2.6%) but when values were expressed per unit dry weight of the phospholipid and glycolipid fractions, it was seen that these fell to some 50% of the control value by the time viability has fallen to the same extent. Those workers also examined fatty acids of the polar lipid fraction in two cultivars from two different harvests, stored at 30% RH and 5° C. These different lots all presented different viabilities and conductivities, but it could not be said with certainty whether the changes were strictly age-related. Lipid hydroperoxides could not be detected and those authors speculate that this could indicate that significant peroxidation was not occurring, or that the hydroperoxides had broken down. The rôle of antioxidants was suggested as a possible reason also, for the absence of hydroperoxides, although from studies on wheat (Fielding and Goldsworthy, 1980) and soybean (Priestley et al., 1980), the absence of a decline in antioxidant levels has been taken as indirect evidence for the idea that lipid peroxidation may not be significant in seed ageing.

Studies on fatty acid levels of extremely old (possibly 466 years) viable and non-viable lotus seeds, showed that cotyledonary levels of unsaturates are comparable to those in freshly-harvested seed (Priestley and Posthumus, 1982), while previously it was noted that while seed lipids may undergo some peroxidative damage, post-mortem, large-scale destruction of unsaturates was not seen in maize

seed aged between 1 and 17 centuries (Priestley et al., 1981). While reasons for these observations are not clear, it does perhaps argue that autocatalytic free radical mechanisms in bulk-phase systems are fundamentally different from those in biological systems. As far as the lotus seed study is concerned (Priestley and Posthumus, 1982), it may well be that coat-imposed oxygen impermeability may allow for free radical mediated deterioration, other than peroxidation.

During senescence in Phaseolus cotyledons, it has been shown that the overall ratio of unsaturates : saturates did not change appreciably in the phospholipid content of rough and smooth microsomes (McKersie et al., 1978). In a later study McKersie and Thompson (1979) showed that pure liposomes exhibited similar properties to those shown by intact microsomal membranes from which they deduced that proteins, per se were not the primary determinants of the altered properties. However, addition of a neutral lipid fraction to a phospholipid preparation from young (liquid crystalline) microsomes, induced gel-phase properties. This rigidifying effect was thought to be brought about by some abundant, but as yet unidentified, product of peroxidation, which may act directly with unsaturated fatty acid chains, possibly intercalating between them (Barber and Thompson, 1983).

Not withstanding the fundamental differences between the dry seed and hydrated, senescent systems such as those described above it is still possible that some peroxidation products may cause phase changes or altered membrane properties in the dry seed during seed deterioration. While loss of membrane lipid unsaturation has been noted in some cases, support for the free-radical theory can only be regarded as substantial when some change is found which precedes fall in viability, possibly occurring concomitantly with vigour loss.

In spite of the contradictions and inconsistencies in the above biochemical studies, several ultrastructural studies have yielded evidence suggesting that varying degrees of membrane damage take place on imbibition.

It has further been established for lettuce seeds that even though chromosome aberrations can attain levels which preclude germination and seedling establishment that radicle protrusion is nevertheless possible (Villiers, 1972).

Scope and purpose of the present study

In the first part of this study lettuce seeds were subjected to controlled ageing conditions and germination and seedling establishment monitored (Chapter 2). On the basis of these observations three broad objectives were set:

1. The development of a technique for the preparation of dry seed tissues for electron microscopy (Chapter 3).

2. An ultrastructural investigation of the localized damage seen in the cotyledons of aged seeds (Chapter 4).

3. An examination of the ultrastructural changes in root tip cells of aged and irradiated seeds to find further support for the suggestions that membrane lesions may be one of the earliest events associated with viability loss (Chapter 5).

In the second part of this study, attempts were made to find supportive biochemical evidence for the suggestion that lipids may be a likely target for free-radical attack. To this end, measurements of lipid hydroperoxides were undertaken, and various chromatographic techniques employed (TLC, HPLC, GLC) to seek changes in phospholipids and fatty acid unsaturation (Chapter 6). Finally, the observation that headspace analysis provides a rapid means of evaluating the extent of peroxidation in model systems (Ross, pers. comm.) prompted an examination of the volatile profiles of seeds during imbibition or during dry heating by packed column and capillary chromatography (Chapter 7). Capillary gas chromatography-mass spectroscopy was found to be an essential technique in the analysis of the complex spectrum of volatile

hydroperoxide breakdown products such as aldehydes, alcohols and hydrocarbons. This latter approach provided indirect, qualitative evidence that peroxidation is a significant event in seed deterioration.

Thesis layout

In this thesis individual chapters are presented as relatively self-contained entities. Because of the widely-differing techniques adopted the thesis may be read as two separate parts, namely, ultrastructural investigations (Chapters 3-5) and biochemical studies (Chapters 6 & 7), each of which contains a concluding discussion. In the last chapter (Overview and Synthesis; Chapter 8) the argument is developed that the results of both parts of the thesis may be viewed within the framework of the free radical theory of ageing.

CHAPTER 2

STORAGE AND GERMINATION STUDIES

Germination studies were undertaken to provide an observational baseline for the design of ultrastructural and physiological investigations. The germination of lettuce seeds was monitored after being subjected to ageing conditions which tended to be milder than many reported in the literature, since it has been suggested that ageing in the long term may be different from accelerated ageing at high relative humidities and temperatures (Priestley & Leopold, 1983). It has been shown that the survival of lettuce seeds in hermetic storage conforms to that of orthodox seeds (Ibrahim & Roberts, 1983) being extended by low temperatures and moisture contents.

In the experiments reported herein, two separate ageing studies were undertaken. In the first, germination was examined for seeds held at a range of constant relative humidities (0, 20, 40, 60%) at 20°C; in the second seeds were held in hermetic storage at 10°, 20°, 30° & 40°C after pre-equilibration at 0, 20, 40, 60, 80 & 100% RH. The pattern of changes observed enabled an ageing sequence to be drawn up, which included some macroscopic markers for the loss of lettuce seed vigour.

MATERIALS AND METHODS

Seed Supplies

Lettuce seeds, c.v. Great Lakes, were purchased from the following Suppliers : Mayford, Johannesburg; McDonalds, Pietermaritzburg and Roode-Lyon, Durban. Seeds were purchased from the above suppliers between 1978 and 1983, and only lots showing greater than 95% germination by 24 hours were used for the above-mentioned experiments. In addition, seeds purchased between 1972 and 1976, were stored as purchased in tins or placed over silica gel at 20°C (Villiers, pers. comm.). These were used for comparative

TABLE 2.1

Moisture contents of two seed lots used in the germination studies described in the text.

Storage relative humidity	Moisture content
0%	2.7
20%	4.2
40%	5.8
60%	6.6
80%	7.7
100%	10.3

TABLE 2.2

Moisture contents of seeds used to study the effect of storage temperature on longevity. Seeds were allowed to equilibrate at the appropriate RH for 16 days before storage in hermetically sealed vials.

Storage relative humidity	Moisture content	
	1978 purchase	1979 purchase
0%	3	2.5
20%	4.7	5.3
40%	5.9	6.9
60%	7.1	8.0
80%	8.5	9.8
100%	11.9	13

illustrative purposes.

Storage at Selected R.H. Values

Seeds were stored in screw-top jars over sulphuric acid of the appropriate specific gravity to maintain a constant RH (Solomon, 1951). Seed moisture contents were determined at the end of experimental periods by drying for 1 hour at 130°C as advocated by the International Seed Testing Association (1976).

Germination Conditions and Data Treatment

Germination studies were conducted on 100 seeds in a 9 cm Petri dish lined with two sheets of Whatman No. 1 filter paper wetted with 5 ml. of distilled water, at 20°C in a walk-in incubator. Lighting was by four 40 watt daylight fluorescent strips at approximately 0.5m from samples. Any visible protrusion of the radicle from the seed coat was scored as germination. Data presented are for single samples of 100 seeds since the large sample thoughput at any one sampling interval precluding any statistical treatments. Germination data was generally not recorded beyond 40 hours. Since these studies involved counts on single samples of 100 seeds for each treatment, without any attempt at statistical treatment, the germination values should be regarded as relative, rather than absolute.

Seedling Establishment Records

Photographic records were made on Kodacolour or Sakura colour print film (ASA 100) using a Wild photomicroscope.

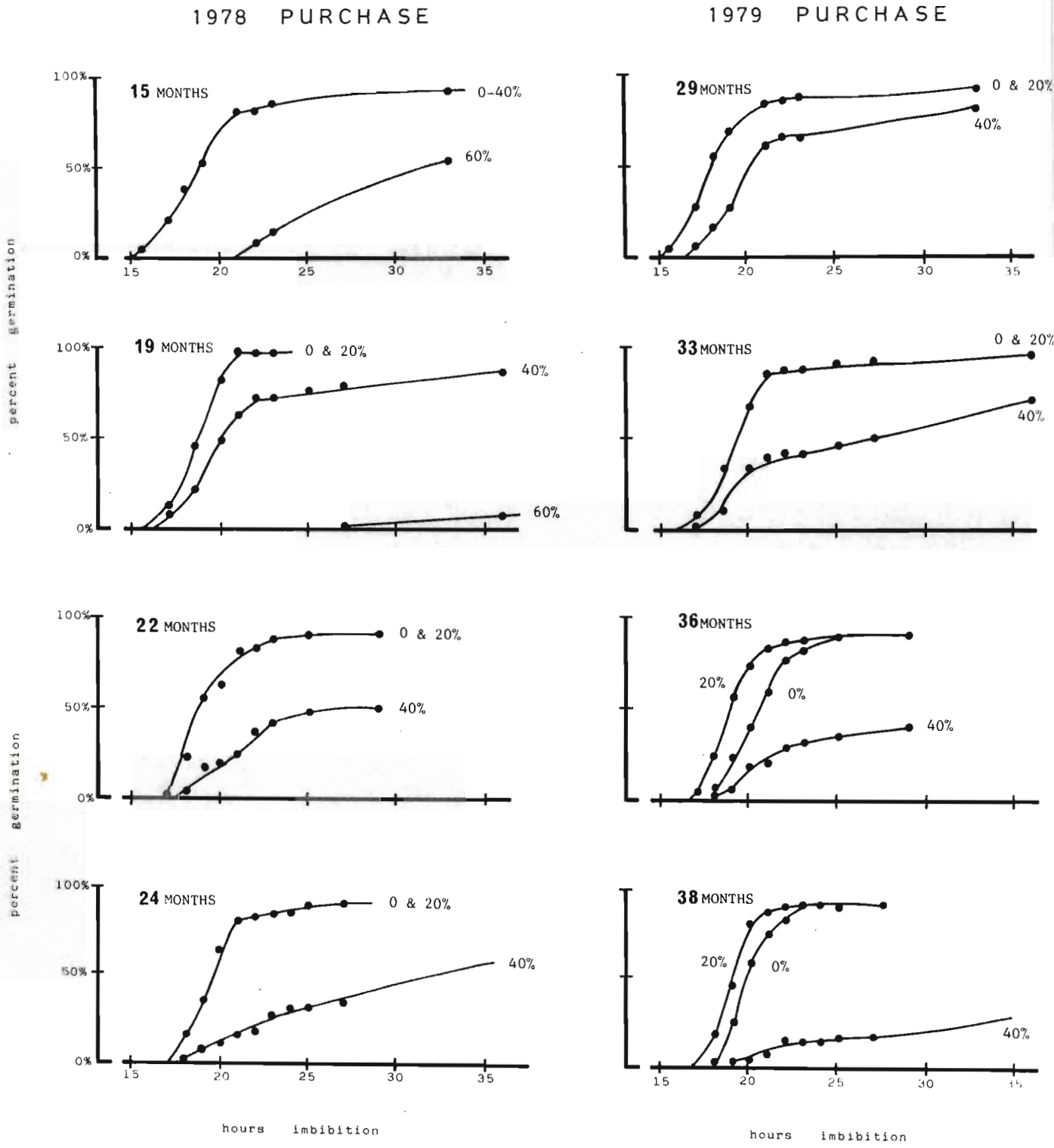

1978 PURCHASE
1979 PURCHASE
15 MONTHS
0-40%
60%
29 MONTHS
0 & 20%
40%
19 MONTHS
0 & 20%
40%
60%
33 MONTHS
0 & 20%
40%
22 MONTHS
0 & 20%
40%
36 MONTHS
20%
0%
40%
24 MONTHS
0 & 20%
40%
38 MONTHS
20%
0%
40%
100%
50%
0%
15
20
25
30
35
percent germination
hours imbibition

FIGURE 1

FIGURES 1 & 2

Illustrates the cumulative percentage germination for two seed lots (1978 and 1979 purchases) of lettuce, cv. Great Lakes. Seeds were held over sulphuric acid to give constant storage relative humidities of 0%, 20%, 40%, and 60% at laboratory temperatures (20°C). Graphs on the left hand side are for the 1978 purchase while those of the right hand side are for the 1979 purchase. In some cases data was recorded beyond 35 hours imbibition and used to present values (e.g. 0% & 20% RH for 35 and 49 months; 40% RH for 38 months).

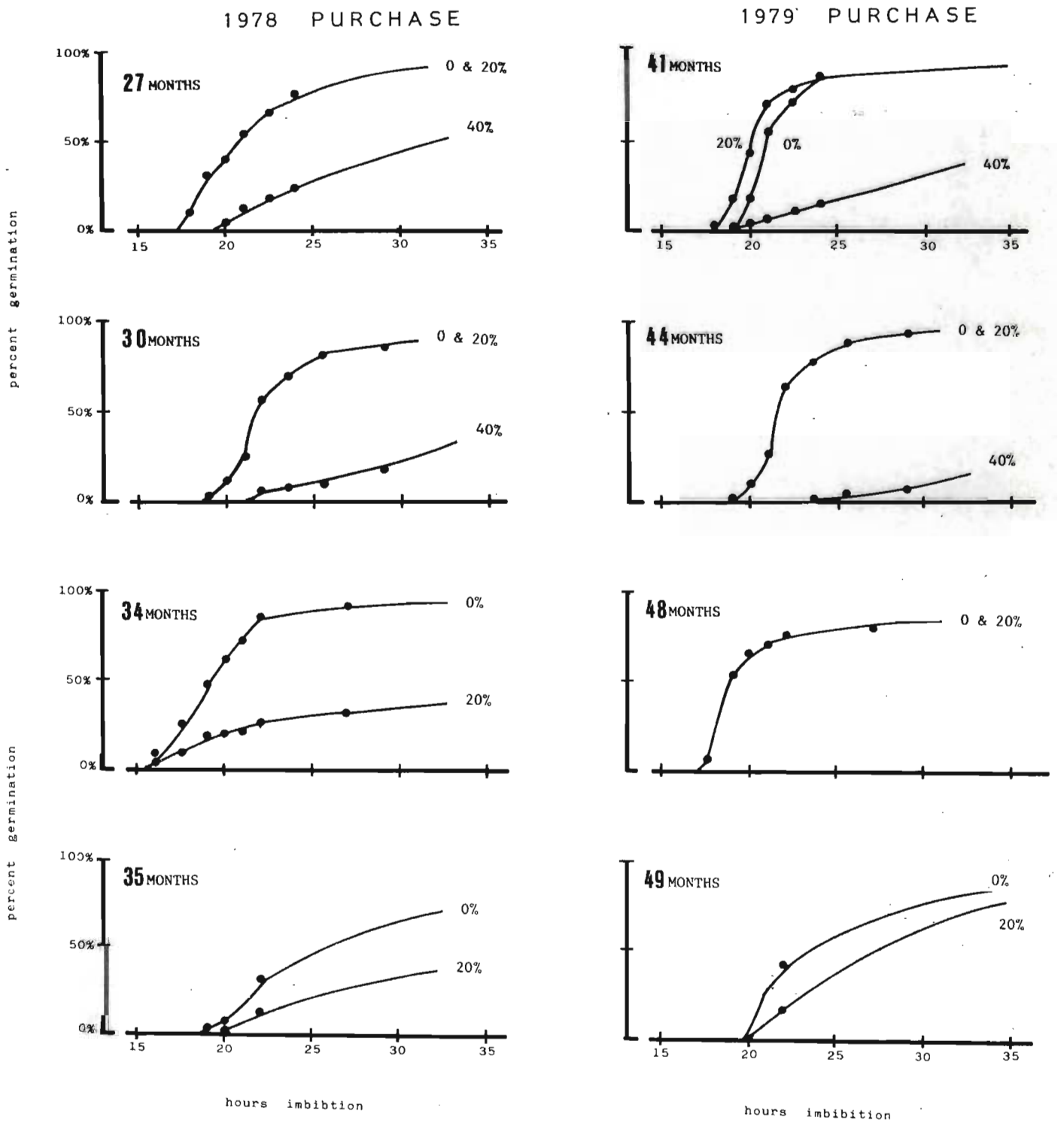

1978 PURCHASE
1979 PURCHASE
percent germination
percent germination
27 MONTHS
0 & 20%
40%
41 MONTHS
20%
0%
40%
30 MONTHS
0 & 20%
40%
44 MONTHS
0 & 20%
40%
34 MONTHS
0%
20%
48 MONTHS
0 & 20%
35 MONTHS
0%
20%
49 MONTHS
0%
20%
100%
50%
0%
15
20
25
30
35
hours imbibtion
hours imbibition

FIGURE 2

RESULTS AND DISCUSSION

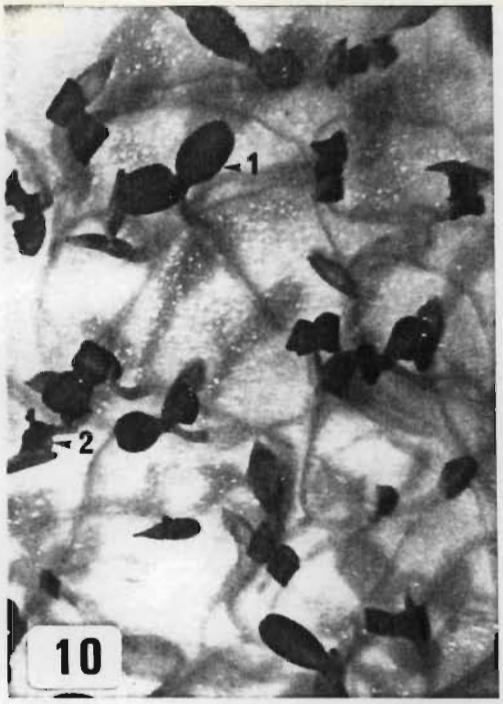
Germination Curves for Different Seed Lots

Moisture contents for the 1978 & 1979 seed purchases are given in Table 2.1, from which it can be seen that the moisture content of the two seed lots varied by no more than 1.3% at the higher storage relative humidities; values obtained are nevertheless in broad agreement with published values (Villiers, 1974; Neergaard, 1977). It has been noted that moisture contents cannot be relied on with an accuracy of greater than 0.5% (Roberts, 1973), and it is not clear whether any differences observed which are greater than this are a reflection of the technique or represent real differences between the two seed lots.

The cumulative percentage germination of seeds purchased in 1978 and 1979 and stored at 20°C over sulphuric acid at relative humidities of 0, 20, 40 & 60% are presented in Figures 1 & 2. For reasons of convenience and notwithstanding the differences between moisture contents of the two lots, values are presented with reference to relative humidity. For some RH values (especially 0 & 20%) a representative value is given to prevent excessive overlapping of data on the graphs, but where values are sufficiently different these are drawn.

Seeds stored at 0% RH, while attaining the same final percentage germination as those stored at 20% or 40% RH, generally showed delays in the time to first emergence of approximtely 2 hours (36 and 38 months, Figure 1; 41 months, Figure 2). The values presented should be regarded as more illustrative of vigour decline rather than viability loss, since the absence of recorded germination


9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2
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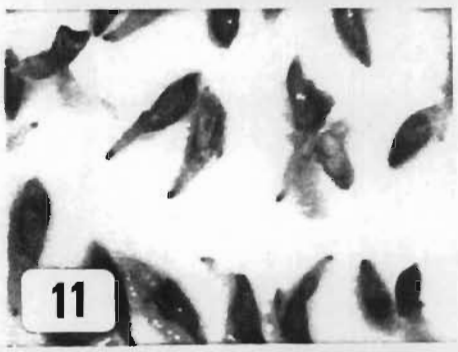
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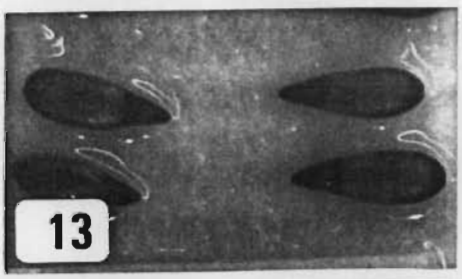
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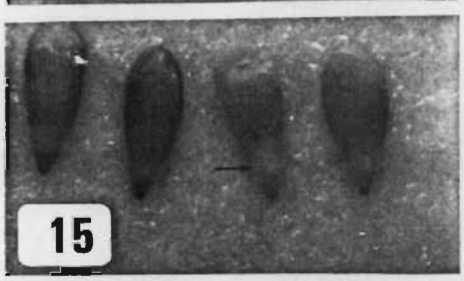
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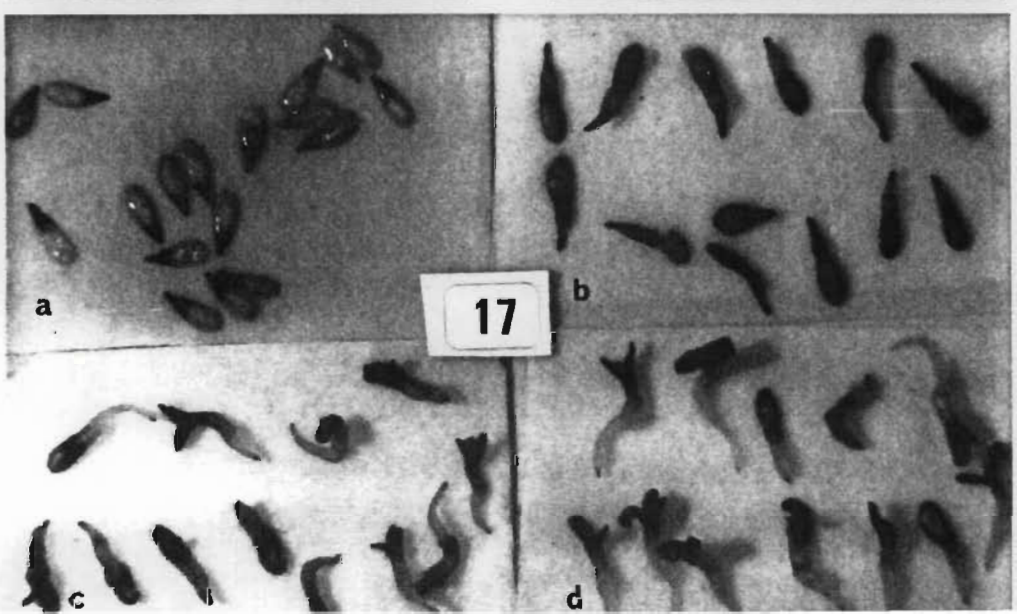
a
17
b
c
d

NOTE: All material illustrated in the Figures opposite were stored at 20°C, except for that illustrated in Figure 15.

Figure 9 : Appearance of seedlings at 10 days after storage of seed for 35 months storage at 20% RH (1979 purchase).

Figure 10 : Appearance of seedlings at 10 days from a 1978 purchase after storage of seed for 49 months storage at 20% RH.

Figure 11 : Appearance of seedlings at 10 days from a 1979 purchase after storage of seed for 35 months storage at 40% RH.

Figure 12 : Appearance of 1979 (left hand side) and 1978 (right hand side) purchases after 60 and 72 months storage at 20% RH, and 10 days post germination.

Figure 13 : Appearance of excised embryos after 48 hours imbibition from seed stored for 9 years 10 months in hermetically sealed tins, as purchased from the supplier.

Figure 14 : Appearance of excised embryos from seed after 48 hours imbibition and 23 months storage at 80% RH.

Figure 15 : Appearance of excised embryos after 48 hours imbibition and 20 days of an accelerated ageing treatment of 30°C and 100% RH. Embryos were stored at 5°C for 23 months before being tested.

Figure 16 : Appearance of excised embryos after 48 hours imbibition and 6 years storage at 20% RH (1978 purchase).

Figure 17 : Appearance of excised embryos at one week post imbibition after storage for :

(a) 9 years 10 months in hermetic storage

(b) 35 months storage at 40% RH

(c) 72 months storage at 20% RH

(d) 8 years 5 months over silica gel.

by 35 hours need not necessarily indicate total loss of viability; neither should a low germination by this time be regarded as indicative of final germination attainable by one week. On the other hand it is doubtful whether such germination values would be attainable in harsher field conditions. If it is assumed that the absence of germination by 35 hours represents viability loss under field conditions, then it can be seen, for example, that seeds purchased in 1979 and stored at 60% RH, lost viability after 19 months storage at 20°C. This value is in agreement with later studies on another seed lot purchased in 1980, where loss of viability occurred between 17 and 22 months. (Figure 7, 20°C data).

A comparison between the 1978 and 1979 harvests revealed a disparity in the loss of viability of seeds stored continuously at 40% RH. The 1979 seed purchase lost viability between 30 and 34 months, while for the 1978 purchase the value lay between 44 and 48 months. This disparity between the two lots became more pronounced at 34 months where seeds stored at 0% and 20% RH began to diverge in germination characteristics (Figure 2) whereas only a slight difference became apparent in the (presumed) chronologically older seeds even at 48 months. Indeed, after 10 days germination, the chronologically older seeds stored at 20% RH were qualitatively marginally superior as judged by the extent of emergence of the cotyledons, and necrosis. (Figures 9 & 10). Quantitatively these seeds were also superior, final germination percentages being 45% and 90% (35 and 49 months, Figure 2).

Cotyledonary Necrosis and Germination

Figure 11 illustrates the appearance of seedlings of the 1979 purchase stored at 40% RH for 35 months after 10 days germination. While it is apparent that these seeds could be scored as viable as judged by the criterion of radicle emergence, cotyledonary development was minimal and further radicle growth did not occur. This is in striking contrast to seeds stored for the same period at 20% RH (Compare Figures 9 & 11). Examination of these same seed lots after 60 and 72 months continuous storage at 20% RH showed that

differences were still evident between the two purchases (Figure 12, left and right hand lanes), and indicated the remarkable resistance of the tissues to necrosis.

Physical rupture and hydrolysis of the surrounding endosperm and integument are considered important components for embryo growth in light-sensitive varieties of lettuce (Ikuma & Thimann, 1963; Nabors & Lang, 1971a&b; Bewley & Halmer, 1980/1981). Cotyledonary necrosis would thus be expected to reduce significantly both the physical thrust attainable by the embryonic axis as well as possibly impairing the putative endosperm hydrolysis signalling system (Bewley & Halmer, 1980/1981).

Lettuce seeds treated with dichloroisocyanurate show embryo expansion without protrusion, possibly due to inhibition of the chemical endosperm weakening mechanism (Pavlista & Haber, 1970). Illustrations of such seeds, and the abnormal germination observed are not unlike those seen in the seeds illustrated in Figure 12. In an attempt to investigate the above possibility, embryos were excised from seeds which had failed to germinate after 48 hours imbibition and their subsequent growth on moist filter paper noted. Seeds were chosen from a range of storage conditions as noted with Figures 13 - 18.

On excision all embryos showed little clear evidence of germination as judged by either radicle extension or geotropic curvature (Figures 13 - 16). Some suggestion of growth was however seen in seeds from two of the above storage regimes in the form of a slight lateral bulge in the hypocotyl region (Figures 15 & 16). The dark brown appearance of the cotyledons proved to be an indicator of subsequent cotyledonary necrosis, although the absence of browning was not itself an indicator of potentially viable tissue (Figures 15 & 18). Browning of the radicle tips at this stage was indicative of no further development and necrosis (Figure 13 & Figure 17a). Growth of radicle and hypocotyl tissue was noted in all cases where these tissues had not browned (Figures 14 - 16 & 17b - d) although growth eventually ceased and embryos became soft and disintegrated.

It is clear from this that intact seeds may be scored non-viable from the viewpoint of radicle emergence, even though embryos may be capable of some further growth on excision. Growth of radicle and hypocotyl tissue was noted in all cases where these tissues had not browned (Figures 14-16 & 17b-d).

It is not possible however to tell whether either enzymatic hydrolysis or physical thrust, or both, are limiting to growth where seed viability is marginal. Ultrastructural evidence will be presented (Chapter 4) which suggests that while disorganisation of the cytoplasm of endosperm cells occurs in parallel with embryonic deterioration, the endosperm walls remain highly structured with little evidence of hydrolysis. It is suggested that this could lead to a situation in which an aged, but potentially germinable embryo may become entrapped by the endosperm.

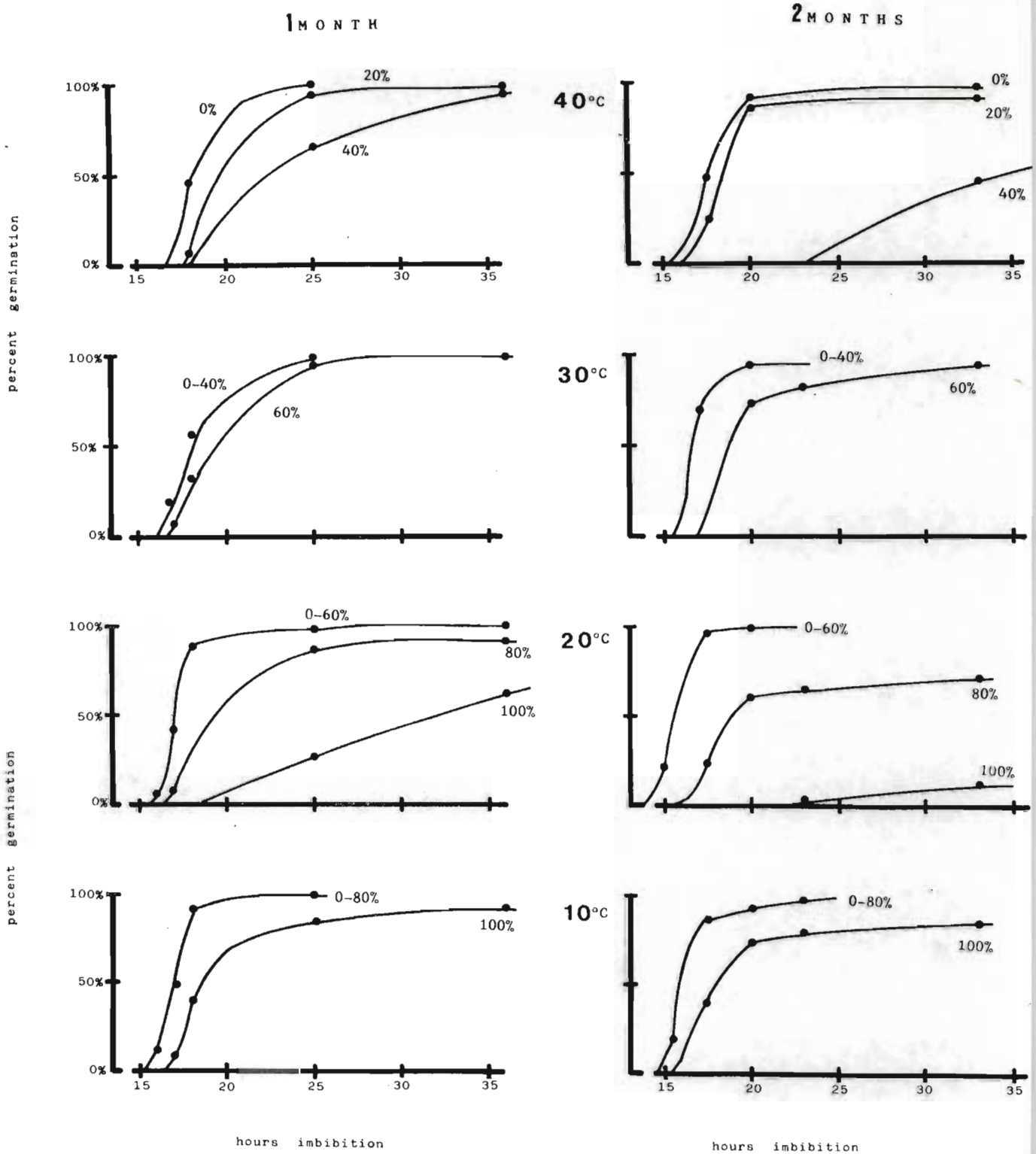
1 MONTH
2 MONTHS
percent germination
100%
50%
0%
0%
20%
40%
40°C
0%
20%
40%
0-40%
60%
30°C
0-40%
60%
0-60%
80%
100%
20°C
0-60%
80%
100%
0-80%
100%
10°C
0-80%
100%
15
20
25
30
35
hours imbibition
hours imbibition

FIGURE 3

FIGURES 3 - 8

Cumulative percentage germination for a single seed lot (1978 purchase) equilibrated at various relative humidities (0%, 20%, 40%, 60% 80% and 100%) before being stored hermetically at a range of temperatures 10°, 20°, 30° and 40°C . Monthly sampling intervals indicated at the top of each set of graphs. After 5 months sampling interval was increased and the experiment terminated at 22 months.

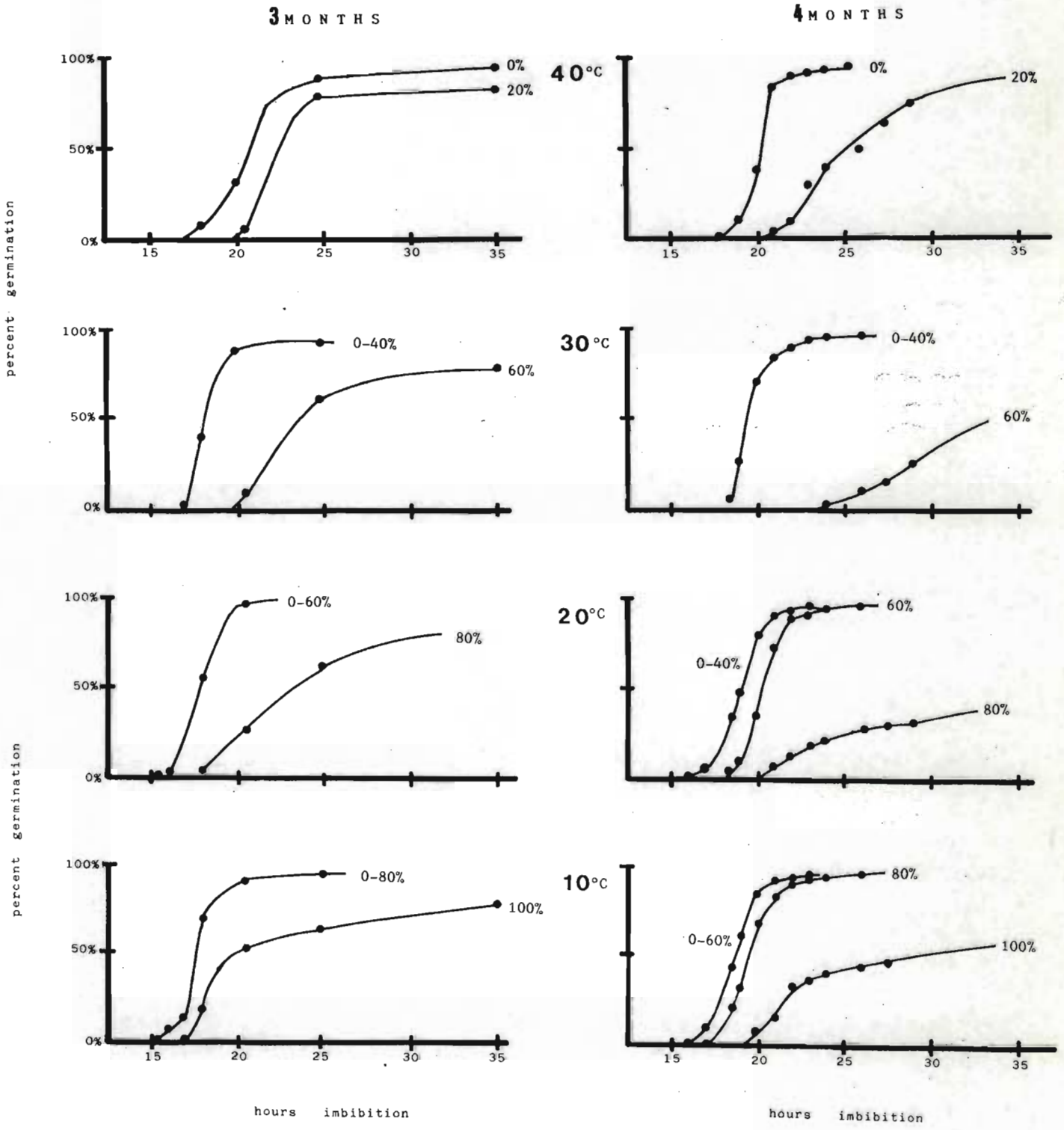
3 MONTHS
4 MONTHS
40°C
30°C
20°C
10°C
100%
50%
0%
0%
20%
0-40%
60%
0-60%
80%
0-80%
100%
15
20
25
30
35
percent germination
percent germination
hours imbibition
hours imbibition

FIGURE 4

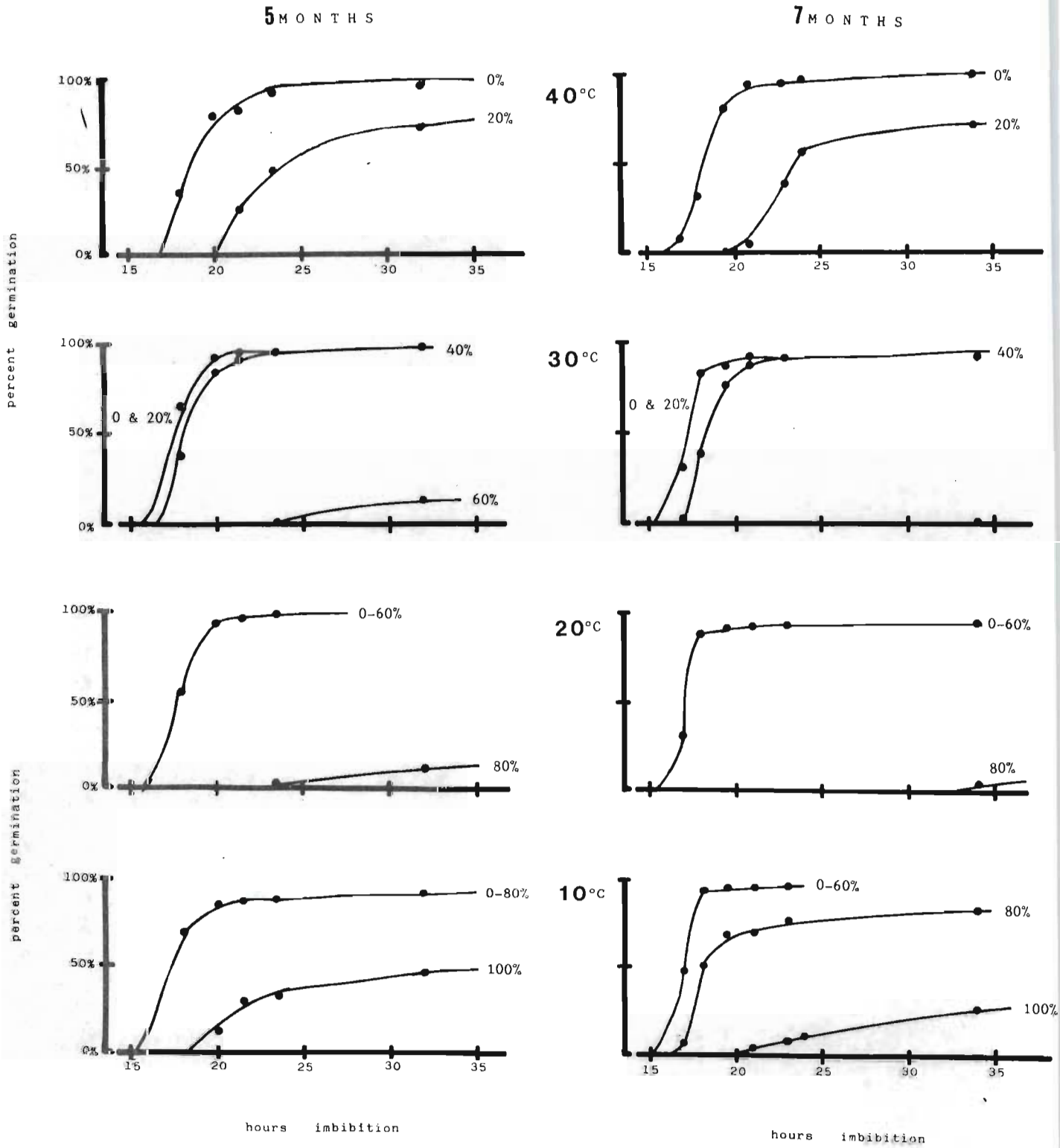
5 MONTHS
7 MONTHS
percent germination
100%
50%
0%
0%
20%
40%
60%
80%
100%
0 & 20%
0-60%
0-80%
40°C
30°C
20°C
10°C
15
20
25
30
35
hours imbibition

FIGURE 5

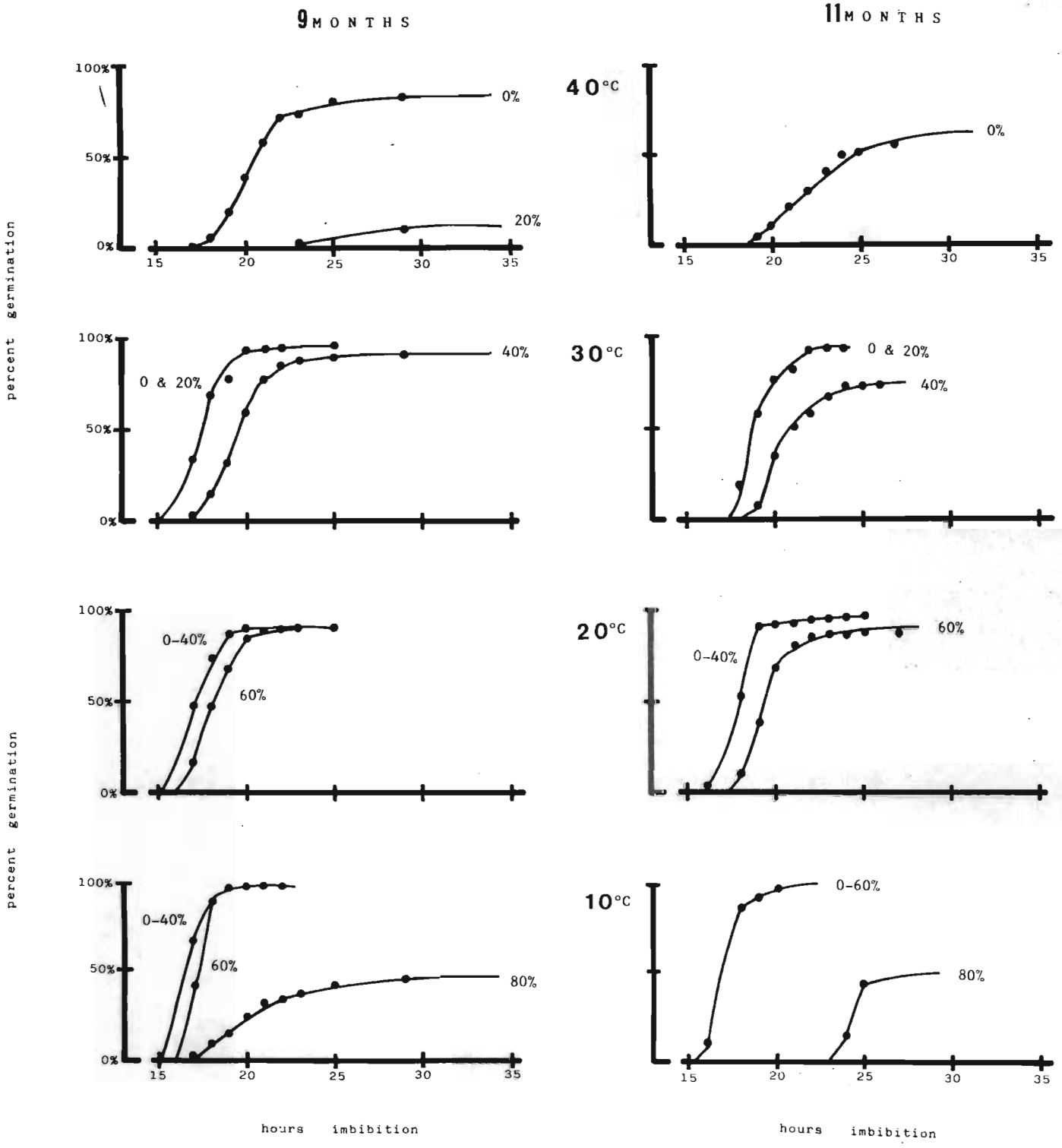
9 MONTHS
11 MONTHS
40°C
30°C
20°C
10°C
100%
50%
0%
0%
20%
40%
60%
80%
0 & 20%
0-40%
0-60%
15
20
25
30
35
percent germination
percent germination
hours imbibition
hours imbibition

FIGURE 6

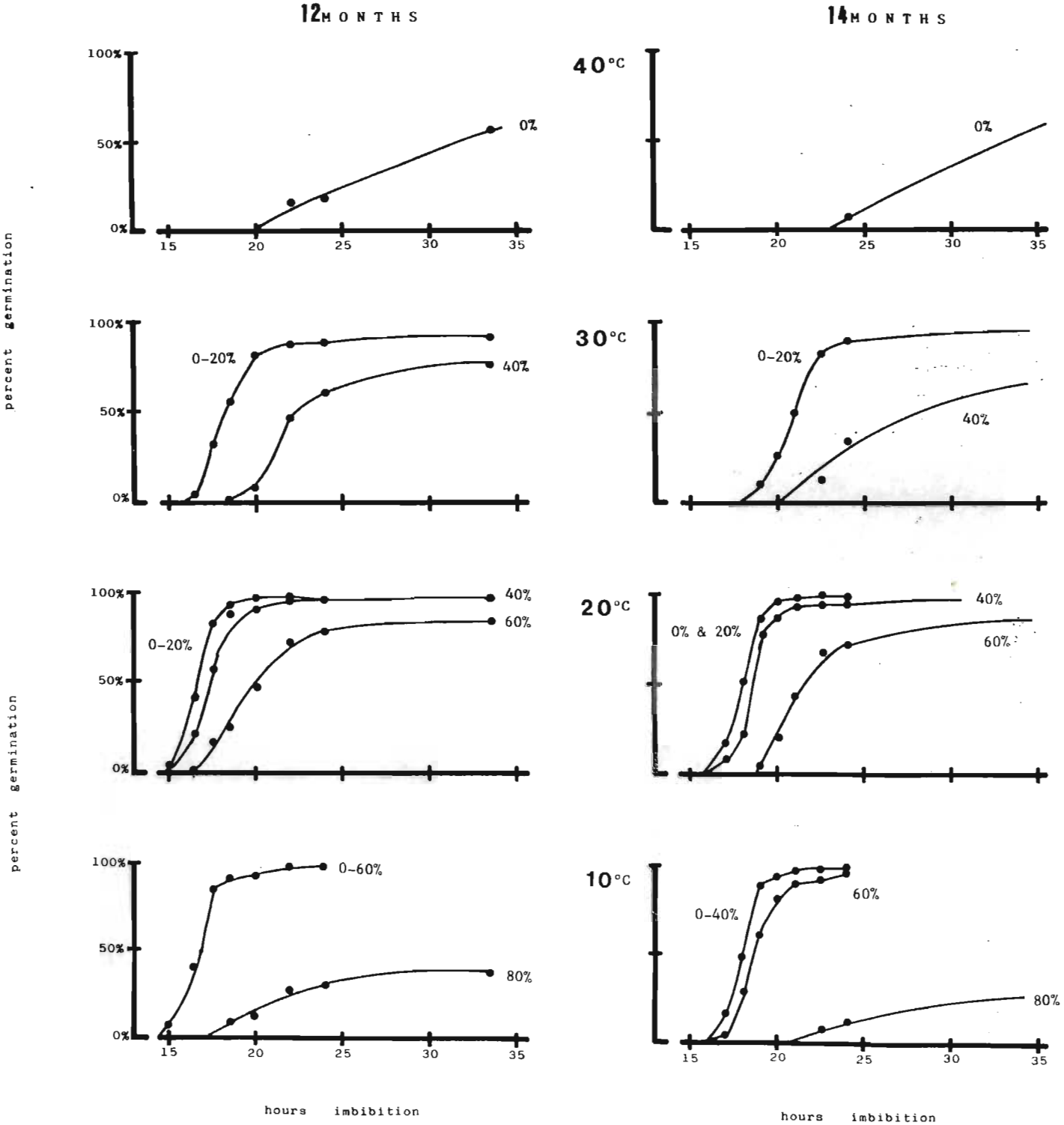
12 MONTHS
14 MONTHS
40°C
30°C
20°C
10°C
100%
50%
0%
0%
0-20%
40%
0% & 20%
60%
0-60%
0-40%
80%
15
20
25
30
35
percent germination
hours imbibition

FIGURE 7

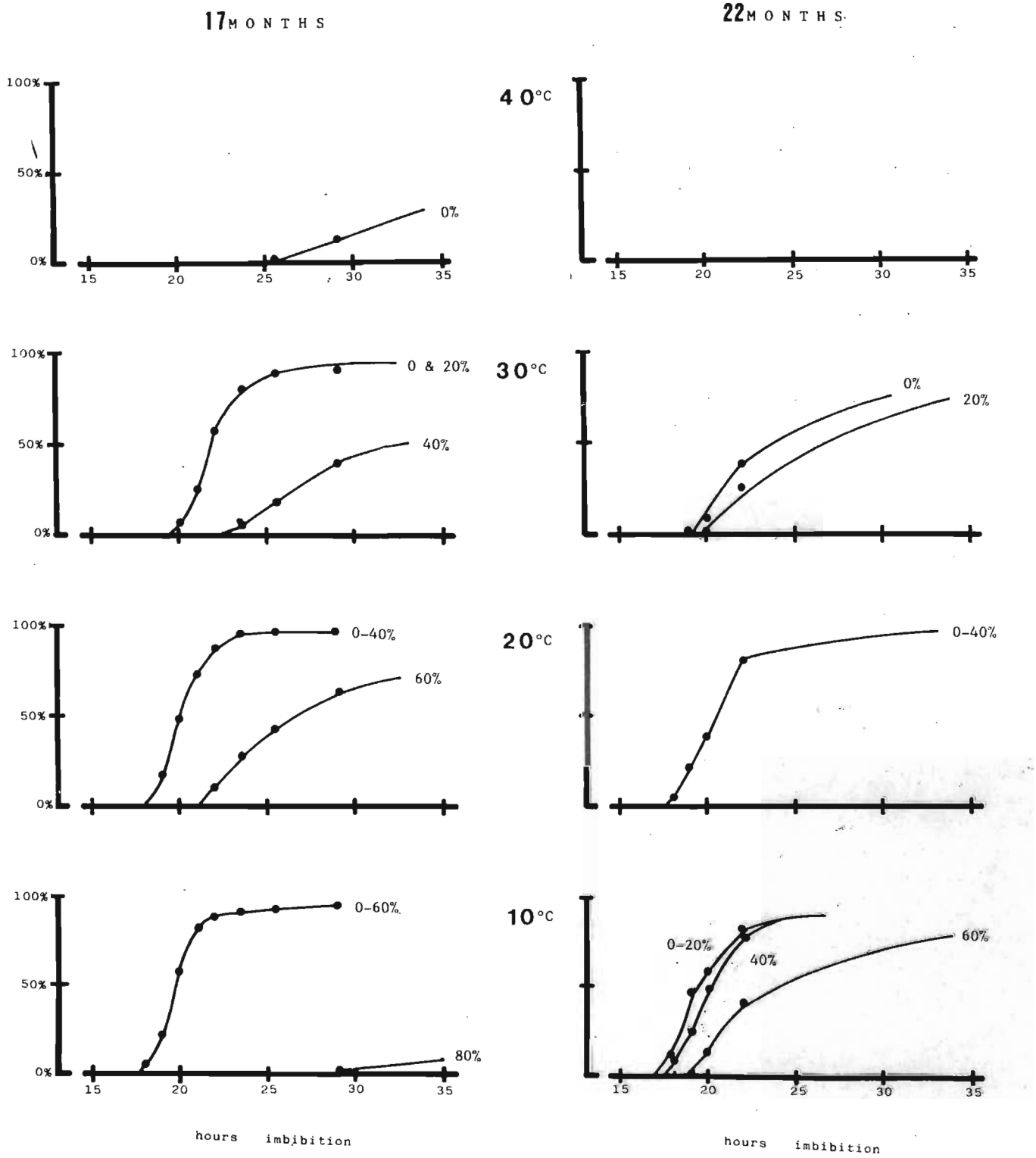
17 MONTHS
22 MONTHS
40°C
30°C
20°C
10°C
100%
50%
0%
0%
0 & 20%
40%
0-40%
60%
0-60%
80%
0-20%
15
20
25
30
35
percent germination
hours imbibition

FIGURE 8

TABLE 2.3

Estimated viability loss for seeds stored at a range of temperatues and relative humidities. Values were obtained by extrapolation of the germination data presented in Figures 1-8.

Storage relative humidity	Months survival at storage temperature			
	10°C	20°C	30°C	40°C
100%	7 - 9	2 - 3	1 *	-
80%	17 - 22	7 - 9	1 *	-
60%	22	17 - 22	7 - 9	-
40%	+	22	17 - 22	2 - 3
20%	+	72 [1]	22	9 - 11
0%	+	72 [2]	+	17 - 22

* loss of viability rapid (less than 1 month)

+ viability considerably extended beyond study period

[1] see Figure 11(c); [2] see Figure 2(b).

Effect of Storage Temperature on Germination

In the second part of this study attempts were made to investigate the influence of temperature on vigour and viability. Lettuce seeds were allowed to equilibrate with the appropriate RH for 16 days at 20°C. Thereafter samples were sealed in serum vials with rubber serum stoppers and held in incubators at 10°, 20°, 30° & 40°C. At selected intervals seed samples were withdrawn for germination studies and the vials resealed and returned to the incubators. Seeds used in this study were from the 1978 purchase and had been stored for 27 months over silica gel at 5°C prior to commencing the study. As before, typical values are presented for certain RH values to simplify presentation; moisture contents are presented in Table 2.2. It is apparent from the germination data of the first sampling interval (Figure 3, 1 month at 10°C and 0% RH) that little observable deterioration had resulted from the 27 months storage at 5°C as adjudged by the shape of the germination curve (Compare with Figure 1, 15 months storage, for instance). The cumulative percentage germination of seeds stored at higher temperatures and relative humidities began to diverge markedly from seeds held under conditions which would be expected to maintain viability (10°C, 1 - 3 months; Figures 3 & 4).

Seeds equilibrated with 100% RH (m.c. = 11,9%) lost viability at 10°C between 7 and 9 months, whereas this occurred between 2 & 3 months for seeds held at 20°C. The pattern of germination decline was seen to be entirely consistent across the range of variables, viz. time, temperatures and moisture content; this may be noted by visual inspection of data or by examination of Table 2.3. Kosar & Thomson (1957) reported that lettuce seed of an homozygous white strain, 3520L, held at 10°C and between 0 and 58% RH, yielded uniformly high germination percentages (90%) after 5 years of storage; it is likely that values of similar magnitude would have been recorded in the present study. Similarly, data presented by Nakamura (1975) showed that lettuce seed stored at room temperature at 2.5% and 6.3% m.c. showed no appreciable loss of viability after 9 years. Since the study was discontinued after 22 months, data are somewhat

Figure 18 : Illustrates excised embryos after 1 week of imbibition from seed subjected to an accelerated ageing regime (30°C and 100% RH). Seed stored for 23 months at 5°C before being tested.

Figure 19 : Appearance of seedlings at 10 days after storage for 49 months at 20°C and 0% RH (1978 purchase).

Figure 20 : Appearance of seedling at 10 days after storage for 22 months at 30°C and 0% RH (1978 purchase).

Figure 21 : Appearance of poorly-developed seedlings at 10 days after storage for 22 months at 30°C and 40% RH (1978 purchase).

Figure 22 : Appearance of poorly-developed seedlings at 10 days after storage for 22 months at 40°C and 0% RH (1978 purchase).

Figure 23 : Appearance of poorly-developed seedlings at 10 days after storage for 22 months at 20°C and 60% RH (1978 purchase).


18


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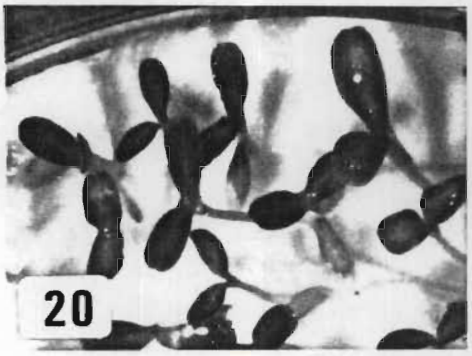
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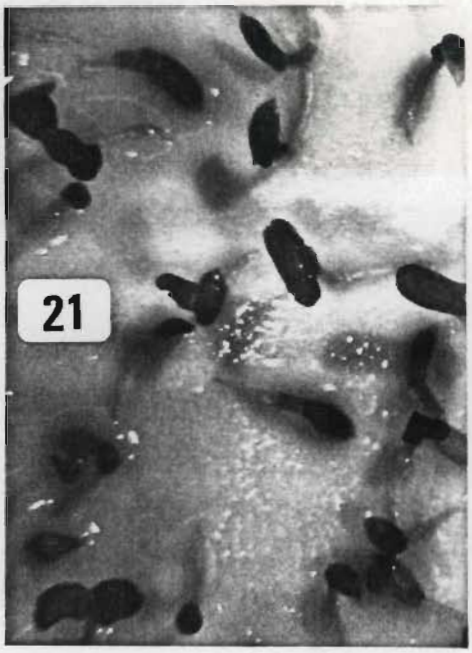
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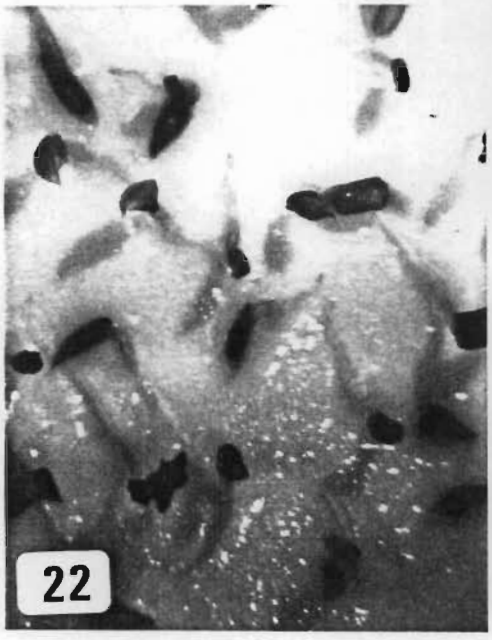
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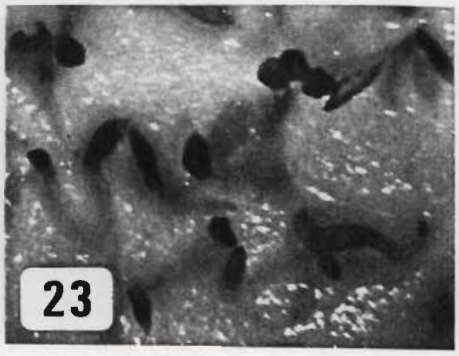
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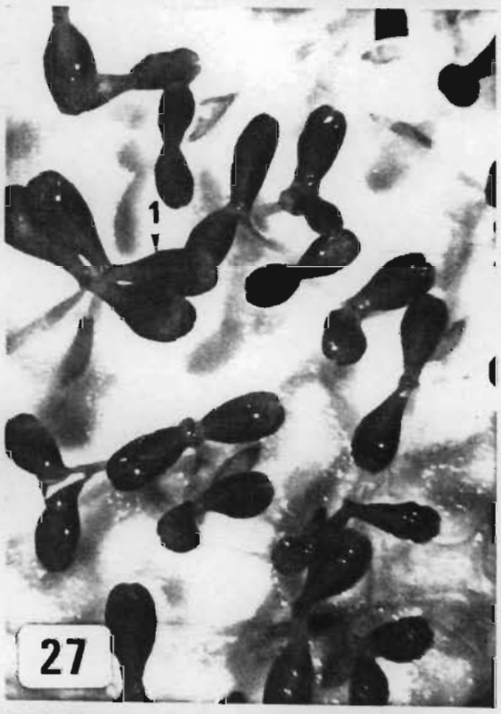

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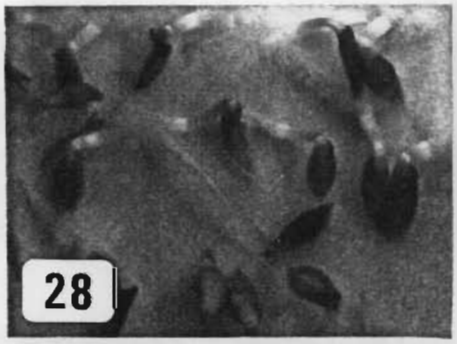

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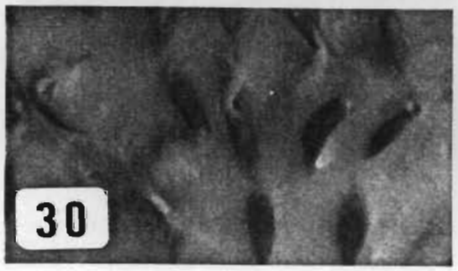

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Figure 32 : Appearance of seedlings at 10 days after storage of seed for 6 years at 20°C and 0% RH (1978 purchase).

Figure 33 : Appearance of seedlings at 10 days after storage of seed for 5 years at 0% RH (1979 purchase).

Figure 34 : Appearance of seedlings at 10 days after storage of seed for 23 months at 60% RH (1983 purchase).

Figure 35 : Appearance of seedlings at 10 days after storage of seed for 23 months at 40% RH (1983 purchase).

Figure 36 : Appearance of poorly-developed seedlings at 10 days after storage of seed for 5 years at 40% RH (upper) and 20% RH (lower).

Figure 37 : Appearance of non-viable seed at 10 days post-imbibition after storage for 22 months at 40°C and 20% RH (1978 purchase). Note absence of browning in the radicle tips. Compare also with Figures 14 - 17.

Figure 38 : Appearance of seedlings at 10 days from seed stored in hermetically sealed tins for 12 years and 10 months. Note the absence of cotyledonary necrosis in this cultivar (Webb's Wonderful) in contrast to the cultivar Great Lakes, where the lesion is evident after much shorter storage periods (Figure 32 - 35).


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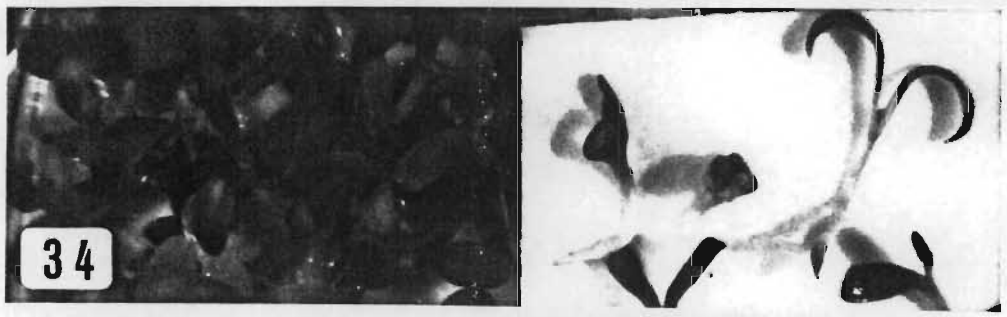
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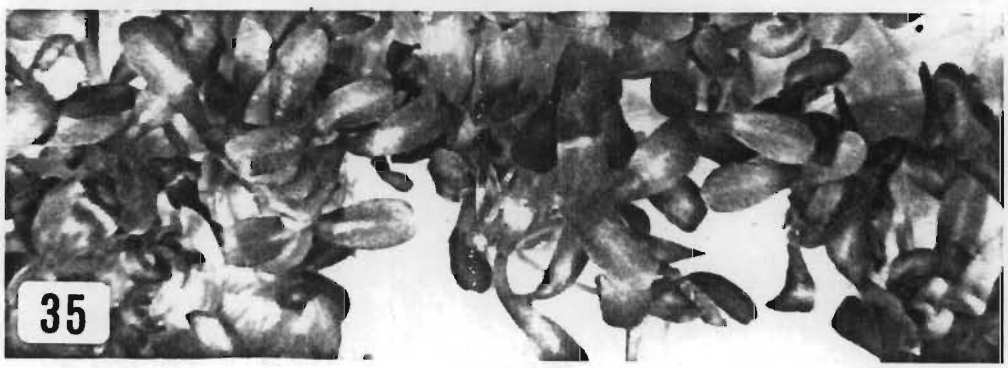
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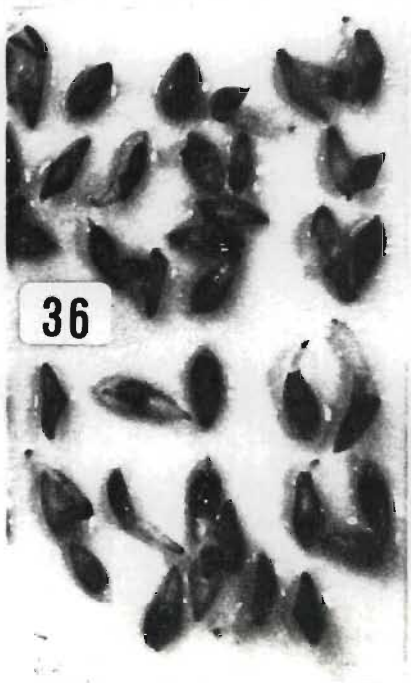
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38

incomplete; at high temperatures and moisture contents loss of vigour and viability was extremely rapid and took place before the first sampling interval.

Comparison of the germination results of seeds stored for 17 months at 20°C (Figure) showed great similarity to those of another seed lot used in the earlier study (Figure 1, 15 months) suggesting these may be viewed as a continuum of a single ageing regime. However, some differences do exist. Examination of the two seed lots after 22 months of ageing treatment revealed that for seeds held at 40% (Figure 1) began to diverge in rate of germination, whereas this was not the case for seeds held in hermetic storage (Figure 7, 20°C). It is not clear whether these results reflect differing storage conditions (i.e. storage at constant RH or hermetic storage) or prematurational and harvest effects. There are several other instances where the cumulative percent germination curves for a particular combination of time, temperature and RH appears remarkably similar to those of some other, quite different, combination. (Compare Figure 2, 49 months at 20°C, with Figure 7, 22 months at 30°C). Further similarities were apparent on examination of cotyledonary development after 1 week of germination (Figures 19 & 20).

Storage Temperature and Seedling Necrosis

It was evident from the ageing sequences studied that the next visible morphological event in deterioration concerned greening of the cotyledons. Examples in support of comparable ageing by particular combination of time, temperature and RH of storage are presented in Figures 21 - 23; 24 & 25. In keeping with suggestions about the gradual nature of events which characterize deterioration, it is evident that the cotyledonary necrosis is first manifest as an inability to green completely in the midrib region at the junction between cotyledon and hypocotyl and spreads progressively basipetally (Arrows 1-5, Figures 24 - 27). Thus although no details of harvest dates were available for the seed lots investigated, it would seem that under optimal dry storage (0 - 20% RH), at ambient temperatures (20° - 25°C), most seed lots of c.v. Great Lakes would

show the beginnings of cotyledonary necrosis. In keeping with evidence that individual seeds in a population do not age uniformly (Roberts, 1972) it is also apparent that while some seedlings may show advanced stages of cotyledonary deterioration, others would appear totally normal. (Figure 10, Arrows 1 & 2). Furthermore, as has been noted (Heydecker, 1972) slower growth and development of the autotrophic plant are one of the many indicators of vigour loss. A delay in cotyledonary greening may be considered the first visible manifestation of deterioration, which became manifest before any other as can be seen by comparing Figures 28 & 29. This syndrome was seen to be time, temperature and RH dependent. Figure 30 illustrates seeds of the 1979 purchase stored at 0% RH for 5 years after 48 hours imbibition. These appear morphologically equivalent to seeds of a 1983 purchase stored at 40% RH for 23 months at 20°C (Figure 28). Harrison (1966) has pointed to significant varietal differences in longevity, the differences being of such a magnitude that they are unlikely to represent preharvest and maturational effects. In the present study it was observed that a 1972 purchase of seeds of c.v. Webb's Wonderful which had been held in hermetic storage at 20°C (12 years 10 months laboratory storage; m.c. = 5.7%) showed little visible deterioration as evidenced by emergence and earlier greening (Figure 31) in comparison with c.v. Great Lakes stored for a much shorter period (Figure 30) at 20°C. Such differences were even apparent in seedlings at one week (Figures 33 & 38). This finding is in keeping with the observations of Bass (1970) who reported cultivar differences with regard to development of necrosis (also termed red cotyledons). This latter author reported that the condition was long recognized, being initiated at the base of the cotyledons as grey, brown or reddish-brown dots. With increasing severity of ageing the spot size increased until most of the cotyledon became necrotic.

The Optimum Moisture Content for Lettuce Seed Storage

It was evident that the longevity of lettuce seed was significantly greater by storage at as low an RH as possible, as can be illustrated by comparison at 1 week post-germination of the 1978 and

1979 purchases after storage at 0% RH (mc = 2-3%) and 20% RH (mc = 4-5%), repectively (Figures 32 & 33; Figure 12). Kosar & Thomson (1957) found that below 25% RH seeds showed a tendency to become dormant and advocated storage above 25% RH. Although seeds with a potential for dormancy may represent a special case as regards storage RH, other studies clearly indicate that lettuce seeds suffer little or no injury by desiccation (Nutile, 1964) and longevity is considerably extended by lower moisture contents (Nakamura, 1975). Extreme drying may bring about injury in seeds of onion, parsley and pepper although much may depend on the manner in which this is achieved; it may further be related to differential removal of free and bound water (Woodstock, Simkin & Schroeder, 1976).

Conclusions - A Morphological Sequence of Deterioration in Lettuce Seeds

The results of the present study suggest that necrosis is part of the mechanism of deterioration which is initially only manifest as a delay in midrib greening (Figures 32, 33, 35). At this stage radicle emergence may be only slightly delayed; growth is unaffected and final germination percentages attained are the same. This leads to a situation in which debilitated axis growth is evident and in which the cotyledons fail to emerge from the coats (Figures 21 & 36). The final deteriorative event is seen when embryonic growth potential is such that radicle emergence cannot take place and the embryo becomes entrapped by surrounding tissues (Figure 37). It will be recalled that limited embryonic growth may nevertheless take place on excision.

It has been assumed, though it is by no means certain, that 'accelerated ageing' of seeds yields results which are the same as those accumulating within the more normal range of storage parameters, and that any differences occurring are of degree rather than kind (Villiers, 1980). The observation that a greater incidence of cotyledonary necrosis was seen in cultivars stored at a low relative humidity rather than at high relative humidity, for the same temperature regimes, may be regarded as a pertinent

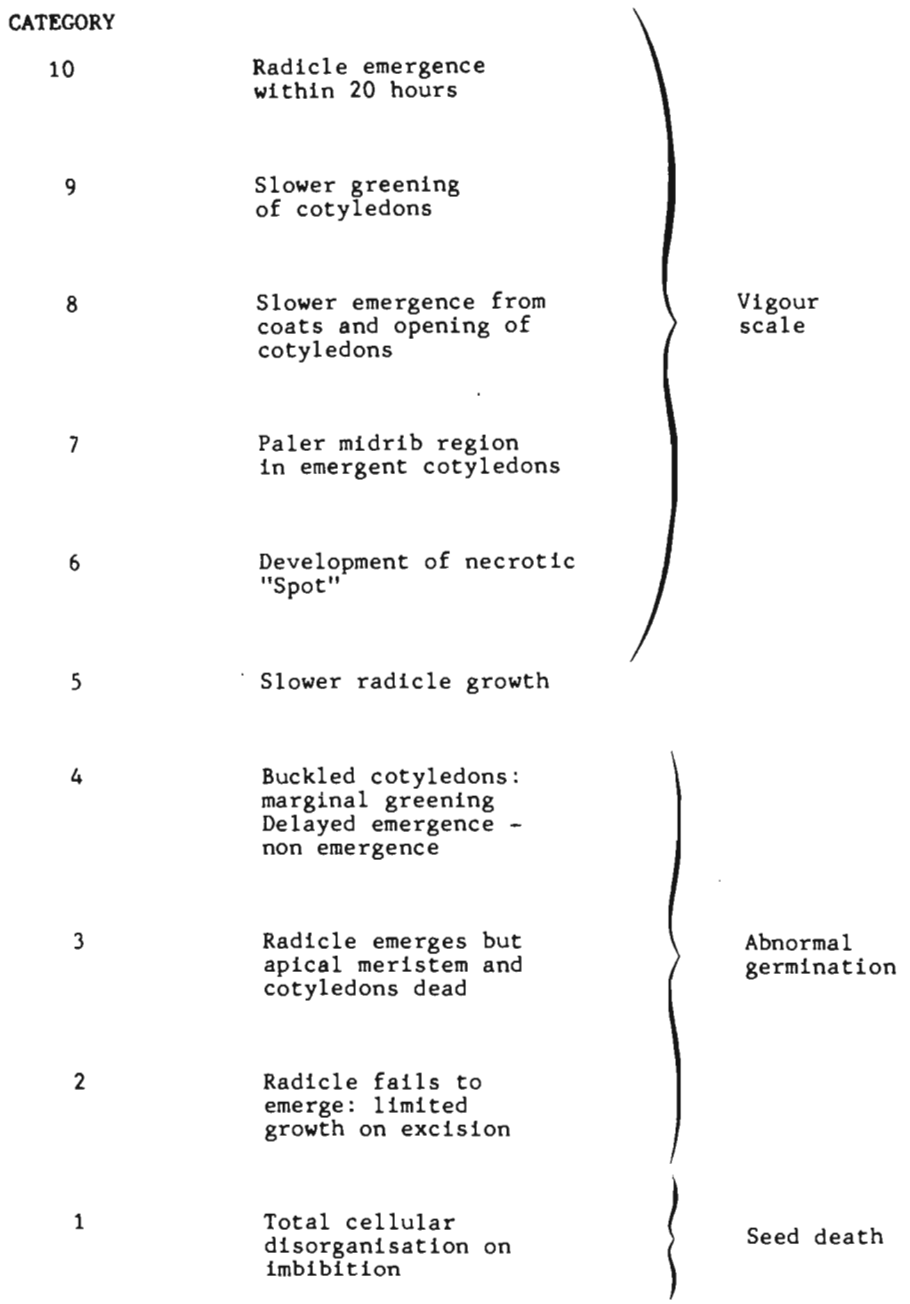


Figure 39: Proposed sequence of events characterizing progressive deterioration in seeds of lettuce. Vigour ratings are included on the scale, with a viability cut-off point being somewhat ill-defined at rating 4. The scale stresses the fact that deterioration is a continuum, as has been noted by previous workers such as Isely, Woodstock, Delouche, and Heydecker.

observation in the light of the above comment by Villiers. It may be, therefore, that under harsher conditions of higher temperature or RH necrosis becomes a precipitous and widespread event with the result that total loss of functionality of cotyledon occurs without the intervening sequences of delayed greening, delayed cotyledonary emergence and a gradual spread of chlorosis before onset of necrosis (Figures 32, 33 & 35). The embryo may thus move from a condition of high viability and vigour directly to a condition such as illustrated in Figure 37. A single anomalous observation which may cast some doubt on this interpretation is presented in Figure 34. This illustrates the appearance of cotyledons and embryonic axes of seedlings at one week from a 1983 purchase of seed (c.v. Great Lakes) stored for 23 months at 60% RH and 20°C. If the above argument is correct cotyledons of seed from the same lot, but stored at 40% RH, should show less necrosis, which is clearly not so (Figure 35).

On the other hand, seedlings from the 1979 seed purchase stored for 22 months were in no way comparable (Figure 23). In the absence of any comparable studies in the literature it can only be speculated that the stunted seedlings reflect a differential sensitivity of axial tissues to the ageing regime which would be soon followed by a rapid cotyledonary necrosis to produce seedlings of the appearance of those seen in Figure 23.

A proposed sequence of events which attempts to summarize the gross morphological events associated with vigour and viability decline is presented in Figure 39.

CHAPTER 3

ULTRASTRUCTURAL STUDIES
ANHYDROUS FIXATION

It has been suggested that in seeds stored at low water contents, the membrane phospholipids exist in a porous, hexagonal arrangement (Simon, 1974). Evidence for this proposal was obtained from several observations. X-ray diffraction had indicated that lipid - water systems could exist in several phases (Luzatti & Husson, 1962), while Stoeckenius (1962), using OsO_4 vapour fixation provided electron microscopic evidence that phospholipids occurred in a lamellar phase at 30% water content, and in an hexagonal phase at 3% water content. The initial leakage of solutes from imbibing tissues could be attractively accommodated in the framework of the above observations by supposing that until the lamellar stage was physically fully reconstituted, the membrane could not function as a selectively permeable barrier. It has further been suggested that peroxidative changes to the membranes in dry storage could exert a profound effect on the reconstitution of the membrane bilayer (Simon, 1974).

On the other hand electron microscope evidence has been obtained suggesting that membranes were clearly lamellar in the dry pea seeds (Perner, 1965), and suggestions that physical damage to cells and membranes could account for leakage have been made by Larson (1968). Villiers (1973, 1980) has drawn attention to the consequences of amplification of damage to membrane systems, enzymes, RNA and DNA on imbibition. It was observed during the early stages of deterioration of lettuce seeds that cells of the embryo radicle appear well-organised, but at the end-point of the deteriorative continuum withdrawal of the plasma membrane from the cell wall and rupture of the vacuoles was a characteristic feature. In the light of the above suggestions of membrane phase changes, attempts were made to devise a satisfactory method for the electron microscope visualization of membrane and general cell organisation in dry seeds as a preliminary to ultrastructural studies on the

radicle and cotyledonary tissues of deteriorating lettuce seeds during imbibition.

MATERIALS AND METHODS

Throughout this study use was made of transverse slices of cotyledonary tissue since these could readily be cut into cubes of predominantly uniform tissue of less than 0.5 mm^3.

Aqueous Fixation

Tissue cubes were fixed by placing directly into 5% phosphate-buffered glutaraldehyde (pH 7.4) for 30 minutes and briefly washed in phosphate buffer for 5 minutes, before post-fixation in 2% aqueous osmium tetroxide. Dehydration through a graded acetone series (25%, 50%, 75%) of 15 minutes each before 3 changes of 100% acetone (5 minutes each) was followed by graded infiltration with epoxy resin (Spurr, 1969).

Technique of Thompson (1979)

In this method tissues were fixed in anhydrous glycerol containing 4% formaldehyde for 18 hours. Thereafter, tissues were washed in phosphate buffer (15 minutes total) before post-fixation in phosphate buffered 2% osmium tetroxide. Dehydration and infiltration were as above.

Vapour Fixation Techniques

Examination of the micrographs presented by Perner (1965) clearly indicated that satisfactory fixation may be achieved entirely in the vapour phase without the need for a fixative vehicle such as anhydrous glycerol. Since use of formalin vapours is a well-known standard procedure for fixation of freeze dried tissues in animal histological techniques (Pearse, 1980), both paraformaldehyde and acrolein vapours were explored as possible fixatives. Neither of these compounds are not regarded as suitable fixatives for lipids, although it should be noted that calcium-formal has evolved

empirically as a fixative for subsequent histochemical localization of lipids (Pearse, 1980) and some evidence exists for chemical interactions between unsaturated fatty acids and formaldehyde (Jones, 1973).

Osmium tetroxide reacts with the unsaturated double bonds of fatty acids and is regarded as both a fixative and stain of lipids (Hayat, 1981). Its suitability was therefore also explored, although always in combination with one or both aldehydes. Penetration by the osmium tetroxide vapour was facilitated by application of a light vacuum from a rotary vacuum pump to a sealable tube, with three repeated evacuations evenly spaced over the 8 day fixation period. Even so, complete penetration by the fixative was not achieved as judged by the white interior and black periphery on halving the tissue cubes at the end of the fixation period. All aldehyde fixations were conducted at 30°C in sealed vials in the presence of silica gel to maintain low relative humidities. Osmium tetroxide vapour fixation was conducted at room temperature and humidity (20 - 25°C; 60 - 85% RH). The following vapour fixative combinations were investigated:

1. Formalin-Osmium fixation (5 and 8 days, respectively)
2. Formalin-Acrolein fixation (5 and 3 days, respectively at 30°C)

Tissue cubes were placed into 100% acetone for one hour before infiltration over 5 days in a graded series of acetone:epoxy resin (Spurr, 1969) followed by infiltration in pure resin over 2 days. Thermal curing was at 70°C for 8 hours.

Studies with Phospholipid Model Systems

Studies were undertaken using protein/phospholipid dispersions as a model system. 500 mg of an acetone-precipitated brain phospholipid (Diagen - Diagnostic Reagents, Oxford) and 500 mg of bovine serum albumin (Miles, Fraction IV) were dispersed in 1.5 ml of distilled water using a glass tissue homogeniser (Kontes), and fixed using conventional aqueous fixation techniques (see above, Aqueous Fixation).

In order to gain familiarity with the appearance hexagonal phase membrane structure use was made of the property of phosphatidylethanolamine to enter the hexagonal phase at 0 - 50°C (Cullis & de Kruijff, 1978) and the fact that aqueous calcium-cardiolipin systems exist in the hexagonal state (Cullis, Verkleij & Ververgaert, 1978). Cardiolipin (Sigma, in ethanol) was added dropwise to 5 ml of 0.1 M aqueous calcium chloride in a centrifuge tube, and the resultant white precipitate centrifuged down with a benchtop centrifuge. Fixation was by 2% aqueous osmium tetroxide (30 minutes) followed by acetone dehydration and embedding in epoxy resin (Spurr, 1969). Phosphatidylethanolamine (Soybean; Sigma, Type IV) was dispersed in bovine serum albumin (25 mg and 250 mg, respectively) in 1 ml of distilled water by homogenisation and small aliquots (200µl) placed in plastic-lined bottle-top caps. These were then incubated at 30°C in a sealed container for 3 hours, before additon of 2% aqueous osmium tetroxide. After 1 hour at 30°C the sample was removed and placed in 100% acetone and embedded as above.

Infuence of the Embedding Medium on Membrane Phospholipids

Sjostrand & Bernhard (1976) reported that in order to preserve membrane structure as close to the native state as possible fixation should be limited to brief fixation by glutaraldehyde. Dehydration agents and osmium tetroxide are regarded as undesirable and increase possible artefacts. These authors advocate that portions of tissue are transferred directly to Vestopal resin and repeatedly centrifuged through the resin to effect infiltration. Alternatively, they may be freeze dried and embedded at sub-zero temperatures (Sjostrand & Kretzer 1975). More recently the development of the acrylic resins Lowicryl K4M and Lowicryl HM20 has led to the development of low temperature embedding techniques with minimal denaturation of protein structure (Kellenberger, Carlemalm, Villiger, Roth & Garavito, 1980).

This technique was investigated using a sample of the formalin-acrolein fixed cotyledonary slices. Dehydration, infiltration and polymerization of the two resin types were conducted in a domestic

deep freeze at -20°C according to the following (Balzers/Union) Schedule:

1 Hour in 30%, 50% & 70% aqueous ethanol; 2 hours in 90% ethanol; 1 Hour in 1:1 and 2:1 resin/ethanol; 1 hour in pure resin, followed by overnight in a fresh change of resin. Polymerization was for 12 hours at 20°C in gelatin capsules under U.V. light.

To investigate the possible interactions between embedding media and phospholipids small portions of the purified brain phospholipid were placed in vials filled with a number of resins commonly used for electron microscopy (Water soluble Durcupan, Vestopal W, hydroxypropyl methacrylate, Spurr's epoxy resin). These were examined for changes in physical appearance and properties of the lipids over a 12 hour period.

Section Staining

It was observed that seed tissues embedded in Spurr's epoxy resin stained poorly with lead citrate after 30-45 mins. Pre-treatment of tissues with aqueous potassium permanganate (0.1 - 1% w/v) for 2 min. was found to reduce the time necessary for staining with lead citrate to 3-5 minutes. After washing with distilled water sections were stained with saturated alcoholic uranyl acetate in 50 or 75% ethanol for 5 mins.

This procedure was also found to be particularly successful with aqueously fixed tissues in agreement with the earlier studies of Bray and Waggenaar (1978). When applied to anhydrously fixed tissues this method produced densely stained protein bodies and darkly staining cytoplasmic constituents. While this produced suitable micrographs at low magnifications, images at higher magnifications were too contrasty for satisfactory photography. Satisfactory alternative staining was obtained by following the aqueous permanganate step with a distilled water wash, before placing sections (on the grids) face-down onto a droplet of 75% ethanol saturated with uranyl acetate or lanthanum nitrate for up to 35 minutes. After rinsing briefly with 50% ethanol, sections were

washed with distilled water before drying and viewing in a Philips E.M. 200 or Jeol 100C electron microscope. In some cases the lanthanum nitrate step was omitted and uranyl acetate used as the only stain after permanganate pre-treatment of sections.

RESULTS AND DISCUSSION

All the resins tested were seen to solubilize samples of brain phospholipid over a 12 hour period, although this occurred to a more limited extent with Vestopal. It proved impossible to obtain sections of cotyledonary tissue embedded in the Lowicryl resins. Although the resin cut satisfactorily, the tissue was apparently not fully infiltrated, since it could be readily deformed and removed from the surrounding resin with the aid of a pair of fine forceps.

No advantage over conventional techniques was obtained by Pongranz when using Lowicryl to embed dry seed tissue (Kellenberger, pers. comm.); consequently no further investigations were conducted using Lowicryl and low temperature techniques.

Many approaches to cell ultrastructure using unconventional techniques have yielded electron microscope images in which membranes appear unstained against a generally darker cytoplasmic background (Tokuyasu, 1976, 1978; Pease 1967a & b; Sjostrand & Bernhard, 1976). Few have attempted to interpret molecular structure from such images or explain why membranes present this appearance. Sjostrand & Kretzer (1975) have noted that while protein-free lipid bilayers have not been reported as being fixed by aldehydes, it is likely that proteins within the membranes would be. Thus if the lipids present were removed or replaced by resin, and provided that proteins retain their position and shape, the membrane would be represented by these remaining structures. The sectioning of tissues fixed with formaldehyde and osmium tetroxide proved to be extremely difficult, as a result of the brittleness of the embedded tissue; the roughly-surfaced nature of the sections was clearly evident from the need to cut sections

somewhat thicker than normal (Figures 1 & 2) in order to obtain retrievable sections.

Plasma membranes did not present the entirely typical tripartite structure of aqueously fixed tissues, possessing a larger central electron translucent region flanked by electron dense layers. (Arrow, Figure 1). In the case of the bounding membrane of the protein body, which is considered to have a half-unit membrane (Yatsu, 1965; Mollenhauer & Totten, 1971), it appeared that abutment of the limiting membrane of the lipid droplets against the "half-unit" membrane of the protein body contributed to an apparent increased thickness of the membrane (Figure 1). Adjacent lipid droplets were never observed to be spherical but frequently assumed closely appressed, irregular and sometimes interdigitating boundries. While the electron dense layer between adjacent lipid droplets appeared superficially membrane-like (arrows, Figure 2) it was evident from fixed, hydrated tissues that this layer represented adjacent lipid droplets membranes between which highly condensed components such as ribosomes were present (Figure 20). It was clearly evident at low magnification that the dominant storage components were numerous electron-dense protein bodies, from which the phytin was occasionally lost on sectioning or in subsequent handling, and numerous smaller lipid droplets. (Figure 3).

Lipid droplets were arranged in a closely appressed shell around the protein bodies and along the cell wall. Occasionally irregularly shaped structures were observed between the lipid bodies which stained extremely darkly, and in which E.R.-like elements could be observed (Figure 2, white arrow). Denseiy-staining stellate nuclei (Figure 3) which were smaller and less conspicuous than those observed in hydrated tissue, frequently possessed complex membrane profiles in close proximity to the nuclear envelope (Figures 4, 6 & 8). The nuclear envelope was not as clearly lamellar as the membranous structures in close proximity to it (Figures 4 & 8). In tissues fixed with combined formaldehyde and acrolein vapours only, the plasmalemma presented a lamellar appearance of less intense contrast to that of tissues fixed in formaldehyde and osmium tetroxide (Compare Figure 1 with Figures 5 & 6). Plasmodesmata,

when observed, possessed a typical "halo" characteristic of the desmotubule (Figure 6).

Tangential sections of the lipid droplets revealed the presence of tubule-like structures (Figures 8, 9 & 11 arrows) whose nature and function must remain unexplained. A survey of the literature has revealed several reports of comparable structures in widely differing systems. "Lipotubuloids" surrounding the osmophilic granules in tissues of Ornithogalum have been described by Kwiatkowska (1972) and were impiicated in the movement of the granules. Freeze-fractured lecithin liposomes have been observed to possess a regularly corrugated surface depending on carbon chain length or holding temperature prior to freezing (Ververgaert, Elbers, Luitingh & van den Berg, 1972; Flick, Henson & Chapman, 1969). Additionally, Buttrose & Soeffky (1973) observed tubular corrugated structures between lipid bodies and the adjacent plasmalemma which was interpreted as a system of lipid rich saccules. The technique of freeze fracturing has shown similar corrugations to be associated with the plasmalemma of soybean axes (Chabot & Leopold, 1982) and with the amyloplasts of pea cotyledons (Swift & Buttrose, 1973).

It was evident from high-resolution studies using holey films that resin granularity could represent a potential source of spurious periodicity in membrane structure (Figure 11). There was however little doubt that membranes appeared as a continuous bounding layer. In some cases, probably as a result of tissue compression on cutting, the plasmalemma presented a wavy appearance (Figure 10). This can be readily interpreted in terms of the deformation of lamellar membranes. Positive staining of cell walls was seen to be dependent on fixation regime, staining technique and duration (Figures 1, 3, 5, 7). Cell walls were irregular and wavy, clearly reflecting the dehydrated nature of the tissues. Nowhere was this more evident than in the deeply-seated provascular tissue of cotyledons fixed by freeze-substitution in an acetone-osmium tetroxide mixture (Figure 13) after the method of Hereward & Northcote (1972). It was evident that one of the consequences of the dehydrated state of the cytoplasm was the closely appressed nature of many of the organelles, particularly in the vicinity of the nuclear envelope.

This consequently made unequivocal identification of organelles difficult (Figures 4 & 6).

The electron microscopical appearance of cardiolipin and phosphatidylethanolamine (P.E.) preparations (Figures 14 & 15) provided further support for the contention that the membranes of dry seeds exist in a lamellar state.

The hexagonal state was predominant in both model systems. P.E. is reported to exist in either a lamellar, or hexagonal state depending on temperature, and source, the latter presumbly reflecting differing unsaturation of the fatty acids. No attempts were made to check the characteristics of this phospholipid and it is suggested that some of the apparent lamellar-hexagonal images seen in the preparations could be a reflection of varying saturation. The P.E. preparation was substantially different from an aqueously fixed bovine serum albumin/brain phospholipid homogenate in which myelin figures were observed which were almost entirely lamellar (Figure 12). The appearance of structures interpreted as being non-lamellar (outlined, Figure 12) is in keeping with current evidence which suggests that non-lamellar structures are a more common element of membrane architecture that was previously believed. (Reviewed by Verkleij,1984).

Results obtained using the technique of Thomson (1979) yielded results which were considered somewhat intermediate between the hydrated and dehydrated states. Since the primary fixative was formaldehyde in glycerol, most aldehyde-reactive substances can be considered to be fixed anhydrously. However, since osmium tetroxide reacts with glycerol, the technique calls for several phosphate buffer washed before post-fixation in phosphate-buffered osmium tetroxide. This would clearly introduce the possibility of tissue swelling since the primary fixative is estimated to contain no more than 5% water (Thomson, 1979). Although tissue swelling was not given any attention by the latter author, it was clear this technique did introduce such artefacts: walls and nuclei were obvious regions where the hydration artefact was noted (Figures 18, 19 & 21). Nevertheless the technique did confirm that organelles were indeed

closely appressed to each other (Figures 18 & 21). The plasmalemmae were continuous intact bilayers (arrows, Figure 18) with some evidence of hydration- or fixation-induced artifacts since they were not always closely appressed to the wall (Figure 19). It was also evident that the membrane-like boundaries seen between lipid bodies by the previously-employed techniques were in effect the half-unit membranes of the lipid bodies closely associated with ribosomes (Figure 21). It was speculated (Thomson, 1979) that the absence of a clearly-defined tripartite membrane was possibly indicative of membranes being structurally different in the anhydrous state. This suggestion cannot be supported on the basis of changes seen in a sample of brain phospholipid fixed by this technique. After the primary fixation step the small cube ($\frac{1}{2}$ mm) was unchanged in appearance. However on commencement of the buffer wash the lipid dispersed, forming long translucent strands in the glass vial used for fixation. The uppermost of these strands poured off with the first buffer rinse, and it was only on addition of osmium tetroxide post-fixative that these blackened, became denser, and settled to the bottom of the processing vial. During the course of acetone dehydration (25, 50, 75, 100%) no further loss of material was noted. On completion of dehydration a portion of the sample was found to be resistant to dissolution by propylene oxide, suggesting that only after osmium tetroxide fixation did the phospholipids became completely "fixed" and insoluble. The electron microscopical appearance of the phospholipid strands suggested that the typical bilayer leaflet was not always present (Figures 16 & 17). At high magnifications the phospholipids were in parts similar to chloroplast lamellae and clearly not tripartite. Where these were sectioned tangentially hexagonal profiles were evident.

The solubilizing effect of the buffer wash on the phospholipid left little doubt that significant artefacts may be introduced by this technique. These include possible expansion- and shrinkage-artefacts as well as modification of membrane structure. The latter was especially evident in mitochondria (Figure 21) where membrane lamellar structure was lost. Further evidence suggestive of the undesirability of using glycerol as a fixative vehicle was obtained from differential scanning calorimetry and X-ray diffraction studies

on the effect of glycerol on phosphatidylcholine liposomes (McDaniel, McIntosh & Simon, 1983). Glycerol was seen to bring about full interdigitation of the bilayer hydrocarbon chains by possible modification of long-range van der Waals forces. The above observation may thus help explain the solubilizing effect on phospholipids and the lack of typical bilayer structure. Yatsu (1983) has observed that peanut seeds underwent little change during the glycerol fixation step when using the method of Thomson (1979) but underwent an 18 - 28% increase in length during the phosphate buffer wash and remained at this increased value at the end of dehydration. This is in keeping with the present observations which suggest that both the cytoplasm and the cell walls undergo expansion (Figure 18). Freeze-fracturing studies by Buttrose (1973) have shown that cells in the scutellum of dry barley seeds are in many respects comparable to the images obtained in the present study using vapour fixation. Numerous approaches to anhydrous fixation of a wide variety of plant and animal systems have, without exception, revealed membranes as bilayer structures. (Morris, 1968; Walz, 1979; Hallam & Gaff, 1978; Opik, 1980). While there will always be some measure of uncertainty about interpreting such images, particularly the interactions of fixatives and resins on the membrane phospholipids, evidence from X-ray diffraction studies has recently become available to support the view that the membranes of dry seeds are in a lamellar state. McKersie & Stinson (1980) have provided X-ray diffraction evidence that membrane phospholipids from Lotus seeds exist in a bilayer at 5% water content and have suggested that solute leakage on imbibition has some explanation other than membrane phase changes. Further support from studies of soybean seeds using ground particles of tissue and isolated membrane phospholipids indicated that although bilayer spacing increased with hydration level, the bilayer arrangement was always maintained (Seewaldt, Priestley, Leopold, Feigenson & Goodsaid-Zalduondo, 1981).

On purely theoretical grounds it might be expected that if two organelles were closely appressed to one another, and if membrane phospholipids were to exist in the hexagonal state, that some evidence of this should be apparent in tissues placed directly into

fixative. It might be argued that although a water front may precede fixative molecules, the potential for large-scale membrane rearrangement would be limited. Thus if the hexagonal state were predominant in the dry seed it might be expected that organelles would show evidence of conjoined membrane systems since, on purely physical grounds, it would be difficult to see how segregation of "self" and "non-self" phospholipids could be achieved.

Contrary to the above findings, and those of the present study, Toivoi-Kinnucan & Stushnoff (1981) observed images in freeze-fracture preparations of lettuce cotyledons at moisture contents of 20 - 25%, suggesting an hexagonal array of lipid rods around the lipid bodies. When tissues were hydrated above 25% moisture content the lamellar phase was seen. It is the opinion of the present author that the micrograph presented of lipid bodies showing an "amorphous" structure in the air-dry state, and another illustrating crystalline structure (Figures 2 & 3 of Toivoi-Kinnucan & Stushnoff, 1981) would be no different if presented at the same magnifications, since replicas are equally grainy. There is some uncertainty about the path of the fracture planes by freeze fracturing. In hydrated tissues it is generally held that the fracture plane passes through the middle of the hydrocarbon region of the bilayer. In air-dry seeds the fracture plane may pass through the centre of lipid bodies and lipids may have an amorphous structure (Buttrose, 1973). However, in the lettuce study by Toivoi-Kinnucan & Stushnoff lipid bodies were seen to be of a uniform granularity which would not be expected if lipid bodies were cross-fractured. Isolated muscle microsomes undergo lateral phase separation into protein and lipid components at 1% water content, with both lamellar and hexagonal patterns evident in the same sample (Crowe & Crowe, 1982). The general absence of such reported separations in anhydrobiotic organisms was attributed to the presence of factors which modulate or prevent such changes (e.g. trehalose, glycerol). In additon those authors draw attention to the considerable forces developed during drying, and cite their own observations in which massive fusion of membranes takes place with concomitant lateral phase separation. On resuspension in water reconstituted vesicles were produced, which were larger than previously, with an altered intra-

membrane particle distribution.

If the above argument is applicable to the membranes of seeds, it might be expected that considerable structural changes may occur to the plasmalemma and other membranes during imbibition irrespective of arguments concerning the possibility of lamellar or hexagonal structure. Chabot & Leopold (1982) have speculated that for the plasmalemma to accommodate the 150% increase in volume seen on imbibition, additional recruitment may come from the membrane vesicles and lipid aggregates which lie adjacent to the plasmalemma. In their study pieces of seed equilibrated at a variety of moisture contents were placed in glycerol for less than a minute before freeze-fractioning commenced. In addition tissue was also prepared using the anhydrous fixation technique of Thomson (1979). They observed that the plasmalemmae were frequently separated from the cell walls but regarded it as debatable whether this image was a reflection of the natural state or an artefact of fixation. Since the studies reported in this thesis strongly favour the latter possibility, the entire foundation of the "membrane addition" hypothesis, as proposed above, rests on validity of static images from carefully pre-equilibrated tissue being representative of changes taking place during imbibition with abundant free water. This may not be so and the results may be equally interpreted as changing fracture planes produced by altered tissue moisture contents, with the exception of changes observed at 24 hours imbibition, where metabolic activity is clearly implicated.

The above interpretations are markedly different from earlier studies (Baird, Leopold, Bramlage & Webster, 1979) where dry and imbibed soybean radicles were investigated using totally aqueous procedures. Dry tissues were placed directly into glutaraldehyde fixative and processed by standard techniques whereas imbibed tissues were soaked (without seed coats) in distilled water for 20 minutes before processing for electron microscopy. Whereas the plasmalemmae of dry radicle cells were frequently characterised by discontinuities, extra-cytoplasmic membrane vesicles and an absence of contiguity with the cell wall, pre-imbibed tissues possessed plasma membranes that were continuous and closely appressed to the cell wall. It was

concluded that the initial imbibition of water into radicle cells results in extensive membrane changes which were primarily physical.

The present study has provided evidence that the membranes in dry lettuce cotyledons were lamellar, and in keeping with the above suggestion it might be expected that the visco-elastic properties of membranes may be fully taxed on imbibition. Any leakage of solutes must accordingly be seen within this framework rather than to suppose that rearrangements from a hexagonal to lamellar state are the root cause of initial leakage and its later abatement. It has been suggested, although supportive biochemical evidence is lacking, that integral membrane proteins may be displaced from membranes during drying. Consequently one of the early metabolic events on imbibition may, inter alia, relate to membrane restitution (Bewley, 1979). Such putative changes are clearly beyond the limits of resolution of the thin-section techniques of this study. While freeze fracturing and etching may be a potentially valuable technique in this regard, alterations in the fracture plane with progressive hydration may complicate interpretation. The possible insertion of considerable phospholipid or protein elements to achieve full membrane functionality, may be one of the physical consequences of the rapid inrush of water on imbibition although there is a dearth of biochemical information in this regard.

CHAPTER 4

ULTRASTRUCTURE OF COTYLEDONARY NECROSIS

It was observed in the germination studies (Chapter 2) that the cotyledons show the earliest morphological manifestations of deterioration in slower and less uniform greening. An obvious sign of damage was an inability to green and the presence of a small reddish-brown patch in the midrib region of the cotyledons on seedlings after one week of germination. Although this lesion has long been recognized and considered an ageing-related phenomenon its underlying cause is unknown (Bass, 1970). The lesion was not immediately evident in imbibed, excised embryos, but took several days to become manifest; ultrastructural studies were undertaken on the cotyledons of such aged seeds in an attempt to obtain information on the etiology of what appeared to be a highly localized form of deterioration.

Materials and Methods

Seeds of a 1979 purchase, which had been stored at 20% RH for 2 years were imbibed on filter paper in Petri dishes under the same conditions as were used in the germination studies. Median transverse slices of cotyledonary tissue were taken from excised embryos at 2, 5 and 7 days, and prepared for electron microscopy using standard procedures (See Materials and Methods, Chapter 3).

Cleaning technique

The clearing technique adopted for examining vascular tissue development in the cotyledons during germination was that of Shih, Peterson and Dumbroff (1983). The only minor modifications were the omission of NaCl and H_2SO_4 steps at the end of the process. Staining was by basic fuchsin (saturated) in 25% ethanol (v/v). Excess stain was removed by repeated washings in 25% ethanol before photography with a Wild photomicroscope on

Ektachrome (ASA 64).

Results and Discussion

Normal Cotyledonary Development

The ultrastructure of cotyledonary cells was consistent with their function as a storage organ, as evidenced by numerous protein and lipid bodies (Figure 1). It was observed that the initial pattern of reserve mobilization was marginal, rather than from the midrib region of the cotyledon. This is consistent with the pattern of greening which began along the leaf margins. Evidence was also obtained for increase in cotyledonary size by lateral and axial growth and cell division (Figures 24 & 25). It was possible in any one cross-section of a cotyledon to obtain a spectrum of developmental change; on the other hand this variability made comparison between control and aged tissue more difficult insofar as categorizing the sequence of events associated with deterioration or normal germination.

As is typical for oilseeds the mobilization of reserves leads to a noticeable increase in glyoxysomes which are fewer in less advanced stages of mobilization (Figures 1 & 3). Electron-dense protein bodies were typically seen to contain phytin-like deposits (Lott, 1970) which were frequently lost from sections (Figure 1). Phytin digestion was seen to be concomitant with the digestion of the protein body matrix (Figure 7), mobilization of the latter apparently being brought about by the fusion of numerous small vesicles with the limiting membrane. This was especially evident in the epidermal cells (Figure 2) possibly because the small cytoplasmic volume of these cells made their detection easier. Protein body digestion and the formation of larger vacuoles by their fusion was seen to occur before large-scale depletion of lipid reserves (Figures 3, 4 & 9).

As might be expected, differentiation of the provascular tissue occurred ahead of palisade, mesophyll or epidermal cell vacuolation and reserve mobilization. Proplastids were numerous with elongate

or irregular profiles suggestive of division (Figures 1 & 12). Possible evidence for division was also observed in the amyloplasts and chloroplasts at later stages of cotyledonary development (Figures 13 & 16).

Although not proven as a general event in germinating oil seeds, it has been suggested that catabolic activity may exceed the load capability of the transport system and that "excess" carbohydrate is channeled into starch as a temporary deposit (Dennis & Miernyk, 1982). The early differentiation of plastids and the appearance of starch (Figures 13 & 15) which is later absent or reduced in functional chloroplasts might be consistent with such an argument. On the other hand one would expect cell metabolism to be finely regulated and this could rather reflect the particular metabolic requirements of the cells or pathways of carbon flow within the cell.

Ultrastructural Characterization of Cotyledonary Necrosis

For the reasons outlined above concerning non-uniformity in the pattern of cotyledondary development in both control and aged seeds, the events will be given without specific reference to the sampling intervals; however such details are provided with the electron micrographs. Examination of cotyledons from seeds of the same seed lot, but stored at 60% & 80% RH, revealed as early as 4 hours after imbibition that the cytoplasm suffered varying degrees of disorganization, although protein bodies, lipid droplets and nuclei were still recognizable. The ultrastructure of cells was consistent with the observation that such cotyledons fail to emerge from the seed coats and green.

No micrographs have been presented of these samples, although Figure 10 might be considered to represent a lesser form of deterioration observed. This may be taken as a convenient end-point of deterioration at which mobilization of reserves and organellar biogenesis did not take place. This contrasted with the material reported herein in which an entire spectrum of deterioration and delayed organelle development was evident. It was also evident

that the necrotic patch, according to its size and severity, represented a region of localized cell death surrounded by tissues of varying degrees of deterioration in which the normal pattern of mobilization was evident or delayed. As was noted in the germination studies this necrosis was predominantly localized in the midrib region and was sometimes symmetrically placed in transverse sections (Figure 21).

The epidermal cells and the provascular cells were two tissues apparently more resistant to deterioration than the palisade or mesophyll cells. The resistance of vascular elements may be a reflection of the fact that xylem and phloem represent tissues which are modified in part by a specialized form of autolysis (sieve tube and the xylem element) and that the steps whereby this takes place need not be achieved in the typical manner. Figure 5 illustrates provascular tissue where membrane damage to the ER and nuclear envelope took the form of membrane dilation. In addition the nuclei have assumed irregular, somewhat amoeboid shapes and possess electron-dense, clumped chromatin. In spite of what must be assumed to be rather significant nuclear damage further differentiation takes place, even if the tissues are sited in the middle of a necrotic "island" (Figures 21, 23 & 26). This vascular continuity would clearly be functionally advantageous since it would permit mobilization of reserves from regions of normal development distal to regions of damage (Figure 23).

Evidence for varying degrees of delay as regards protein body and lipid mobilization may be seen in Figures 4 & 7-11. The presence of substantial amounts of membranous inclusions (Figure 4) and evidence of small membrane-limited pockets of cytoplasm including mitochondria within the vacuole (Figure 6) suggested that some measure of cellular autophagy had occurred. Such an observation would be consistent with the suggested role of the vacuole as a lysosome-like organelle (Berjak, 1968; Matile, 1975) and is further supported by a recent biochemical and electron microscope study which has suggested that the protein bodies of mung bean cotyledons play a role in cellular autophagy (van der Wilden, Herman &

Chrispeels,1980). In addition to such striking differences between cotyledons sampled on different days, contiguous cells were seen to differ greatly as regards cellular integrity and extent of reserve mobilization (Figures 9, 18 & 19).

While some measure of cell death by schizogeny was seen as a part of normal cotyledonary expansion in unaged tissues, a greater incidence was noted in aged tissues. In some cotyledons a buckling of cell walls was evident, with similar distortion of cytoplasmic contents (Figures 19 & 22). This was interpreted as a result of localized cell expansion or division by cells in the cotyledon which had not suffered damage. This could lead to an uneven force being exerted on cells in which turgor was reduced by membrane-related damage; additionally some buckling may be attributed to the restraint imposed by endosperm tissue.

In addition, evidence for dehydration-induced damage was evident to some cells. These either took the form of discontinuities in the cell wall which appear as "splits" (Figures 7 & 18) or irregular profiles to the lipid bodies (Figure 19).

Confluence of lipid, which was also observed in aged root tip cells during imbibition (Chapter 5), was seen in cells where evidence of mobilization was lacking or extremely delayed (Figures 6, 11 & 19). In other instances overall ultrastructural features consistent with normal catabolism could be observed, the presence of microbodies and mitochondria (Figures 6 & 17) adjacent to such lipid pools suggested that mobilization and metabolism could perhaps take place although possibly at a slower rate. Visible manifestations of membrane damage, such as breaks in bilayer continuity or myelin-like figures adjacent to the plasmalemma, were not evident; indeed clear visualization of the plasmalemma was not always apparent (Figures 1, 2, 4, 7, 16, 20).

Thus there was little evidence to suggest that delayed mobilization was the result of membrane lesions and that once repair had been effected reserve mobilization could commence. The possibility

therefore exists that damage to the genome might significantly impair transcription of information for reserve mobilization and organelle biogenesis until the necessary repair had been effected. Clear evidence for some form of damage was seen in amyloplasts and chloroplasts of aged cotyledons. This took the form of localized dilations to the lamellae of these organelles and an absence of granal development (Figures 14, 16, 17 & 20). Several speculative suggestions for the abnormal appearance of these organelles may be proposed:

1. That damage to the nuclear or genome plastid (or both) could lead to faulty or defective information for organelle development or chlorophyll biosynthesis.

2. That some disturbance to membrane synthetic pathways leads to the production of lipids of altered fatty-acid structure. This might alter physical properties (e.g. fluidity) or increase susceptibility to peroxidative attack.

3. That the protective machinery normally present in the plastids to circumvent possible peroxidative assault is defective in some way.

None of the above are necessarily mutually exclusive, and in the absence of comparable studies in the published literature these proposals must remain conjectural.

It will be recalled that evidence has been obtained to suggest that cell extension precedes cell division in the radicle and that consequently the primary event(s) which lead to loss of germination (i.e. cell extension and hence radicle protrusion) are likely to be extranuclear. On the other hand the somewhat protracted delay in reserve mobilization and cotyledonary greening with ageing, and the fact that some cell division is presumably a normal part of cotyledonary expansion (Figures 13, 24 & 25), lead to the viewpoint that both nuclear and extranuclear deterioration are concurrent. The above features would accordingly make it extremely difficult to

separate cause from effect and suggest a single event which leads to cotyledonary death.

The development of cotyledonary necrosis as an early morphological manifestation of ageing may relate to the inhomogeneity of lipid distribution between different parts of the embryo. These may be quantitative (greater total lipid), or qualitative (greater abundance of unsaturated fatty acids). These could predispose cotyledonary tissue to lipid peroxidation, other factors being equal. It has been reported for wheat that lipids constitute 1 - 2% of the endosperm, 8 - 15% of the germ and about 6% of the bran (Harwood, 1980). Such patterns of differential lipid distribution in seed tissues might explain differences in the results of studies where whole seeds or organs (e.g. radicles) were used to assess possible peroxidative involvement in ageing and changes in fatty acid levels (Priestley & Leopold, 1979; Stewart & Bewley, 1980).

The E.R. was reported to commence membrane synthesis in the cotyledons of mung bean seedlings within 12 hours of imbibition (Gilkes, Herman & Chrispeels, 1979), while synthetic activity is much more delayed in cucumber cotyledons (Macher, Brown, McManus & Mudd, 1975). The present ultrastructural investigation revealed little evidence of extensive E.R., and clear evidence of synthetic activity was only evident 2 days post-imbibition. This may indicate that lettuce, like cucumber is slow to mobilize cotyledonary reserves and commence synthetic activity. Since the radicle tip is abundantly endowed with lipid and protein reserves it may be speculated that cotyledonary reserves contribute to the later stages of radicle growth.

Cotyledonary expansion by cell division appears to be an important component of post-germination growth, a feature not seen in cotton (Kunce, Trelease and Doman, 1984), cucumber (Becker, Leaver, Weir and Reizman, 1978), squash (Lott, 1970) mustard (Weidner, 1967) and watermelon (Becker, Leaver, Weir, and Reizman, 1978).

CHAPTER 5

ULTRASTRUCTURAL CHANGES DURING IMBIBITION

This Chapter is divided into four sections (A - D) the last of which, being concerned with irradiation studies, contains its own combined results and discussion since it stands somewhat apart from the other sections. A fuller discussion of the earlier sections (A - D) has been held over until the end of Chapter 5 (page 94), since many comments are applicable to the studies of all four sections.

(A) Studies using tissue slices

A number of studies have been undertaken in which dry seed tissues have been placed directly in fixative, and it is generally assumed that images obtained represent the state of cellular organisation in the non-active condition. The basic premise has been that if air-dry tissues are prepared by conventional electron microscope techniques tissues will hydrate before fixative molecules can enter, but that the amount of overall cellular re-organisation would be limited. While the membrane lipids are likely to remain fluid until after the osmium tetroxide post-fixation step (Jost, Brooks & Griffith, 1973), significant information may still be obtained about membranes, and the form and disposition of organelles, all of which may have a significant bearing on seed viability (Villiers, 1980). Material fixed in this manner will be referred to as imbibe-fixed, in contrast to others who have referred to material prepared in this manner as "dry" (Paulson & Srivastava, 1968; Swift & O'Brien, 1972; Webster & Leopold, 1977; Baird et al., 1979).

It has been reported that while tissues from non-viable lettuce seeds contain distinguishable organelles shortly after imbibition, a functional plasma membrane is not readily distinguishable (Villiers,

1980) In seeds of low vigour, with partially impaired germination, the above degenerative changes can be seen in isolated cells or groups of cells.

This may be seen as clear evidence implicating membrane changes in loss of viability and was taken as the starting point for the present ultrastructural studies on seed deterioration.

MATERIALS AND METHODS

Seeds from a single lot which had been stored at 60% RH and 20°C for 8 and 22 months were chosen for study. Germination was 89% and 0%, respectively. Although entire root tips were imbibe-fixed for comparative purposes, most of the investigations were conducted on transverse slices (1mm) of the radicle tip region which were either placed on a moistened filter paper strip for 30 minutes before fixation, or were imbibe-fixed and prepared for electron-microscopy using standard techniques (see Materials and Methods, Chapter 3). In an attempt to exclude any effects which physical cutting might have on ultrastructure, lettuce seed powders were imbibe-fixed, and prepared for electron microscopy. In this case, entire seeds were crushed in a mortar and pestle under liquid nitrogen, a condition presumed to be comparable to freeze fracturing, insofar as physical shearing would be reduced or avoided. As earlier encouraging results were obtained using a freeze-substitution technique (Chapter 3, Figure 13), attempts were made to use fix tissue slices after 10 minutes imbibition with an ethylene glycol-aldehyde mixture at freezing temperatures. This technique, modified after Pease (1967 a & b), was seen as overcoming possible membrane changes induced by aqueous solutions of differing osmolarity, since tissues remained at -20°C until after the secondary fixation step with osmium tetroxide. Tissue slices, imbibed on small slivers of wetted filter paper, were plunged into "Freon" cooled to liquid nitrogen temperatures. After quenching, the samples were transferred to a small volume of solution at approximately -50°C. The fixative solution was prepared by mixing 70ml ethylene glycol with 28ml of 25% aqueous glutaraldehyde and 2ml of Acrolein.

Tissues were held in this fixative for 3 days during which time the temperature was allowed to gradually rise to -20°C in a chest deep-freeze. The fixative was then decanted and samples held overnight in cold (100%) acetone at -20°C. After a further rinse with acetone (1 hour) samples were transferred to 2% osmium tetroxide in acetone for 6 hours. After a 2 hour rinse in acetone samples were held in 50:50 Acetone:resin (Spurr's) overnight before being transferred to a further 50:50 Acetone:resin mixture at room temperature. Gradual infiltration with resin followed (Spurr, 1969).

RESULTS

Transverse sections through imbibe-fixed tissue slices of seed of high viability revealed little evidence of gross cytological damage to cells immediately adjacent to the cut surface (Figure 2 arrow) and the plasmalemma was generally continuous and closely appressed to the cell wall. Surface cells which had been cut were seen to be partly freed from the remaining cell walls, but some cytoplasmic elements were nevertheless retained. Intact plasmalemmae were evident on the uncut face of the cells, although plasmodesmatal links were evidently severed. Localized regions of the cytoplasm in deeply-sited cells were seen to contain a sparse distribution of ribosomes, suggesting physical dispersion of the ground cytoplasm, while immediately adjacent cells were apparently unaffected (Figure 1). In contrast to the occasionally dispersed nature of the ground cytoplasm the nucleoplasm and nucleoli showed comparative resistance to changes in shape or structure (Figures 1 & 2). The nuclear envelope on the other hand was seen to show evidence of localized discontinuities (Arrows, Figures 3 & 6), suggesting differential fragility. This view was amply confirmed in more deeply seated nuclei of samples from non-viable material which showed clear evidence of damage to the membrane and a disorganised nuclear matrix (Figures 7 & 8). For reasons which were not evident paler-staining regions of nuclear envelope (Figure 3) were observed which may have preceded actual physical discontinuity (Figure 5). Apart from a greater incidence of plasmalemma discontinuities, cell plasmolyis was evident in samples drawn from the non-viable seeds. (Compare Figures 5 & 11 with 20,

21 and 24). Cytological indications of deterioration ranged in severity within each sample (Figures 20 & 21), in keeping with the predictions and observations that viability loss within a population would be expected to show a normal distribution in time (Roberts & Ellis, 1982).

The severity of damage was notably greater in samples imbibed for 30 mins. before fixation and processing for electron microscopy than in imbibe-fixed samples (Figure 5), in keeping with the suggestion that the damage accumulated in dry storage becomes amplified with progressive imbibition (Villiers, 1980).Where the plasmalemmma was not continuous (Figures 10, 11, 14) a shell of closely appressed lipid bodies was observed which produced an almost continuous boundary. This observation is in keeping with other studies which have observed the inner membrane surface to have a lipid-rich covering (Swift & Buttrose, 1973; Buttrose 1973; Buttrose & Soeffky, 1973). It has even been suggested that lipids may be added to the plasmalemma on imbibition to accommodate the substantial increase in membrane area (Chabot & Leopold, 1982). Circular whorls of membrane in close proximity to cell walls of the kind described by Baird et al. (1977) were rarely observed. Similarly, large masses of convoluted membrane invaginations such as those reported for rice coleoptiles (Opik, 1972) were not observed.

These latter elements have been interpreted as contributing to the plasmalemma the surface of which presumably increases substantially on imbibition. The presence of an electron-translucent ground cytoplasm sparse in ribosomes (Figures 1 & 15), dilated vacuoles (Figure 4) and the presence of apparently distended mitochondria (Figures 5, 8, 15 & 20) may suggest that rapid imbibition-induced changes may be responsible for these features. On the other hand an explanation for these observations may not only lie in the inability of the membranes to keep pace with the rapidly expanding protoplasm but also a differential expansion and shrinkage during processing for electron microscopy. Such changes have been documented in the animal literature (Nordestgaard & Rostgaard, 1984) and may be presumed to be an unavoidable consequence of fixation

and preparation for electron microscopy in most cells.

It is also likely that a broaching of the plasmalemma out of the plane of section might produce without evident cause, cytoplasm of patchy or electron translucent appearance, (Figure 15) unlike cases where such cause-effect relationships could be inferred (Figure 1). Plasmalemma profiles seen in thin sections clearly represent an exceedingly small proportion of the total surface area of the plasmalemmae of cells, and the probability of detecting smaller, infrequent ruptures would be extremely slight.

Mitochondrial profiles were rounded and generally with completely intact membranes. Few cristae were evident and the matrix of the organelles were mostly electron opaque except for patches of sparsely distributed fibrillar material (Figures 5, 8, 13). In cases where membrane deterioration was more severe, discontinuity of the outer mitochondrial membrane was seen (Figures 12 & 15), but this was not invariably associated with general cytoplasmic disorganisation.

Clusters of mitochondria, with relatively straight sections of contiguous membranes were commonly observed in aqueously fixed samples of high viability material (Figure 13) a feature which was less obvious in freeze-substituted tissue (Figures 18 & 19). Although not specifically commented on, similar clusters are evident from the micrographs of other workers (Figure 6 of Webster & Leopold, 1973; Figure 2 of Buttrose & Soeffky, 1973). While the reason for this arrangement remains enigmatic it is notable that during the later stages of imbibition the mitochondria are distinctly spherical and separate.

The cryptic and evidently non-spherical appearance of mitochondria in the cotyledons of anhydrously fixed tissues (Chapter 3, Figures 4 & 21) clearly suggested the considerable expansion which these organelles underwent on imbibition. The rare observation of mitochondria with discontinuous membranes (Figure 12) in a sample from the high viability seed lot was considered a possible manifestation of rapid swelling rather than a localized symptom of

ageing. Such symptoms of deterioration have been reported in non-viable rye (Hallam, 1973), and rupture of plastids and mitochondria has been seen in root tip cells of non-viable lettuce seeds (Villiers, 1973). Further evidence suggestive of extensive expansion of mitochondria on imbibition was obtained from examination of tissue prepared by freeze-substitution (Figures 18 & 19). The matrix of mitochondria appeared uniformly floccular, possessing occasionally elongate cristae almost equal in length to the mitochondrial diameter. The limiting membrane was less evidently trilaminar than when prepared by conventional techniques. Cristae were of similar appearance with evidence of a regular array of uniformly-sized particles on the surface (Figure 18, Arrows). Since tissue slices had been imbibed for 10 minutes before fixation full hydration might have occurred, although this may have been somewhat modified by processing. In more deeply-seated regions of freeze-substituted tissue walls surrounded protoplasts of plasmolysed appearance were seen (Figure 17), the latter being typically indicative of freezing damage (Lyons, 1973). In surface cells which were less likely to suffer injury, a continuous triliminar plasmalemma was observed which was closer, though not fully appressed, to the cell wall (Figure 16). In keeping with the observations obtained using anhydrous fixation, the limiting membranes of adjacent lipid droplets were closely appressed to the plasmalemmas and regions between adjacent lipid droplets were seen to contain regular arrays of ribosomes in a close-packed or quasi-crystalline array (Figure 16). Where the membrane of the lipid droplets contacted the plasmalemma the impression was gained that the two elements became fused (Figure 14, Arrows). This was also observed for imbibe-fixed cells (Figure 10) or cell tissue slices imbibed for 30 minutes before fixation (Figure 11). This was not as clearly evident in tissues fixed after 1 hour of imbibition (Figure 22), lipid droplets tending to be separate membrane-limited structures close to, but rarely contacting the plasmalemma.

Table 5.1

Germination data for the seed lot used in the study described in the text.

STORAGE CONDITIONS ALL AT 20°C	PERCENTAGE GERMINATION AT: 13hrs.	17.5hrs.	21hrs.	34hrs
Control (silica gel)	0	61	76	96
60% RH	0	24	29	43
100% RH	0	0	0	0

(B) Studies on intact radicle tissues after 12 hours imbibition

From the foregoing section evidence was obtained suggesting that altered membrane properties may be significant features of deterioration. Membranes of the nucleus and plasmalemma were two subcellular components particularly susceptible to damage on imbibition although the possibility that the images obtained were a direct consequence of using tissue slices could not be totally excluded. It has been argued that the entry of water into dry seeds is more complex than simply bulk flow through a porous material, especially if the tissues involved are bulky cotyledons Waggoner & Parlange, 1976; Vertucci & Leopold, 1983). Such a situation would clearly not prevail in the tissue slices used in the above study, although it has been suggested that some regulation of water uptake may be effected by the endosperm in intact seeds (Speer, 1974).

The tissue slices used represented a small, and not necessarily reproducible collection of cells from the radicle tip and observations would clearly have limited predictablity as regards the actual germination of the embryos from which the sample was taken. This is especially so in view of the fact that any ameliorating effect of the hydration shell on the inner tissues would have been largely removed by using tissue slices. It has been argued that, as a possible consequence of free-radical attack to susceptible molecules, particularly membranes, threshold levels of damage are reached, beyond which repair becomes impossible and loss of viability ensues (Villiers, 1973). While the observations of the previous section do not address this particular proposal directly, they do show that even samples of high viability may suffer membrane damage on imbibition. Seeds which had lost viability were seen to have greatly increased damage, and to which the term osmotic fragility may perhaps be applied. Further studies were therefore undertaken to investigate the validity of the above proposals by examining ultrastructural changes in the radicle tip of embryos from seeds

after several hours imbibition. In this study seeds (from a lot which had been held over silica gel for 18 months) were subjected to ageing regimes of 60% and 100% RH for 3 months at 20°C. Germination data are presented in Table 5.1.

Radicle tips were excised from seeds after 13 and 17½hours imbibition and prepared for electron microscopy as outlined in Materials and Methods (Chapter 3).

RESULTS

In control, viable embryos clear evidence of metabolic activity was evident in root cap cells between 13 and 17 hours imbibition. Nucleoli with vacuoles, extensive sheets of rough ER, vesicles fusing with the plasmalemma and numerous starch grains within the plastids were clearly evident (Figures 23, 25 & 74). The development of vacuolation by progressive expansion, and fusion, of smaller vacuoles, in addition to an origin by distension of the lumen of the ER, were characteristic of root cap cells.

The above features were strikingly different from those observed in low viability and non-viable embryos, which were characterized by obvious dilation of the ER and Golgi bodies (Figures 26 & 29). In addition, mitochondria were enlarged (Figure 29) with electron translucent matrices and poorly-defined cristae. Amyloplasts in deteriorated seeds were seen to undergo a progressive reduction in electron density of the matrix (compare Figure 25 with 27) and loss of the bounding membrane so that free-starch grains came to lie in the cytoplasm (Figure 28).

In control, viable tissue plastids and mitochondria in the meristematic and epidermal tissues were elongate or dumbell-shaped suggesting possible division or increase in size (Figures 30 and 73). This situation was uncommon in deteriorated tissue and when observed was interpreted as either a compensatory mechanism to increase respiratory sufficiency or as an indication of resistance and the relative competency of these organelles in spite of other evident

damage (Figure 32).

In the cortical tissues of control, viable embryo radicles, a "shell" of lipid droplets surrounded the electron-dense protein bodies which showed only limited evidence of digestion (Figure 36 & 39). ER with associated ribosomes was seen to be associated with protein bodies (Figure 38). In low viability samples fusion of the lipid bodies with each other, or with the protein body membrane was observed. Initially larger-than-normal lipid droplets were observed in cells of otherwise normal appearance (Figures 34). With further deterioration, protein and lipid body masses became confluent in an obviously less structured cytoplasm (Figures 40- 42, 47 & 49).

In the final stages of cytoplasmic disorganisation the plasmalemma appeared to lose functionality or break down and large confluent pools of lipid came to lie within a floccular disorganised cytoplasm (Figure 44) which may or may not be withdrawn from the cell wall. Occasionally lipid aggregates came to lie to the outside of the withdrawn plasmalemma. It is possible that the lipid droplets which were typically distributed along the plasmalemma were released to the exterior at the time of collapse of plasmalemma (Figure 44).

From the above it was evident that while germination studies may yield relatively clear-cut data, ultrastructural features associated with loss of viability constituted a continuum of deterioration, varying in severity between different samples, tissues of the root tip and even between adjacent cells (Figures 46 & 48). It was also observed that this differential deterioration could also extend to the endosperm (Figures 53) even though this layer is 2 - 3 cells thick. While no membrane system could be unequivocally recognised as the "first" to deteriorate, plasmalemma and nuclear envelope lesions were amongst the earliest identified in the proposed deteriorative sequence (Figure 5.1). The dilated appearance of the nuclear envelope along its entire length in sectioned profile (Figures 32 & 33) may have originated by the separation of the two members of the envelope. Evidence for the early stages of the genesis of this lesion may perhaps be seen in the root cap cells (Figure 26), in

which the two halves of the membrane bilayer are clearly separate. Further evidence for nuclear envelope changes were presented earlier (Figures 3 & 8). The inability to resolve a typical trilaminar membrane was, however, not only restricted to non-viable samples, being evident also in the nuclei of control viable tissue (Figure 30). In such cases the association of darkly-staining heterochromatin may have obscured membrane detail (Figure 25).

The appearance of sparse, interrupted plasmalemma profiles or the absence of a clearly discernible plasmalemma were taken as evidence for damage to this membrane system (Figures 29, 33, 40, 43 & 47). Occasionally the wall appeared deformed in the cortical tissues, presenting a zig-zag pattern with little observable membrane detail, while the cytoplasm was otherwise apparently normal. This was interpreted as a consequence of a softening of the cell wall matrix coupled with reduced cell turgidity as a result of impaired plasmalemma function. Thus if nearby adjacent cells showed limited extension growth or could develop greater turgor, this may bring about physical deformation of the cells of reduced turgor. A close association of the lipid bodies with discontinuous portions of plasmalemma was sometimes seen (Figure 37) in keeping with earlier observations of this type (Figures 10, 11 & 24). Further evidence for this association was also seen in non-viable samples (Figures 28, 44 & 47) suggesting that this may help retain the cytoplasm of a otherwise moribund cell (Figure 49). A diagrammatic representation of some of the sequences of deteriorative events is presented in Figure 5.1.

The single fortuitous observation of a cell in which nuclear division was abnormal and incomplete (Figure 45), and in which numerous enlarged vacuoles and abnormal membrane profiles were evident was seen as implicating both nuclear damage and membrane abnormalities associated with the mitochondria and vacuoles in the inability of the cell to complete normal division. It was notable however that no obvious plasmalemma abnormalities were evident, which suggests that extension growth might have been possible even though the cell division mechanism was defective. The appearance of an

apparently lysed cell (Figure 45, centre) with numerous myelin-like figures serves to highlight further the variability in extent of damage between adjacent cells. Since this observation was made on a sample from seed of high viability, and there was no evidence of any other cell damage except for this localized area, it is presumed that germination would have been normal.

The possible significance of endosperm deterioration to seed viability

Although it was not the intention to undertake a study on endosperm tissue, the adherent endosperm sheath was sometimes present when radicle tips were sectioned for microscopy. Observations reported here are of a limited nature but nevertheless of considerable significance.

The endosperm layer which is typically two cells thick, has walls which are thickest distal to tissues of the root tip (Figures 51 & 55). Cells at 20 hours of imbibition were typically characterized by darkly-staining protein bodies, many small lipid bodies, numerous mitochondria, and rough and smooth ER (Figures 52 & 56). Digestion of protein bodies was inferred from the numerous vesicles within, and apparently fusing with, the limiting membrane of the protein body (Figure 50). The presence of peg-like wall ingrowths (Figures 52 & 54) were typical of endosperm tissue and have been suggested (Jones, 1970) to be a means whereby the surface to volume ratio is increased for reserve mobilization.

It was evident that during deterioration one or both cell layers of the endosperm (Figures 53, 54 & 55) could become disorganised. This was apparently no different from the general pattern of deterioration seen in the radicle and cell plasmolysis, lipid coalescence and nuclear deterioration were typical features (Figure 54). In spite of total cytoplasmic disintegration it was apparent that walls of the pericarp and endosperm nevertheless remained

structurally intact (Figure 55).

A considerable research effort has been undertaken on the mechanisms which operate in the germination of light-sensitive lettuce seeds. It will be assumed, though this is by no means proven, that the mechanisms which operate in light- and non-light requiring seeds differ only by degree. Of particular interest to the present study are observations that embryos develop growth potential in order to break free of the restraining influence of the endosperm (Ikuma & Thimann, 1963; Nabors & Lang, 1971; Takeba 1980 a,b & c) and the production of enzymes which digest the endosperm wall as a post-germinative event (reviewed by Bewley, Leung & Ouellette, 1983). When these two aspects are viewed against the background of the ultrastructural observations it is possible to speculate that:

1. Declining viability leads to decreased growth potential by, amongst others, altered membrane properties (e.g. solute leakage).

2. Endosperm wall digestion may assume increasing significance in the absence of adequate growth potential being produced by the embryonic axes.

3. If the chain of events leading to wall digestion were to be impaired or fail, a potentially germinable embryo may be entrapped within an unyielding endosperm.

Observations of atypical germination in which the embryo emerges from the seed coat at the cotyledonary end of the seed, and embryonic axes of buckled appearance within a distended seed coat might be considered as partial support for the above arguments (Germination Studies, Chapter 2). Further support was obtained from the striking increases in germination obtained for a deteriorated seed lot (1978 purchase stored for 33 months at 60% RH and 20°C) in which possible endosperm restraint was reduced or eliminated by

Table 5.2

Improvement in the final germination attained by removing a small portion of tissue from seeds of low viability.

	PERCENT GERMINATION AT DAY		
	2	3	5
SLICED	42	60	72
CONTROL	0	8	20

Table 5.3

Germination data for two lots of lettuce seeds stored for 2 and 15 months at a range of temperatures and relative humidities. Note that all the seeds gave a high percentage germination, and that the emergence was delayed by a few hours.

SEED STORAGE REGIME Control	HOURS IMBIBITION									
	15	16	17	18	19	22	24	34	42	60
1 Month at 10°C & 0% RH	5	13	36	82	98					
2 Months at 40°C & 40% RH	0	0	0	0	0	0	0	40	44	79
2 Months at 40°C & 20% RH	0	0	8	19	33	67	83	92	92	98
2 Months at 20°C & 100% RH	0	0	0	0	0	0	0	7	10	78
2 Months at 20°C & 80% RH	0	4	17	29	37	61	68	75	79	93
15 Months at 20°C & 60% RH	0	0	0	0	0	0	15	31	36	88
15 Months at 20°C & 40% RH	0	2	14	30	38	68	78	90	92	97

Figure 5.1:

Diagrammatic representation of some of the subcellular events which may accompany loss of seed viability (not necessarily in order of appearance) in an idealized cell.

A. Withdrawal of the plasmalemma from the cell wall.

B. Confluence of lipid droplets (stippled) and apparent engulfment into the protein body matrix (solid).

C. Confluence of lipid droplets (stippled) but pooling of material in the extracytoplasmic space.

D. Loss of electron-density of the matrix of the amyloplasts; discontinuity of the outer envelope; disappearance of starch grains (light grey stippling) and the membrane delimiting the organelle from the cytoplasm.

E. Dilation of the membrane of the protein body (solid) and subsequent fusion of such structures to yield large vacuole-like bodies with laterally disposed protein reserve.

F. Dilation of mitochondria and/or loss of internal structure and membrane to yield a small vacuole-like body.

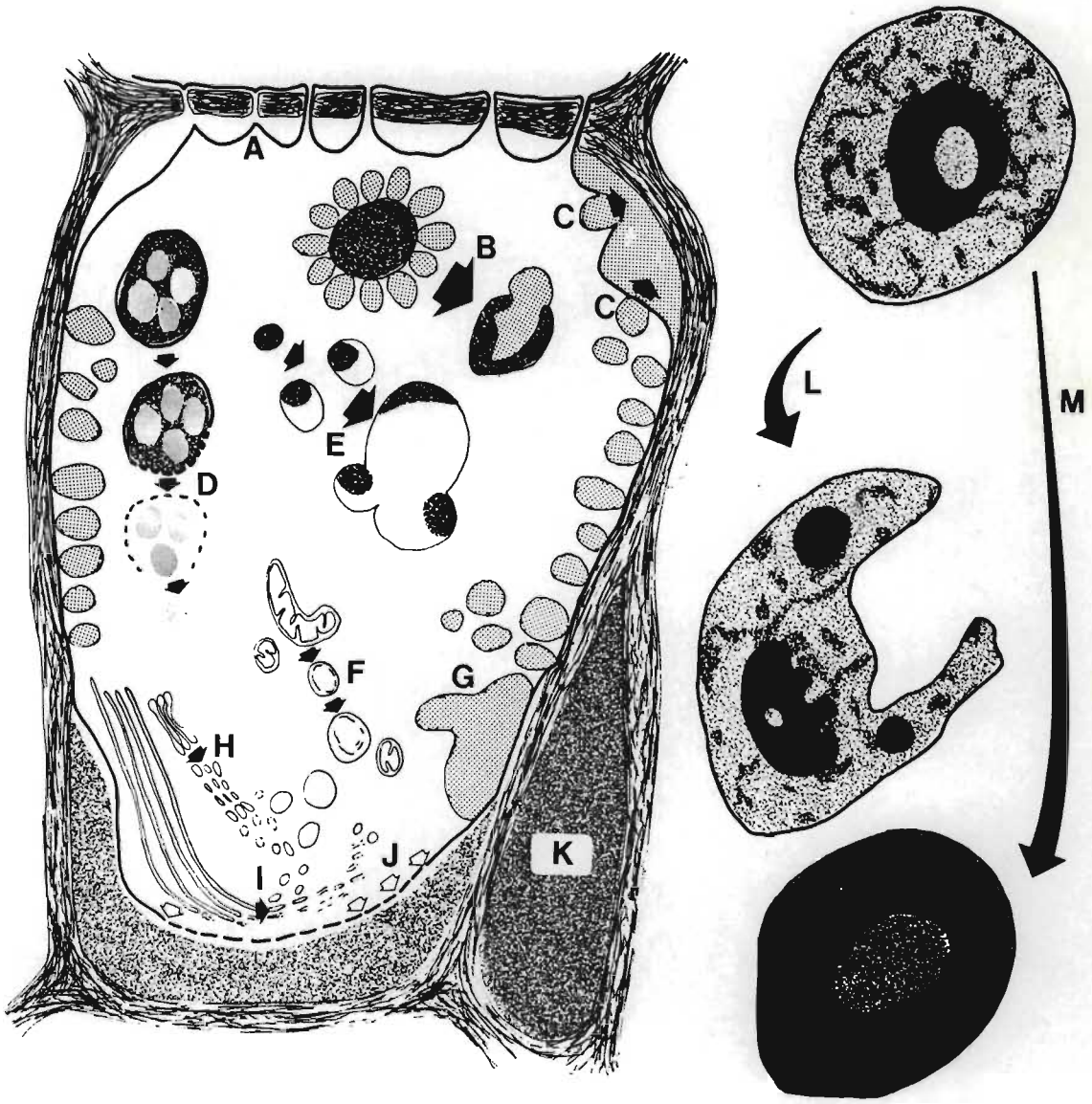
G. Confluence of lipid droplets within the cytoplasm to produce droplets of varying size.

H. Dilation of elements of the Golgi apparatus and unstacking of cisternae to produce numerous small vesicles, possibly indistinguishable from those of mitochondria and E.R.

I. Vesiculation of the E.R. to yield numerous small vesicles, the latter giving the cytoplasm a "frothy" appearance.

J. Physical breaks in the plasmalemma (discontinuous line) leading to the appearance of finely floccular material to the extracellular space (possibly by loss from the cytoplasm). This material may not always be seen but the cell may nevertheless show evidence of plasmolysis. Floccular deposits may also be seen in intercellular spaces (K).

Nuclear changes, illustrated on the right of the diagram, include: lobing and clumping of the heterochromatin normally distributed in the nucleoplasm around the nucleolus (L), or a direct and total loss of discernible structural components, leading to an amorphous dense sphere (M).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longitudinally slicing off a small part (less than 10% of dry seed weight) of the endosperm and cotyledon of seeds. Germination in this case was characterized by a lateral emergence of the embryo, and geotropic curvature of the radicle.

The data presented in Table 5.2, indicate that the conventional laboratory germination test is not a true measure of embryo viability, tending rather to measure the ability of debilitated embryos to attain a threshold growth potential.

It has recently been noted that studies with isolated axes lay emphasis on the growing part of the seed, which can simplify interpretations and provide more rapid assessments of embryonic condition when conductivity measurements are made (Woodstock, 1983 - Pre-print No. 69, 20th ISTA Congress, Ottawa, 1983).

(C) Comparative studies on embryos of differing vigour

The foregoing studies have provided clear evidence that a wide range of membrane lesions are associated with seed deterioration. Studies with tissue slices provided evidence that imbibition can be severely disruptive to membranes in seeds of low viability, while examination of root tip cells from non-viable and viable seeds after 13 hours imbibition provided further evidence of membranal changes, and the sequence of cytological events leading to cellular disorganisation (Figure 5.1). However, these two studies represent extreme cases, in as much as viability was low for all samples investigated. Ultrastructural studies were therefore extended to include seeds in which vigour was clearly reduced but in which viability remained high.

MATERIALS AND METHODS

Two seed lots subjected to a variety of storage conditions were selected and samples of radicle tissue prepared for electron microscopy at 4, 12, 24 and 36 hours imbibition. Germination data

are presented in Table 5.3. Generally only a single sample from each regime was examined to reduce microscopy to manageable proportions and cannot be regarded as a fully exhaustive investigation. It was argued that if any particular membrane system showed visible damage with early vigour loss, ahead of any other that this might indicate the primary site of damage in ageing.

Electron microscope techniques were as previously described (Chapter 3).

OSMIUM-ZINC IODIDE IMPREGNATION

(After Harris & Chrispeels, 1980)

Tissue root tip slices were fixed in 5% phosphate-buffered glutaraldehyde at pH7. After 5 hours fixation at room temperature, tissues were given three washes with distilled water over 30 minutes, before being placed in the osmium zinc-iodide complex for 6 hours at room temperature in darkness. This was followed by a further 18 hours in freshly prepared mixture before dehydration in a graded acetone series and embedding in Spurr's resin (Spurr, 1969). Thick sections (blue-green interference colours) were collected on small mesh grids and viewed without further staining at 120 kV in a Jeol 100C transmission electron microscope.

RESULTS

With the exception of one sample examined, which was evidently from a non-viable embryo (Figures 57, 58 & 59) overall cytoplasmic organisation was not grossly changed at 12 hours imbibition.

As previously observed (Chapters 5A & B) there was a limited fusion of lipid bodies in both earlier and later stages of imbibition (Figures 63 & 72) but by far the most striking feature observed were irregularities at the plasmalemma. These ranged in apparent severity, the simplest being breaks in continuity with numerous plasmalemma-derived vesicles in close proximity to the membrane

(Figures 60 & 66). In more extreme cases membranes apparently infolded into the cytoplasm producing whorled myelin-like figures (Figures 62, 64 & 65). The increase in size of such structures by fusion with the small plasmalemma-associated vesicles was also evident (Figure 61). These whorled structures may have become part of, or become included into, the vacuolar system. However similar membranous structures were also observed within vacuoles of unaged embryo samples (Figure 68) and was entirely consistent with the concept of the vacuole functioning in cellular autophagy (Matile, 1975). If it is assumed that the observed membrane profiles were rounded structures, it becomes apparent that a significant proportion of the plasmalemma could be in such whorled structures, with a corresponding loss of cell integrity. Dilation of ER was seen in root cap cells (Figure 71) and was also evident in a single sample taken from cotyledonary tissue (Figure 70). The plasmalemma breaks and ER dilation observed suggested that membrane susceptibility was not greatly different between the two organs. The separation of the two membrane leaflets of the nuclear envelope (Figure 70) strongly reinforces earlier observations (Figures 26, 32 & 33).

Examination of tissues at 36 hours of imbibition yielded evidence of increased metabolic activity. Especially notable were well-differentiated nucleoli, evidence of organelle differentiation, ribosome-rich cytoplasm and a dramatic reduction in lipid bodies. In spite of this inferred activity the plasmalemma was seen to be withdrawn from the cell wall at localized regions. Extensive vacuolation was noticeably absent and some evidence for cell division was noted (Figure 72).

It is generally held that cell extension precedes cell division in lettuce (Haber & Luipold, 1960). Images of such partially plasmolysed cells suggested that full cytoplasmic turgour might be limiting to extension growth, and may therefore be preceded by nuclear division. This view was supported by the observation that cells in the meristematic and procortical tissues were lacking in vacuolation and possessed ribosome-rich cytoplasm which might be expected in dividing tissues.

On the basis of the above observations it would appear that mild membrane lesions, insufficient to produce gross changes in ultrastructure, may lead to a delay in germination of several hours. It has been argued that in the absence of a fully functional cellular organisation, complete repair of DNA lesions is not possible (Osborne, 1982). An undefined element in this arguement is the precise nature of membrane repair. At present there is no quantitative biochemical measure for repair, although ion leakage may be taken as one such indirect measure.

From the results of this ultrastructural study of root tip cells it is suggested that there may be two different orders of cell membrane repair. The first is sufficient to retain "essential" ions at a level which permits DNA repair and general synthetic activity (including cell division). The second is one which may influence viscoelastic properties of the plasma membrane and permit attainment of the necessary turgor for cell extension. It is suggested that this latter category of repair may be less rapid than the first, and could explain the observed lower stress resistance of seeds of low vigour in field conditions (Abdul- Baki & Anderson, 1972; Heydecker, 1977).

TABLE 5.4 : SOME EFFECTS OF IONIZING RADIATION

CHARA	Membrane depolarization; K^+,Cl^- efflux	Doughty & Hope (1976)
DNA IN AQUEOUS MEDIUM	Hydrogen bond breaks Strand breaks Degradation of bases	Reviewed by Myers (1973)
WHEAT ROOT VESICLES	K^+ stimulated ATPase imbibition ahead of malondialdehyde production	Wright, Murphy & Travis (1981)
SOYABEAN LECITHIN LIPOSOMES	Increase in glucose efflux and MDA formation with dosage	Nakazawa & Nagatsuka (1980)
CULTURED TOBACCO CELLS	$^{86}_{Rb}$ efflux, but not glucose	Wright & Murphy (1978)
BEETROOT TISSUE	Loss of betacyanin	Siegel & Corn (1974)
BARLEY SEEDS	Detectable free radicals at 3% m.c. Resistant enzymes : amylases, glucosidase, galactosidase Sensitive enzymes : phosphomonoesterase, ATPase, phosphodiesterase	Kurobane, Yamaguchi, Sander & Nilan (1979)
PEANUT SEED COTYLEDONS	Decreased nucleic acid synthesis after storage Leucine in intracellular spaces by ARG;proteins "broken down"	van Huystee & Verna,(1969)
MAIZE WHEAT SORGHUM RADISH SEEDS	Reduced respiration	Woodstock & Justice,(1967)
LETTUCE SEEDS	Inhibition of rRNA synthesis implicated in germination delay Detectable free radicals	Yealy & Stone (1975) Haber & Randolph, (1967)

TABLE 5.4 continued

SOME EFFECTS OF IONIZING RADIATION

ERYTHROCYTE MEMBRANE	K^+, Na^+ permeability Inactivation of ATPases Decrease in SH groups Lipid peroxidation Osmotic fragility	Numerous studies cited in : Ottolenghi & Ellory (1983) Grzelinska, Bartosz, Gwozdzinski & Leyko (1979) Miller & Raleigh (1983)
RAT LIVER MITOCHONDRIAL FRACTION	Lipid peroxidation increased, measured as MDA Higher cytochrome oxidase & NAOH-cytochrome reductase activities, lower SOD and catalase activity	Kergonou, Braquet & Rocquet (1981)
RAT LIVER LYSOSOMES	Become leaky - release hydrolytic enzymes	Wills & Wilkinson (1966)
CULTURED MAMMALIAN CELLS	Nucleolar lesions Nuclear invaginations and associated membrane structures	Barham & Walters (1978)
RAT SALIVARY GLAND	Increase in serum amylase Dilation & vacuolation of ER Sequestered, degenerating cell membranes Intramitochondrial particles	El-Mofty & Kahn (1981)
CHLAMYDOMONAS CELLS	Nuclear envelope swelling Condensation of nuclear material Plasmalemma rupture Vacuolation of cytoplasm & chloroplast	Gruber (1978)

(D) Ultrastructural changes during imbibition in seeds after irradiation

Although ionizing radiation has been excluded as a possible cause of seed ageing (Roberts, 1972) there are many reasons why irradiation studies with seeds are particularly attractive. Sax & Sax (1963) were the first to suggest that irradiation studies with seeds may be of value in understanding mechanisms in seed ageing, although this suggestion has apparently not received much attention.

Certainly attempts to attribute loss of seed viability to nuclear factors have many historical and methodological parallels with radiation studies. Chromosome studies provided a clear and simple technique for linking structure with function. The good correlations which exist between DNA content per cell and radiation sensitivity strongly suggested that nuclear or genetic disturbances may be the primary site of radiation injury. While this did not exclude a role for possible disturbances of cytoplasmic structure, difficulties with accurately determining the precise nature of the injury and whether the changes were primary or secondary may have led to excessive emphasis being laid on the nucleus.

The search for other "targets" has led to other suggestions in radiation research, including the membrane and the sulfhydryl hypotheses (Okada, 1970). However only in recent years has radiation damage to cell membranes been investigated in sufficient depth to provide a reasonable alternative in animals (Koteles, 1982) or plants (Murphy, 1983) to the "nuclear target" theory. It is well-documented that dry seeds, spores and cysts show considerably greater radioresistance than comparable hydrated systems possibly because of restricted mobility of free radicals.

While comparisons between dry and hydrated systems might seem questionable, it is evident from Table 5.4 that the number of parallels seen in many diverse biological systems are too numerous to ignore when compared with changes reported in seed deterioration. As far as the author is aware there are no published studies which

Table 5.5

Percentage germination of lettuce after γ irradiation. Samples in sealed vials were placed in a ^{60}Co source and removed at daily intervals. At day 5 (last treatment) samples of all the treatments were set to germinate.

DAYS IRRADIATION	% GERMINATION AFTER :			
	1 Day	2 Days	4 Days	5 Days
0	100	100	100	100
1	25	100	100	100
2	-	25	48	51
3	-	-	4	7
4	-	-	6	16
5	-	-	7	30

Table 5.6

Hydroperoxide levels of the total lipid extract from two seed lots after γ irradiation in air. Germination data for unaged seed given in Table 5.1. Seed held at 80% was non-viable. Storage temperature 20°C.

DAYS IRRADIATION	RECENTLY PURCHASED STORED AT 0% RH	2 YEARS AT 80% RH
1	0.14	1.29
2	0.12	1.51
3	0.14	1.24
4	0.16	1.13
5	0.16	1.36
Control non-irradiated	0.11	1.27

have examined the ultrastructure of irradiated dry seeds on subsequent imbibition. While the absence of biochemical and physiological measures of the effect of irradiation treatment was considered likely to cause difficulties in interpretation, it was felt that comparable changes in the ultrastructure of aged and irradiated seeds could provide some indirect support for the free-radical involvement in seed ageing.

MATERIALS AND METHODS

Seeds of high viability which had been stored at 0% RH for 1 year (3% m.c.) were placed in stoppered hermetically sealed vials and subjected to irradiation from a ^{60}Co source. Doses ranged from 3.4 kGy (1 day) to 23.8 kGy (7 days). Root tips were excised from imbibed seeds between 3 and 24 hours after imbibition and prepared for electron microscopy using standard procedures (Chapter 3).

RESULTS AND DISCUSSION

Germination data for treated and control seeds is given in Table 5.5. The apparently anomalous results obtained for days 3 - 5 are presumed to reflect the immediate testing of the day 6 sample, whereas days 3 and 4 treatments were held at laboratory temperatures for 2 and 1 days respectively before the germination testing. Post-irradiation storage effects have been reported and may be the result of complex interactions (Atayan & Gabrielan, 1978).

Whilst there are naturally difficulties in interpretation in the absence of corroborative biochemical evidence, the basic premise in this study has been that should free radical production be significant in deterioration, then some ultrastructural parallels should be evident between natural ageing and irradiated seeds. Where reference is made to irradiation studies on hydrated systems it should be remembered that water radiolysis produces highly reactive OH groups with a potential to act over a much broader front than in

seeds where water contents are limited. Thus molecules which may be targets in hydrated systems may be relatively insensitive in the dry seed. This may be part of the reason for the radioresistance of anhydrobiotic organisms.

The lowest radiation dose used (1 day treatment, 3.4 kGy) markedly retarded the rate of germination but not viability. Observable changes were only noted in root cap and provascular cells at this dosage and were manifest as partial coalescence of some lipid droplets and the appearance of a "halo" around some plastids caused by an absence of cytoplasm. (Figures 1 and 2). In some instances portions of what appeared to be cytoplasm were contained within the amyloplasts. This was interpreted as an enclave of cytoplasm within the organelle. Similar structures have been noted in root tip cells of long-dormant embryos (Villiers, 1976).

Visible damage to the plasmalemma was evident after 2 days irradiation treatment, having the appearance of either membrane lamellae which lay parallel to the wall (Figure 4), vesicles or discontinuities. Cytolysome-like structures were evident in which E.R. with electron-dense contents partly or wholly enclosed portions of cytoplasm (Figures 3 & 9). The presence of alternate layers of E.R. and ribosomes was sometimes evident in these structures (Figure 9). Since these features were noted after 3 hours imbibition it was concluded that they represented a rapid response to irradiation initiated on imbibition, independent of metabolic involvement. Protein bodies failed to undergo normal vacuolation and fusion and instead sequestration of portions of cytoplasm and lipids was observed (Figures 5, 10 & 14). Nuclear and nucleolar morphology showed no gross pathology (Figures 6, 12 & 14) although the absence of nucleolar vacuoles suggested that these organelles were relatively inactive. In root cap cells nuclei were mildly lobed with small inactive nucleoli (Figure 10). After 3 days irradiation the lability of cap cells to damage was particularly evident and changes suggesting dilation of mitochondria and the E.R. were seen (Figures 7 & 8). Disorganisation of amyloplasts was apparently initiated by deterioration of internal membrane structures. (Figures

7 & 11). This probably represented an irreparable form of damage, unlike that observed earlier where internal cytoplasmic pockets would have increased the surface area to volume ratio, and may have facilitated organelle repair. Large-scale plasmalemma irregularities were seen in the root cap being especially evident after 3 hours imbibition (Figure 8). Evidence for some mitochondrial damage was observed after 1 day of irradiation treatment, particularly in the root cap, and took the appearance of dilution of the mitochondrial matrix and loss of cristae. Generally, however, the organelles showed remarkable resistance to damage and after 4 days irradiation were seen to possess internal vesicle-like profiles, presumably derived from the membranes of the cristae, in addition to developing occasional protuberances with associated dilution of matrix density (Figure 15). Seeds subjected to 4 days irradiation attained 50% germination at 11 days which represented a considerable retardation of germination. All seedlings were however remarkably uniform and erect, but dwarfed. Although normal greening was observed, full cotyledonary expansion did not occur, and primary leaves did not develop.

Meristematic activity was thus clearly limited or absent and all seedlings ultimately became necrotic and died. Ultrastructural examination of one of these "gamma plantlets" (Haber & Foard, 1969) revealed highly vacuolate cells which had apparently undergone some measure of normal differentiation. Xylogenesis had occurred and few gross cytoplasmic abnormalities were seen. Although nucleoli appeared inactive, numerous polysomes could be seen in phloem cells and elsewhere. This tended to suggest that at some earlier stage nucleoli might have been much more active. Degenerate cell cytoplasm noted between developing conducting tissues was interpreted as a normal manifestation of development rather than a specific radiation-induced lesion (Figure 16). In contrast to this was the striking absence of vacuolation or wall changes in the endosperm (Figure 13). This suggests, as was observed earlier, that embryo viability may be significantly underestimated by the possible restraining influence of the endosperm.

There would appear to be no comparable ultrastructural studies on seeds in the published literature. In general seeds have been seen as experimental systems in which various parameters such as time, temperature and humidity may be manipulated in order to study the radiation response. Although many of these studies were conducted in the 60's, they have been pursued until quite recently (e.g. Atayan & Gabrielian, 1978).

In keeping with problems noted earlier in attempting to distinguish between primary and secondary ultrastructural lesions associated with deterioration, radiobiological studies have similarly been complicated by intervening reactions which may amplify the primary damage. This is illustrated as follows:

Primary radiation damage to molecules	→	Amplification process or processes	→	Observable radiobiological phenomenon (e.g. death, chromosome aberrations).

It has further been suggested (Okada, 1970) that amplification of damage may occur through coupled reactions of the redox type, via the production of "nonsense information" from damaged DNA, or increasing susceptibility of proteins and membranes to hydrolytic attack. Additionally the interlocking of metabolic pathways may lead to small amounts of damage being greatly amplified. This serves to highlight the difficulties inherent in attempts to suggest cause-effect relationships, and all possible interpretations offered must be seen as highly tentative in the absence of corroborative biochemical studies.

The partial coalescence of lipid droplets, plasmalemma and other membrane abnormalities might be seen as evidence of some changes in membrane properties. This may be the result of free-radical induced peroxidation of lipids, since measurement of lipid hydroperoxides showed evidence of a slight increase over control

values (Table 5.6). A small increase was also observed in non-viable seeds suggesting that peroxidation could progressively increase post-mortem. The apparent engulfment of cytoplasm by protein bodies and cytolysome-like structures might be interpreted in terms of altered membrane properties, but could also indicate repair by removal of damaged cytoplasmic constituents.

The apparent resistance of mitochondria to irradiation damage was a notable feature which is not without parallels in other irradiation studies. Evidence has been obtained to suggest that mitochondria of pear and cherry fruits are markedly resistant to radiation (Romani, Fischer, Miller & Breidenbach, 1967). Those authors did note a reduction in the number of organelles in the mitochondrial pellet with increasing dose, and an increase in membrane-bound "empty bodies". However, neither transaminase, cytochrome oxidase or fatty acid levels were altered by irradiation. This observation is entirely consistent with the present ultrastructural study which suggests that although organelles offer some resistance to free-radical damage, they may nevertheless undergo changes in membrane properties as manifest by vesiculation and altered morphology. The "empty bodies" noted in the study by Romani et al. (1967) may thus reflect rupture of functional, but osmotically fragile organelles during tissue fractionation.

Changes to plastids may reflect similar changes in membrane properties and offer direct parallels with the earlier studies (Chapters 5 A, B & C). Notwithstanding complications of possible amplification discussed earlier, evidence suggests that damage may occur at successive levels and that membranes may be significant targets. Studies on E. coli by Yatvin (1972) demonstrated that membrane repair in a radiation-resistant strain occurred ahead of single stranded DNA repair. It has further been suggested that DNA does not uniquely determine the survivability of irradiated cells (Yatvin, Gipp & Werts, 1984). Evidence was provided that "packaging" of DNA (as membrane - DNA complexes), and changes in phospholipid composition by incorporation of cardiolipin, possibly to effect membrane stabilization, may be major aspects of radiation

survival.

Studies with yeast cells have shown that doses of radiation, too small to affect cell survival (2.5 Gy), altered membrane properties (Khare, Trivedi, Kesavan & Prasad, 1982). These included stimulation of amino acid uptake, loss of membrane sulfhydryls and lipid peroxidation. The membranes of nuclei appeared among the last to show damage, an observation in keeping with the present study. Evidence is available from animal studies to indicate that phospholipids of nuclei are more saturated than those of the E.R., mitochondria and plasmalemma (Schlager & Ohanian, 1980; Keenan, Berezney & Crane, 1972).

There is evidence that lipid peroxidation induces changes in membrane permeability, particularly in model systems such as liposomes where analysis is considerably simpler. Nakazawa & Nagatsuka (1980) have shown that glucose permeability changes in phosphatidylcholine liposomes and this could be directly related to malondialdehyde formation, increasing with radiation dosage (20 - 80 Gy).

It has become evident that membrane-protein interactions may be as important as the formation of conjugated double bonds or breaks in the fatty acid chains (Grzelinska et al., 1979). Of particular significance are studies which have shown that increased K^+ efflux in cultures of Rosa after irradiation by U.V. light occurs as a result of K^+/ATPase inactivation (Murphy, 1983). While ATPases might be one of the early sensitive targets in hydrated systems, higher doses (5-25 Mrads) are required for inactivation of dried in vitro enzyme (Ottolenghi & Ellory, 1983).

It has been pointed out (El-Mofty & Kahn, 1981) that the release of lytic enzymes is more likely to be a late event in radiation induced deterioration. While isolated lysosome preparations can be shown to leak enzymes on irradiation, the doses required are several times higher than those required for cell killing.

While many of the changes induced by irradiation of seeds are comparable to those seen during the imbibition of aged seeds, autophagy was uniquely a radiation-induced response. Early disturbances of membrane function were noted in salivary gland cells at low dosage (200 rads), including cellular swelling, dilation and vacuolation of E.R., and discrete areas of sequestered, degenerating and disintegrating cellular membranes (El Mofty & Kahn, 1981). Considering the widely disparate systems involved, the parallels with changes in the root tissues are particularly striking.

Biochemical responses to irradiation

While the gulf between biochemistry and ultrastructure remains wide in radiobiology it is nevertheless profitable to consider some of the consequences of irradiation to seed metabolism on subsequent imbibition. Studies in the area are somewhat limited but nevertheless serve to direct attention to the damage of components other than membranes and DNA.

(a) Enzyme changes

Observations by Kurobane et al. (1979) that carbohydrate metabolizing enzymes were resistant in barley seeds while those involved in phosphate metabolism such as ATPase, and phosphodiesterase were more sensitive, could lead to a situation where an imbalance between glycolysis and phosphorylation might occur. Since amylases remained relatively unaltered, starch breakdown could occur, although the early pumping-priming phosphorylation of glucose might presumbly be limited.

(b) RNA Synthesis

Yealy & Stone (1975) have attributed a delay in the germination of lettuce seeds to impairment of rRNA synthesis by irradiation. While there is no evidence to preclude this possibility in the present study, there was no evidence of altered nucleolar morphology except at higher radiation doses, membrane changes being the first

observable changes. Machaian & Vakil (1980) have provided details of changes in protein and nucleic acid synthesis in irradiated wheat seeds. While the rate of total RNA synthesis decreases with irradiation (50 - 200 k rads) total RNA levels were higher than controls during the period investigated (first 6 hours of imbibition). Ribonuclease activity declined to approximately half the control levels at the highest irradiation dose employed. Incorporation of amino acids into proteins and total protein levels were markedly depressed although uptake was not affected. It was suggested that a low re-utilization of newly synthesized RNA occurred, as reflected in a steadily increasing specific activity of this fraction. Since rRNA comprises the bulk of cellular RNA it may be assumed that the above observation predominantly reflect synthesis of ribosomes or their precursors. The observed engulfment of cytoplasmic ribosomes in vacuoles and cytolysome-like bodies at a comparatively later stage than the above studies might be related to decreased RNAse levels and represent a compensatory mechanism to bring about nucleotide turnover in lettuce seeds.

(c) Membranes

Radiation-induced peroxidation of membrane lipids was shown in membrane cytochrome P_{450} reconstitution experiments to lead to a decrease in cytochrome P_{450} by destruction of the haeme group. This suggested that membrane-bound enzymes could be influenced by the physical or chemical properties of membranes (Yukawa, Naatsuka & Nakazawa, 1983). This provides further evidence for the difficulties in separating direct and indirect membrane irradiation effects unless constituents components of the systems are thoroughly examined.

CONCLUDING DISCUSSION : ULTRASTRUCTURAL STUDIES

1. Interpretation of Ultrastructure

Before considering the results of the present investigations in the light of published findings it is perhaps well to give some attention to certain aspects of methodology. A necessary prerequisite for the critical interpretation of electron micrographs is a thorough appreciation of the influence of preparative procedures and the likely artefacts.

In both the present study and all published ultrastructural studies on seed ageing it has been tacitly assumed that the ultrastructural appearance of tissues is a true reflection of *in vivo* condition. From the limited number of studies which have been undertaken it is possible that this assumption may not be entirely valid. Indeed it has been noted (Jost, McMillen & Griffith, 1983) that the criterion for successful fixation has until now not involved any direct measure of molecular motion, but rather the appearance of the specimen after fixation. This not surprisingly leads to sujectivity and difficulties in selecting suitable criteria for judging "what specimen ought to look like from the final micrograph alone".

Aspects which have been identified as important in both plant and animal studies include:

1. Tonicity of buffers and fixative solutions.

2. Relative penetration rates of aldehyde fixative and buffers.

3. Cell and tissue sensitivity to fixation and embedding regimes.

4. Possible tissue swelling on primary fixation, and shrinkage artefacts during dehydration and embedding (Reviewed by Lee, 1983).

That the tonicity of fixative solutions and buffer washes should closely approximate to the water potential of the cells is self-evident if net movement of water into or out of the cell is to be avoided. It is well-documented that membranes retain selective permeability after the primary aldehyde fixation step, and only when fixed by glutaraldehyde and osmium tetroxide in sequence, do they loose the capacity to swell (Kirschner & Hollingshead, 1980). Isolated hepatocytes have been shown to swell to 119% of control volumes after glutaraldehyde fixation, increasing still further to 136% after the osmium tetroxide post-fixation step (Nordestgaard & Rostgaard, 1984).

In the case of plant cells it may be assumed that the wall will limit any likely expansion, but if the membrane shows increased permeability (as is suggested to occur in ageing) a greater loss of water, inter alia, is likely to occur. Consequently greater shrinkage of the protoplast may ensue during subsequent processing, leading to cells of plasmolysed appearance. Thus observations that the protoplast of aged seeds is not in contact with the cell wall during the imbibition period (Villiers, 1973) and those of the present study (Chapter 5 : Figures 20, 21, 23, 44, 48 and 70-72) may be interpreted as artefacts of preparation rather than a reflection of the in vivo appearance of cells.

It has been known for some time that senescent and highly vacuolate cells are profoundly influenced by conditions of fixation (Mohr & Cocking, 1968). Abu-Shakra & Ching(1967) reported that the mitochondria from aged seeds were more fragile and less amenable to fixation by electron microscopy. The only recent study in this area appears to be that by

Lawton & Harris (1978). Those authors found it necessary to distinguish between natural senescent features of developing fibre cells and those resulting from fixation. Tonoplast intactness was deemed to be a useful criteria for evaluating the quality of fixation. The observations may have serious implication for suggestions that the bursting of the vacuolar membrane may bring about precipitous cell death by uncontrolled release of hydrolytic enzymes (Berjak & Villiers, 1970; Villiers, 1973).

A finding deemed to be of particular significance in the present study is the differential response of phospholipid monolayers to progressive peroxidation (van Zutphen & Cornwell, 1973). Initial peroxidation was seen to lead to an expansion of phospholipid monolayers, possibly through the introduction of peroxide groups, but with further oxidation a contraction of the films was observed. This observation may explain the results of a recent study (Rifkind, Araki & Hadley, 1983) in which the relationship between the distribution of osmotic fragilities and the distribution of cellular ages were investigated for human erythrocytes. Contrary to expectations it was observed that ageing did not lead to a gradual increase in osmotic fragility; older cells were seen to show both increases and decreases in osmotic fragility. Those authors noted that the observation of greater dispersion in cellular ageing had only been previously reported for ageing, entire organisms. This dispersion has also been noted in connection with seed ageing (Roberts & Ellis, 1982). Dimitrov and Jain (1984) have recently reviewed the topic of membrane stability and concluded that molecular models of membrane rupture are at a rudimentary level. Two types of rupture of the red blood cell were recognized, and these are considered relevant in the context of the present studies.

In hypotonic solutions the cells swell, and membranes become stretched as a result of increasing tensicn. At a certain critical level, the membrane breaks, holes are produced in

membrane and haemoglobin is lost from the cell. The loss of the osmotic pressure difference leads to closure of the holes, and cytoskeletal elements permit the cells to return to their original shape, albeit as ghosts. Those authors recognize that several stages can be identified in this haemolytic process, and point out that a 2-3% increase in surface area leads to a significant 50-60% increase in volume.

Fragmentation, which results from "breakage of cytoskeletal proteins, leads to a destruction of the membrane". Unfortunately no further details of this latter process were provided, but it may be assumed that after membrane cleavage by the cytoskeletal system the rupture of the membrane is irreversible.

Significantly, the role of the cytoskeleton in plant cell damage has been a relatively recent development (Sargent et al., 1981 Berjak et al., 1985). The above observations have clear relevance to the studies discussed below (Section 3 : Membrane damage and seed ageing). There are parallels between the above observations and those of Villiers (1973), see below.

Although the electron microscope is theoretically capable of resolving details of molecular structure under optimal conditions, this cannot be claimed for the majority of plant ultrastructural studies. While 10Å has been cited as a typical resolution level, this is not considered attainable with combined glutaraldehyde and osmium tetroxide fixation (Sjostrand & Kretzer, 1975). Thus any studies where membrane lesions are seen in thin section must presumably represent either higher-order levels of molecular change or artefacts of the preparative technique.

While the assumption that discontinuities in the plasmalemma indicate leakage would seem a valid interpretation, it must be assumed that membranes can be "leaky" without evident physical discontinuity. Palta & Li (1980) have suggested that

complete loss of semi-permeability or membrane rupture is probably one of the last events in the sequence of events leading to cell death from chilling injury. Conformational alterations to membrane carrier proteins, such as ATPases, lateral phase separations, or changes in the fatty acid levels of phospholipids would seem obvious examples which may not bring about detectable ultrastructural changes and yet could cause profound alterations to membrane, and hence cellular, function.

2. Tissue, Cell and Organelle Variability

Cells of the root tip represent the classical example of progressive differentiation of cell and tissue lineages and it may perhaps be assumed that the cytological differentiation seen is also a manifestation of changes at the molecular level as regards cytoplasmic lipids, proteins, and the protein:lipid ratios in membranes. (Obroucheva, 1975).

The observations that individual membranes appear to deteriorate at different rates may therefore not be altogether surprising presumably reflecting inter alia greater susceptibility to peroxidative damage. The arguments that populations of seeds show greater dispersion and differences in quality during deterioration can presumably also be applied to populations of cells, to accommodate the observations that deterioration was not uniform between cells of the same tissue. It is not clear at the present time what the precise physiological basis for these differences might be, although it is notable that differential deterioration has also been observed in senescent leaves and other tissues (Barton, 1966; Varner, 1961).

This could be a manifestation of the organised nature of deterioration but it may also reflect intrinsic differential resistance of sub-cellular components. It has been proposed by Leshem (1981) that free-radicals are involved in tissue senescence. Since these may well be difficult to control, the apparently controlled nature of deterioration may merely be a reflection of differential cell and membrane susceptibility to uncontrolled free-radical attack.

The meagre biochemical data on the fatty acid composition of plant membranes (Harwood, 1980) does not permit the electron microscope observations to be interpreted in terms of, for instance, differential membrane susceptibility to peroxidative damage. The high level of unsaturates in chloroplasts is well-known, but apart from the apparent absence of 18:3 fatty acids in nuclear membranes from onion tissue few clear trends are apparent (Mudd, 1980).

Mitochondria, it may be argued, are organelles at high risk because of the potentially damaging effect of electrons (Vladimirov, Olenev, Suslova & Cheremisina, 1980), and that higher levels of free-radical scavengers or special protective mechanisms may exist in these organelles compared with other membranes. Additionally the higher protein:lipid ratio of the cristae may offer some amelioration against lipid peroxidation by spacing phospholipids more widely apart and thus lessening the possibility of free-radical propagation.

Mitochondria with well-defined limiting membranes but sparse, ill-defined cristae were observed in root-tip cells of both imbibe-fixed tissues or those which had been imbibed for 13 hours before fixation. (Chapter 5 : Figures 5, 13, 20, 30, 32). Similar featureless mitochondria have been observed in many seed tissues shortly after imbibition, including an earlier study on lettuce (Srivastava & Paulson, 1968). This simplification of structure has been attributed to lack of metabolic competence, although Nir, Klein & Poljakoff-Mayber (1970) have suggested that this may reflect the altered ability of membranes to react with osmium tetroxide post-fixative. This view was not supported by the ultrastructural

appearance of mitochondria after fixation by freeze-substitution. Although cristae were sparse, the mitochondrial matrix was seen to be uniformly electron-dense, with evidence of particulate elements aligned along cristae. (Chapter 5 : Figure 18). These superficially resembled the coupling factor F_1 reported from negatively-stained preparations by Racker and co-workers in the 1960's (Ernester & Shatz, 1981).

That mitochondria were not entirely separate structural entities was seen from the apparently conjoined membranes which may be a heritage from the desiccation stage of seed maturation. (Chapter 5 : Figure 13). This observation may explain why attempts to extract mitochondria from dry or recently imbibed seeds has not always been entirely successful as regards marker enzymes or respiratory control. It has been reported that mitochondria extracted from lettuce seeds during the first 24 hours of imbibition were unable to incorporate ^{32}P into ADP (Pradet, 1982). Hourmant & Pradet (1981) have shown that the synthesis of ATP take place during the first minutes of imbibition in intact lettuce seeds and that the full cytochrome pathway is operational.

3. Membrane Damage and Seed Ageing

Many cytological and biochemical changes have been detected in the chain of deterioration which leads to seed death, but so far it has proved difficult to establish the primary cause of ageing. While observing that evidence for free radical involvement was not great, Roberts (1983) nevertheless noted that support for membrane deterioration in ageing continues to grow. In seeds of maize subjected to an accelerated ageing regime it was noted that, apart from the obvious damage to mitochondria and the possibility of repair of damage (Berjak & Villiers, 1972a), cells of the root cap underwent precocious senescence instead of following the normal pathway of differentiation upon imbibition (Berjak & Villiers, 1972b). In particular, acid phosphatase activity (originally confined within pro-vacuoles) became dispersed throughout the cytoplasm, suggesting that the membranes of these organelles had failed to retain the

enzyme(s). Whilst those observations were restricted to the inner root cap cells, it was considered likely that their localized death foreshadowed a spread of necrosis throughout the root. Using autoradiographic techniques, those workers were able to show during the earlier period of accelerated ageing (10 - 14 days out of a maximum of 30) that an increase in thymidine incorporation took place into DNA of the non-meristematic zones. Later during the ageing sequence (18 - 20 days) incorporation declined. It was suggested that this pattern might possibly indicate recruitment of new, quiescent cells to replace damaged meristematic cells (Roberts, 1972). On the other hand, it was argued that this thymidine incorporation might represent some form of breakdown of the control of the DNA replicative mechanism in normally non-meristematic cells (Berjak and Villiers, 1972 b, c). Those studies clearly need further elaboration, especially in view of the suggestion of Osborne (1983) that DNA repair is one of the earliest events following seed rehydration.

In a later study, Berjak et al. (1985) provided further evidence for the involvement of genetic and membrane damage as the most important lesions leading to loss of seed viability. Deteriorative changes were examined in maize seeds of the same variety all of which had moisture contents in the range 8 - 11%. The seeds had been harvested in different years and stored for 5 months (ambient temperature) and 8, 12 and 15 years (cold storage), respectively. Examination after 12 hours of imbibition was made on root tips from individual samples shown to be fungus-free, while samples were additionally assessed for viability and percentage infection by storage fungi. This long-term ageing study is significant, for it shows that progressive membrane- and nuclear-associated changes are directly comparable with those obtained from earlier studies where 40°C and 14% moisture content were used to accelerate the ageing progression. An additional observation which has bearing on membrane activity, and cytoplasmic organisation was the progressive clumping of cytoskeletal elements. This, along with collapse of the nuclear skeletal framework, could lead to altered properties of vacuoles, clumping of lipid bodies and nuclear lobing. The observed presence of what was assumed to be a cytoskeletal

element (Chapter 5 : Figure 41) may also implicate changes in the cytoskeletal system with loss of cytoplasmic structure in lettuce tissues. While the above studies have implicated both nuclear and membrane damage in maize seed ageing, it has been proposed that for lettuce seeds some extranuclear element, such as membrane damage is the primary determinant leading to viability loss (Villiers, 1973).

Evidence in support of this proposal was suggested by the following:

1. Cell division is not essential to the process of germination since the initial emergence of the radicle in lettuce takes place by elongation. No mitotic figures were detected in 1mm radicle tips, although these increased exponentially when the radicles were between 2.5 - 3mm long, at which point values tended to become constant (Villiers, 1974). Furthermore seeds subjected to high but sublethal doses of γ irradiation are nevertheless capable of forming seedlings (albeit "gamma dwarfs") in the absence of cell division (Haber & Luipold, 1960).

2. The technique for evaluating chromosome aberrations is a gross and highly selective technique for evaluating the extent of damage to the genome. It has been suggested that chromosome aberrations may influence cellular differentiation and seedling morphogenesis rather than germination (Villiers, 1973). Indeed the scoring of an abnormal mitotic figure does not in itself give information on the likely functional activity of the cell in question or, more importantly in the present context, the ability to undergo cell extension. Since cells with chromosome aberrations are already committed to part of the process of cell division, it is perhaps the cells which fail to enter prophase or which cannot produce relevant, functional mRNA which may be more important determinants in germination. There are admittedly studies which have shown good correlations ($r = 0.93$) between percentage germination and aberrations (Murata et al., 1980; Murata et al., 1981), but these findings are not in themselves proof of causality. In addition poor relationships have been observed between chromosome

abnormalities and germination in lettuce and onion seeds (Harrison & McLeish 1954; Harrison, 1965). This is apparent also from an examination of the data presented by Villiers (1974) for lettuce which militates against any simple cause-effect relationship.

3. An examination of the ultrastructure of root tip cells from embryos which died shortly after emergence showed organised cytoplasmic ultrastructure during early imbibition although clumping of the heterochromatin was evident (Villiers, 1973). Subsequent disorganisation became apparent some 24 hours after imbibition, and was attributed to the loss of integrity of the vacuolar membrane. Using the Gomori test for acid phosphatase as a marker for vacuolar integrity, it was shown that enzyme activity was initially confined to vacuoles, but the enzyme reaction became spread throughout the cytoplasm during later imbibition. The disintegration of mitochondria and plastids was seen to follow vacuolar membrane breakdown. On the other hand, seeds which had been aged for a longer period were seen to suffer large-scale membrane damage at imbibition with the immediate onset of subcellular disorganisation.

It has earlier been noted that the hypothesis of Bacq & Alexander (1961) implicating lysosomal rupture (the "suicide bag" hypothesis) in radiation-induced cell lysis can no longer regarded as a satisfactory explanation for cell death. While the observations of the present study implicate possible plasma membrane damage as an early cytological event in seed deterioration, no evidence was obtained in support of the above lysosomal rupture hypothesis. This is thought to be a reflection of fact that seeds in the present study were subjected to milder ageing conditions of 20°C and 60% RH rather than the somewhat harsher 30°C and 100% RH used by Villiers (1973).

The two categories of damage described in the above studies may be accommodated within a framework of increasing peroxidative damage to membranes. In the one case it is possible to ascribe the delay in vacuolar breakdown to post-imbibitional increases in membrane

peroxidation, possibly as a result of autocatalysis (See Chapter 7). On the other, total membrane breakdown on imbibition may be explained by supposing extensive peroxidation to have taken place in all membranes in the dry state, and hydration is thus seen as merely permitting expression of this damage. It has been stressed that these observations should be seen as representing part of the continuum of deterioration (Villiers, 1973) a view supported by the present studies.

Plasmalemma damage was identified as the earliest lesion in the study on embryos of differing vigour but high viability (Chapter 5c). However it cannot be assumed that this damage is the cause of physiological deterioration in the seeds for the reasons outlined at the outset of this discussion (Section 1, Interpretation of ultrastructure). The physiological significance of such visual evidence for membrane damage cannot be fully understood at the present time. Minor membrane leakage could, for instance, exert a "cascade effect" with losses of intracellular potassium leading to depressed protein synthesis. This could lead to an impairment of repair or general synthetic functions, and could explain the delay in germination noted in low vigour seeds. While direct evidence for such a suggestion is lacking for seeds, a connection between potassium levels and protein synthesis has been established for erythrocytes and pollen grains (Cahn & Lubin, 1978; Bashe & Mascarenas,1984). Some other suggested consequences of membrane leakage include the mixing of enzymes or substrates which are normally spatially separate (Bewley & Black, 1982) and the metabolic imbalance hypothesis which has been proposed for soybean seeds (Woodstock & Taylorson, 1981 a & b) which is seen by the present writer as the beginning of a new direction in viability research. Relevant ideas in this latter area are presented in Chapter 8 of this thesis.

The inconclusiveness of the above observations and discussion serve to highlight the urgent need and particular value of studies based on structural-functional relationships rather than either biochemical or electron microscopical observations on their own.

4. Membrane Damage and Repair

The results of the present study (Chapter 3) have suggested that seed membranes retain a bilayer structure in the dry state, and are not in the porous, hexagonal state suggested by Simon (1974). Reasons for solute leakage in early imbibition must thus be sought elsewhere. That rapid water uptake can be damaging to seed tissues has been demonstrated many times (Parrish & Leopold, 1977; Powell & Matthews, 1978; Tilden & West, 1985) and the plasmalemma must perforce be one of the first sites of such possible injury. Bewley (1979) has speculated that full membrane functionality may require repair mechanisms to replace structural or enzymic elements, and that this may occur over several hours of rehydration. This possibility would be particularly relevant as regards mitochondrial function, although similar repair mechanisms could also operate at the plasmalemma. A further speculative possibility, directly related to the above suggestions, may be a transient loss of overall cellular compartmentation during early imbibition. Although speculative possibilities are almost limitless, it may be that lipolytic enzymes, lipoxygenases, and pro-oxidant, iron-containing proteins may have access to substrates with which they are rarely in contact. It is suggested that unaged seeds would be equally liable to such an assault, but would re-establish compartmentation more rapidly than aged seeds.

Galliard (1980) has drawn attention to the fact that the activity of lipid acyl hydrolases are enhanced by the release of free fatty acids. This in effect leads to a reaction on membrane lipids which is autocatalytic, since released free fatty acids provide greater enzyme access to membrane substrate. While the potato tuber system upon which these suggestions are based is probably one of exceptional activity, these observations may have relevance in the present context in explaining the initiation and spread of membrane deterioration seen by electron microscopy.

It is unfortunate that evidence for repair to membranes and other macromolecules has generally been inferred (Berjak & Villiers, 1972a; Villers, 1973) although Osborne (1982) has provided biochemical

evidence in support of unscheduled DNA synthesis as an early post-imbibitional event in aged rye seeds. Biochemical experiments attempting to demonstrate membrane repair would be fraught with technical and interpretational difficulties.

In the studies of Berjak & Villiers (1972a) it was noted that mitochondrial abnormalities found in the root cap cells of seeds aged for 12 days were no longer evident after 24 hours imbibition, while damage was largely reversed at 48 hours for embryos aged for 20 days. In the present studies it was observed that root tip cells from seeds of low vigour were of normal ultrastructural appearance at 24 and 36 hours but some were partially plasmolysed (Chapter 5 : Figures 71 & 72). This may reflect changed membrane properties on ageing which are only slowly reversed.

There is a paucity of information on membrane lipid turnover against which the above observations can be evaluated. Morre et al.(1979) have indicated that turnover is very slow (of the order of days), while for non-dormant and dormant embryos of Avena the half-life values were between 35 and 57 hours (Cuming & Osborne, 1978). If these values can be considered anywhere near representative it could indicate a significant delay in the replacement of peroxidized lipid or their reaction products, unless some mechanism exists for their selective removal.

On the other hand, a figure of less than 30 minutes is given for ER–GA–plasmalemma movement by membrane "marker" proteins in studies cited by Robinson & Kristen, (1983) although this value must be considered atypical since the examples chosen were active wall or slime secreting plants. Villiers (1973) has suggested that the repair mechanisms may themselves be impaired in ageing and could thus considerably delay return to full membrane functionality. The finding by Mullet & Wilkinson (1979) of a significant negative correlation between mean conductivity per unit seed weight and mean plant height at 24 days might be seen as a long-lasting influence of membrane damage on plant growth. While it is impossible to exclude the possible involvement of the genome, it was observed that seed yield showed no correlation with electrolyte loss.

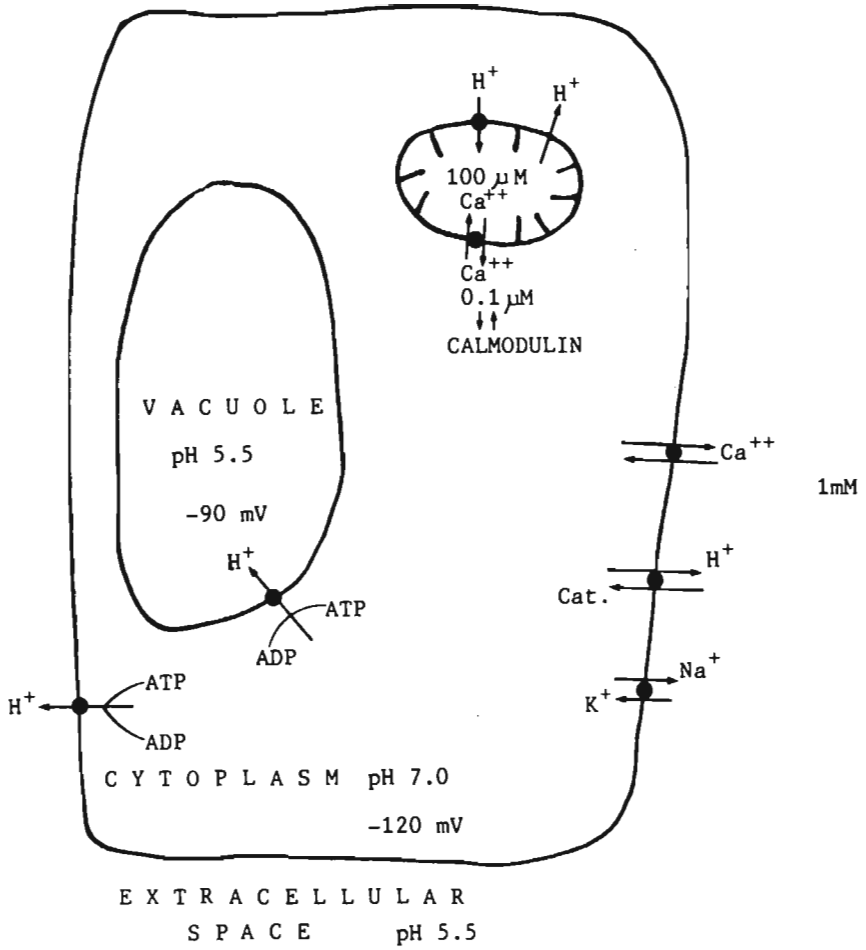


FIGURE 5.2

Illustration of an idealized cell protoplast based on proposals and suggested models by Sze & Churchill (1983); Hanson (1983) and Smith et. al. (1984). The energy-dependent proton extension mechanism and other plasmalemma pumps are illustrated (includes calcium, proton-cation (Cat.) exchange and Na^+/K^+ pump). Compartments shown include the vacuole and with an inwardly-directed proton pump, and mitochondrion with proton and calcium pumps. It is assumed that intracellular levels of free calcium require fine adjustment by calmodulin and the pumps indicated.

5. Membrane Flow and Repair

The concept of membrane flow, thought to be valid for all eukaryotes, suggests that dictyosomes serve as the site of transformation of membrane from an ER-like to plasmalemma-like membrane type (Robinson & Kristen, 1982). While it is doubtful whether membrane traffic is likely to be significant during the early imbibition of seeds, two points in the above review warrant attention.

The first concerns the observation that significant ultrastructural changes occur in plant cells with an altered ionic status. Griffith & Ray (1982) have shown that elevation of cytosolic Ca^{++} levels by the ionophore A23187 leads to premature vesicle fusion in pollen tubes. The ability of Ca^{++} to modify membrane properties may be through rigidification induced by binding and screening, so minimizing electrostatic repulsions between headgroups (Rossignol & Grignon, 1982).

The second concerns changes in cytoplasmic pH by the ionophore nigericin which resulted in an accumulation of Golgi-derived vesicles within the cytoplasm. Extrapolation of the above two observations to seed ageing may offer a ready explanation for some of the cytoplasmic changes observed in the present study. It is suggested that changes to the normaliy finely regulated ionic status of the cell (such as typified by Figure 5.2) could precipitate:

1. Fusion of lipid bodies.

2. Vesiculation of the ER and Golgi apparatus to produce cytoplasm of frothy appearance (Chapter 5 : Figures 26, 27 & 29).

   The apparent association of phospholipase D with ER (Roughan & Slack, 1976) is considered significant in view of ultrastructural observations which indicate dilation of the ER to be an early observable symptom of deterioration in root cap

cells. If the ER is intimately involved in phospholipid synthesis, as is the case in castor bean endosperm (Beevers, 1975), then the morphological alterations seen in this membrane system could have profound effects on membrane synthesis and repair. Such an argument is complicated by the transfer of lipids from microsomes to other intracellular organelles. For instance, peroxidative damage to polyunsaturated fatty acids the inner mitochondrial membrane would require import of both PC, PE and the relevant 18:2 and 18:3 fatty acids from the cytoplasm, since these organelles cannot synthesize these molecules (Mazliak, Douandy, Demandre & Kader, 1975).

3. Vacuolar engulfment of lipid bodies. The finding that lipid bodies enter the vacuole of yeast cells in stationary phase growth at areas depleted of intramembranous particles (Moeller & Thompson, 1979) could implicate membrane phase separation as a result of peroxidative damage or altered cytosolic ionic status as a significant modifier of membrane properties.

4. Mitochondrial changes, since the mitochondrion may be seen as a further ion regulating compartment in the cell.

Quite apart from the above suggestions, all the reactions in membrane biosynthesis, except for the phosphorylation of choline, are membrane bound with clear involvement of the ER and the Golgi apparatus. Since these membrane systems were shown to be especially labile in ageing, the consequences for phospholipid biosynthesis could be significant. The recruitment of new membrane would be especially important to germination, and more particularly for extension growth.

6. The Contribution of Cotyledons and the Endosperm

Much of the ultrastructural emphasis undertaken in the present study has been concerned with changes in the radicle tip, since the emergence of this organ was defined as the criterion for germination. As was evident from the germination studies (Chapter 2) the

cotyledons may play an important role in the overall germination process by influencing embryo emergence and the establishment of the seedling, a view which was reinforced by the restricted development of necrotic cotyledons (Chapter 4). In addition evidence was obtained suggesting a possible restraining influence of the endosperm as evidenced by the substantial improvement in germination of low viability seeds when these were sliced before imbibition.

7. The possible role of seed storage fungi

The incidence of fungal infection was not investigated in the present study and the possibility thus exists that some of the ultrastructural changes reported in the embryonic tissues may be attributed to fungal activity. Factors which are likely to cause proliferation of storage fungi include high temperatures and storage relative humidities (Christensen & Kaufmann, 1969).

Certain ultrastructural features have been noted in wheat and pea seeds innoculated with Aspergillus spp. (Anderson et al., 1970; Harman & Granett, 1972) including: coalescence of lipid bodies, plasmalemma rupture, cytoplasmic shrinkage, and mitochondrial damage. Since these changes were noted in the present study and during ageing in seeds presumed to be free of fungi (Berjak & Villiers, 1972 and; Villiers, 1973; Simola, 1974) it may be difficult to separate the effects of ageing per se, from fungally-mediated activity unless experiments are specifically designed to consider this possibility. Some studies of this kind have recently been forthcoming (McLean, 1986; Berjak, Dini & Gevers, 1986; Samuel, 1986) and indicate that deteriorative changes are similar but more profound in fungally-infected seeds than the equivalent non-infected seeds than the equivalent non-infected material.

CHAPTER 6

PEROXIDATION IN SEED AGEING

While there have been some direct investigations on the possible involvement of free-radicals in seed deterioration (Conger & Randolph, 1959, 1968; Priestley et al., 1980; Buchvarov & Gantcheff, 1984) results have been equivocal. Other studies have sought indirect evidence for free-radicals by examining unsaturated fatty acid levels since, it is argued, these molecules would be susceptible to free-radical attack. This approach has also yielded equivocal results (Priestley & Leopold, 1979; Stewart & Bewley, 1980). In these latter studies it has been suggested that differences in technique may have been responsible for the contradictory results obtained when soybean seeds were subjected to similar accelerated ageing regimes (Stewart & Bewley, 1980). In a later study Priestley & Leopold (1983) suggested that accelerated ageing fails to bring about a loss in unsaturated fatty acids, but under milder conditions loss of unsaturation is noted.

Another more direct approach has been to assay for the presence of lipid peroxides. Van Staden, Davey & du Plessis (1976) examined oils from viable and non-viable seeds of Protea by both U.V. spectroscopy for conjugated dienes, and iodometrically for the presence of hydroperoxides. They reported no appreciable differences between viable and non-viable seeds by either of these techniques. Changes seen later during seed imbibition were regarded as a result, and not the cause, of viability loss. This contrasts markedly with the work of Rudrapal & Basu (1982) which showed a clear negative correlation between hydroperoxide levels and viability in seeds of wheat and mustard. In addition Pearce and Abdel Samad (1980) were able to show declines in fatty acids with loss of viability in peanut seeds, but were unable to detect the presence of hydroperoxides.

In the study reported here fatty acids and hydroperoxide levels were examined in seeds held in storage at a range of relative humidities and temperatures. The modified iodometric technique of Beuge & Aust (1982) was the method of choice for the determination of lipid peroxidation, as it was reported to be particularly sensitive. In addition, some attention was given to possible changes in lipid unsaturation and peroxide levels during imbibition. It has been suggested from ultrastructural studies on lettuce seeds (Villiers, 1973), and inferred from measurements of malondialdehyde levels in imbibing soybean axes (Stewart & Bewley, 1980) that the effects of peroxidation in the dry seed become evident, or are possibly amplified on imbibition.

MATERIALS AND METHODS

(1). SEED GRINDING TECHNIQUES AND LIPID EXTRACTION

(a) Dry Seeds:

Dry seeds were ground in a mortar and pestle with liquid nitrogen until a fine homogenous powder was obtained when examined with the naked eye. Powders were placed in beakers over silica gel and allowed to warm up to room temperature to prevent possible hydration. 2g of seed powders were placed in screw-cap bottles and the total lipids extracted for 20 minutes with 20ml of chloroform: methanol (3:1) containing BHT (butylated hydroxytoluene) as an antioxidant. Folsch washing and further treatment were as described below (c).

(b) Imbibed seeds:

Two lots of 2.5g of dry seed were placed in two 9 cm diameter Petri dishes lined with two sheets of Whatman No. 1 filter paper. 10 ml of water was added and seeds allowed to imbibe for appropriate intervals. At required times seeds were mixed with an equal volume of acid washed sand (BDH) and the contents of the two Petri dishes transferred to a dry mortar which

had been held in a boiling water bath. 5 ml of boiling water-saturated butanol was pipetted onto the seeds, followed by rapid grinding with a heated pestle. A further 5 ml of boiling water-saturated butanol was added and grinding continued until a thick paste was obtained. To extract lipids this was washed with 50 ml of chloroform : methanol (3:1) containing 2% BHT as an antioxidant (Christie, 1973). Folsch washing and other treatments were as described below in (c) and (d).

(c) Lipid cleanup:

After filtration the crude lipid extract was subjected to a Folsch wash with 0.1% aqueous NaCl. The aqueous layer and non-lipid contaminants were removed by aspiration after centrifugation in a bench-top centrifuge. After drying with anhydrous Na_2SO_4, the extract was further cleared of further fine debris by filtration by passage through a Millipore pre-filter membrane. Solvents were removed by placing samples in test-tubes in a water bath at 45°C and bubbling with N_2. The total extracted lipid was used for hydroperoxide determination as outlined in (d) below.

(d) Hydroperoxide determinations (After Beuge & Aust, 1978).

1 ml of acetic acid : chloroform (3:2 v/v), previously depleted of oxygen by bubbling with nitrogen at 4°C, was added to the extracted lipid. This was then followed by the addition of 0.05 ml of potassium iodide solution (made up fresh each day by adding 6 g of potassium iodide to 5 ml of water previously bubbled with nitrogen at 4°C). The reaction mixtures were then placed in the dark for 5 minutes before the addition of 3 ml of cadmium acetate (0.5 g in 100 ml of water). The solution was then transferred to a centrifuge tube and spun at maximum Xg in a benchtop centrifuge for 5 minutes. 1 ml of the upper phase was diluted with 9 ml of distilled water and the absorbance at 353 nm read against a blank containing complete assay mixture without the lipid. A standard curve was prepared using cumene hydroperoxide as the peroxide standard.

(2) LIPID FRACTIONATION

(a) Silicic acid Column Chromatography:

Lipids were washed from test tubes with a small volume of chloroform (1 ml) and applied to the top of the column (Biorad Econo-columns 160mm x 20mm O.D) of 3.5 g of silicic acid slurried in chloroform. Successive elutions with 13 ml of chloroform, acetone and methanol yielded neutral and free-fatty acids, glycolipids and phospholipids, respectively (Beutelmann & Kende, 1977).

(b) Silicic acid Batch Fractionation:

0.5 ml of total lipids extracted according to methods (a) and (c), above, were dissolved in an equal volume of chloroform and the solution applied to 3.5 g of dry activated silicic acid in a 100 ml flask. The silicic acid was successively washed with two 10 ml volumes of chloroform, acetone and methanol, the solvents being decanted after each washing. The last solvent fractions were pooled and taken to dryness under nitrogen at 45°C.

(c) Phospholipid precipitation:

15 ml of acetone were added to a 1 ml of dried total lipid and the mixture kept overnight at 5°C. 50 µl of methanol saturated with $MgCl_2$ were added to the cold solvent and the mixture centrifuged at maximum Xg in a benchtop centrifuge. The pelleted precipitate was taken up in 1 ml of chloroform : methanol (3:1) from the centrifuge tube. Two further washings were made of the tube to yield a final volume of 3 ml. This represented approximately 100 µg/ml phospholipid; 10 - 20 µl were applied to TLC plates. Phospholipid was estimated to be approximately 1.6% of the seed dry weight.

(d) Thin Layer Chromatography:

Extracted lipids were applied with a fine capillary 1.5 cm from the

edge of 20 x 20 cm glass plates coated with Kieselgel 60 (E. Merck). All plates were heated to 120°C for 2 hours and used on cooling. The following solvent systems were employed for two dimensional development .

i. Chloroform/Methanol/Ammonium Hydroxide (25%) 65 : 35 : 4 for $3\frac{1}{2}$ hours.

ii Chloroform/Methanol/Acetic Acid/Water (25 : 15 : 4 : 2) for $2\frac{1}{2}$ hours.

For comparative purposes a sample of purified brain phospholipids (Thames Reagents, Oxford) was run at the same time to assist with identification and selection of run times.

Phospholipids prepared by acetone precipitation or by silicic acid fractionation were subjected to development in one direction with either of the following solvent systems:

i. Chloroform/Acetone/Methanol/Acetic Acid/Water (10 : 4 : 2 : 2 : 1) for 2 - $3\frac{1}{2}$ hours.

ii. Di-isobutyl ketone/Acetic Acid/Water (40 : 25 : 3 : 7) for 2 hours.

Where appropriate phospholipid standards were purchased from Sigma Chemicals and run with extracted phospholipids.

(e) <u>High Performance Liquid Chromatography</u> (After Chen & Kou, 1982)

Chromatography was carried out using a Waters Associates system consisting of a Model 6000 solvent delivery system, Wisp autosampler, variable wavelength detector and data module. The chromatographic column was a 30 cm x 3.9 mm I.D. prepacked stainless steel column packed with 10 µm particles of silica gel (Waters microporasil).

The solvent system of acetonitrile-methanol-85% phosphoric acid (130 : 5 : 1.5 : v/v) was delivered to the column at a flow rate of 1

ml/min at a pressure of 34 bar at room temperature (23°C).

Detection was at 203 nm with recorder response set at 0.4 a.u.f.s. Phospholipids prepared by silicic acid column chromatography after evaporated to dryness in a stream of nitrogen (see (2) (a) above) were taken up in 1 ml of chloroform : methanol (3 : 1) and placed in auto-sampler vials. Analysis was undertaken within 24 hours of preparation.

(3). TOTAL LIPID FATTY ACID ANALYSIS

(After Priestley & Leopold, 1979)

Ground seed samples (0.5g each, prepared as described in 1(a), above) were placed in pear-shaped flasks (15 ml) fitted with a water-cooled glass condenser. 4 ml of methanolic potassium hydroxide (0.5M) was added and samples boiled for 5 minutes. Thereafter 4 ml of boron trifluoride in methanol (14% w/v, E. Merck) was added through the condenser and the sample boiled for a further 2 minutes. The flasks were then removed and placed in iced water and 5 ml of saturated aqueous sodium chloride added. The methyl esters were partitioned into 3 ml of n-hexane which was clarified by centrifugation in a benchtop centrifuge.

1 µl samples were injected into a Pye 104 gas chromatograph fitted with a glass column (1.5m x 4mm I.D.) and flame ionization detector. The oven was operated isothermally at 180°C, with nitrogen as the carrier gas at 30 ml per minute. Column packing material was Celite (B.D.H.) with a 10% loading of polyethylene glycol succinate. Peak integration was initially by triangulation but in later studies use was made of an electronic integrator (Hewlett-Packard 3380). All results were expressed as area percentages.

Table 6.1

Total fatty acid levels of dry and imbibed lettuce seeds after 34 months storage at 20°C and a range of relative humidities. For additional comparison the fatty acid levels are given for two seed lots after 7 hours imbibition. Values expressed as area percentages, calculated by triangulation from gas chromatographs. Data are the mean of 2 injections

+, * moisture contents not determined.

- no detectable level.

	Storage R.H.	FATTY ACID 16:0 Palmitic	18:0 Stearic	18:1 Oleic	18:2 Linoleic
DRY SEED	0%	6.7	1.4	21.8	69.9
	20%	7.4	1.2	24.1	67.1
	80%	7.6	2.0	21.7	68.5
7 HOURS IMBIBITION	0%	6.0	1.7	24.4	67.8
	20%	6.6	1.2	26.2	65.8
	40%	6.8	2.7	21.9	68.4
	60%	6.6	1.7	23.7	67.8
	80%	6.5	2.0	25.9	65.4
NEW PURCHASE	+	5.8	-	25.2	68.9
10 YEARS OVER SILICA GEL	*	7.3	-	23.5	69.1

Table 6.2

Percentage germination for 1978 purchase of lettuce seed, cv. Great Lakes, stored for 34 months at 20°C and the relative humidities indicated.

Storage Relative Humidity	% germination (61 hours)
0%	98
20%	97
40%	94
60%	0
80%	0

Table 6.3

Hydroperoxide levels of three separate seed lots of lettuce after various storage regimes. The total extractable lipid was fractionated by silicic acid column chromatography into 3 fractions and the peroxide values determined. Values are expressed as absorbance units and as a percentage of the total (sum of absorbance of 3 fractions), in parenthesis. Note discrepancies are evident between the unfractionated lipid (unfractionated value) and the values for individual fractions or their sum (total absorbance value).

CONDITIONS OF STORAGE	FRACTION 1 NEUTRAL LIPID	FRACTION 2 GLYCOLIPIDS	FRACTION 3 PHOSPHOLIPIDS	TOTAL ABSORBANCE VALUE	UNFRACTIONATED VALUE
9 Years over silica gel at 20°C	0.82 (70%)	0.33 (29%)	0.01 (1%)	1.168	0.27
50 Months at 60% RH and 20°C	1.23 (85%)	0.15 (10%)	0.07 (5%)	1.448	2.45
22 Months at 80% RH and 30°C	0.04 (41%)	0.01 (12%)	0.04 (47%)	0.094	0.85

Table 6.4

Hydroperoxide levels in the total lipid extract of lettuce seed before, and at various times after, imbibition. Levels are expressed as molar equivalents of cumene hydroperoxide.

* Same seed lots reported in Figure 2.1.

\- Values not determined.

STORAGE CONDITIONS (ALL AT 20°C)	HYDROPEROXIDE LEVEL, MOLAR			
	DRY SEED	HOURS IMBIBITION 7	8	9
Recently purchased high viability lot (1981) Strain 659	6×10^{-6}	7.8×10^{-5}	4.8×10^{-5}	8.6×10^{-5}
10 Years over silica gel (Big Boston)	1.8×10^{-5}	-	8.3×10^{-5}	8×10^{-5}
34 Months at 0% RH *	1.8×10^{-5}	1×10^{-4}	-	6.6×10^{-5}
34 Months at 40% RH *	7.5×10^{-5}	1×10^{-4}	-	3×10^{-4}
34 Months at 80% RH *	-	4×10^{-5}	-	-

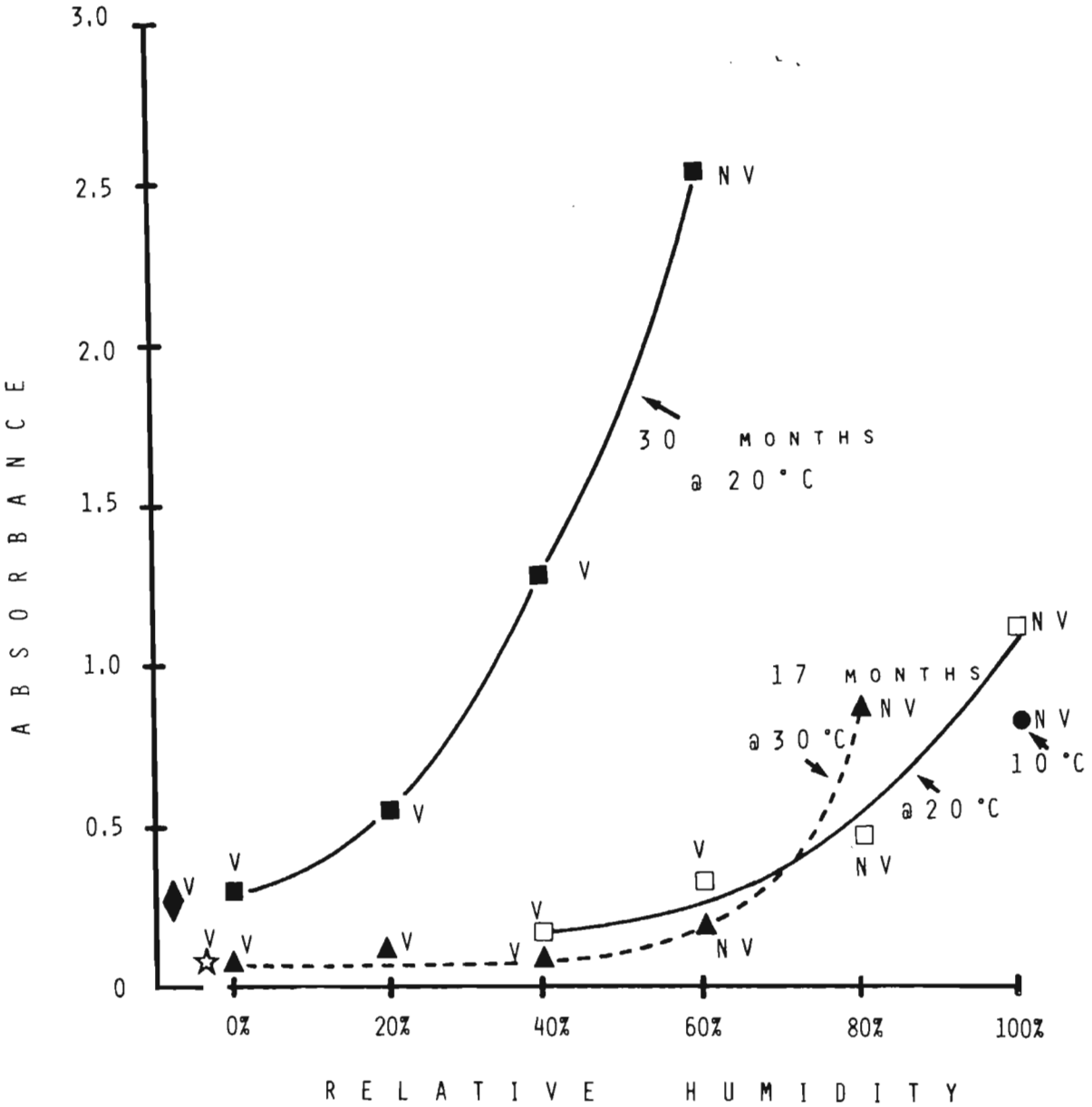


FIGURE 6.1

Hydroperoxide levels of the total lipid extract of two lots of lettuce seeds after storage at a range of relative humidities for 30 and 17 months. Storage temperatures are indicated on the Figure, and whether seeds were viable (V) or non-viable (NV). Only a single value is presented for seed stored at 10°C (solid circle). Note also the low hydroperoxide values for other seed lots held for 6 & 12 months at 20°C (asterisk) or for 8 years over silica gel at 20°C (cv. Big Boston, solid diamond) which lie at the lower limits of detection by the assay system.

RESULTS AND DISCUSSION

(A) Lipid Hydroperoxides and Fatty Acid Levels

No obvious decline was evident in the total lipid fatty acid fraction of either dry or imbibed seeds (Table 6.1) which could be correlated with either loss of viability or vigour (Table 6.2). Furthermore, after 7 hours imbibition it was seen that fatty acid levels of a recently purchased high viability seed lot (1982 purchase, 98% germination) were in no way different from seed which had been stored for 8 years over silica gel (1973 purchase, c.v. Big Boston, germination 53%).

Hydroperoxide levels for the total lipid of two seed lots stored at 10°, 20°, and 30°C and a range of relative humidities for 17 and 30 months showed a clear increase in a time and RH-dependent manner (Figure 6.1). The kinetics of peroxide development at the temperatures investigated suggest some similarities to the classical pattern of autoxidation, viz. an induction period followed by a phase of rapidly accelerating oxidation (Swern, 1964). However this relationship was more strongly dependent on the prevailing RH of storage than on time. It was especially noticeable that above 60% RH the induction period was considerably reduced as seen by the rapid peroxide increases for seeds held for 17 months at 20° and 30°C.

It was further evident that seeds could not be assigned as viable or non-viable on the basis of an attainable hydroperoxide value, *per se*. Thus comparatively low peroxide levels were seen in seeds which were non-viable after storage at 60% RH and 30°C, while for seeds held at 20°C and 40% RH exceptionally high values were recorded although seeds were still viable. Significant peroxidation was also evident *post-mortem*.

Since the peroxide determinations presented in Figure 6.1 were those

of the total lipid extract, an attempt was made to evaluate the contributions made individually by the neutral, glycolipid and phospholipid fractions. Fractionation of lipids was undertaken as described in Materials and Methods, and the hydroperoxide levels were determined as absorbance for the individual fractions without reference to the mass of each fraction (Table 6.3). If peroxidation were to occur uniformly throughout all lipid fractions of the seed it might perhaps be expected that the percentage contributions of each fraction would remain relatively constant between samples; this was clearly not the case. An increasing severity of ageing regime was seen to bring about a progressive rise in the peroxide level of the phospholipid fraction. It should, however, be noted that only the first sample (9 years at 20°C over silica gel) was viable, and the trend observed is one of progressive post-mortem changes. A feature typical of edible oil oxidation is a decline in the peroxide value at an advanced stage of oxidation (Swern, 1964). Some evidence for this pattern may be evident in the values for the unfractionated lipid (Table 6.3). The low level of 0.85 for the seeds stored for 22 months may however represent the combined interactions of the higher temperature and RH.

It is also possible to infer from the data presented that phospholipids are relatively resistant to peroxidation in relation to the other lipid fractions. This may be a reflection of the activity of membrane-associated anti-oxidants such as tocopherols. While the importance of membrane peroxidation is likely to be of profound importance to cellular compartmentation and membrane functioning, it is nevertheless possible to envisage peroxidation of the neutral storage lipids as having a profound effect on metabolism at germination. Most of the neutral lipid fraction is likely to be contained within the lipid bodies and although relatively discrete structures, they are nevertheless membrane bound and their properties could thus be profoundly influenced by peroxidation of the lipid body contents. As was observed in the ultrastructural studies, altered properties including movement and/or fusion of the lipid droplets could be identified as one of the early deteriorative events (Chapter 5). On the other hand, free-radical or

hydroperoxide damage produced in dry storage may lead to increased damage on imbibition, either as a result of autocatalysis, deficiencies in the defence mechanisms, or through the action of damaging breakdown products. Although there are no direct studies in this area there are some studies unrelated to seed ageing per se, in which these possibilities have been raised (Pauls & Thompson, 1980; Senaratna McKersie & Stinson, 1985).

Further evidence for the above suggestion is reported in the study below in which hydroperoxide levels were determined for dry seeds and after 5, 8 or 9 hours imbibition (Table 6.4). It is evident that hydroperoxide levels can increase significantly on imbibition, even in unaged seeds of high vigour. The overall trend from all these results is of a progressive reduction in hydroperoxide levels between 7 and 9 hours post imbibition. Whilst the vigour of the seeds stored at 40% RH was reduced when compared with seeds of the same lot stored at 0% RH, comparable germination was achieved (94% and 98%, respectively, at 61 hours). However, from the germination studies it is evident that cotyledonary necrosis had become a noticeable feature in seedlings stored at 40% RH (Chapter 2). It remains to be seen what relative contributions of the cotyledons and the radicle are to overall seed peroxidation. Although not extensively investigated, the single determination for seeds stored at 80% RH, and which were non-viable, was low. This might indicate that in post-mortem material hydroperoxides may break down either during storage or on imbibition. Such a proposal would be in keeping with the evidence from model studies in peroxidizing oils (Swern, 1964) and is partially supported by the results obtained in Table 6.3.

Overall, the results presented show that while lipid peroxidation may occur in storage, the level of which may increase with severity of storage, no simple relationship could be seen between viability and hydroperoxide level. Further, analysis of lipid fatty acids suggested, as did those of Priestley & Leopold (1979) that loss of unsaturation was unrelated to ageing in lettuce seeds. This conclusion is, however, subject to certain reservations.

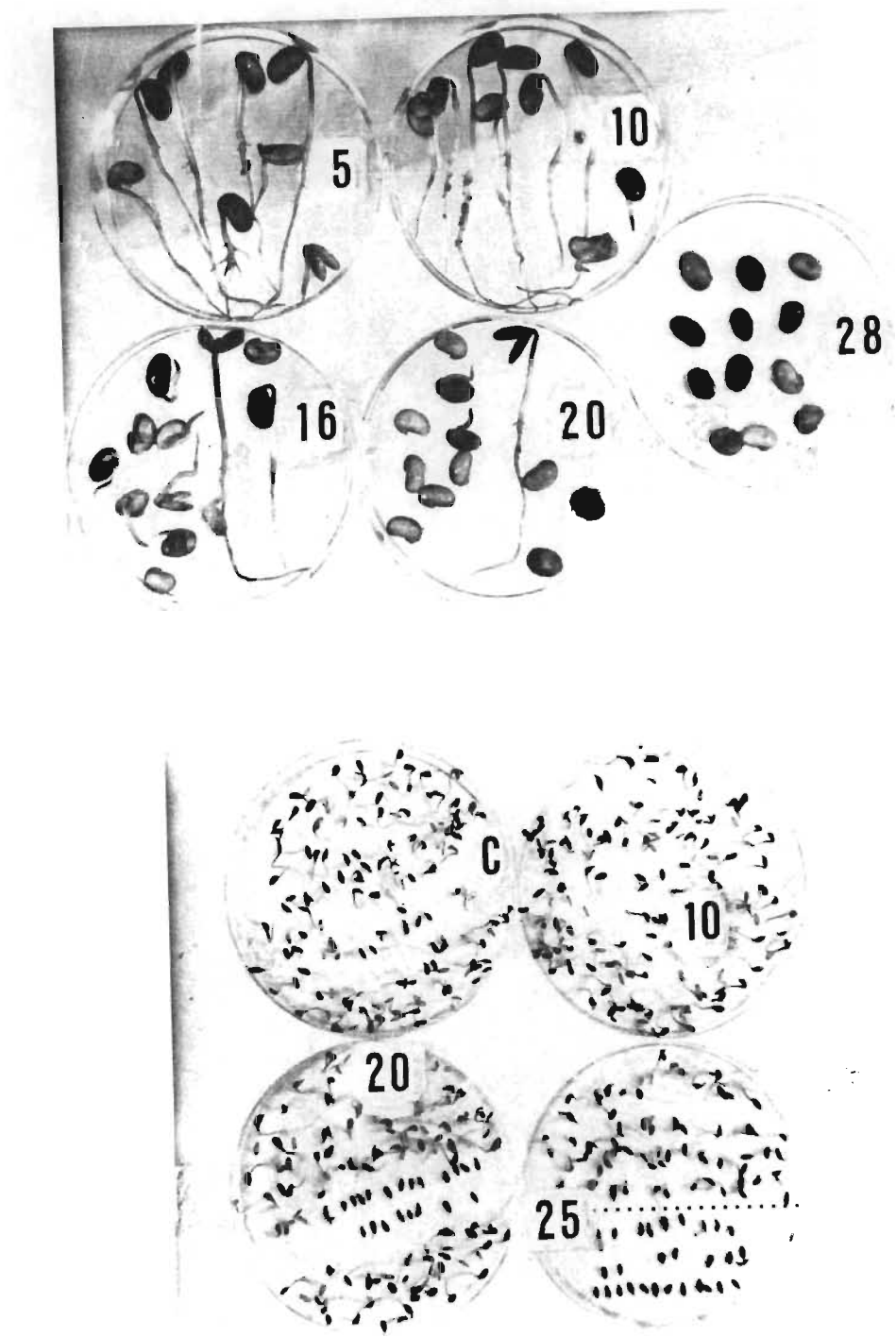


FIGURE 6.2

Illustrates the appearance of seedlings of soybean (upper) and lettuce (lower) after being subjected to an accelerated ageing regime of 30°C and 100% RH for the number of days indicated (C, control). Soybean seedlings were germinated in vermiculite in a greenhouse and exhumed, while lettuce seeds were germinated on filter paper and photographed after 1 week.

Note that although some of the lettuce seeds after 25 days ageing treatment show radicle protrusion, (lower portion of Petri dish), further growth did not take place.

Table 6.6

Levels (as area percentages) of the 5 major fatty acids present in seeds of soybean (cv. Hartebeest) after an accelerated ageing regime of 30°C and 100% R.H. Standard deviations are given in parenthesis, and the total number of separate determinations indicated. Superscripts denote significance levels obtained by the Student's t test between the control and the successive sampling intervals of the ageing treatment. A - .001 ; B - .002 ; C - .01 ; D - .02 ; E - .05 ; F - .1

DAYS AGEING	CONTROL	10	16	20	28
GERMINATION AT 5 DAYS	83%	83%	58%	25%	0%
Fatty Acid					
16:0	11.3 (0.23)	11.16 (0.45)	11.25 (0.51)	11.28 (0.08)	12.35 (0.24) ^{A}
18:0	3.2 (0.15)	3.36 (0.05)	3.42 (0.27)	3.22 (0.13)	3.51 (0.1) ^{C}
18:1	20.8 (0.38)	21.4 (0.45)	21.7 (0.77) ^{F}	21.56 (0.21) ^{C}	22.04 (0.13) ^{A}
18:2	53.2 (0.62)	53.2 (0.05)	52.82 (0.68)	53.14 (0.11)	52.42 (0.19) ^{B}
18:3	10.86 (0.2)	10.5 (0.05) ^{E}	10.37 (0.22) ^{D}	10.46 (0.11) ^{C}	8.85 (0.35) ^{A}
Number of separate Determinations	3	3	4	5	6

Table 6.7

Levels (as area percentages) of the 5 major fatty acids present in seeds of lettuce (cv. Great Lakes) after an accelerated ageing regime of 30°C and 100% R.H. Standard deviations in parenthesis, and total number of separate determinations indicated. No significance was found between any successive sampling intervals when applying the student's t test.

DAYS AGEING	CONTROL	5	10	16	20	25
GERMINATION AT 4 DAYS	99%	97%	97%	88%	73%	65%
Fatty Acid						
16:0	7.42 (0.15)	7.26 (0.23)	7.28 (0.37)	7.43 (0.34)	7.28 (0.2)	7.23 (0.29)
18:0	1.88 (0.06)	1.8 (0.1)	2.1 (0.35)	1.96 (0.17)	1.9 (0.2)	1.3 (1.02)
18:1	22.2 (0.6)	22.62 (0.16)	22.8 (0.97)	22.5 (0.28)	22.8 (1.3)	23.18 (1.07)
18:2	66.7 (0.82)	66.6 (0.15)	66.46 (0.81)	66.3 (0.27)	66.47 (0.69)	66.8 (0.42)
18:3	1.49 (0.19)	1.56 (0.11)	1.61 (0.24)	1.55 (0.05)	1.72 (0.2)	1.46 (0.1)
Number of separate Determinations	4	5	4	6	4	5

Since data was not treated statistically, and area percentages varied by approximtely 2% it was not possible to exclude the possibility that very small changes in fatty acid levels were occurring. Furthermore, the total lipid fatty acid levels were examined by direct hydrolysis/methanolysis of ground seed powders. Priestley & Leopold (1979) believed this to be a satisfactory technique which yielded almost identical fatty acid levels to those obtained on esterification of solvent-extracted lipid, but the validity of this assumption for lettuce was not tested.

An examination of the fatty acids of the polar lipid fraction of whole seeds of soybean revealed no change with ageing although levels of phosphatidylcholine and phosphatidylethanolamine declined when measured as phosphate (Priestley & Leopold, 1979). In a later study it was suggested that accelerated ageing produced no change in fatty acid levels, while ageing in the long term (40°C, low RH for 44 months) resulted in loss of unsaturation (Priestley & Leopold, 1983).

However since the studies on lettuce seed fatty acid levels may be regarded as ageing in the long term, the absence of change was not as expected (Priestley & Leopold, 1983). This may of course have been a reflection of differences between the two genera, and in order to investigate this possibility seeds of soybean and lettuce were subjected to accelerated ageing at 30°C and 100% RH for up to 28 days. It was argued on the basis of results obtained thus far with lettuce and those of Priestley & Leopold for soybean that no large changes would be expected; if any changes were evident in the fatty acid levels of lettuce these would probably be of the order of 2% (see above). In order to detect any small differences which may have been present, many separate determinations (3-6 per ageing treatment) were made on seed powders using the direct hydrolysis/methanolysis technique of Priestley & Leopold (1979). Furthermore a minimum of two injections per isolation were made. Data from this study are presented in tables 6.6 & 6.7, and a photographic record of the germinating seeds is presented in Figure 6.2.

For soybean, analysis of the changes in the 18:3 fatty acids between

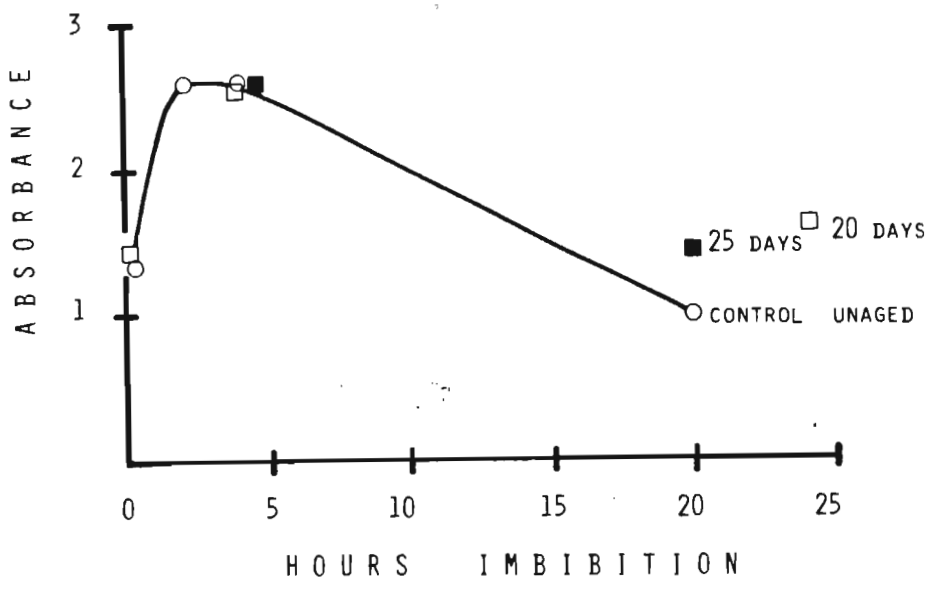


FIGURE 6.3

Changes in lipid hydroperoxide levels of lettuce seeds during the course of imbibition. Seeds were aged for 20 and 25 days at 30°C and 100% RH.

the control and the successive sampling intervals by the Student's t test revealed a progressive increase in the significance levels; furthermore in the latter stages of ageing a significant increase in 18:1 fatty acids was seen. On the other hand, there was little significant change in 18:2 levels (Table 6.6). Thus a complete loss of viability by the 28th day was accompanied by a 2% loss of 18:3 fatty acids in total extractable lipids.

Lipid hydroperoxide levels were seen (Table 6.5) to rise progressively with the duration of ageing treatment, rising substantially over control values with total viability loss.
On the other hand for lettuce seeds there was no clear decline in the 18:3 fraction (Table 6.7) and rises in hydroperoxide levels were small although possibly of some value as an indicator of deterioration (Table 6.5). Since hydroperoxide levels of seeds were seen to be capable of substantial increase on imbibition (Table 6.4), this possibility was also investigated for lettuce seeds subjected to accelerated ageing. However, since the hydroperoxide values obtained from the dry seeds were low the assay technique was modified as outlined in Materials and Methods. Seeds which had been imbibed for 10 minutes served as a control in order to allow for any possible artefactual peroxidation caused by the heating and grinding treatments. Results indicated (Figure 6.3) as had been previously observed (Table 6.5) that there was litle, if any, difference between the hydroperoxide levels of dry unaged seeds and those subjected to 20 days of the ageing regime where germination had fallen to 73%.

Hydroperoxide levels rose rapidly following imbibition, peaking by the 7th - 8th hour and although the values presented were single determinations for each sample there was clear evidence to suggest that hydroperoxides declined more slowly in seeds which were subjected to ageing treatments. This might suggest that cellular mechanisms for the elimination of hydroperoxides may be impaired in some way compared with unaged material. Further discussion of the results in this section will be held over until the end of this chapter.

Table 6.8

Hydroperoxide levels (as absorbance units) for the acetone-precipitated phospholipids of three seed lots stored as indicated. In order to increase absorbance readings (cp. Table 6.3) 1 ml of the upper reaction phase was diluted with 3 ml of water (see materials and methods). * denotes the same seed lot.

STORAGE CONDITIONS (ALL AT 20°C)	GERMINATION	HYDROPEROXIDE VALUE (absorbance)
1½ Years over silica gel	91% at 30 hours	0.17
30 Months at 40% RH *	54% at 40 hours; 88% at 63 hours	1.224
30 Months at 80% RH *	20 Months post-mortem	0.12
10 Years over silica gel cv. Big Boston	45% at 40 hours; 55% at 65 hours	0.35

(B) Thin Layer Chromatography of Phospholipids

There are numerous studies which have shown changes in the phospholipids of dry seeds in ageing, either as a decline in unsaturation of fatty acids or as phospholipid phosphate and these are presented in summary form in Table 6.10 at the end of this Chapter. The possibility of using thin-layer chromatography for the separation of phospholipids prior to further analysis was therefore explored. Total seed lipids were extracted and the phospholipid fraction subjected to two dimensional thin layer chromatography (2D TLC) as outlined in Materials and Methods. A sample of purified brain phospholipids was run for comparative purposes. For reasons which were not immediately apparent, it proved difficult to obtain reproducible separations. It was nevertheless possible to tentatively identify phospholipids present in lettuce seeds on the basis of representative, published chromatograms (Kates, 1975; Christie, 1973) as being: phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidic acid and phosphatidylserine.

However because of the lengthy development times (5 - $5\frac{1}{2}$ hours total) and the lack of reproducibility, 2D TLC was abandoned in favour of single solvent development. After loading 15µl of the chloroform : methanol solution of the acetone-precipitated phospholipids (approximtely 25 mg) onto the plate, the remaining sample was taken to dryness under nitrogen and the hydroperoxide values determined as described in Materials and Methods. These results indicated (Table 6.8) that peroxidation of phospholipids in seeds of high vigour was low, while values for seeds of reduced vigour stored at 40% RH and 20°C for 30 months were approximately sevenfold higher. The lowest values were recorded for the phospholipids of post-mortem seed, which is in keeping with the earlier observations concerning long-term declines in the peroxide values of autoxidizing oils (Swern, 1964) and total hydroperoxide data presented in Table 6.3. Although the viability of a seed lot stored over silica gel for 10 years was subtantially reduced, hydroperoxide levels were only twice that of phospholipids from high

FIGURE 6.4

A. Thin layer chromatography plates of seed and phospholipid standards.

Lanes 1-4 standards, lanes 5-12 seed phospholipids.

lane 1 : purified brain phospholipids (Folsch technique) comprising phosphatidylethanolamine (PE) cerebrosides and others.

lanes 2 & 4 : Diphosphatidylglycerol (DPG) standard (Sigma C 3760).

lane 3 : Phosphatidylethanolamine (PE) standard.

lanes 5 & 6 : 1980 seed purchase of high viability (20µl & 10µl loadings respectively).
PS tentative identification of phosphatidylserine.

lanes 7 & 8 : 1973 seed purchase (cv. Big Boston) after 8 years storage over silica gel at 20°C (53% germination).

lanes 9 & 10 : 1979 purchase of seed stored for 21 months at 80% RH (non-viable).

lanes 11 & 12: 1979 purchase stored for 2 years at 40% RH (86% germination).
Development time 3½ hours in a solvent system consisting of chloroform : acetone : methanol : acetic acid : water (10:4:2:2:1).

B. Lanes 1-9 standards; lanes 10-15 seed phospholipids.

lanes 1-3 : Phosphatidyl inositol (PI) standard (Sigma P6636; 50% by TLC containing PE and phosphatidylinositol (PI) as impurities.

lanes 4 & 5 : Phosphatidylserine (PS) standard (Sigma 86641). BP? possible breakdown product.

lanes 6 & 7 : Phosphatidylcholine (PC) standard (Sigma P6638, Bovine).

lanes 8 & 9 : Diphosphatidylglycerol (overloaded).

lanes 10 -12 : 8 years storage over silica gel, cv. Big Boston (see also lanes 7 & 8, Figure a).

lanes 13-15 : 1979 purchase, 2 years storage at 80% RH (non-viable). Compare also with lanes 9 & 10, Figure a
Development time 2 hours, solvent system as in A, above.

C. Lanes 1, 2 & 11-16 Phospholipid standards; lanes 3 & 4 viable seed; lanes 6 & 7 non-viable seed.

lane 1 : Phosphatidylcholine

lane 2 : Phosphatidylethanolamine

lanes 3 & 4 : 8 years storage over silica gel, cv. Big Boston (20µl & 10µl loading respectively).

lane 5 : Phosphatidic acid (PA) standard (Sigma PO 394 D,L dipalmitoyl PA).

lanes 6 & 7 : 1976 purchase, 5 years storage at 60% RH for 2 years, thereafter in hermetic storage. Non-viable.

lane 8 : Phosphatidylcholine (PC).

lanes 9 & 10 : 2 years storage at 30°C & 80% RH (1979 purchase), seed non-viable.

lanes 11 & 12: Diphosphatidylglycerol.

lane 13 : Phosphatidylethanolamine.

lanes 14 & 15: Phosphatidylethanolamine.

lanes 16 & 17: Phosphatidylinositol.

Development time 2 hours in solvent system of Di-isobutyl Ketone: acetic acid: water (40:25:3.7).

Seed phospholipids in Figures A, B & C prepared by acetone precipitation.

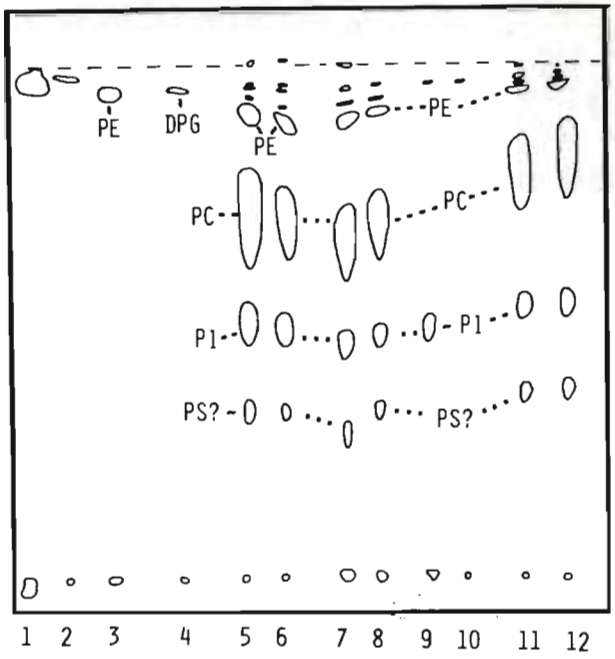
D. Evaluation of comparative efficacy of different methods of phospholipid preparation.

lanes 1-3 : Acetone precipitated phospholipids of : lettuce (lane 1), peanut (lane 2), soybean (lane 3).

lanes 4-6 : Silicic acid column fractionation of phospholipids of: lettuce (lanes 4 & 6) and peanut (lane 5).

lanes 7-9 : Silicic acid batch isolation of phospholipids of: soybean (lane 7), lettuce (lane 8) and peanut (lane 9).

Development time 2½ hours in same solvent system as C, above.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DPG
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PC
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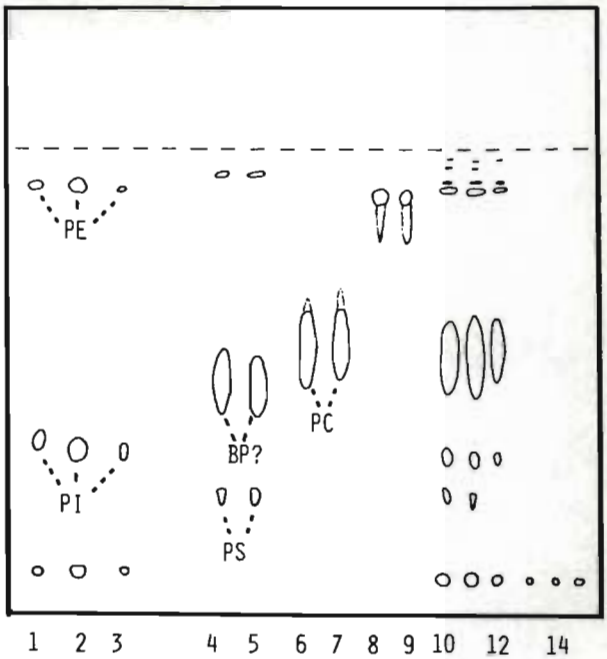
PS?
1 2 3 4 5 6 7 8 9 10 11 12

A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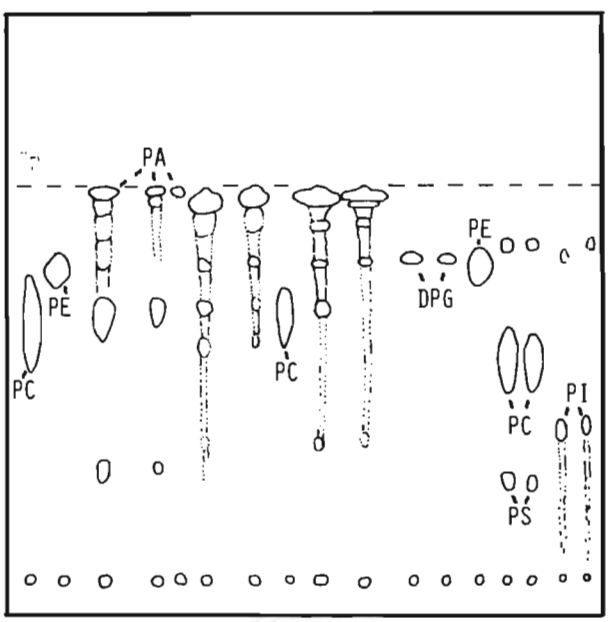
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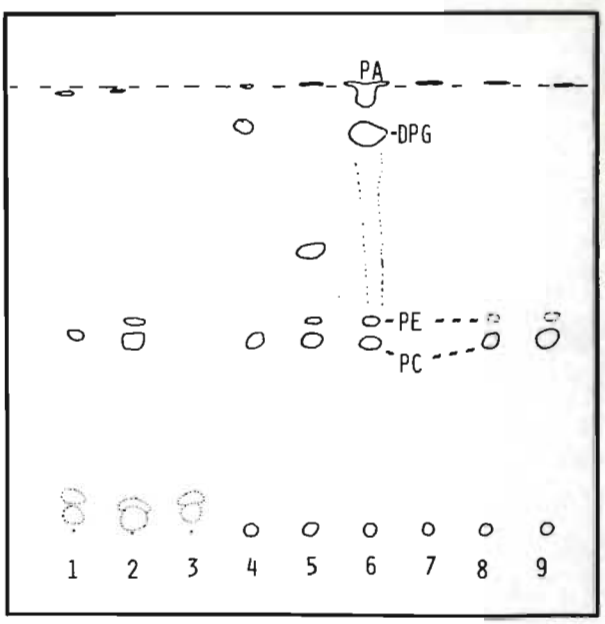
PA
DPG
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1 2 3 4 5 6 7 8 9

D

vigour seed. This observation is consonant with data presented in Table 6.1 which point to only moderate increases in peroxidation at relative humidities below 20%.

Representative chromatograms from the one-dimensional thin layer chromatography studies are presented in figure 6.4 A - D. Diphosphatidyl-glycerol (cardiolipin) and phosphatidic acid were identified close to the solvent front (Figure 6.4A; Lane 4; Figure 6.4C, Lane 5) although these were widely separated by the 2D TLC solvent system. As was found in the 2D TLC studies, non-viable samples (49 months at 60% RH and 20°C; 21 months at 80% RH and 30°C) also yielded fewer identifiable phospholipids by one dimensional development (Figure 6.4A, lanes 9 & 10; Figure 6.4B, lanes 13-15). When greater loadings were made using precipitated phospholipids from two non-viable samples satisfactory resolution of specific phospholipids was obtained. (Figure 6.4C, lanes 6 & 7; lanes 9 & 10). Although a standard for phosphatidylcholine (PC) gave unequivocal identification (Figure 6.4B, lanes 6 & 7), phosphatidylserine (PS) was not satisfactorily resolved. The standard for phosphatidylserine was known to be of low purity (Sigma P6641) and yielded three spots on chromatograms (Figure 6.4B, lanes 4 & 5). One, at Rf 9.3, was assumed to be a breakdown product, or phosphatidylethanolamine (PE) as an impurity. The major component of the sample run was considered as a breakdown product (Rf 0.45) which tended to co-chromatograph with PC (BP? in Figure 6.4B). Likewise phosphatidylinositol (PI) yielded two spots on plates those of higher Rf being tentatively identified as an impurity (P E) or a breakdown product. Levels of PI have been reported to constitute a significantly greater proportion of the membrane phospholipids than PS in carrot, tobacco and sunflower tissue cultures (Phillips & Butcher, 1979). Similarly no PS was reported for soya or pea seeds (Powell & Matthews, 1981; Priestley & Leopold, 1979) or in the cotyledons of mung bean seedlings (Gilkes, Herman & Chrispeels, 1979). While there was evidence for both PS and PI in Figure 6.4A, it was concluded that the compounds of low Rf in Figure 6.4C (lanes 3, 4 8 & 9) were probably PI.

It has been stated (Kates, 1975), though without reference to specific published work, that acetone is not effective in precipitating seed phospholipids. Both Koostra & Harrington (1969) and Priestley & Leopold (1979) used this technique to recover polar lipids from the total lipid fraction of cucumber and soybean seeds, respectively. It has more recently been reported (Touchstone, Levin, Dobbins & Beers, 1983) that the supernatant in the acetone precipitation technique contains phospholipids. If this were a constant, small proportion it may not be unduly significant, but if any differential losses were operating this could possibly explain discrepancies of the kind reported by Priestley & Leopold (1979, 1983). Thus, if phosphatidic acid or lysophospholipids produced as a consequence of ageing were lost to the supernatant phosphate levels might decline without necessarily being reflected by a loss of unsaturation. In the above studies a decline in both PC and PE was observed, though with a minor decline (17%) in total extractable phospholipids for soybean and an 81% decline for cucumber when expressed as µg phosphate per unit weight. In these latter studies total viability loss had not yet ensued. The two lettuce samples used in the present study were essentially post-mortem, which may thus have led to a significant loss of phospholipid, either reflecting the in vivo situation or being an artefact of precipitation technique. It is clear from the studies of Stewart & Bewley (1979) that significant post-mortem changes are possible in soybean seeds subjected to accelerated ageing. Linoleic acid declined by 26% with loss of viability, but additional losses of 44% and 77% (of control) were seen at days 1 and 2 post-mortem.

Some evidence for the undesirability of using acetone precipitation is presented in Figure 6.4D where the phospholipids obtained from total lipid extracts of peanut, soybean and lettuce seeds were compared when fractionated by either acetone precipitation or silicic acid. Two silicic acid fractionation techniques were employed, the traditional column method or a batch extraction method involving solvent washing in a flask (See Materials and Methods). Using the same volume of oil, acetone precipitation gave a limited yield of resolvable phospholipids (Figure 6.4D, lanes 1-3), whereas column

fractionation yielded 2 or more compounds (Figure 6.4D, lanes 4-6). The batch method (Figure 6.4D, lanes 8 and 9) appeared comparable to the acetone method. Batch and acetone prepared phospholipids soybean oil failed to produce iodine spray-detectable phospholipids on TLC plates. A feature common to all acetone precipitated samples was the presence of a spot at the origin which failed to stain with iodine spray. This could indicate carry-over of some non-lipid products, and may in part explain the presence of the immobile spot observed at the origin in the studies of Koostra & Harrington (1969).

Qualitative comparisons between peanut and lettuce oil phospholipids by the three methods, using the colorimetric technique of Raheja (1968) indicated that less phosphate was obtained by acetone precipitation than either of the silicic acid methods. From these rather incomplete studies it is clearly evident that acetone precipitation should never be employed to obtain phospholipids, and results of reported studies which have done so should be evaluated with extreme caution. Some studies which fall into this category may be identified in Table 6.10 in which specific details of technique, and of some of the findings, are presented in summary form.

(C) High Performance Liquid Chromatography of Phospholipids

Since difficulties were encountered with identification and reproductibility of phospholipids by TLC methods, phospholipids were isolated by fractionation on silicic acid from the total extractable lipids prior to using HPLC techniques to identify possible changes in individual phospholipid species with ageing. Chromatographic conditions on a silica column were after the method of Chen & Kou (1982) using U.V. detection at 203 nm (See Materials and Methods). Consequently all values presented may reflect changes in double bonds rather than loss of phospholipids per se. Although all data presented is for a single extraction with an average of two injections per sample the superiority of the technique was clearly

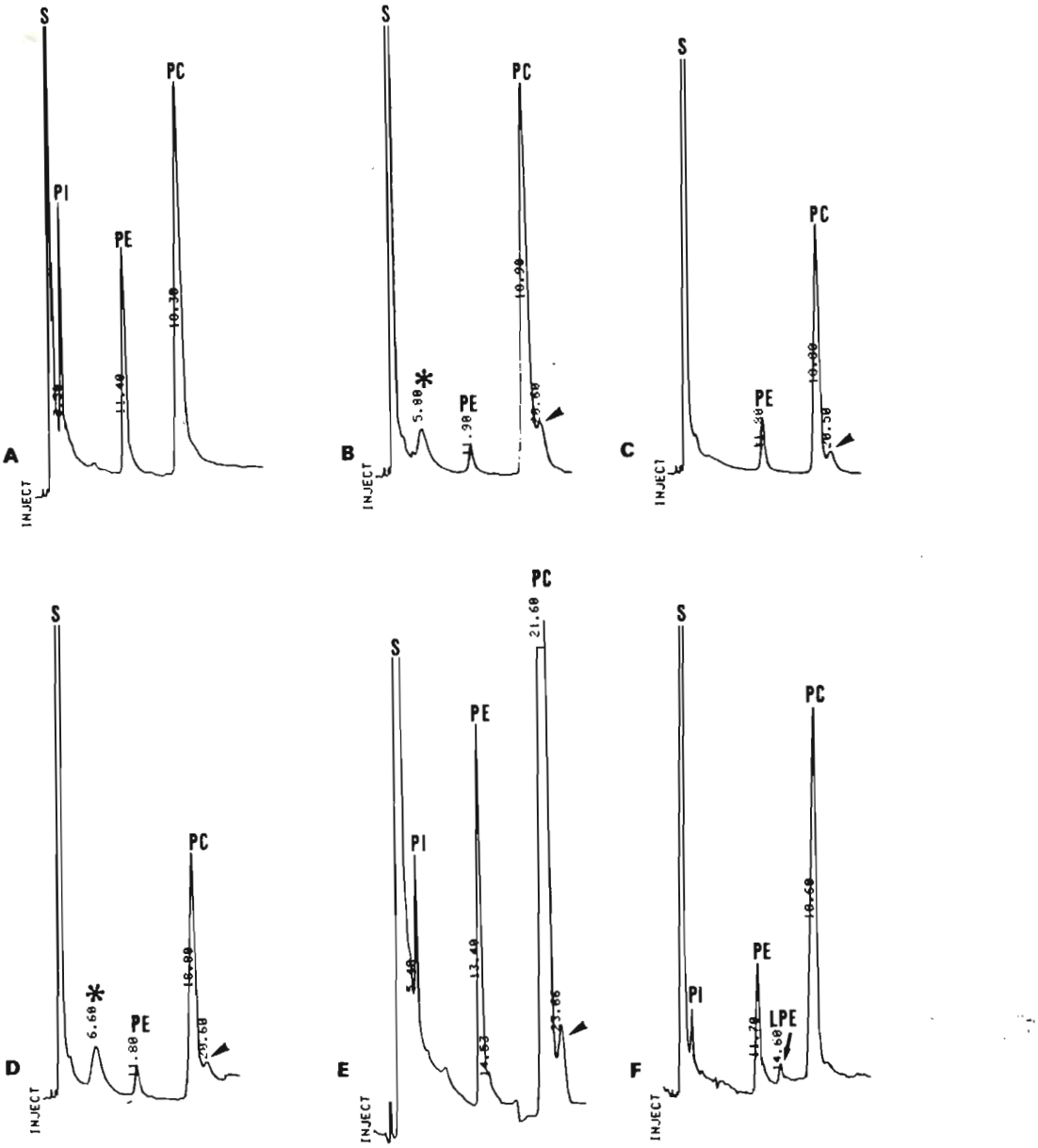


FIGURE 6.5

Representative chromatographic traces of phospholipids separated by HPLC and detected by U.V. absorbance. Tentative peak identification as follows: S, solvent; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PC, phosphatidylcholine. Note shoulder present on tail of PC peaks (arrowheads) and small unidentified peak (asterisk) thought to be an oxidation product on some chromatographs. Storage conditions for differing seed lots given as in table 6.9:

A. 2 years at 0% RH, 1983 purchase.

B. 5 years at 20% RH, 1979 purchase.

C. 2½ years over silica gel, cotyledonary necrosis on purchase (1982).

D. 6 years over silica gel, 1978 purchase.

E. 2 years at 40% RH, 1983 purchase.

F. 2 years at 60% RH, 1983 purchase.

Table 6.9

HPLC separation of the phospholipids of different lots of lettuce seed after storage at 20°C for the times indicated. Phospholipids were fractionated on a column of silicic acid (see materials and methods) before HPLC separation according to the method of Chen & Kou (1982). Values expressed as area percentages, using U.V. absorbance for detection. Peaks identified by retention times and co-chromatography of phospholipid standards. + & * denote same seed lots. Except for seeds stored for 6 years all values are the mean of 2 injections. $*^{o}$ & $*^{3}$ denotes sampling immediately on preparation (o) and after 3 days storage of extracted phospholipids in chloroform : methanol at room temperature.

STORAGE CONDITIONS ALL AT 20°C	PERCENTAGE GERMINATION & SEEDLING CONDITION	AREA PERCENT OF PHOSPHATIDYL -			
		- ETHANOLAMINE	-CHOLINE	-CHOLINE 'SHOULDER'	UNKNOWN
6 Years over silica gel	30 Cotyledonary necrosis	7^{*o} 5^{*3}	69^{*o} 46^{*3}	6^{*o} 36^{*3}	18^{*o} 10^{*3}
5 Years at 20% RH +	9 Poorly Developed Cotyledons	4	75	13	8
5 Years at 0% RH +	97 Normal Cotyledons	4.5	88	7	N.D.
2 Years at 0% RH *	98 High vigour	16	83.6	N.D.	N.D.
2½ Years over silica gel	75 Cotyledonary necrosis at purchase	12	78.5	9	N.D.
2 Years at 40% RH *	98 Cotyledonary necrosis	12	82	4.5	N.D.
2 Years at 60% RH *	68 Dwarf Seedlings	21	76.6	T.R.	N.D.

evident, quite apart from the rapidity of analyses. Seed samples chosen for investigation differed greatly as regards vigour and viability.

PI which eluted just after the solvent front was frequently undetected by the integrator. Estimates by triangulation indicated that it comprised as much as 17% (on an area percent basis) in a sample of high vigour (Figure 6.5 A) being nearly equivalent to that for PE (23%). In most analyses the peak was smaller, being absent in seeds from long-term storage (Figure 6.5, B) or a seed lot of high viability which was observed to produce seedlings with cotyledonary necrosis (Figure 6.5, C). Consequently in Table 6.9 PI has been omitted to permit comparison of other phospholipids. The seed lots chosen for study appeared to fall into two categories as far as these results were concerned. In the first, seeds stored for 5 or more years at low relative humidity appeared to contain lower levels of PE. This phospholipid was thus either more susceptible to double-bond attack or lost in the extraction and work-up technique.

The following speculative possibilities are suggested:

1. Highly polar peroxy- or hydroxy- fatty acid may have led to losses during silicic acid fractionation.

2. Selective de-esterification of the fatty acid chain from the glycerol/PE moeity may occur.

3. Cross reactivity between the peroxidized fatty acids or the PE headgroup and other molecules, such as proteins, may lead to unextractability by lipid solvents.

An additional feature evident from two seed lots of low germination (9% & 30%) was the appearance of a compound with retention time of approximately 6 min., which eluted between PI and PE (Figures 6.5, B & D; Table 6.9). According to the data presented by Chen & Kou (1982), using identical chromatographic conditions, PS elutes in this region. As noted earlier PS appears rare in seed tissues, and this

product was therefore assumed to be some shorter chain breakdown product of PC or PE. Because of use of U.V. detection it is not possible to interpret changes as losses of phospholipid, and further analyses of column effluent would be required before meaningful interpretations can be made of the observed changes. Perhaps the most significant feature to emerge from HPLC studies was the presence of a 'shoulder' with an elution time close to PC. It was evident in nearly all seeds examined, but was minimal in a high viability sample (2 years storage at 0% RH; 98% germination) and essentially absent in a sample stored 60% relative humidity of reduced vigour and viability. However in this latter case PE levels were proportionately the largest recorded which could indicate a greater stability of this phospholipid and a greater lability of PC. However the absence of the supposed PC "shoulder" product may indicate complete loss of PC rather than partial oxidative modification. This might then weight area percentages in favour of PE. Based on retention data of Chen & Kou (1982) lyso - PE may have been present (2.4% at RT 14.6 mins., Figure 6.5 F) which may argue for deteriorative mechanisms being fundamentally different at relative humidities above 60%.

DISCUSSION

Porter (1984) has drawn attention to the fact that fatty acid oxidation products cannot be satisfactorily separated by HPLC unless special derivatizations are undertaken, such as reduction of the OOH group and the production of methyl esters. Not withstanding such comments it is noteworthy that the present HPLC studies have shown marked (though as yet chemically unidentified) changes in phospholipid profiles. When this is seen in the light of the earlier results (Table 6.7) which showed no significant decline in the fatty acid levels of the total lipids seeds of lettuce seeds subjected to accelerated ageing it becomes necessary to consider the following proposals:

1. **That loss of unsaturation is not in itself directly relevant to seed deterioration.** Such a view has been supported by studies

which show no loss of unsaturation with viability loss in accelerated ageing (Priestley & Leopold, 1979). On the other hand later suggestions that ageing in the long term may bring about changes (Priestley & Leopold, 1983) might be seen as evidence for accelerated aging acting in some different and as yet undefined way to more natural ageing treatments. It is difficult however to reconcile these suggestions with studies which have shown loss of unsaturation in ageing.

2. That changes are smaller than previously considered important, and that certain phospholipid species may be more labile than others. PC has been identified in many studies as an especially labile phospholipid (Koostra & Harrington, 1969; Priestley & Leopold, 1979; Powell & Matthews, 1981; Petruzelli & Taranto, 1984; Francis & Coolbear, 1984).

   Other minor changes include increases in breakdown products such as phosphatidic acid or lysophosphatidylcholine (Koostra & Harrington, 1969; Powell & Matthews, 1981; Petruzelli & Taranto, 1984). The results of the HPLC studies may be seen as possible support for this suggestion. PE has been observed to decline also, although in one case it appeared symptomtic of deterioration (Powell & Matthews, 1981).

3. That direct hydrolysis/methanolysis of seed powders is an unsatisfactory method for examining changes in unsaturated fatty acids, especially if changes in the smaller phospholipid fraction were important to seed deterioration. This argument cannot be upheld since there are a number of studies in which this technique has yielded evidence for loss of unsaturation of fatty acid with ageing (Harman & Mattick, 1976; Priestley & Leopold, 1981; Flood & Sinclair, 1981). It has been noted that boron trifluoride/methanol will effect dehydration of hydroperoxide and hydroxyl groups to esters with addition of one more double bond (Morrison & Smith, 1964). Since there is clear evidence for the presence of lipid peroxides in seeds aged at high RH this property should be borne in mind as a

	RESEARCHERS	SEED & AGEING REGIME(S)	EXTRACTION AND ANALYTICAL TECHNIQUE	FINDINGS	OTHER COMMENTS
1	Koostra & Harrington (1969	Cucumber - different harvests long term aged in dry storage for 1-9 years. Accelerated ageing: 24°C/100 RH for 1-4 weeks	After Ch : MeOH extraction phospholipids precipitated with acetone : Satd. $MgCL_2$. TLC separation of PL species, quantification as PO_4.	Decline in weight of polar ppt. & PO_4 in accelerated ageing; not evident in long term ageing	PO_4 loss more marked than PL weight loss. Seed weight decline: 72% of control at 50% germination
2	van Staden, Davey & du Plessis (1976)	Protea - viable and non-viable (No details)	Petroleum ether soxhelet extraction of lipids. * fatty acids by G.C.; free fatty acids measured; U.V. absorbtion for conjugated dienes and peroxide value (P.V.)	No loss of unsaturation in fatty acids or increase in free fatty acids. Dienes unchanged. P.V. differed by 20 meq./kg	*AOAC "official" method selective for certain lipids (Sahasrabudhe & Small bone, 1983). Weight of extractable lipid differed by 2% between viable and non-viable seeds
3	Harman & Mattick (1976)	Pea - accelerated ageing: 30°C, 92% RH, 0-10 weeks	Seed powders : total lipid hydrolysis/ methanolysis	Slow decline in linoleic (18:2) in whole seeds, not so for axes. Linolenic (18:3) more closely parallels viability decline	Speculated relationship between fungi and initiation of oxidation. Small 18:2 changes masked by greater overall level
4	Priestley & Leopold (1979)	Soya - accelerated ageing: 40°C, 100% RH, 0-5 days	Seed powders : total lipid hydrolysis/ methanolysis; also methylation of extracted total lipid & polar PL by MeOH : Benzene : H_2SO_4. GC of fatty acids Acetone ppt. PL. TLC separation of PL species, quantification as PO_4	No change in unsaturated fatty acids by either method. Marked PC decline ahead of viability loss, less so for PE	Concluded that lipid-oxidation unrelated to ageing Unexplained why PL declir observed by none noted ir fatty acid levels
5	Stewart & Bewley (1980)	Soya - accelerated ageing: 45°C, 100% RH, 0-6 days; viability lost in 2 days	After Ch : MeOH extraction lipids fractionated on silicic acid with acetic : chloroform & methanol to give polar fraction. GC of fatty acids	Polar lipids showed decline in linoleic (18:2) and linolenic (18:3) with viability loss; notable post-mortem losses	Increases in malondialdehyde with imbibition : Post imbibition peroxidation. SOD activity after 1½ hours. Excessively harsh ageing regime?
6	Pearce & Abdel Samad (1980)	Peanut - long term aged: 5°C storage of two Seasons' cultivars for 2 & 38 months. Accelerated ageing: 38°C/ 90% RH 0-28 days	Propan-2-ol extracted lipid fractionated n florisil into neutral glycolipids with phospholipids. GC of fatty acids Total lipid hydroperoxides determined*	Natural ageing: No clear trend in fatty acid levels with natural ageing. Negative relationship to leakage. Accelerated ageing : fatty acids in phospholipids & glycolipids precede viability loss None detected	Fatty acid levels and leaching more related to harvest conditions than ageing? * Method not sufficiently sensitive?
7	Flood & Sinclair (1981)	Clover - 20 years old permeable & impermeable coated compared with freshly harvested	Seed powders : total lipid hydrolysis/ methanolysis	19% decline in linoleic (18:2) and 54% decline in linolenic for permeable seed	Exclusion of oxygen prevented fatty acid oxidation in impermeable seed; contradictory to Ohlrogge & Kiernan (1981)
8	Powell & Matthews (1981)	Pea - Surface sterilized accelerated ageing 25°C/94% RH, 0-9 weeks; vigour loss rather than viability decline	24 Hours imbibition: Folsch extraction Ch : MeOH : H_2O* TLC separation of PL species, quantification as PO_4, conductivity & ^{4}TTZ staining	Relationship between TTZ staining, conductivity and PL loss. Major loss of PC	*Undesirable possibility because of lipolytic activity PE appears with ageing: artefactual?
9	McDonnell, Pulford, Mirbahar, Tomos & Laidman (1982)	Wheat - high and low vigour but same viability	Water saturated, Butan-1-ol extracted lipid. Silicic acid fractionation into neutral, glycolipids, phospholipids; quantification as PO_4 & sugars. GC of fatty acid levels of above lipid categories and sterol levels	High and low vigour embryos have same levels of PL & glycolipids. Lower sterol : PL levels in low vigour samples, but fatty acids show no differences	Low vigour embryos commence membrane synthesis later in imbibition: reason(s) not clear
10	Priestley & Posthumus (1982)	Ogha Lotus collection possibly 40D yrs. old : 4 seeds compared with freshly harvested	Ch : MeOH : MeOH extracts of cotyledonary powders; as 4 above: GC of fatty acids	3 of the 4 seeds grew. No differences between fresh and old seed fatty acids	Little evidence of oxidation in spite of extreme age
11	Priestley, Galinat & Leopold (1981)	Recently grown seed of teosinte and maize compared with seed 78 - 1,700 years old	As above	Post-mortem material contained appreciable 18:2 although lower than recently grown seed	Sufficient polar lipid to maintain bilayer?
12	Niklas, Tiffney & Leopold (1982)	Fossil fruits and seeds of eocene & oligocene, 750 Myr.	Total lipid : Saponification; BF_3 : MeOH TLC for polar fraction	9 - 17% of total lipids still unsaturated; preferential oxidation of polar lipids	18 : 2 comprised 2% to 6% of total fatty acids 16:0 & 18:0, 35 - 48% & 34 - 49% respectively

13 Priestley & Leopold (1983)	Soya - long term ageing: 44 months at 4°C & low RH Accelerated ageing: 2-7 days at 40°C, 100% RH	As 4 above, 2 cultivars tested (a & b opposite)	a Approx. 2% loss of 18:2 & 18:3 with 12% loss of viability for whole seed total lipids b 2.9% loss of 18:3; 4.9% loss of 18:2 with 70% loss of viability for whole seed total lipids. 13% loss of 18:3 and an 8% increase in 18:2 from axis total lipids. No changes in PO_4 or tocopherols No changes in unsaturates with accelerated ageing. No changes in tocopherols	Peroxidation of storage and membrane lipids may exert influence on much of cellular metabolism in natural ageing. Accelerated ageing may give misleading biochemical differences Fatty acid data from only single chromatographic run; agrees with the present study (Table 6.5).
14 Francis & Coolbear (1984)	Tomato - controlled deterioration : imbibed to 70% m.c. (dry wt.) 45°C for 1-7 days; air dried to 11% m.c. and held at 5°C before use.*	Lipid extraction as (9) above; TLC separation of PL species; identification as PO_4	PL loss parallels viability decline. 28% PC loss at 90% germination. "Presowing" (blocking germination of imbibed seeds by 10°C for 21 days) unable to restore losses	PI synthesis important to germination? *Unorthodox ageing regime - enzymic activity?
15 Petruzelli & Taranto (1984)	Wheat - long term ageing: 20°C, 50 - 70% RH. Accelerated aging: 35 & 42 days, 30°C, 12.5, 14.5, 16.5% m.c.	Boiling propan-2-ol, TLC separation of PL species, quantification as PO_4	Accelerated ageing: decline in PC and increase in PA especially with higher m.c. ahead of viability loss.* Natural ageing: changes followed viability loss	*Possibility of lipase activity?
16 Powell & Harman (1985)	Pea - accelerated ageing for 0-6 weeks at 94% RH and: (a) 25°C or (b) 30°C "Short term" ageing at high m.c. & temperature: (c) 20% m.c., 45°C, 0-18 hours	Folsch extracted lipid, TLC separation of PL species; identification as PO_4. Hydroperoxides by A.O.C.S. method; TTZ staining cotyledons; electrolyte leakage	a 25°C: No evidence of deterioration by TTZ and conductivity fall. PC fall unrelated to physiological ageing b 30°C: Increased conductivity although TTZ initially unchanged; later onset of dead-tissue c Short term: increase in total PL until 12 hours; fall at 18 hours	Lipid hydrolysis by week 6: no peroxide increase Slight increase in hydroperoxides; oxidation of protein SH? Membrane repair/turnover? Slight decline in peroxides Rapid ageing not comparable to long term ageing

Table 6.10

Chronological summary of the many studies which have examined phospholipid and fatty acid levels in relation to seed ageing. A wide variety of seeds, ageing regimes and analytical techniques have been employed yielding an almost equally wide range of findings.

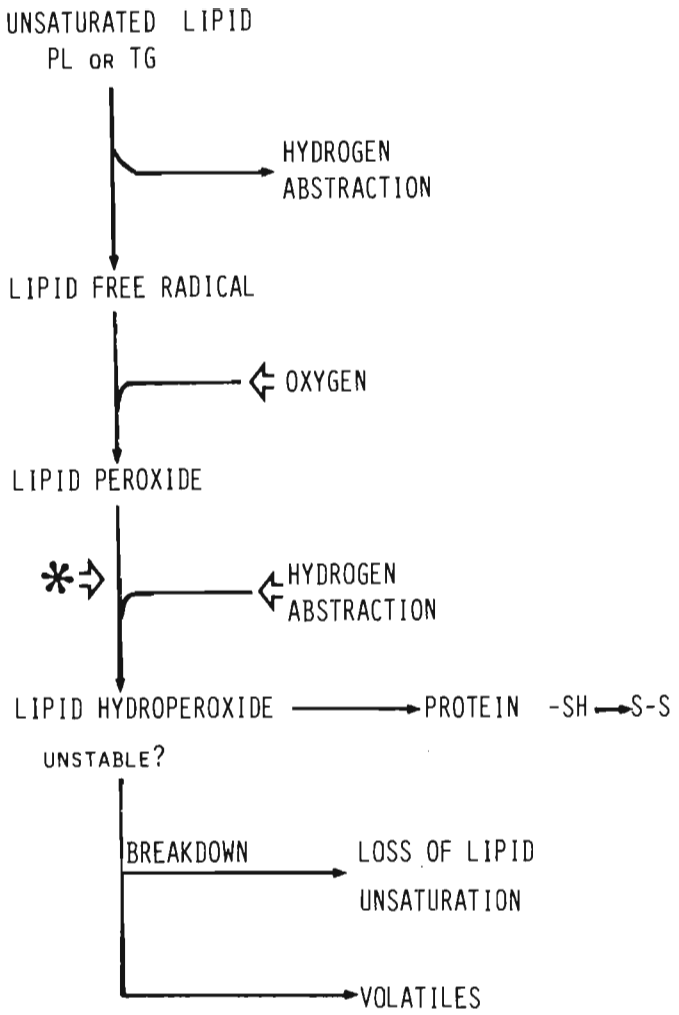


FIGURE 6.6

Simplified diagram of the sequence of events which might characterize lipid peroxidation of phospholipid (PL) or triglyceride (TG). After initial hydrogen abstraction the lipid free radical reacts with oxygen to form a lipid peroxide. At this point further reaction may be terminated through the agency of an antioxidant (asterisk), failing which, the peroxide abstracts a hydrogen atom from an adjacent molecule to produce a hydroperoxide. this has the potential to oxidize protein sulphydryl groups, or may be unstable. Breakdown may lead to loss of fatty acid unsaturation in the lipid or the production of volatile breakdown products. A more detailed account of fatty acid peroxidation and breakdown products is given in the Discussion, Chapter 7.

potential pitfall. However no obvious evidence of apparent increases in unsaturates was noted when compared to unaged material, and it is assumed that under the milder conditions used in the present study that this was not a major consideration.

4. It is impossible at present to find a common denominator between the many studies (Bewley & Black, 1982). Until an unambiguous relationship can be established between lipid peroxidation, the extent of loss of unsaturated fatty acids (or lipid phosphorus) and the likely consequences to membrane function, support for free radical involvement in seed ageing remains limited. This is especially evident on examination of Table 6.10, which summarizes the results of many of the relevant studies to date.

A cornerstone of free-radical theory of seed ageing has been the assumption that formation of the hydroperoxides or their subsequent breakdown must lead to the loss of unsaturation of fatty acids (Figure 6.6). However such a proposed mechanism of autoxidation should be seen against the backdrop of studies by Leopold and co-workers on seeds of 400, or more, years of age which clearly demonstrate how resistant fatty acids may be to total loss of unsaturation (Priestley, Galinat & Leopold, 1981; Priestley & Posthumus, 1982; Niklas, Tiffney & Leopold, 1982).

Apart from attempts to seek evidence for the presence of free-radicals by possible losses in fatty acid unsaturation, attempts have been made to correlate loss of viability with declines in the anti-oxidants such as tocopherols (Priestley, McBride & Leopold, 1980; Fielding & Goldsworthy, 1982). These have shown no loss of antioxidant with fall in viability, from which it has been concluded that free radicals are unlikely to be involved in seed ageing. This argument can be faulted for two reasons:

1. While there is litle doubt that tocopherols are efficient free radical quenchers, it has been observed that high oxidative

stability may be imparted to oils by substances other than tocopherols. These include other phenolics or phenolic glycosides, and phospholipids, although the latter are thought to act in concert with antioxidants (Swern, 1964). Significantly, Mead (1980) has reported that less unsaturated fatty acids such as oleic when dispersed in a monolayer retard autoxidation. This might suggest that cell membranes, by virtue of particular fatty acid ratios may have greatly increased oxidative stability. Tocopherols (AH) terminate free radical propagation by the reaction:

$$ROO^{\bullet} + AH \longrightarrow ROOH + A^{\bullet}$$

the electron entering the phenol ring as a resonance hybrid.

It should be noted that lipid hydroperoxides may nevertheless build up at a slow rate while the tocopherols exert their protective effect. Mobility has been invoked to explain the efficacy of the antioxidant - fatty acid interaction since it has been calculated that one molecule of α-tocopherol protects 2,000 - 20,000 molecules of linoleic acid (Wu, Stein & Mead, 1979).

2. The observation that antioxidants are rapidly depleted at the end of the induction period has been contradicted by many observations (Swern, 1964).

Witting (1975) has noted that the rate of tocopherol destruction is not simply a matter of the rate of initiation of free radicals. The degree of fatty acid unsaturation is also important. Linolenate causes destruction of tocopherol at five times the rate of linoleate, at equimolar concentrations of antioxidant. Likewise, at the end of the induction period, 30 - 50% of the original tocopherol level remains. It has been noted that the fate of antioxidant molecules is a complex problem, yet few studies have been undertaken (Kurechi and Yamaguchi, 1980).

Lettuce seeds have been shown to possess high levels of chlorogenic acid (Butler, 1960) which is likely to exert antioxidant activity because of the presence of many hydroxyl groups on the phenyl rings and the vinyl group in the straight chain portion of the molecule (Swaroop & Ramasarma, 1981). Phenolic compounds have tended to receive more attention in plants as regards possible roles in IAA metabolism (Schneider & Wightman, 1974) and in host-pathogen interactions (Friend, 1979) than as possible antioxidants. The cellular site of localization of these compounds is regarded as the vacuole, and consequently they may play a minimal role in general membrane protection. Indeed, the lipid insolubility of these compounds has been a serious hindrance to their efficient use as antioxidants in non-aqueous food systems (Pratt & Birac, 1979). On the other hand they are likely to provide some protection to the vacuolar membrane, a not entirely inconsequential role in view of the lytic nature proposed for this structure.

Apart from the measurement of free radicals, which has yielded equivocal results, several methods have been employed to assess lipid peroxidation. Slater (1984) has reviewed the methods used for detecting lipid peroxidation, pointing out some associated pitfalls and although directed exclusively at animal-orientated studies, many of the comments are equally relevant to seed ageing studies. Some of these will now be considered

1. Loss of Lipid

Slater (1984) has suggested that McCay and co-workers were amongst the first to apply gas chromatography to monitor changes in PUFA's of membrane suspensions in vitro. However the dynamics of metabolizing systems should be borne in mind when the technique is applied to in vivo systems. This may be clearly seen in the cotyledonary studies of Thompson and co-workers (see page 34) where loss of unsaturation is not observed during senescence and was not identified as a contributory cause of membrane phase changes. Furthermore, for reasons which are not immediately apparent, this technique has tended to be the most widely used method in seed studies. As a general rule it appears

that when loss of unsaturation has been noted in dry, deteriorated seeds, the percentage decrease of the polar fraction has generally always been small.

2. Oxygen Uptake

This method finds greatest application in plant studies as an assay for lipoxygenase, and in studies on cyanide and cyanide insensitive respiration rather than for lipid peroxidation. Presumably it should be equally applicable to the measurement of free-radical induced peroxidation but has been somewhat overshadowed by the former applications. Some evidence will be presented later that measurement of oxygen levels of dry seeds in hermetic storage may permit a non-destructive measurement of the course of peroxidation (Chapter 7).

3. Diene Conjugation

This method has been used extensively in animal peroxidation studies but relies on distinguishing a small "shoulder" on a background absorbtion. This method has been used by oil chemists also, but is generally only satisfactory when significant peroxidation has occurred. This technique has found limited use in seed ageing (van Staden et al., 1976) being largely semi-quantitative.

4. Lipid Hydroperoxides

"In principle this should be a reasonable and direct approach to the measurement of lipid peroxidation. However, this is complicated by the rather easy breakdown of hydroperoxides catalyzed by metal ions, by the reduction of hydroperoxides by thiols, and the metabolism of hydroperoxides by peroxidases" (Slater, 1984). While the above may apply to hydrated animal or plant systems these objections may not be of major importance in the dry seed. Slater notes that this method of assessment has not been as extensively utilized as the measurement of diene conjugation or malondialdehyde; such a viewpoint is equally applicable to plant studies. While the

method of Beuge & Aust (1978) used in the present study has advantages of reasonable sensitivity and rapid sample throughput, more sensitive but time consuming methods have been developed. They include time-course measurements in a spectrophotometer cell with a reported lower sensitivity limit of 1n mole (Hicks & Gebicki, 1979). Studies which have examined hydroperoxides, hydroxy fatty acids or hydroxyepoxides are relatively few (Radrupal & Basu, 1982; Moll et al., 1979; van Staden et al., 1976). These have generally yielded results consistent with the free-radical scheme in Figure 6.6, the only exception being the work of Pearce & Abdel Samad (1980). These workers employed a titrimetric technique which involved prior boiling of the lipid : acetic chloroform mixture with potassium iodide. Although no reference for the particular technique employed was given the use of heating might be contra-indicated, and it is certainly not advocated in the standard A.O.C.S. (1969) method. Hamilton (1974) has drawn attention to the fact that peroxides do not dissociate spontaneously at physiological temperatures, although this misconception is widely held. It is argued that oxygen-oxygen bond energy is too high (43 kcal/mole) for the spontaneous reaction:

$$ROOH \longrightarrow RO^{\bullet} + OH^{\bullet}$$

Conditions which would favour this reaction were cited as:

(a) Temperatures above 100°C,

(b) Transition metals of lower valency state,

(c) The possible formation of a resonance hybrid:
RO would thus have a lower energy requirement.

(d) High energy irradiation

Furthermore, should the above reaction occur it is argued that the $OH^{\bullet}$ would be such a highly reactive species that it would react with just about any molecular species (including alkanes, alkenes, aromatics or enzyme active sites). The cell therefore has no specific defence mechanism for such a reaction and presumbly a hit-or-miss quenching mechanism must apply. Halliwell & Gutteridge (1984) have also noted that pure lipid hydroperoxides are fairly stable at physiological temperatures. Haemoproteins such as peroxidase and cytochromes were seen as possible contributors to propagation of peroxidation in membranes in vitro. In terms of stochastic models of ageing the hydroxyl radical is pre-eminently suitable for providing an increasing number of "hits", dependent on hydroperoxide levels.

5. The Thiobarbituric Acid Reaction (TBA)

This procedure is probably the most widely used in both animal and plant systems, since it is extremely simple. After acid precipitation of cellular proteins, the centrifuged supernatant is heated with TBA and, after cooling, absorbance is measured. While Slater (1984) presents evidence that the technique compares well with other techniques for measuring peroxidation, Benge & Aust (1978) suggest that *in vitro* applicability is poorer than *in vivo*.

It was argued that *in vitro* metabolism and protein reactivity could give a distorted picture of peroxidation. It was further noted that tissue aldehydes and sugars are also able to react with TBA, and metal-enhanced catalysis may lead to spuriously high increases in TBA-reactive material. This latter viewpoint has been reinforced by a study which suggests that significant errors are inherent in the TBA assay when applied to leaf tissues (Matsuo, Yoneda & Itoo, 1984). The TBA assay of Beuge & Aust (1978) was seen to considerably overestimate levels of MDA (possibly a hundred fold), raising some doubts about earlier leaf senescence studies (eg. Dhindsa, Plumb-Dhindsa & Reid, 1982).

Since the technique supposedly measures *break-down products* of unsaturated fatty-acids with 3 or more double bonds, variations between seeds or tissues may reflect differences in fatty-acid composition rather than peroxides formed. Frankel & Neff (1983) have indicated that TBA values are a poor reflection of the oxidation products formed from thermal decomposition of monohydroperoxides, hydroperoxy epidioxides and other secondary products of lipid oxidation.

"It may be prudent where the TBA reaction is being used to cross-check its relation to lipid peroxidation by reference to as many other methods as is feasible" (Slater, 1984). Indeed, many of the studies on seed ageing have tended to examine only one parameter by a limited number of techniques, and on the basis of these results attempted to infer causality. This tendency, like the technique of accelerated ageing, probably represents experimenter convenience rather than rigorous experimental design.

CHAPTER 7

VOLATILES AND SEED AGEING

(A) Volatile production during seed imbibition

The production of volatile aldehydes and alcohols are thought to be responsible for the characteristic odour produced by plants on wounding, or by tissue extracts (Hatanaka, Kajiwara & Harada, 1975; Galliard & Phillips, 1976) and lipoxygenase has been implicated in the initial stages of the pathway leading from C_{18} fatty acids to such volatiles (Galliard, 1980).

Aldehydes and oxidizable compounds were produced from seeds of pea, bean, cucumber and pine during the course of normal germination (Stotzky & Schenck, 1976). These were regarded as a component of normal germination without reference to deterioration. Volatile metabolites (identified by GC as acetaldehyde, ethanol and acetone) were produced in large amounts by weed seeds under conditions of reduced oxygen supply and were considered to exert a possible role in the dormancy of buried seeds (Holm, 1972). The production of volatile fatty acid peroxidation products in pea seeds has been implicated in stimulating the germination of fungal spores (Harman, Nedrow & Nash, 1978; Harman, Mattick, Nash & Nedrow, 1980).

Soybean seeds subjected to accelerated ageing (40°C, 100% RH) produced acetaldehyde and ethanol on imbibition, reaching a maximum 4 hours after imbibition (Woodstock & Taylorson, 1981). Anaerobic conditions and respiratory inhibitors were seen to bring

about comparable rises in these compounds. It was suggested that as a result of membrane deterioration an imbalance developed between glycolysis and the TCA cycle. The possibility of measurement of volatiles by GC as a means of assessing seed quality, either as a result of peroxidative damage or metabolic perturbations by some other cause, was investigated for seeds of lettuce; particular attractions were simplicity and the non-destructive nature of the technique.

Two of the three sections of this Chapter (Sections A & B) were undertaken using packed column gas chromatography for the separation of volatiles. During the later part of the study access was gained to a capillary GC-MS facility with an automatic headspace sampler. While the results of the latter technique are strikingly apparent, such facilities are unlikely to be available in many seed testing laboratories. The present writer has therefore chosen to present the earlier packed column studies in some detail to maintain the chronological perspective of the study and to provide some indication of experimental parameters for other researchers to follow. The reader may thus omit Sections A & B and begin at Section C, page *153* without serious loss of either continuity or detailed discussion.

MATERIALS AND METHODS

(a) Headspace volatiles on imbibition:

Dry lettuce seeds were placed in 10 ml serum vials, the walls of which were lined with a cylinder of filter paper. Vials were sealed with butyl rubber stoppers secured with crimped aluminium caps. At the start of the experiments distilled water was introduced with a syringe and needle into the vials. In preliminary studies 0,5 g of seed and 2 ml of water were used, but this was later increased to 3.5 g seed and 5 ml of water. At intervals 50 µl of headspace was withdrawn from the vials using a 400 µl Precision gas-tight syringe. Samples were injected into the column of a Pye-Unicam series 104 gas

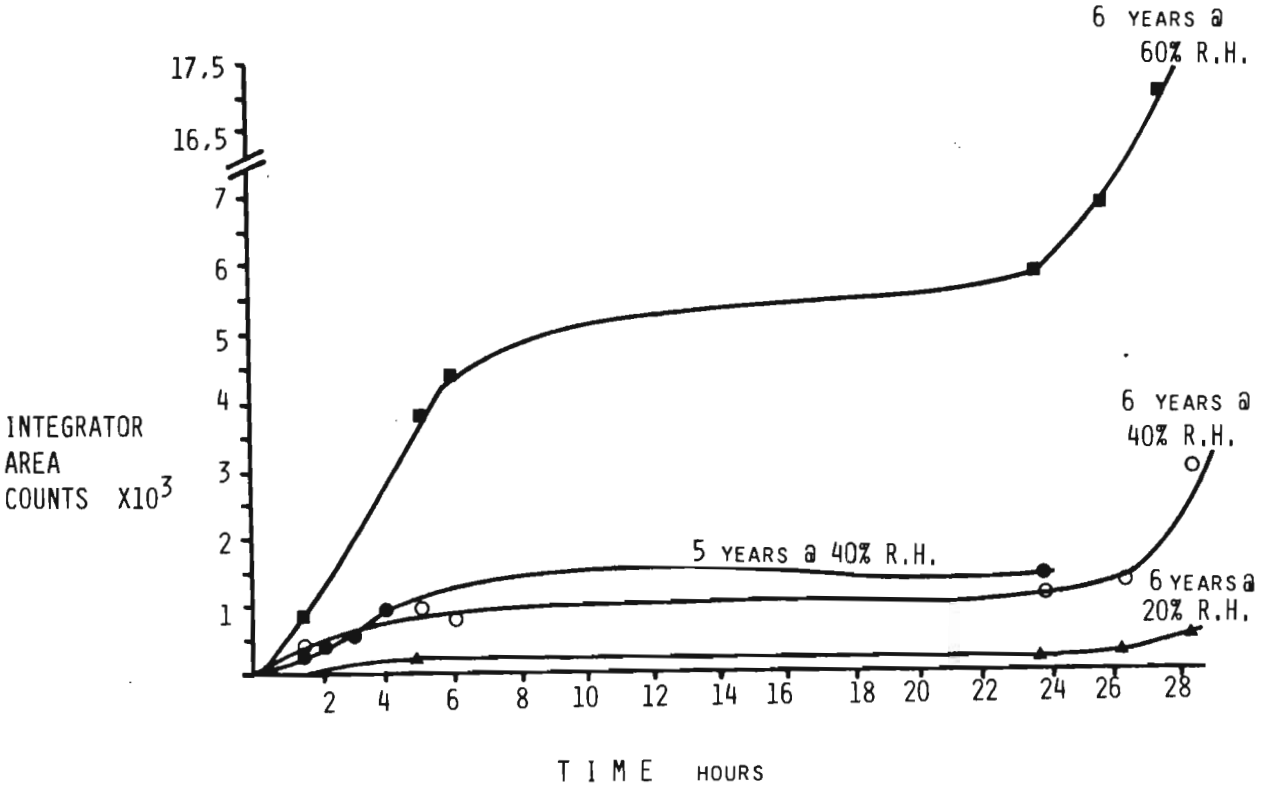


Figure 7.1

Production of a pentane-like volatile (tentatively identified on the basis of co-chromatography) by seeds imbibing in hermetically sealed vials at ambient temperatures at the times indicated. Prior storage of the seeds was at 20°C at the relative humidities indicated. Germination data for 6 years are given in Table 7.1. The retention time for the pentane-like volatile was 0.46 - 0.54 minutes.

Table 7.1

Relative increase (as area percentage) in an unidentified volatile produced with decreasing R.H. of storage, and increasing viability, at 28½ hours imbibition in hermetically sealed vials.

STORAGE R.H.	AREA PERCENTAGE AT RETENTION TIMES (MINS.)		% GERMINATION AT 48 HOURS
	0.46 - 0.54	1.10	
60%	99.6	Not detected	0
40%	81.2	18.7	0
20%	36.7	63	28
0%	29	71	63

chromatograph equipped with a flame ionization detector. A 1,5 m x 4 mm I.D. glass column packed with 2% carbowax 20M on chromosorb W was used and the column operated at 80°C with nitrogen as carrier gas. Flow rate was 25 ml/min. and ionization amplification was between 1 - 10 x 10^2. A Varian 4270 integrator was used with attenuation settings between 1 and 32.

(b) Determination of N_2/O_2 and N_2/CO_2 ratios:

A Varian Model 3700 chromatograph fitted with a thermal conductivity detector and two columns in the series - across - detector (SAD) configuration was used. The first column was a 2 m x 2 mm I.D. stainless steel column packed with 80/100 mesh Poropak N, maintained at 80°C in the oven. The second was a 5 m x 2 mm I.D. stainless steel column packed with 60/80 mesh Molecular Sieve 5A, at ambient temperature outside the oven. Helium was used as the carrier gas. A Varian 4270 Integrator was used for peak recording and integration. Positive peaks for the compounds eluting from both columns were obtained by switching polarities.

RESULTS AND DISCUSSION

Preliminary studies were undertaken on a 1977 purchase of seed which had been stored for 6 years at 20°C and at a range of RHs from 0% to 60%. Seeds which had been held continuously at 40% and 60% RH were non-viable. For comparative purposes another seed lot, which had been stored for 5 years at 40% RH and which was considered approximately 1 year post-mortem, was also investigated. Results are presented graphically in Figure 7.1. In contrast to non-viable seed for which significantly greater integrator counts were observed, counts were marginally greater than background in the case of seeds stored for 6 years at 20% RH (28% germination at 48 hours). Seeds held at 0% RH (63% germination at 48 hours) were indistinguishable from background. Up to 24 hours imbibition the dominant and first-eluting peak contained a volatile product which

Table 7.2

Influence of different gaseous environments on the production of a pentane-like volatile in imbibing seeds in hermetically sealed vials after storage as indicated. Values expressed as integrator counts (x 10^3).

* Peak at RT = 1.9 - 2 Mins. only 2 percent of total

\+ Minor peaks observed for N_2 samples at RT = 1.11 - 1.13 Mins. accounting for 15, 17, 3 & 0.5%, of total counts for seeds stored at 0, 20, 40, & 60% R.H., respectively. Germination data not recorded.

STORAGE CONDITIONS 8 YEARS AT 20°C & RH INDICATED	HOURS IMBIBITION								
	4.5 - 5 Hours			11 - 11.45 Hours			22 - 23 Hours		
	Air	O_2	N_2	Air	O_2	N_2	Air	O_2	N_2
60%	63.7	63.5	66.5	100	99.8	104	104*	101*	101+*
40%	14.4	14.2	14.2	25	24	24.2	28.5	25.7	23.4+
20%	2.4	1.9	2.7	4.8	3.4	6.4	6.9	3.9	5.7+
0%	2.7	2.8	3.0	4.6	4.1	6.3	5.0	3.6	4.5+

Table 7.3

Area percentage, contributed by two peaks at the retention times indicated, after 23 hours imbibition in hermetically sealed vials. Seeds stored for 8 years at 20°C and R.H. indicated and imbibed in oxygen, air or nitrogen.

STORAGE R.H.	OXYGEN		AIR		NITROGEN	
	0.46 Mins	1.1 Min	0.46 Mins	1.1 Mins	0.46 Mins	1.1 Min
60%	100	N.D.	98	0.8	98	0.4
40%	100	N.D.	91	6	97	2
20%	100	N.D.	73	26	82	17
0%	100	N.D.	76	23	84	15

co-chromatographed with pentane. (RT = 0.46 - 0.54 minutes). This pentane-like volatile was seen to increase during the first 6 hours, followed by a lag phase which ended after 24 hours imbibition. This latter phase was not considered physiologically relevant, since when vials were opened, the contents smelled malodorous. Nevertheless, headspace samples from seeds at $28\frac{1}{2}$ hours imbibition revealed an increase in a second peak with RT = 1.98 minutes. Although unidentified, this latter peak constituted a greater area percentage in viable samples. (Table 7.1). These preliminary studies suggested that headspace analysis of imbibing seeds and the pattern of volatile evolution might be a useful indicator of conditions of seed storage and viability. Since the nature of the volatiles produced and their relationship to normal metabolism was complicated by decreasing oxygen tensions in the sealed vial system, and possible acidification as a result of increased CO_2 levels, the influence of different gaseous environments on volatile evolution was investigated. Vials containing 2 gm of dry seeds were flushed with high purity oxygen or nitrogen and sealed for 18 hours. Thereafter vials were re-flushed with the appropriate gas before final securing of septa with crimped aluminium caps. Distilled water which had been thoroughly gassed with nitrogen or oxygen was introduced into vials with a syringe and needle. Headspace samples were taken at 4.5, 11 and 22 hours for the same seed lots used earlier; integrator counts are presented in Table 7.2. It was evident that overall increases in the pentane-like peak were similar irrespective of gaseous environment, and showed a similar trend to that observed earlier (Figure 7.1). However the progressive appearance of a second peak with RT = 1.1 minutes was evident in all samples except those held in oxygen. In keeping with the preliminary study (Table 7.1) values were lower in post-mortem material as indicated in Table 7.3. Although the evolution of the pentane-like volatile appeared to be independent of the gaseous environment, the enzyme lipoxygenase is known to operate equally well under anaerobic conditions (Galliard & Chan 1980). Indeed, studies have shown that pentane was produced under anaerobic conditions in a lipoxygenase - fatty acid model system, although greater amounts are produced under aerobic conditions (Gardner, 1979). Purified lipoxygenases are

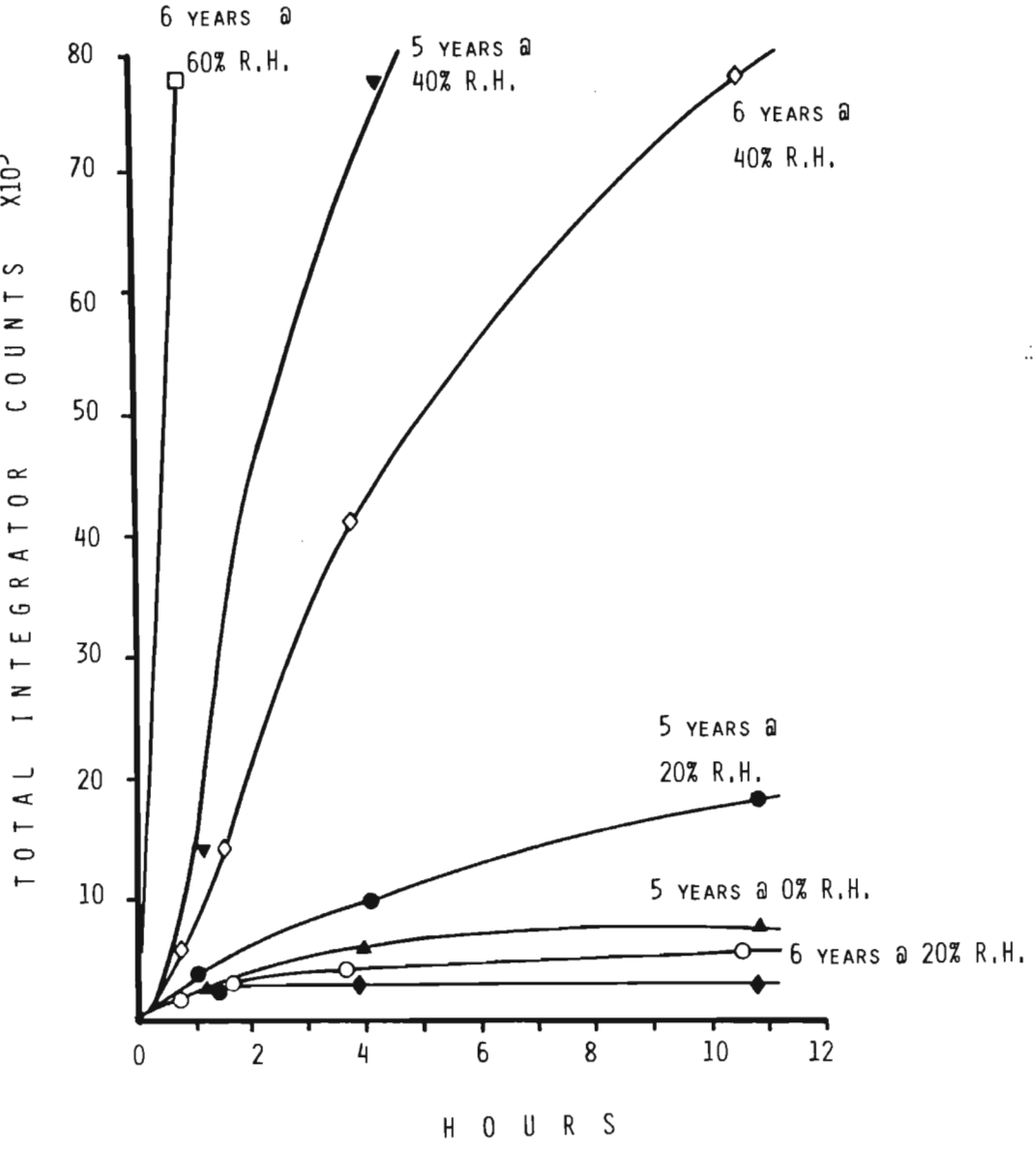


Figure 7.2

Integrator counts for a pentane-like volatile produced by two seed lots during the course of imbibition in hermetically sealed vials. Seeds were stored for 5 and 6 years at the R.H. indicated. Values close to background were recorded for seeds stored for 5 or 6 years at 80% (solid diamond). Germination data given in Table 7.5.

Table 7.4

Integrator counts (x 10^3) for a pentane-like volatile produced by seeds heated to 90°C for 45 minutes before imbibition. Determinations made at 11 and 22 hours (compare with Table 7.2)

8 YEARS STORAGE AT RH	HOURS OF IMBIBITION	
	11	22
60%	105.8	100.7
40%	24.3	28.3
20%	8.2	8.7
0%	7.4	7.2

Table 7.5

Germination percentages of two seed lots of lettuce cv. Great Lakes stored at a constant temperature of 20°C and the relative humidities indicated. Germination scored at 48 hours.

STORAGE R.H.	PERCENTAGE GERMINATION	
	5 years storage 1979 purchase	6 years storage 1978 purchase
80%	0	0
60%	0	0
40%	20	0
20%	48	36
0%	89	86

rapidly denatured at 70°C, although more drastic treatments are used in the food industry (Galliard & Chan, 1980). If this enzyme were wholly responsible for the production of the pentane-like volatile, it was felt that heat treatment of dry seeds (90°C for 45 mins.) might lead to a partial or complete diminution of such activity on subsequent imbition. After heating in sealed vials, water was introduced by means of a syringe and needle, as before, and headspace samples taken at 11 and 22 hours (Table 7.4). When these values were compared with Table 7.2 it was observed that higher counts were obtained for viable material (stored at 0% & 20% RH), whilst values for post-mortem material were unchanged. This suggests that the lipoxygenase enzymes might not be implicated in volatile evolution and increased levels noted in viable seeds might be a consequence of heat-induced cell damage or thermal acceleration of the autoxidative reactions in seed tissues. The possible value of examining volatiles from heated seeds has been suggested as a possible means of evaluating seed quality (Fielding & Goldsworthy, 1981). This possibility is explored further in the second part of this chapter.

For the pentane-like volatile to have relevance in evaluating the performance of viable seed it was evident that counts should be maximized for such samples. In order to achieve this, 3 g of seed were placed in 10 ml vials and 5 ml of water added. Two different seed lots were used in this study (1979 & 1978 purchases) and which had been held continuously at 0, 20, 40, 60 & 80% RH at 20°C for 5 & 6 years respectively. Results of this study are presented graphically in Figure 7.2; germination data are presented in Table 7.5. As was evident earlier, integrated counts for the pentane-like volatile were several fold higher in low viability samples. The earlier observation that chronologically older seed yielded higher viability (see Germination Studies, Chapter 2 was upheld by the volatile evolution counts. Seeds stored for 6 years at 60% RH yielded a substantial quantity of pentane-like volatile within one hour of imbibition (78×10^3 counts); these values increased further (379×10^3 counts) at 10.5 hours. During the first hour of imbibition the rate of increase of the volatile pentane-like compound

A
.46
.57
1.06
B
.43
.50
C
.45
.56
1.08
D
.42
1.09
E
.43
1.10
F
.44
1.17
2.03
G
.43
.57
1.07
H
.45
I
.45
1.17

Figure 7.3

Representative chromatographs for a number of lots of lettuce seed at various times during the course of imbibition in hermetically sealed vials. Storage temperature 20°C.

A. 3 years storage over silica gel, cotyledonary necrosis on purchase in 1982. 11:22 hours imbibition.

B. Same seed lot as A, above but after 1:32 hours imbibition.

C. 5 years storage at 0% RH, 10:55 hours imbibition.

D. 6 years storage at 20% RH, 3:48 hours imbibition.

E. Injection of air immediately after the above sample using the same syringe. It is evident that there has been some volatile carry-over by the syringe, especially noticeable at RT = 1.10.

F. 6 years storage at 60% RH, 3:49 hours imbibition. Compare this non-viable sample with Figure D, of the same seed lot where 48% germination was observed.

G. 2 years storage at 60% RH, 11:28 hours imbibition. Germination value of 77% recorded for this sample.
Comparisons may be made between Figure E, a blank syringe showing volatile carry-over and Figure C, a sample of 89% germination but stored at 0% RH for 5 years.

H. 5 years storage at 80% RH, post-mortem sample after 11:13 hours imbibition. Note appearance of single pentane-like peak.

I. 2 years storage at 80% RH, non-viable seed (1983 purchase) at 11:22 hours imbibition. Note also the appearance of a single pentane-like peak.

was 1.3 - 1.5 x 10^3 per minute, whilst over the experimental period this value was reduced to 0.52 - 0.58 x 10^3 counts per minute. This is in keeping with the earlier study (Figure 7.1) where counts level-off after some 6 hours imbibition. Background values for water-filled vials were high (mean = 1830, S.D. = 298) and it became apparent that volatile carry-over by syringes between successive injections was a potential source of error. This is illustrated in Figure 7.3, A & E where, following injection of 100 µl of air from a water-filled vial after a sample, 2062 counts were recorded at RT = 0.43 mins. in addition to the artefactual peak at RT = 1.10 mins. which accounted for 32% of total integrator counts.

It would appear therefore, that for any given seed lot, evolution of the pentane-like volatile increases progressively in a time- and RH-dependent fashion and reflects the viability of the seed lots. Thus seeds which were held at 80% relative humidity were seen to give lower values after 5 and 6 years storage than comparable material stored at 60% RH. The observation possibly has a parallel in model lipid oxidation studies where, after an induction period, peroxide values increase exponentially before declining to very low levels (Swern, 1964). Further support for this suggestion was seen in non-viable seeds from a 1983 purchase which had been held at 80% RH and 20°C for 2 years were also seen to have a small pentane-like peak which was not greatly different from background. (1868 counts vs background 1830 (± 298 ; Figure 7.3 I). In seeds from this same lot which had been stored at 60% RH, and where germination was 77% at 48 hours, the pentane-like peak was also not greatly different from background (2123 counts). In the former case the peroxide value may have decline post-mortem, while in the latter any lipid peroxidation present may have been insufficient to bring about a rise in the level of the putative pentane-like volatile on imbibition.

It was noted earlier in the preliminary studies that another unidentified volatile was evident after 20 hours imbibition at RT = 1.08 minutes. While this was considered more a product of anaerobiosis or carbon dioxide acidification (Table 7.1), the fact that the relationship was observed for seeds imbibed in a nitrogen

Table 7.6

Changes in the integrator counts (X 10^3) and area percentages for a second, unidentified volatile peak seen in two seed lots of lettuce stored for 5 and 6 years at 20°C and the RH. indicated. Retention time approximately 1.08 mins (see Figure 7.3).

*A Small Peak Present but not integrated, estimated 7%

*B Chromatograms illustrated in Figure 7.3

*C Chromatograms illustrated in Figure 7.3

*D Data not available

STORAGE R.H.	5 YEARS STORAGE				6 YEARS STORAGE	
	4 - 4.5 HOURS POST IMBIBITION		10.5 - 11 HOURS POST IMBIBITION		10.3 - 10.5 HOURS POST IMBIBITION	
	Counts	Area %	Counts	Area %	Counts	Area %
80%	0	0	0	0 *B	0	0
60%	0.9	0.3	1.8	0.5	1.1	0.3
40%	2.2	2.8	9.6	6	6.4	7.5
20%	2.5	18	12.7	40	10.2	6.2
0%	-	- *A	11	57 *C	-	-*D

atmosphere (Table 7.2) suggested the information might nevertheless have diagnostic value. The appearance of this second volatile was investigated in 1978 and 1979 purchases of seeds stored at selected relative humidities, during imbibition; results are presented in Table 7.6. At 4 hours imbibition the volatile at RT = 1.08 minutes was seen to be a minor component (Figure 7.3, D), both in terms of counts and when expressed as an area % of total counts, being smallest in non-viable samples. By 11 hours imbibition this peak represented a major component of evolved volatiles in viable and early post-mortem material (Figure 7.3, A, C, G). It could be argued that the conspicuous absence of this second peak in seeds held at 80% RH was the result of a combined interaction of time and relative humidity in bringing about post-mortem deteriorative changes. That this may not be so is indicated by the absence of such a peak in seeds deemed to be early post-mortem (2 years at 80% RH; Figure 7.3,I) although it was present in seeds stored for 6 years at 60% RH which might be expected to have accumulated at least comparable damage (Figure 7.3, F).

A feature of particular significance was the presence of a comparatively large pentane-like peak (7.4×10^3 counts) for a 1982 purchase of seeds which had been stored over silica gel for 3 years (Figure 7.3, A). This was comparable with that of the 1979 purchase which had been held for 5 years at 0% RH (Figure 7.3, C; 7.7×10^3 counts). Percentage germination at 48 hours was comparable being 80% in the 1982 purchase and 89% in the 1979 purchase. An additional peak, regarded as a post-mortem feature, was noted at RT = 2.03 mins. in non-viable material which had been stored for 5 or 6 years. Although apparently significant (Figure 7.3, F) in terms of counts, this was only 2% on an area percent basis at 3.5 hours imbibition for the 1978 purchase. In seed of this same lot stored at 80% RH (chromatogram not illustrated) the peak represented 28% of counts on an area percent basis at 4 hours imbibition, but this was the result of a substantial decline in counts for the pentane-like peak. This peak was absent in seeds of the 1979 purchase, which had been stored at 80% RH, although it may have been obscured by signal noise (Figure 7.3, H).

Thus on the basis of the above studies, the possibility existed that the two major headspace volatiles identified may show some relationship to seed viability. These may be related to the extent of lipid peroxidation (the pentane-like volatile) or some overall measure of metabolic competence (the unidentified volatile at RT= 1.08 minutes).

Since co-chromatography alone was clearly not an entirely satisfactory method of attempting to identify the headspace volatiles, packed column studies were extended to an examination of the profiles obtained from heating dry seeds in hermetically sealed vials. Peaks of the same retention times would suggest that metabolic involvement might be excluded as the possible source of the volatiles.

(B) Volatile production on heating dry seeds

Results in the previous section (7 A) suggested that a pentane-like compound was the principal volatile produced during seed imbibition, the amounts of which could be directly related to RH and duration of storage. However, the ideal objective of chromatographic profiling would be to detect small differences in viability or vigour; use of a gas chromatogram to distinguish different degrees of seed deadness would clearly serve little purpose.

It had been suggested that volatile compounds released on heating dry wheat seeds for 24 hours at 60°C in nitrogen may be a sensitive indicator of physiological age and potential vigour (Fielding & Goldsworthy, 1982). Although the extent of peroxidation was not measured, indirect evidence was obtained that one of the peaks thought to be of diagnostic value may have been derived from seed lipids. There is a great deal of evidence to support such a suggestion in the lipid oxidation literature.

An association has long been made in the food science literature between the off flavours in foodstuffs and lipid oxidation (Schultz, Day & Sinnhuber, 1962). It has been suggested that these may either be caused by hydroperoxide breakdown products (Keeney, 1962)

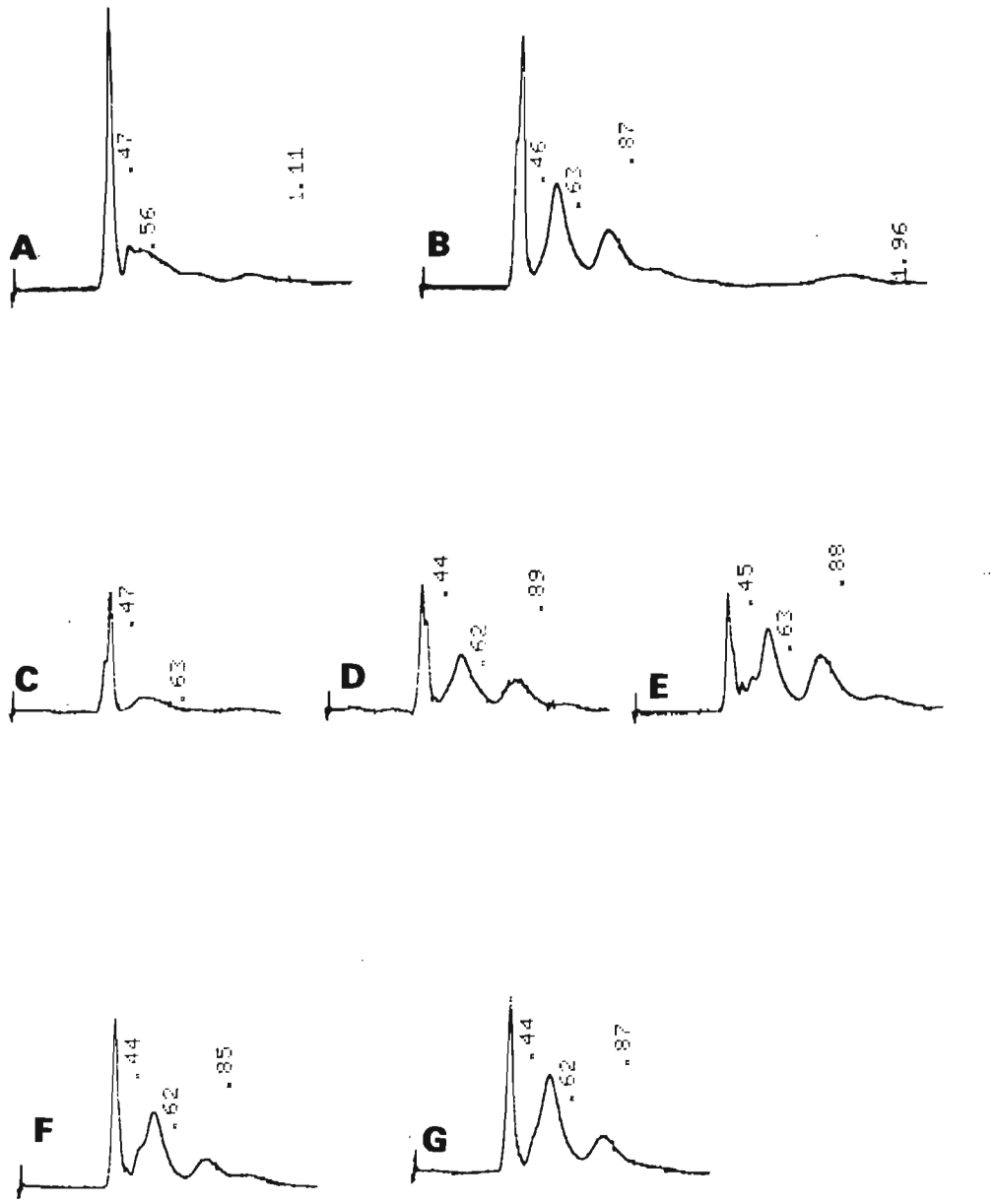


Figure 7.4

Volatile profiles obtained from three seed lots stored under slow (A - E) or accelerated ageing conditions (F & G). Dry seeds were removed from storage and heated at 90°C for 45 minutes; 50 µl of vial headspace was injected into the gas chromatogram using gas-tight syringes. Chromatographic conditions identical to those used earlier (Figure 7.3). Storage conditions were:

Storage conditions were: 6 years at 20°C and 0% RH (A) or 20% RH (B);

2 years at 20°C and 0% RH (C), 40% RH (D), 60% RH (E);

10 days at 30°C and 100% RH (F) ; 20 days at 30°C and 100% RH (G).

See also Table 7.7.

Table 7.7

Volatiles identified by gas chromatography from seeds of different storage regimes and viability after heating in hermetically sealed vials at 90°C for 45 minutes. Data presented as total integrator counts and relative area percentages for integrated peaks.

*A Values corrected for background by subtraction

*B Mean and standard deviation (parenthesis) for four injections

*C Seeds stored in hermetically sealed vials at 5°C for 2 years after ageing treatment

*D Seed imbibed for 1 hour after heat treatment.

See Figure 7.4 for representative chromatograms.

R.H. of Storage	Duration of Storage	Area % of Peak at RT					Total counts *A	% germination
		0.47 min.	0.62 min.	0.88 min.	1.11 mins	1.96 mins.		
60%	2	10	53	35			1911	77
40%	2	30	66	33			1295 (186) *B	97
0%	2	49	50				465	95
100%	20 Days @ 30°C *C	16	55	27			2355	2
100%	10 Days @ 30°C *C	21	51	26			1600	71
40%	6	29	40	23		8	2822	0
0%	6	81	9		9		921	63
		77	23				1523 *D	

or epoxy- or hydroperoxy - fatty acids (Moll, Bièrmann & Grosch, 1979). Thermal decomposition of such hydroperoxides at high temperatures in model fatty acid autoxidation systems has indicated that a wide range of highly characteristic products, principally aldehydes, hydrocarbons and fatty acids are produced depending on the particular position of the hydroperoxy group on the fatty acid chain (Reviewed by Frankel, 1982). Although earlier studies in which lipid hydroperoxides were measured failed to show a clear relationship between level, per se, and viability (Figure 6.1) it was felt that investigation of the volatiles produced on heating could be profitably undertaken. This could provide a one-step method for determining both hydroperoxides, their breakdown products and any radical species produced, which might give a better indication of overall peroxidative damage to the seed.

MATERIALS AND METHODS:

In view of the exploratory nature of the study and the varied experimental parameters used, appropriate details will be given in the text so as to place the development of the investigation into chronological perspective. Initially investigations were undertaken using the same chromatographic conditions as were used earlier (Chapter 7(A)). In addition the use of other column packings were explored, namely Poropak QS (Water Associates) and S.E. 30 liquid stationary phase (Ohio Valley Specialities) on Chromosorb WAW (50/75 mesh).

RESULTS:

It was initially observed that when 3.5 g of seed were placed in sealed 10 ml. vials, and heated at 90°C for 45 minutes, that peaks tended to be small and poorly resolved (Figure 7.4). Nevertheless within each seed lot the total integrator counts showed a negative relationship with germination (Table 7.7). These promising findings prompted attempts to find suitable chromatographic conditions which would improve resolution of the peaks and integrator counts. The simplest approach was to increase temperature and the

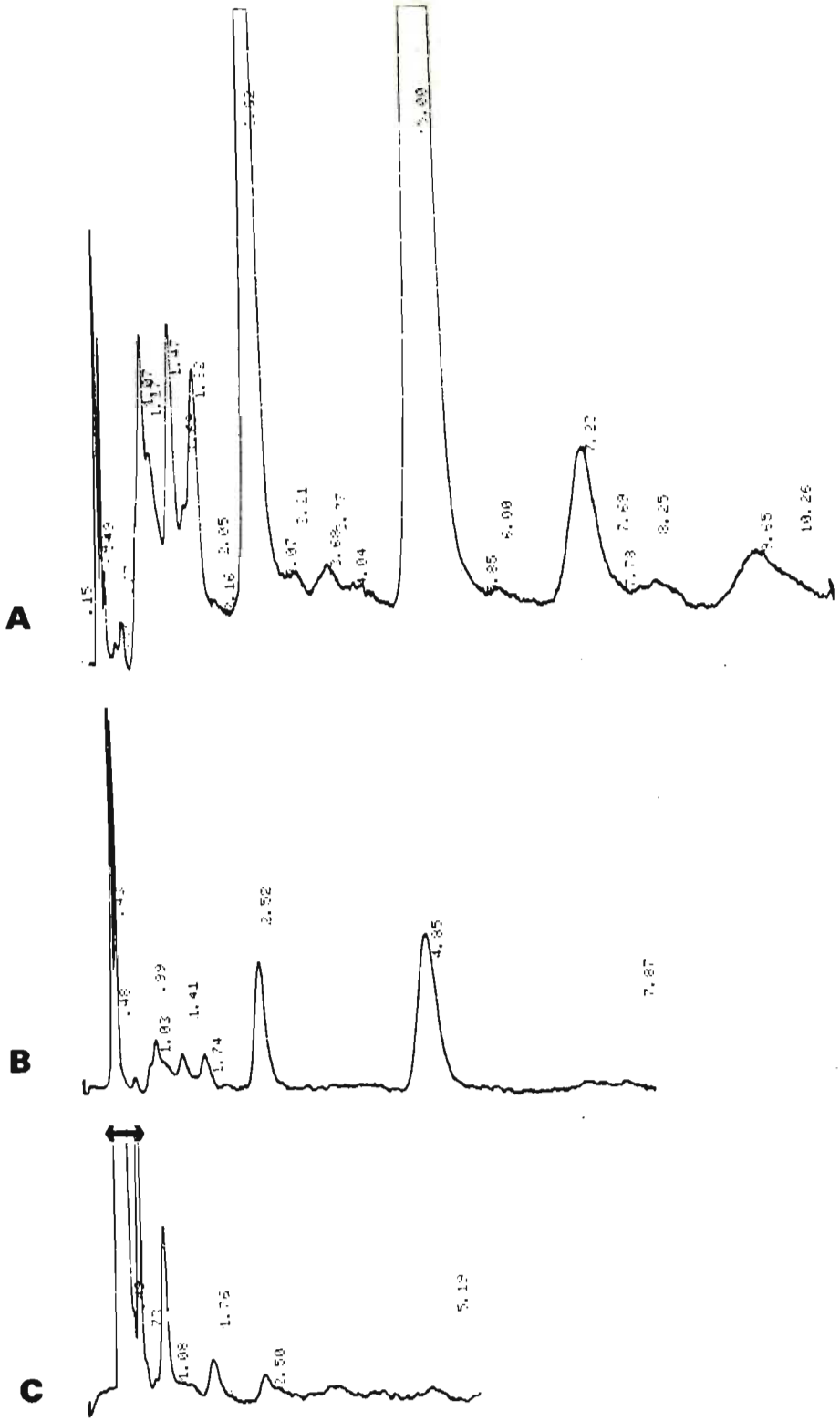


Figure 7.6

Volatile profiles from two lots of heated lettuce seed illustrating the better resolution obtained by gas-solid chromatography on Poropak QS (compare Figure 7.5). The differences between injection of hot headspace volatiles (A) and the chromatographic profile obtained on cooling (B & C) are clearly evident, as are differences between non-viable seed (A & B) stored for 10 years in hermetically sealed tins at 20°C, and viable seed after 12 years hermetic storage at 20°C in cans (C). 500gm tins of seed were heated at 120° for 20 minutes and 100µl of headspace withdrawn by piercing the metal foil seal with the syringe needle (A), or on cold injection on the following day (B & C).

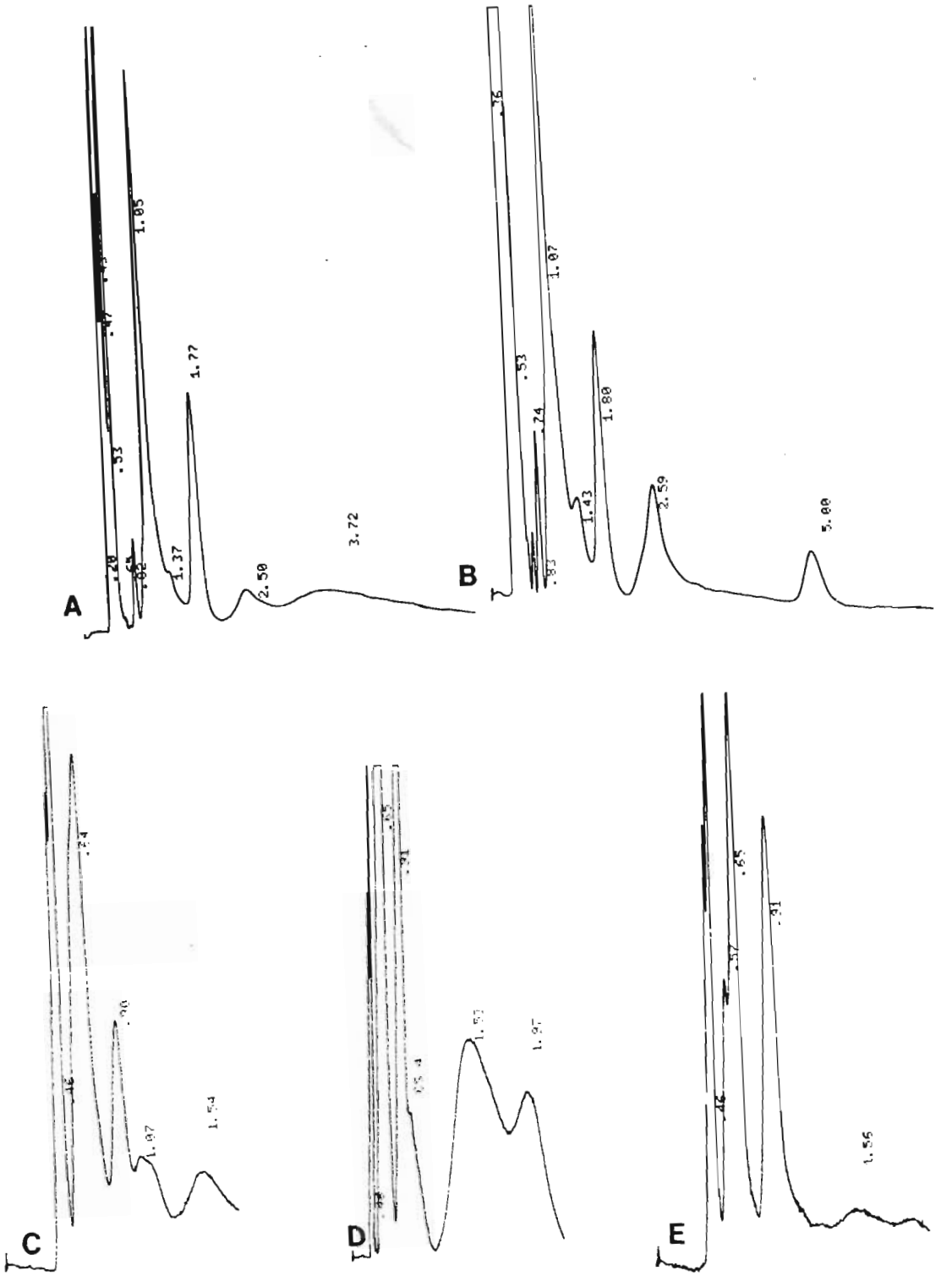


Figure 7.5

Volatile profiles for seeds heated at higher temperatures and in which the seed : headspace ratio was increased by adding 10 gm of seeds to vials. Clear qualitative differences are evident between seeds of high (A) and low (B) viability. Vials heated to 160°C for 1 hour and cooled before injecting 100µl of headspace.

Peaks are poorly separated with short heating periods (5 minutes at 100°C) when injected hot (C & D). With a longer heating period of 100°C for 30 minutes and cooling peaks are still fused (E) but not as poorly resolved as earlier (D).

2 years storage at 20°C and: 20% RH (C)

and 60% RH (D & E)

seed:headspace ratio by increasing the weight of seeds added to the vials to 10 g . The results of this approach are illustrated in Figure 7.5, and show that under chromatographic conditions identical to those used earlier (Table 7.9), substantially greater peaks were obtained. It was evident from these results that the volatile profiles changed significantly depending on whether samples were injected from hot vials (Figure 7.5, D) or whether these were allowed to cool before injection (Figure 7.5, E). The decline of the peaks at RT = 1.53 and 1.97 minutes presumably reflected the low volatility of these compounds, although chromotographic separation was not entirely satisfactory. Nevertheless, injection of headspace samples from vials which had been allowed to cool after heating revealed significant differences between seeds of different viability (Figures 7.5, A & B). It was apparent that when comparing a sample of high viability (97% germination, Figure 7.5, A) with another seed lot (63% germination, Figure 7.5, B) that the peaks at RT = 2.59 and 5.00 mins. were possibly indicative of deterioration. The suggestion that the first major peak represented pentane (as based on co-chromatography) was less apparent in these studies than the earlier investigations since this peak was often composed of additional fused elements.

In order to try and improve the probability of identification of the unknown peaks, Poropak QS was used as a stationary packing phase. Hermetically sealed tins of seed were heated to 160°C for 20 minutes before injection of a headspace sample or allowed to cool overnight before injection. Pentane co-chromatographed with RT = 7.2 minutes, and constituted 4.5% of total integrator counts when injected as a hot sample (Figure 7.6, A). Surprisingly, on cooling, the pentane-like peak was absent, (Figure 7.6, B) which suggested that the volatile may have become re-partitioned within the seed tissue. Pentane would not however be expected to disappear without trace at room temperature and this suggested that some other product may have co-chromatographed with pentane. Additionally two dominant peaks at RT = 2.6 & 5 minutes declined substantially on cooling, although other peaks were nevertheless reduced proportionately, with the exception of the first eluting peak(s) RT = 0.48 mins (Figure

7.6, B). The seeds used in this study had been held in the tins, as purchased from the Supplier, for 10 years at 20°C (cv Great Lakes R200). These latter seeds were non-viable in contrast to another cultivar stored for 12 years in sealed tins at 20°C (cv Webb's Wonderful) for which 95% germination was recorded. (Figure 7.6, C).

For this lot and cultivar a much larger first eluting peak was a striking feature of a hot injected sample, and was almost totally lacking the two indicator peaks which were considered likely indicators of deterioration. These two cases investigated were for viable and non-viable seed samples stored under comparable conditions for long periods. To be of any possible predictive value it would be necessary to demonstrate that the putative "indicator peaks" were able to indicate smaller differences in viability loss without the need to use excessively large amounts of seed. The small peak seen at RT = 2.5 mins. (Figure 7.6, C) represented less than 1% of total integrator counts. The clearly better peak resolution obtained using Poropak compared to Carbowax prompted a re-examination of the two seed lots examined earlier (Figure 7.5, A & B), where cool injected volatiles on carbowax had shown differences in volatiles which could be related to viability.

10 g of seeds were placed in 10 ml vials and these were heated to 120°C for 30 minutes. Vials were removed from the oven and allowed to cool to room temperature before injection. A better separation was clearly achieved (Compare Figures 7.5 A & B with 7.7 A & B). The pentane-like peak was identified at RT = 7.8 mins., and appeared to show a clear relationship to seed viability, being greater in the sample with lower (63%) germination (Figure 7.7, B). The appearance of a large peak with RT = 1.97 mins. was anomalous, in that it was much smaller in a sample of the same seed lot which had been held at 20% RH (data not presented). The volatile patterns were essentially indistinguishable for all samples during the first 3 minutes of the chromatographic run; differences which were likely to be indicators of viability were only seen after this time.

From the results presented it is evident that there may be as many as 10 compounds present in the volatile profile of heated seeds. Poropak is a highly uniform porous polymer which readily separates volatile compounds from strongly polar sustances, the latter always appearing ahead of analogous hydrocarbons. Hydrocarbons ranging from ethane to butane would be expected to elute before pentane. In addition, methanol, formaldehyde, acetaldehyde, ethanol, formic acid, propionaldehyde, acetone, isopropanol, acetic acid, methylacetate and propanol would elute before pentane. Compounds likely to elute after pentane include isobutyraldehyde, butanone, isobutanol, propionic acid and pentanol (Packard-Becker Catalogue). While some of these compounds have been identified as being present on thermal decomposition of lipid hydroperoxides, considerable emphasis has been laid on aldehydes (Frankel, 1982). Unsuccessful attempts were made to identify the peaks on these chromatographs with the aid of standards, when these became available (See page 151). A clear disadvantage of Poropak was the relatively long run times required (10 minutes), and in later studies the use of S.E. 30 (silicone oil) as liquid stationary phase was explored which permitted rapid analyses to be undertaken, and hence increased sample throughput.

It may be concluded from these studies that qualitative and quantitative comparisons (as integrator counts, only) could be meaningfully made between seeds of differing viability, and between different seed lots. In a sense the heat treatments required to produce the volatiles could be considered a form of heat-stress of the kind used to evaluate seed storage potential (Powell & Matthews, 1984). Although the conditions used in the studies reported above were lethal, there seems no reason why, with longer heating times at lower temperatures, this could not be used to achieve meaningful results. Roberts (1982) has argued that for seeds all temperatures other than absolute zero could be regarded as a form of heat treatment. This being so, there should a priori be no reason why seeds in normal laboratory storage should not produce volatiles, although the time required for their detection might be months or years rather than hours. Some support for this argument has been

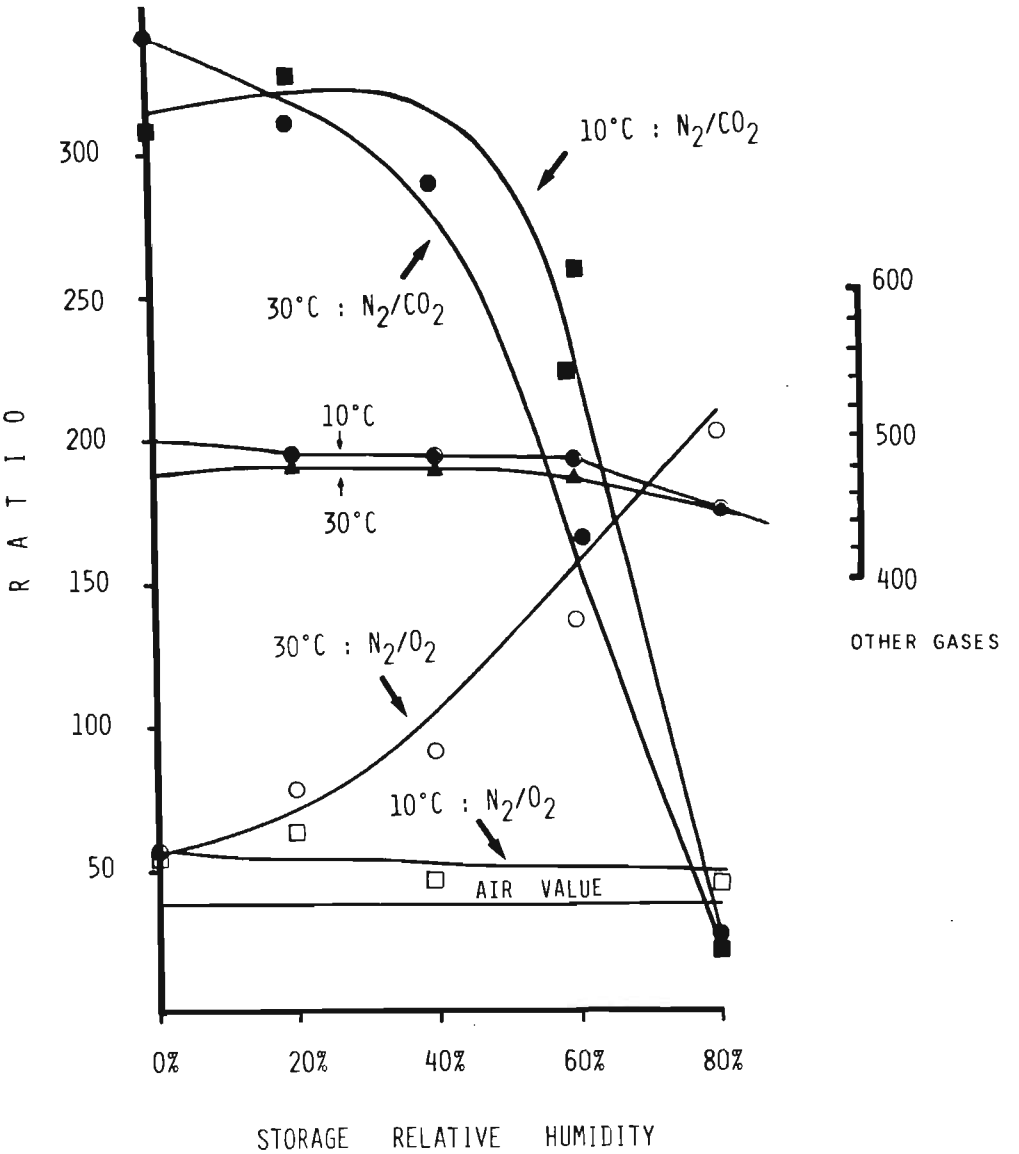


Figure 7.8

N_2/O_2 and N_2/CO_2 ratios for a single seed lot stored hermetically for 2 years at 10°C and 30°C and a range of RH, as indicated. In addition, total integrator counts for all other gases are given, although these show little change.

Open squares N_2/O_2 ratios at 10°C;

Solid squares N_2/CO_2 ratios at 10°C and

Ringed solid circle total counts for other gases at 10°C.

Solid circles N_2/O_2 ratios at 30°C;

Open circles N_2/CO_2 ratios at 30°C and

Triangle total counts for other gases at 30°C.

N_2/O_2 ratio for air denoted as AIR VALUE. Note: Values on the ordinate for N_2/O_2 should be reduced by one tenth.

obtained by sampling the headspace of seeds sold in hermetically sealed packets which differed in viability (Hailstones, 1983). However the total volatile count was found to be more closely related to date of packing than viability. This might be expected if some volatiles produced are lost in open storage. In addition volatiles may be produced which are unrelated to deterioration per se, such as those from dry seed respiration, of which CO_2 is likely to be the most abundant (Stanwood & Bass, 1981). Although CO_2 would not be detected by the flame ionization detector, it could perturb the air peak sufficiently to give spurious integrator counts.

(i) Carbon dioxide production and oxygen utilization during dry storage

Further support for the production of volatiles in storage was sought by examining the headspace of seed samples which had been used in the earlier germination studies. In these studies, seeds equilibrated to a range of RH's were stored in 35 ml hermetically sealed serum vials, and the loss of viability recorded over 22 months (Chapter 2). On the termination of these experiments the sealed vials were returned to the appropriate temperature regimes. After 2 years, during which time the vials had remained sealed, samples were withdrawn from vial headspace and N_2/O_2 and N_2/CO_2 levels determined by GC using a two column system of Poropak and molecular sieve with a thermal conductivity detectors (See Materials and Methods). This technique permitted the measurement, as integrator counts of all gases, as well as those for O_2, N_2 and CO_2. Since nitrogen levels remain constant, it was possible to measure changes in the ratios of CO_2 and O_2 relative to the nitrogen value. (The N_2/CO_2 ratio for air is large at $\pm$ 400, whereas the N_2/O_2 level is small at $\pm$ 3.9; production of CO_2 in hermetic storage would be reflected by a fall in the ratio, whereas if O_2 is consumed the ratio will rise). The contribution of other gases was determined by difference, being total integrator counts minus the values for CO_2, O_2 and N_2. For air this value would normally include the noble gases but in the present system could include smaller hydrocarbons, alcohols and aldehydes. The values obtained are presented graphically in Figure 7.8.

Unfortunately it was only possible to make analyses on seeds held at 10°C and 30°C since sufficient and equal quantities of seed were only available for these two regimes. It was observed that for seeds held at 10°C the oxygen levels remained relatively constant while at 30°C levels of O_2 decreased with increasing RH of storage. By contrast N_2/CO_2 levels were very similar at 10° and 30°C, with CO_2 production showing a progressive and rapid increase beyond 40% RH. In striking contrast to these changes, the contribution made by all other gases tended to remain constant irrespective of temperature over the ranges 0 - 60% RH, with a slight decline evident at 80%. It was notable that these counts were greater than the levels recorded for air, and yet were not greatly different at the two temperatures; these results are not readily explained. This could be regarded as indirect evidence for the production of other volatile products in storage.

In seeking to explain the differences between the CO_2 and O_2 levels at the two temperature regimes the relative contributions of respiratory and non-respiratory sources should be considered. There is however a limited literature on the topic of respiration in dry seeds. An early study by Bartholomew and Loomis (1967) showed that production of CO_2 in maize seeds increased exponentially with increasing moisture content. CO_2 output was also shown to be temperature dependent with Q_{10} values within the range expected for enzymatic reactions. However, dead-sterile seed produced 30-50% more CO_2 than comparable viable seed, from which it was concluded that non-respiratory sources could also be important. Stanwood and Bass (1981) have reported a progressive increase in CO_2 by safflower seeds stored for 17 years at temperatures between -12°C and 32°C (moisture contents not mentioned), which was also suggested to be the result of respiratory activity. Certainly there is some metabolic evidence based on radio-isotope studies to suggest that limited metabolic activity is possible in seeds (Edwards, 1976; Chen, 1972). It will be assumed for the sake of the present argument that enzymic decarboxylations, even if they do not include a complete pathway, also fall into the latter category.

A further possible non-respiratory source of CO_2 not considered in the seed literature is that derived from peroxidation. Loury (1975) has identified aldehydes, alcohols, hydrocarbons, CO_2 and water from the autoxidation of oleic acid and nonanal in a model system at temperatures between ambient and 40°C. It was suggested that complex reactions could take place, including the autoxidation of aldehydes to produce an unstable hydroperoxyaldehydes which may cleave to produce formic acid. The latter may be subjected to further free radical attack and decompose to water and CO_2. Although these results do not provide an immediately obvious solution to the differing results seen in Figure 7.8, the following highly speculative suggestion is advanced: if the oxygen requirement for respiration is presumed to be negligible at 10°C, then the low, and almost constant level of oxygen used may be implicated in the complex peroxidation reactions proposed by Loury. After the initial peroxidation it may be assumed that the autocatalytic reactions may be independent of temperature and further oxygen consumption, but significantly influenced by RH. At 30°C oxygen consumption reflects increased respiratory activity (by perhaps seeds and microflora) in addition to peroxidation, which results in a slightly greater production of CO_2 with increasing RH.

(ii) Further studies on volatiles produced by heating dry seeds

In the preliminary studies thus far comparisons were of a limited nature and rather severe heating conditions were employed. In the light of the encouraging results obtained, the study was extended, with the specific purpose of investigating different seed lots of different viabilities. To ensure a more rapid and uniform heating of seeds, vials were placed in a sand bed which had been allowed to equilibrate to oven temperature (90°C). Several gas-tight syringes were used in rotation, and between samplings the syringes were stripped and held in the oven so as to eliminate any possible cross-contamination of volatiles. Vial septa were of silicone rubber to reduce possible septum contamination by outgassing. The chromatographic column was changed to a 1.5m x 4mm. I.D. glass column with SE 30 as the liquid stationary phase.

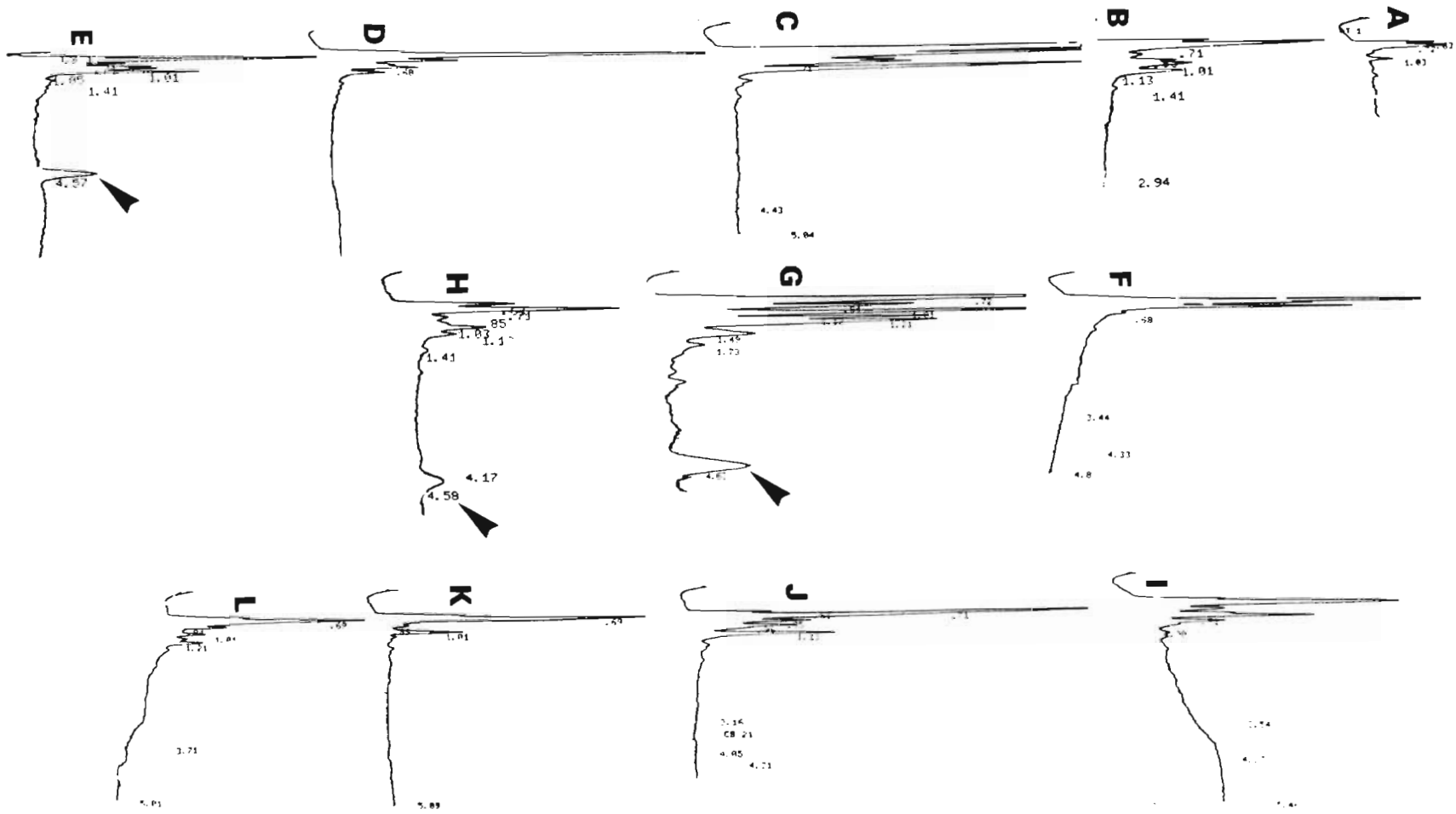

E
1.41
4.57
D
C
4.43
5.84
B
1.71
1.01
1.13
1.41
2.94
A
1.01
H
1.03
1.41
4.17
4.58
G
1.73
F
2.44
4.33
L
1.71
3.71
K
1.01
J
4.95
4.21
I

Figure 7.9

Volatile profiles for several seed lots on heating in hermetically sealed vials at 90°C for various lengths of time. Although early peaks are rapidly eluted and make qualitative comparison difficult, some relationship may nevertheless be seen between seed age, and duration of heating.

A putative hexanal peak is indicated (arrowheads).

Storage conditions:

A 6 years at 0% RH, 1½ hours heating

B 6 years at 0% RH, 5 hours heating

C 2 years at 80% RH, 3 hours heating

D 6 years at 40% RH, 3½ hours heating

E 6 years at 40% RH, 1 hour heating

F 20 days at 30°C and 100% RH*, 1 hour heating

G 11 years storage over silica gel, long term post-mortem, 1 hour heating

H 3 years over silica gel, necrotic cotyledons on purchase, 1½ hours heating

I 9 years over silica gel, 1 3/4 hours heating

J 13 years in sealed tins (cv. Webb's Wonderful), 1 1/4 hours heating

K 2 years at 0% RH, 3 1/6 hours heating

L 2 years over silica gel, high vigour seed, 1 1/4 hours heating

* Seed held for 2 years at 5°C in hermetically sealed vials. Except for (F) all seeds held at 20°C.

Oven temperature was 220°C with a carrier gas flow of 50 ml/min. 200µl of headspace was injected using hot syringes. It should be noted that silicone oil (S.E. 30) is a liquid stationary phase characterized by low McReynolds constants with a C.P. index of 5 (Squalene reference = 0) which contrasts with that of carbowax 20 M which occurs essentially in the middle of the polarity scale (C.P. index = 55 out of a total of 100).

Under the chromatographic conditions applied, all major compounds eluted within 1.7 minutes (Figure 7.9). While it was obvious that the many rapidly eluting individual peaks were not individually integrated, a total integrated count was obtained. It became evident that a relationship existed between total counts, conditions of storage and seed germination. During the early stages of heating (less than 1 hour) the first eluting peak was seen to be two fused peaks with RT = 0.63 and 0.72 minutes (Figure 7.9 A & B).

With further heating of vials, the second volatile increased progressively, and tended to obscure the smaller first peak. This latter peak appeared to remain constant and possibly represented the dead time for emergence of air. This dominant peak co-chromatographed with pentane. A peak with RT = 4.5 minutes, and which co-chromatographed with hexanal was evident in some samples (Figure 7.9 E, G & H). Although produced by some viable seed samples, it was generally in the range of 2 - 4% of total peaks, and appeared after 3 hours heating. On the other hand, samples which were characterized in later seedling growth by necrotic cotyledons, but yielded a high germination (79%) the hexanal-peak represented 9.5 - 12% of total peaks, and could be detected within 1 hour of heating (Figures 7.9 & 7.10). For long-term post-mortem material the hexanal-peak represented 13% of total integrator counts, being some 6,280 counts (Figure 7.9, G). This peak was conspicuously absent from samples of two seed lots which had been aged at 80 & 100% RH, the viability of samples being 0% & 3%, respectively (Figure 7.9). While the former sample was clearly post-mortem material (2 years storage at 80% RH) this feature could not be used to explain the absence of this peak since it was evident in a post-mortem sample from a seed lot held at 40% RH for 5 years (Figure 7.9 E).

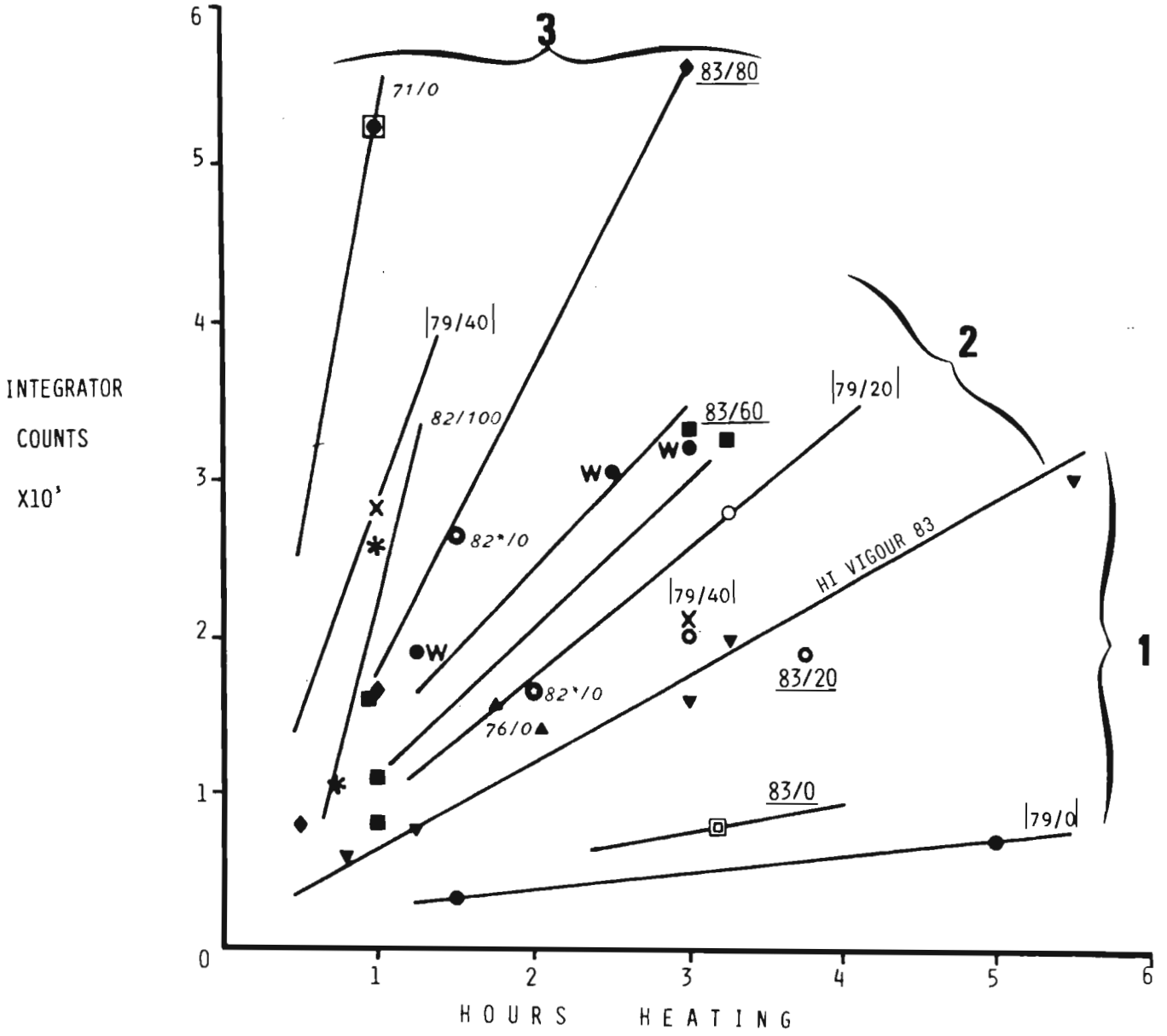


Figure 7.11

Total Integrator counts for volatiles derived from heating seeds in hermetically sealed vials for various periods of time. Values presented are for 7 different seed lots stored for between 2 and 14 years.

Date of purchase is followed by RH of storage, eg. 71/0. All cultivars are Great Lakes, except for Webb's Wonderful denoted as W .

Duration of storage may be obtained by subtracting date of purchase from 85.

It may be note that three broad categories of seed quality may be distinguished (1-3).

Additional data pertinent to this graph may be seen in Figure 7.9 and 7.10. The inverted, solid triangle denotes an additional seed lot purchased in 1983 which was considered to show high vigour (HI VIGOUR 83).

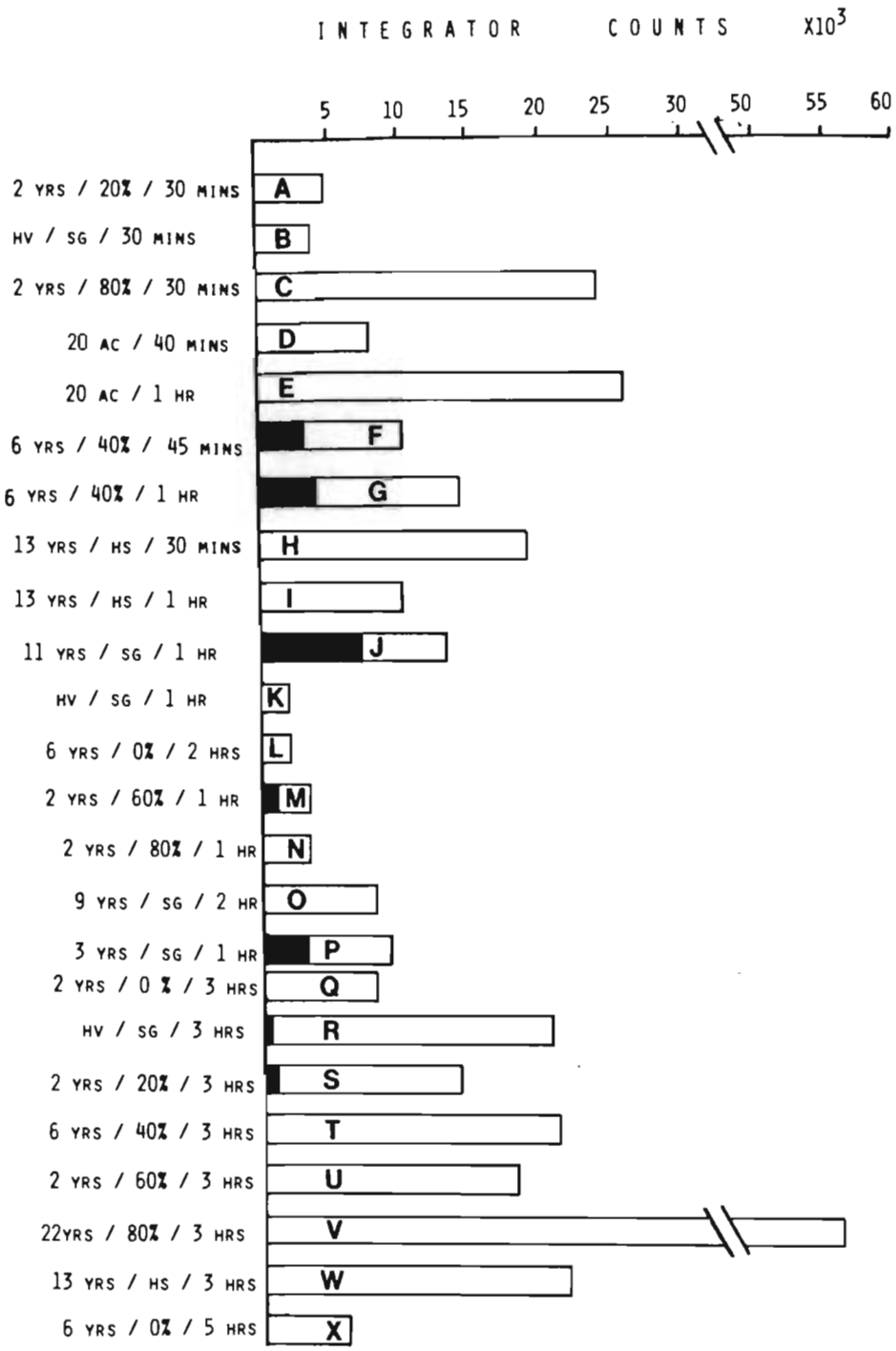


Figure 7.10

Erratum: for HR and HRS read hours

Total integrator counts for volatiles from heating seeds in hermetically sealed vials at 90°C. (See also representative chromatographs in Figure 7.11). A putative hexanal peak and its contribution to peak counts is indicated by solid shading in the histograms. To facilitate comparisons storage data are indicated adjacent to the histograms, eg. 2yr/20%/30 mins 2 years storage at 20% RH, 30 mins heating. Except for heating times of less than 1 hour, which are given in minutes, all other times are rounded to whole hours.

Abbreviations:

HV - High vigour seeds, 2 years storage in silica gel (SG)

20 AC - 20 days accelerated ageing at 30°C and 100% %H

HS - Hermetic storage in tins, cv. Webbs Wonderful

In order to facilitate the interpretation of results obtained from the many seed lots investigated in this study, total integrator counts are presented graphically in Figure 7.11, straight lines are drawn between the points or cluster of points obtained to facilitate interpretation. Where only a single value was obtained a line was drawn which extrapolates to the intercept. This was considered to be 2000 on the ordinate. This value was obtained from the projected straight line obtained for a high viability sample stored for 2 years over silica gel at 5°C for which 5 determinations were available. Using this approach it was possible to group seed lots into 3 categories:

1. Seeds of high germination percentage germination which were free of necrosis.

2. Partially deteriorated seed where germination was between 30% and 95%. Cotyledonary necrosis may or may not be present.

3. Long-term post-mortem, non-viable or low viability seed.

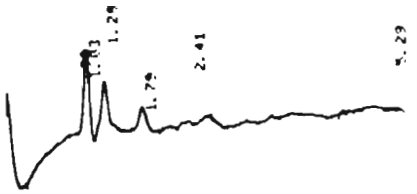
The only exception observed was a seed lot purchased in 1979 for which 90% germination was recorded and in which evidence of necrosis was seen in 20% of seedlings.

Whilst the relationship between counts and viability was not entirely consistent between seed lots, it was noticeable that a progressive increase in counts with RH was seen. (e.g. 1979 & 1983 purchases which were stored at 0 & 40% RH and 0% - 80% RH for 5 and 2 years, respectively). As can be seen Figure 7.10, attempts to resolve inconsistencies by referring to the pentane-like peak yielded no trend which was not already apparent from the total counts.

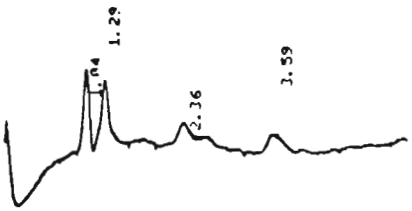
In some cases the pentane-like volatile could be seen to increase over a short timespan (Figure 7.10; D & E, F & G). In some instances smaller increases occurred over a longer heating period (Figure 7.10; L & X, G & T). Reduced output of the pentane-like

volatile was noted in samples of high viability or partially reduced viability (Figure 7.10; A & B, K & I) but low levels were sometimes evident in non-viable samples when compared with viable samples heated for the same time (Figure 7.10; G, I & J, R & T). The pattern of evolution by non-viable or low viability seed lots stored at high relative humidities was less predictable, generally increasing with duration of heating treatment. Reference to the hexanal-like compound (Figure 7.10; F, G, J, M & P) was seen to provide a better relationship to seed viability than total counts alone. However, the presence of small amounts of hexanal in viable material after 3 hours heating (Figure 7.10; R & S) and its apparent absence in later stages of heating (Figure 7.10; T) was inexplicable unless decomposition or thermal reactivity is invoked.

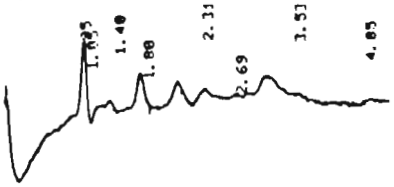
In order to more clearly resolve the rapidly-eluting, fused peaks noted above, oven temperature was reduced to 150°C at a N_2 carrier gas flow of 35 ml/min. Further 6 g quantities of a more limited selection of seed lots were placed in vials, as before, and heated to 90°C. Several peaks were now more clearly distinguished when 50 µl of headspace was injected per sample but excessive detector noise necessitated lower ionization amplification (5×10^2). Total integrated counts were thus much lower and consequently the pattern evident in Figure 7.11 was less apparent from this data (Table 7.8, Figure 7.12). Since 4 - 6 peaks were clearly discernible, an attempt was made to identify particular peaks with viability decline in the different seed lots. However, shortly after this was done the author was able to gain limited access to a GC-MS facility. From the results of these studies, which are considered in full later, it became evident that some of the single peaks in the packed column studies were, in all probability, several co-eluting compounds. (For comparison, see capillary GC chromatograms in Figures 7.16 & 17.18). Attempts to use aldehyde standards in the packed column studies to identify some of the peaks was not entirely satisfactory. The first-eluting peak at RT= 1.03 minutes was considered to be an air peak upon which was superimposed some unknown volatile, since counts varied with both time of heating and seed sample. No relationship could be seen between this peak and



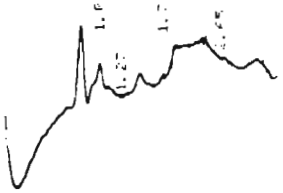
A. 2:53 HRS HEATING; 2 YEARS @ 0% R.H.



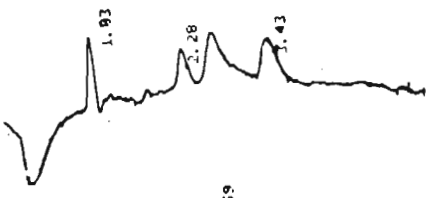
B. 3:00 HRS HEATING; 5 YEARS STORAGE OVER SILICA GEL



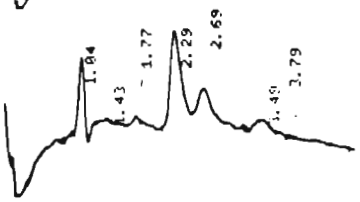
C. 2:44 HRS HEATING; WEBB'S WONDERFUL 13 YEARS IN HERMETIC STORAGE



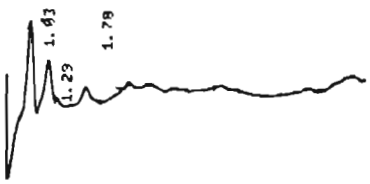
D. 6:05 HRS HEATING; 2 YEARS @ 40% R.H.



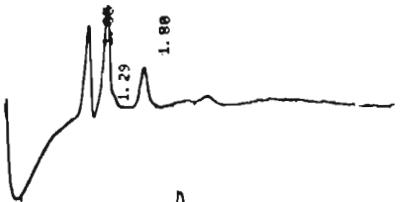
E. 3:57 HRS HEATING; 2 YEARS @ 60% R.H.



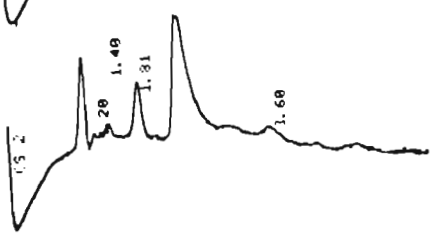
E. 2:31 HRS HEATING; 2 YEARS @ 60% R.H.



F. 5:24 HRS HEATING; 3 YEARS STORAGE OVER SILICA GEL; NECROTIC COTYLEDONS



A. 5:37 HRS HEATING; 2 YEARS @ 0% R.H.



G. 1:48 HRS HEATING; 11 YEARS POST-MORTEM STORAGE OVER SILICA GEL

Figure 7.12

Erratum: for HR and HRS read hours

Chromatographs of volatile profiles obtained from heating seeds in hermetically sealed vials (See also Table 7.8). Heating time followed by storage conditions, all at 20°C, given on right hand side.

Table 7.8

Total integrator counts (x 10^3) and peak counts at relevant retention times for seeds heated in hermetically sealed vials. Seeds heated at 90°C for duration indicated; chromatographic conditions differ from earlier (Figures 7.9 - 7.11). Relevant chromatographs are shown in Figure 7.8 for the storage regimes listed (A - G).

\+ poor peak integration

* Septum fault, only first peak recorded.

Storage conditions (20°C unless otherwise stated)	Heating time (h)	Total counts X10^3	Retention time (minutes) 1.03	1.29	1.39	1.8	2.32	2.75	3.54	5.3	7.72
2 years at 0% RH	2:53	5.56	1.99	2.31		.86	.52				
A	5:30	13.27	6.93	3.95		2.38	.52				
2 years over silica gel at 5°C (High Vigour)	3:0	4.19	1.73	2.17			.43		.75		
B	5:45	7.73	2.24	2.42			.59		2.61		
13 years in sealed tins (Webbs' Wonderful)	2:44	7.72	1.68		.93	.66	.58	.86	2.9		
C	5:14	5.7	2.22		1.01	.69	.68	-	1.08		
2 years at 40% RH	2:03	14.8	3.28	3.03		3.14	2.52	2.12			
D	6:05	15.8	6.57	3.19		3.41		5.42			
2 years at 60% RH	3:57	5.5	1.59				.79	1.65	1.5		
	2:31	8.3	1.57		.66	.08	3.06	2.3	.7		
E	6:10	*	3.4	-	-	-	-	-	-	-	*
3 years over silica gel (necrotic cotyledons)	1:30	6.67	2.50	3.75				.41			
	2:14	5.24	2.37	2.29		.106		.46			
F	5:56	6.39	4.6	1.4		.36					
	6:30	3.78	2.5	.79		.49					
11 years over silica gel	1:00	5.57	2.13	.61	.63	.89	.29			.97	
G	1:48	9.05	1.66	.12	.16	.98	6.0		.12		1.1

viability. A butanal standard yielded a major peak at RT = 2.5 minutes and a smaller peak at RT = 1.2 mins. Since butanal was not identified as a major component of the volatile profile (Table 7.9), it is suggested that the compound at RT = 1.29 may be hexanal. While this compound was evident in material of high viability (A, B and F in Figure 7.12), it was present in seed of lower viability (C, D and E in Figure 7.12) and long-term post-mortem seeds (G in Figure 7.12). This putative hexanal peak was seen to decline with further heating (Table 7.8), which is not in agreement with the earlier observations (Figure 7.10). It was not possible, therefore, to relate this peak to viability in any way. GC-MS analysis indicated that methylpropanal and methylbutanal were evident at high levels only in seeds stored for 13 years, whilst for those stored for 2 years these totalled less than 2%. A methylpropanal standard yielded 3 equal peaks at RT = 2.32, 2.75 and 3.54 minutes in addition to a very small peak at RT = 1.4 mins. on the packed column, while propanal yielded a single peak at RT = 2.32 mins. and a smaller peak at 2.75 mins.

Seeds from a lot stored hermetically in tins for 13 years contained 4 volatile peaks which corresponded to those of the above aldehyde standards, as did seeds stored for 2 years at 60% RH, although relative contributions from each peak differed with time of heating (Table 7.8). Pentane was tentatively identified at RT = 1.8 minutes on the basis of co-chromatography and was noticeably absent in a sample of high vigour held for 2 years at 5°C over silica gel. By contrast seeds stored for 2 years at 20°C and 0% RH, in addition to the seeds held in hermetic storage for 13 years possessed pentane-like peaks. Levels were higher in seeds held for 2 years at 40% RH and in which some evidence of mild cotyledonary necrosis was seen (32% of seedlings). Seeds in which necrosis was evident on purchase (3 years over silica gel at 20°C; 32% germination) had noticeably lower levels, as did material held at 60% RH for 2 years (68% germination). These results are not entirely consistent with those of the earlier study, possibly as a result of integrator performance with rapidly eluting peaks, or the broad peaks and baseline drift in this study.

While the interpretation of these results appears difficult when attempted identifications were made using aldehyde standards, there is nevertheless a qualitative trend apparent from the chromatograms. In all the samples investigated which had been either stored long term (C and G in Figure 7.12) or which were likely to have impaired germination (B, E and G in Figure 7.12) were seen to possess a greater number of peaks with RT = 2.32 minutes and above. One notable exception was a sample showing cotyledonary necrosis (F in Figure 7.12). Examination of quantitative data (Table 7.8) for total integrator counts, counts at particular retention times and changes with time did not reveal any trend which was thought to be of likely diagnostic value.

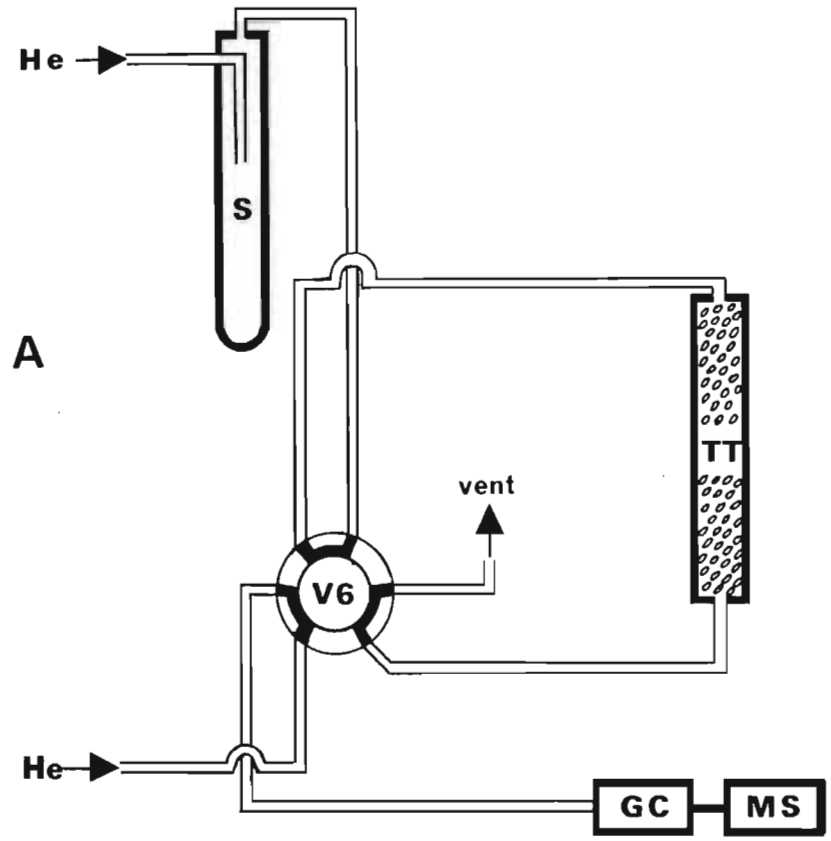
(C) GAS CHROMATOGRAPHY - MASS SPECTROMETRY STUDIES

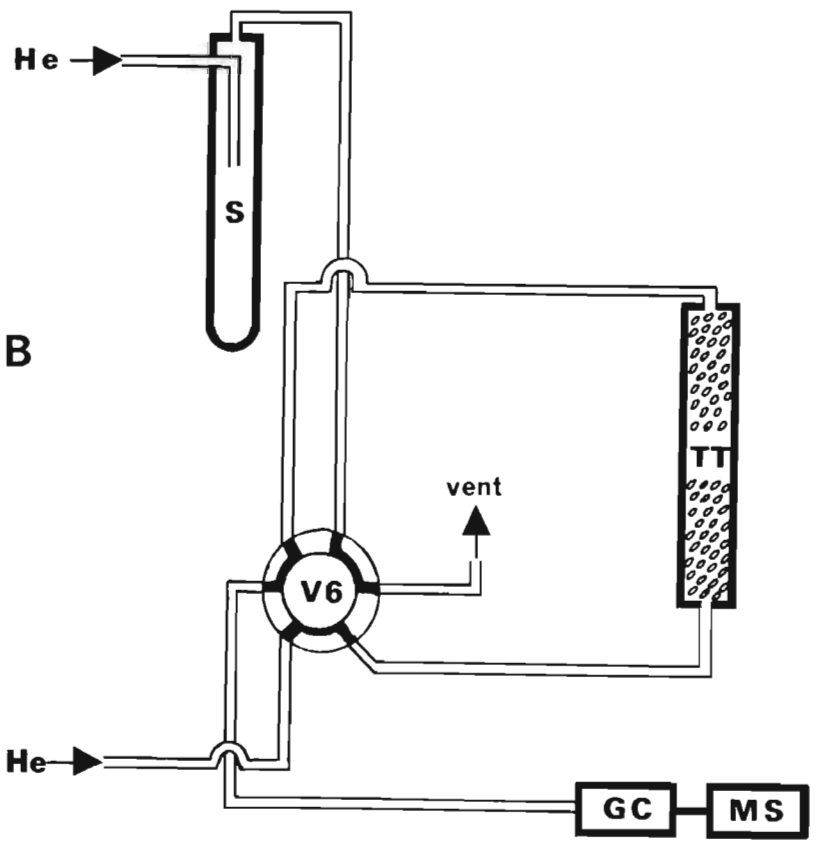
The use of volatile levels and patterns as a possible indicator of seed viability was clearly evident from the foregoing studies. However, a major difficulty encountered was the aldehyde standards which did not yield single peaks, suggesting possible breakdown products present as impurities which made identification, at best, tentative. The benefit of GC-MS in the identification of decomposition products is evident from many studies of model hydroperoxide systems (Frankel , 1982). Limited access was gained to such a facility which additionally possessed a headspace concentrator-sampler which permitted rapid, accurate heating of samples. Full aspects of the principle and mode of operation used are given below since it is necessary to fully appreciate differences between this system and that previously employed using serum vials.

Principles of Operation of Headspace Concentrator

A simplified schematic diagram of the Tekmar 4000 purge-and-trap headspace concentrator is presented in the figure overleaf. Samples are placed in a sample tube (S) through which helium carrier gas (He) passes at a rate which can be controlled by the operator. In-line hydrocarbon traps ensure that any possible contaminants are removed from the incoming helium carrier gas. The swept headspace

Diagrammatic representation of operating principles of Tekmar model 4000 purge and trap system used in GC - MS studies.





of the sample tube is carried via heated transfer lines (190°C) and a six-port valve (V6) to a "Tenax" porous polymer trap (TT) at ambient temperature which absorbs all volatiles, the scrubbed helium gas being vented to atmosphere via the six-port valve (V6). This is illustrated diagrammatically in Figure A, opposite.

After a pre-selected interval, appropriate solenoid valves isolate the trap from the transfer lines, during which time the trap is electrically heated to 190°C to desorb volatiles. The solenoid valves are used to regulate the duration of the desorb cycle to a short period (usually set at 0.5 minutes) to limit column loading. This operation takes place after electrical switching of the six-port valve which re-routes volatiles along a heated transfer line to the injector of the gas chromatogram (GC). After separation on the capillary column, effluents pass to the mass spectrometer (MS). This is illustrated diagrammatically in Figure B, opposite.

An electrically operated heating mantle may be placed around the sample tube, if desired, to bring about rapid, controlled heating of the contents. During chromatographic runs the "Tenax" is subjected to a 5 minute bake cycle at 220°C in a helium gas stream to remove any remaining volatiles and to reactivate adsorbent. After the automatic cool-down cycle has been effected another sample may be run.

GC/MS operating conditions

A Finnigan 1020 series gas chromatograph/quadrupole mass spectrometer/data system fitted with a 25m x 0.3mm i.d. fused silica capillary column coated with SE 30 was used in conjunction with the headspace sampler. Helium carrier gas was at 3 cm^3/min in a splitless injector mode. The column oven was held at 50°C for 5 minutes and was then linearly programed to 220°C over 15 minutes. The column exit led directly to the ion source of the mass spectrometer. Scans were performed from m/e 33 - 350 at 1-sec scan rate. Identification was made by reference to computer library standards, except for hexanal, for which a spectrum was obtained

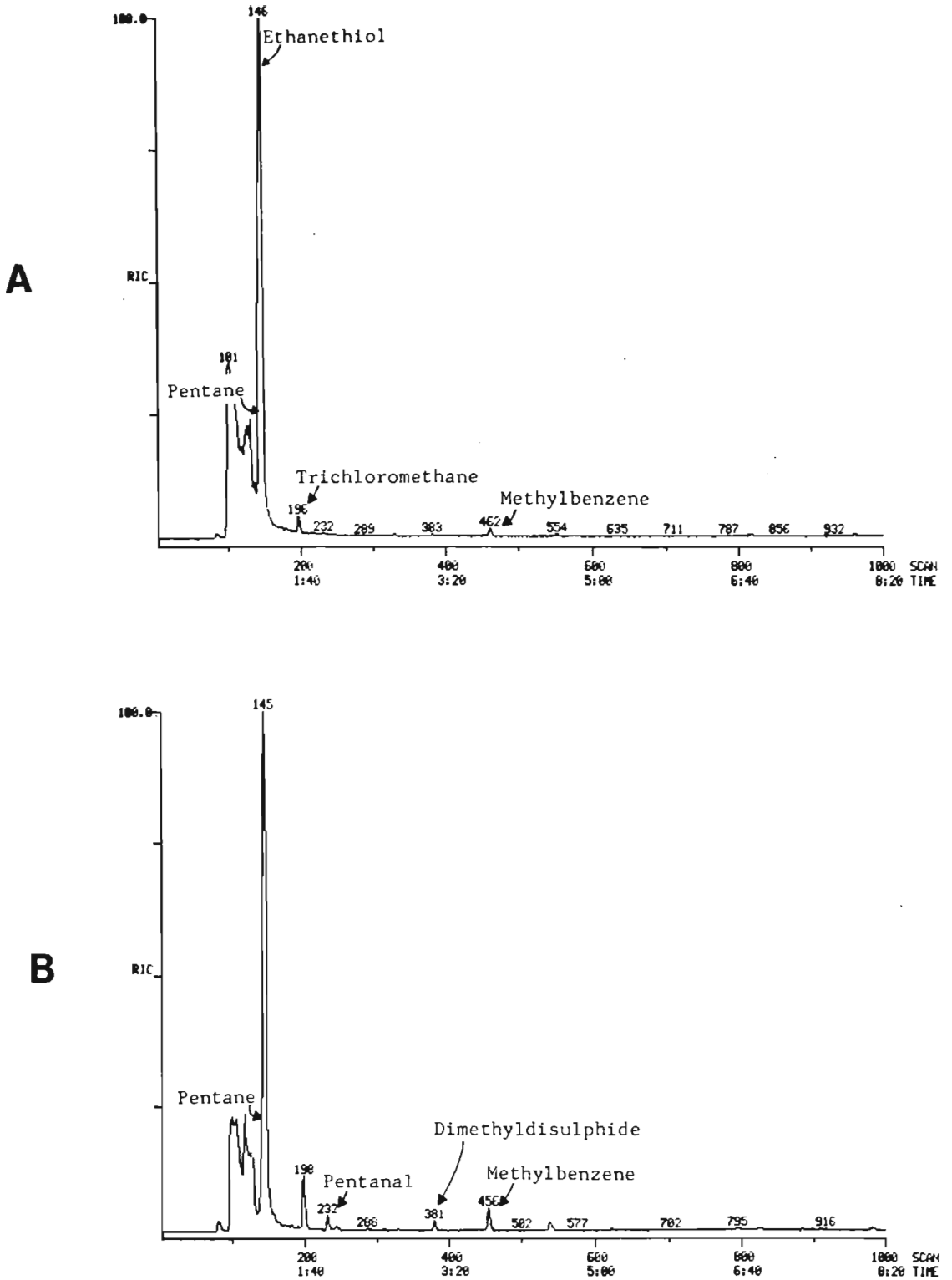


Figure 7.13

Volatiles identified by GC - MS in the headspace of imbibing seeds of lettuce:

A After 12 hours imbibition. Seed stored for 2 years at 0% RH, 97% germination.

B Same sample as above, but heated to 70°C for 4 minutes before analysis.

Pentane and ethanethiol co-elute; trichloromethane from use of an aerosol spray used for cryogenic focussing.

by injection. At the time of writing several standards had been obtained but these had not been added to an "operating library" of spectra. Thus all identifications, except for hexanal, must be regarded as tentative until subjected to verification with authentic standards. Compound quantitation for some of the runs was made using the Finnigan data system to determine areas under each of the chromatographic peaks in the reconstructed ion chromatograms. At the time of writing response factors had not yet been made pending a decision on the precise nature of quantitation to be undertaken. (Problems associated with headspace quantitation are considered in the discussion Page 167).

(i) Volatile production during seed imbibition

In the first part of this study, 3.5 g of seed from the same lot which had been stored at 20°C for 2 years at 0% and 60% RH were allowed to imbibe for 12 hours in 5 ml of distilled water in a thick-walled test tube (total volume 35 ml). The lip of the tube was sealed with polyethylene film (Glad Wrap) which was only removed before samples were connected to the headspace sampler. The headspace of the high viability sample (stored at 0% RH; 97% germination) was seen to contain a single large peak composed predominantly of ethanethiol fused with a smaller pentane peak (Figure 7.13, A). A small peak of freon was observed at scan 196, being the result of the use of this cryogen to effect cryofocussing on the capillary column. Although results were entirely qualitative, it was nevertheless clear that ethanethiol was the dominant volatile. Since the possibility existed that volatiles may have been present in the tissue but were not sufficiently volatile to enter the headspace in sufficient amount to be detected, the sample was subsequently heated for 4 minutes at 70°C and re-analysed. Little overall increase in volatiles was observed, with the exception of a small peak of pentanal at scan 232 (Figure 7.13, B). On the other hand when a sample from the same seed lot (germination 68%) which had been stored at 60% RH was similarly heated prior to headspace analysis, some substantial differences were observed in the volatile

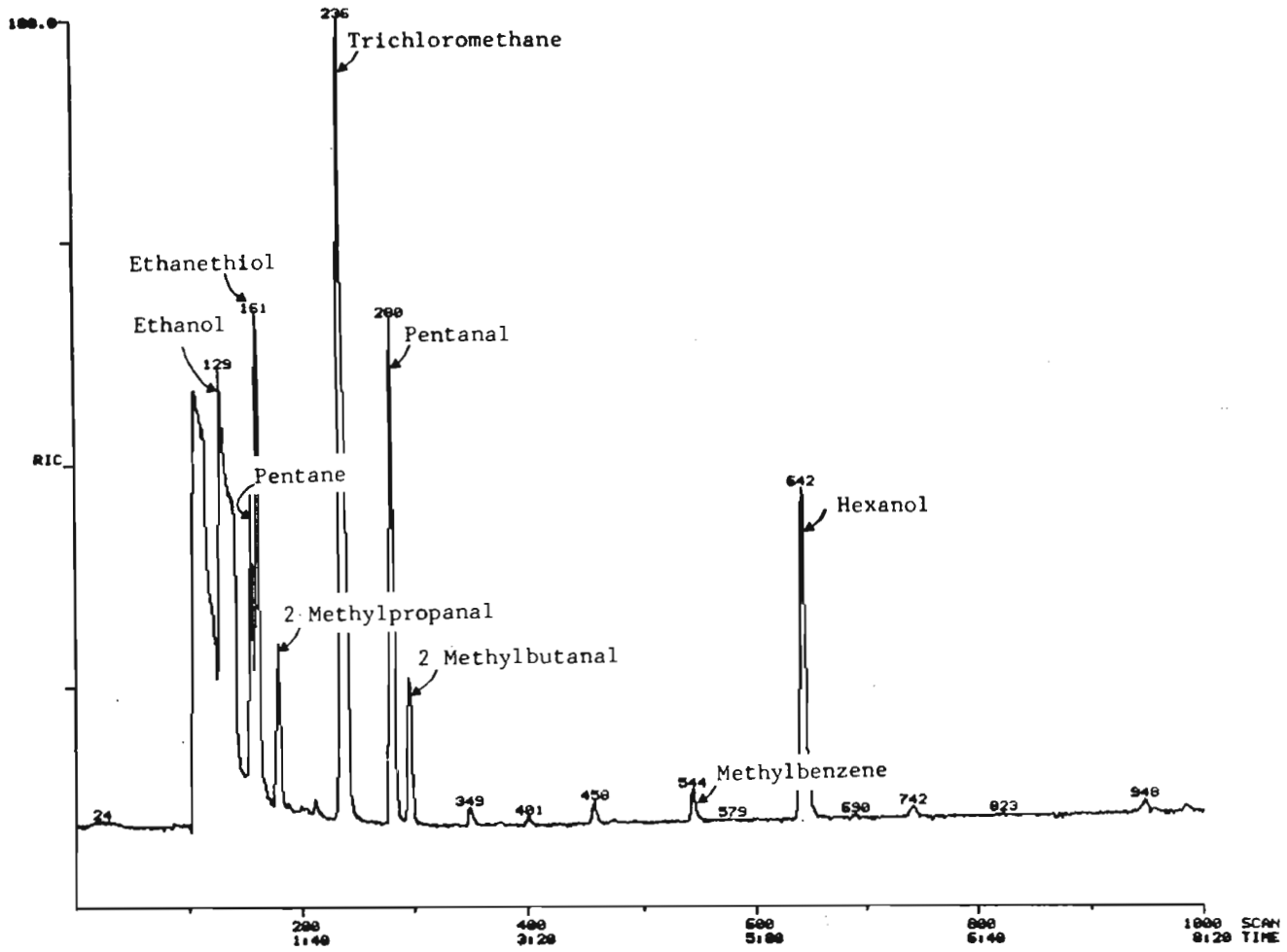


Figure 7.14

Seed of the same lot as illustrated in Figure 7.13 but stored for 2 years at 60% RH, germination 68%. Seeds were imbibed for 12 hours and then heated to 70°C for 4 minutes before sampling of headspace volatiles (compare with Figure 7.13 B).

profile (Figure 7.14). A number of straight chain and branched aldehydes were present as a dominant element of the volatile profile. As before, pentane and ethanethiol were present as closely fused peaks, confirming the suspicion that the earlier identification of pentane as the dominant volatile on packed columns was probably incorrect.

It was even possible that methylpropanal would have been co-eluted in the packed column studies with the pentane-like peak. The presence of ethanol may possibly be explained as a result of metabolic imbalances between glycolysis and the Krebs cycle, as has been suggested by Woodstock & Taylorson (1981) for soybean seeds. Ethanol was not observed in any samples of dry seeds which were heated (Section (ii), below), which further suggests this product may have been derived from respiratory activity rather than hydroperoxide decomposition.

Taylorson & Hendricks (1980/1) in reviewing anaesthetic release of seed dormancy have presented results which show that exogenously applied ethanol at 320 µM stimulated dark germination of Grand Rapids lettuce seeds, although the seedlings were seen to possess short radicles. It is suggested that an "alcohol overload" either as hexanol (Figure 7.15 C) or ethanol (Figure 7.14) may be responsible for reduced growth. (Other speculative consequences are considered later, page *161* - Further Physiological Consequences). The additional presence of aldehydes (Figure 7.14) may cross-link proteins and exacerbate cell damage. This could conceivably lead to the appearance of the stunted seedlings in which cotyledonary greening was seen to be normal (Figure 39, Chapter 2).

The conversion of aldehydes to their corresponding alcohols through the action of a possible oxidoreductase has been suggested from studies on blended, ripe tomatoes. Apart from reports of unsaturated compounds, which were not observed in the present study, acetaldehyde, n-butanal, 3-methylbutanal, pentanal, hexanal, methanol, ethanol, n-butanol, n-pentanol, n-hexanol, 3-methylbutanol and 2-methylpropanol have been identified in many studies (Reviewed

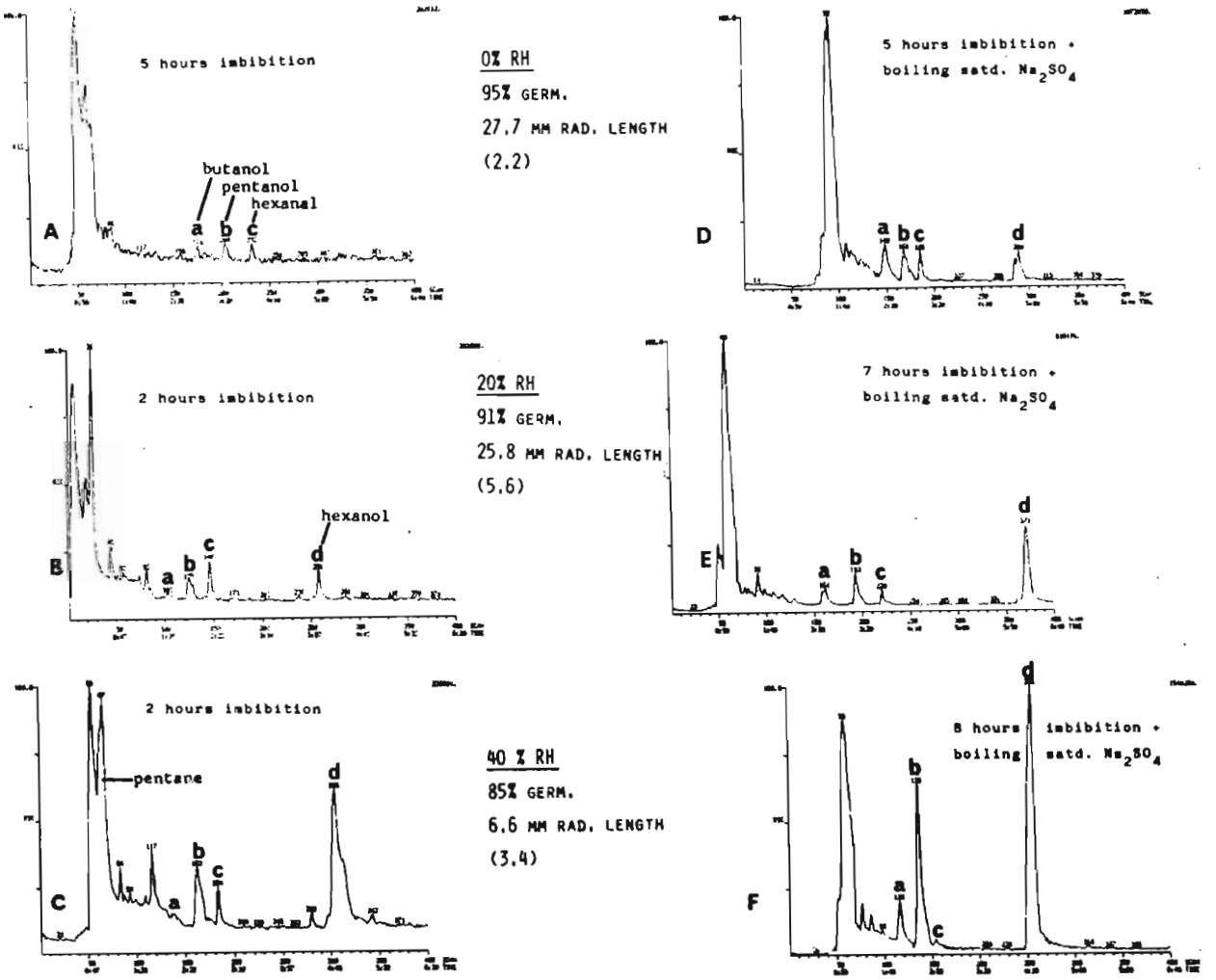


Figure 7.15

Seeds of the same lots as used earlier (Figures 7.13 and 7.14) but subjected to analysis after 2.75 years storage at 20°C and the RH indicated. In addition to germination data, mean radicle length at 5 days and standard deviation (parenthesis) are given. R.I.C. on left (A,B,C) show peaks identified after periods of imbibition indicated. R.I.C. on right (D,E,F,) are for seeds imbibed for periods indicated and then "salted out" with boiling saturated sodium sulphate solution.

by Eskin, Grossman & Pinsky, 1977).

It may be that hexanal, produced as a result of activation of the lipoxygenase pathway on wounding (Theologies & Laties, 1981; Galliard 1980) is nonspecifically removed through the action of the oxidoreductase. In vivo, a more tightly controlled system may operate in order to ameliorate possible damage, and interconvertibility between alcohol and aldehyde cannot be ruled out.

In the second part of this study, samples from the same seed lot were again analysed for headspace volatiles after a further year in storage. Certain experimental parameters were changed: 3.5 g of seed and 6 ml of water were placed in 50 ml conical "Quick fit" flasks fitted with a two-piece "Quick fit" PTFE stopper which, on assembly, would accept a 5 mm. o.d glass tube and effect a gas-tight fit by means of 0 ring seals. This glass tube could then be locked onto the sparging accessory of the autosampler, permitting positive sweeping of the seeds by the 37 ml/min. helium carrier gas. As before, the tube was sealed from the atmosphere at imbibition using polyethylene film (Glad Wrap).

Imbibition periods were shorter than previously being 2 or 5 hours but were sufficient to obtain headspace volatiles. (Figures 7.15 A, B, & C). In addition, samples were treated with 40 mls of boiling saturated sodium sulphate (Figures 7.15 D, E & F). The combined effect of the latter treatment was intended to release any volatiles from seed tissues by both heating and by increasing headspace concentration of water-insoluble volatiles (Hachenberg & Schmidt, 1971). Germination data were recorded for samples, as was radicle length at 48 hours, these being presented with the results. Satisfactory separation of lower molecular weight volatiles was not achieved probably since cryofocussing was not undertaken on the capillary column at the start of the chromatographic analyses, as had been done previously (Figures 7.13 & 7.14). The benefits of this latter technique are well-documented in the literature for improving separation of lower boiling point volatiles (Jennings & Rapp, 1983).

Pentane was however identified in one instance (Figure 7.15 C), and was presumably present in all samples in the first broad peak. The two samples stored at 0% & 20% RH which showed high germination and only minor differences as regards radicle growth (Figures 7.15 A & B) yielded only minor peaks of butanol, pentanol and hexanal which were not greatly different. Hexanol was however present in the headspace of the more deteriorated sample (Figure 7.15 B), which was increased by salting out (Figure 7.15, B & E). For the higher viability sample stored at 0% RH hexanol was only manifest by the hot salting-out treatment (Figures 7.15 A & D).

Seeds which had been stored at 40% RH and for which 85% germination was recorded were severely retarded as regards radicle growth and yielded substantially larger hexanol and pentanol peaks (Figure 7.15 C) which was dramatically heightened on hot salting out (Figure 7.15 F).

The predominance of short-chain alcohols (butanol, pentanol, and hexanol) and a presence of hexanal (Figures 7.15 A & D) was in striking contrast to the absence of such compounds when analysis was undertaken on the same seed lot 1 year earlier. (Figures 7.13 A & B). While there may be other reasons for the observed differences, one obvious inference is that these alcohols and hexanol are early manifestations of physiological ageing, even though viability was high (95%) and radicles were of uniform length with litle dispersion (mean 27.7 mm and a standard deviation of 2.2 mm). Such a view is supported by the qualitatively greater peaks seen in more deteriorated seed (Figure 7.15 C). While the diagnostic potential of the headspace volatiles was most striking, it is difficult to envisage how such volatile profiles, taken between 2 and 5 hours of imbibition can be a reflection of radicle protrusion or growth some 24-48 hours later. Confirmation of these results will be needed for other seed lots before this technique can be seen as a useful diagnostic technique in vigour evaluation, although recent studies on pea seeds (Gorecki et al., 1985) may be seen as additional support for volatile profiling as a diagnostic technique (Gorecki et al., 1985). It will be recalled that intrinsic factors identified in seed

ageing have been suggested to include:

1. Chromosome aberrations and damage to the DNA
2. Changes in RNA and protein synthesis
3. Changes in enzymes and food reserves
4. Defective respiratory activity and ATP production
5. Membrane changes and leakage

There is little reason for believing that the above volatiles could originate from chromosome aberrations and damage to the DNA, or changes in RNA and protein synthesis. Equally, quantitative changes in food reserves, with the possible exception of lipids, and ATP production could also not be readily implicated in the production of volatiles. On the other hand likely systems where lesions might conceivably lead to the production of volatiles might include changes to enzymes, respiratory activity and membranes.

It was Harman, Nedrow & Nash (1978) who first noted a significantly greater production of volatile carbonyl compounds from aged or fungally infected pea seeds during germination (24 - 48 hours). They speculated that an increase in carbonyl compounds might be one of the consequences of peroxidation during seed deterioration and that it may play a role in the loss of vigour and viability. In a subsequent study emphasis was placed on the possible role of fatty acids and their peroxidation products in stimulating fungal spore germination (Harman, Mattick, Nash & Nedrow, 1980). Among the compounds identified as having possible regulatory roles were pentanal, 1-penten - 3-one, hexanal, 2, 4 hexadienal, hexene-1-ol, octanal and nonanal. Only saturated aldehydes were identified in the headspace of dry-heated seed samples by GS-MS (see below) and this may be regarded as partial supporting evidence for their proposals.

It was noted in earlier packed column studies (page *137*) that total integrator counts of the pentane-like peak were not significantly different during imbibition in either air, nitrogen or oxygen atmospheres. In the light of the later capillary GC/MS studies this interpretation was almost certainly confounded by co-elution of many peaks, notably ethanethiol, pentane and possibly ethanol. However,

even with the wisdom of hindsight it was clearly evident that total integrator counts may offer a simple and relatively rapid method of assessing seed condition provided measurements were made up to 12 hours of imbibition. One unresolved issue which emerged from these studies was whether the evolved hydrocarbons at such an early stage of imbibition have the potential to predict long-term developmental deteriorative events such as cotyledonary necrosis or impaired radical growth which are only evident many hours later. From the ultrastructural studies it was evident that cotyledonary necrosis represents a slow and progressive cell death and was not an event precipitated by imbibition, per se, except when ageing was extreme. (Chapter 4).

It is important to note that many volatiles produced by heating dry seeds were common to those obtained from either imbibing seeds or imbibing, heated seeds. It was assumed that these arose by identical mechanisms, although this is by no means certain. For the purposes of simplicity of presentation the putative mechanisms for the origin of these compounds will be given in the discussion which follows at the end of this chapter (page *168*)

On the other hand, ethanethiol, uniquely associated with imbibition-associated treatments has not been previously reported in the seed literature, and its origin must remain highly speculative. Methanethiol has been recognised as an important component in flavour and off-flavour production in certain foodstuffs. Prolonged sterilization in the canning of beef led to the development of an off-flavour which was attributed to methanethiol (Ziemba & Malkki, 1971) and 1 Methylamino-1-ethanethiol has also been identified by Brinkman et al.(1972) in beef broth.

Apart from lettuce, ethanethiol was identified in the present study by GC-MS in imbibed heated seeds of carrot, radish and cabbage (data not presented) which suggests that a common mechanism may be involved in their genesis. The presence of volatile sulphur compounds in cooked cabbage has been well documented (Dateo, Clapp, McKay, Hewitt & Hasselstrom, 1957).

Since sulphur containing amino acids are probably the only likely source of SH groups in seeds of lettuce one possibility may be that ethanethiol is derived in toto from thermal decomposition of cysteine, cystine or methionine. However, none of these amino acids is so structured such that simple cleavage would yield ethanethiol. Furthermore, stochiometry would demand comparable levels of nitrogen-containing compounds, yet none were noted in the volatile profiles. Exceptionally small traces of hydrazines were seen in dry heated seeds .

It is however well documented that lipid hydroperoxides react with proteins, especially the sulphur containing amino acids (Finley & Lundin, 1980). Protein-lipid adducts might conceivably yield this compound on decomposition, but since this compound is equally evident in high viability samples it may be an artefact of heating and unrelated to deterioration.

It has been suggested that hydrogen abstraction by a pentane-free radical leads to the production of pentane in the thermolytic cleavage of the 13 hydroperoxide of linoleic acid (Evans, List, Dolev, McConnell & Hoffman, 1967). Ethane free-radicals have been suggested as intermediates in aqueous systems where peroxidative degradation of polyunsaturated fatty acids occurs (Frank, Hintze & Remmer, 1983).

In this latter case the radical is seen as abstracting hydrogen from another molecule, although it is possible that an SH group may be abstracted instead, so leading to the production of ethanethiol.

It is generally held that the C - S bond is considerably weaker than the S - H bond, although when molecules are in excited states, cleavage of the bond to hydrogens occurs preferentially (Barltrop & Coyle, 1975).

Further Physiological Consequences:

It has been suggested (Priestley & Leopold, 1980) that alcohol-stress

to seed membranes may occur at several levels. At low concentrations alcohols may intercalate within the bilayer and alter membrane fluidity and enzyme function. These perturbations may be reversible. At higher concentrations irreversible damage may occur by the alcohols causing the production of micells in the membrane bilayer. Reynolds (1977) has shown that 1-hexanol is highly inhibitory to lettuce seed germination, a concentration of 0.86 mM being required to bring about a 50% reduction in germination. Ethanol, propanol, butanol and pentanol brought about comparable imbibition at 117, 51, 5.3 and 2.1 mM respectively. Thus, although the results of one study indicated that substantially higher levels of ethanol were produced during seed imbibition, more significant perturbation of membrane function may be brought about by the smaller levels of longer chain alcohols. Reynolds further showed that hexanal was a potent inhibitor of germination, bringing about 50% imbibition at 1.8 mM. Propanal, butanal and pentanal were effective at 13.7, 9.5 & 4.1 mM respectively.

It will be appreciated that while exogenous application of alcohols and aldehydes may bring about inhibitory effects, these may well differ in mode of action from those presumably generated within the tissue at particular sites such as membranes and organelles.

In addition to the above studies there is a considerably greater body of information on the effect of n-alkanols on enzymic and transport functions in animal cells and model membranes. Many of these have been summarized by Fourcans & Jain (1974). They observed that up to a given chain length, as the lipid solubility of alkanols was increased by hydrophobicity, so bilayer mobility was increased. This is expected to modify the conformational stability of membrane-bound enzymes. Some observed effects include:

1. Increased water permeability and membrane expansion (C_4-C_8).
2. Increased phospholipase activity (C_4-C_{12}). At concentrations greater than 50 mM the effect is reversed. The response is

biphasic.

3. Altered Na^+/K^+ ATP ase activity (C_4-C_{10}), a further biphasic response.
4. Reduced electron transport in Euglena mitochondria (C_4-C_{12}).
5. Altered resting potentials and action potentials in axons; Na^+/K^+ permeability reduced and action potentials blocked.

Denaturation of proteins (Massicotte-Nolan, Glofcheski, Kruuv & Lepock, 1981) and inactivation of membrane ATPase (Grisham & Barnet, 1973) have been emphasized in other studies, although recent suggestions (Wilson & Dahlquist, 1985) tend to favour a general non-specific effect of alcohols on membrane proteins. Thus the above reports probably reflect only a small proportion of membrane proteins likely to be affected. Alcohols could thus cause or exacerbate the lesions which were identified as being important targets in seed deterioration. As noted earlier however alcohols were seen as being causes, rather than effects of vigour and viability decline (p 154).

Table 7.9

Relative abundance of volatiles identified by GC - MS on heating dry seeds of lettuce to 100°C for 8 minutes. Values have been normalized relative to the most abundant compound identified which is given a value of 100. All seeds stored at 20°C. A complete printout of all compounds identified by computer library search is given in the Appendix at the end of the thesis. N.D. not detected.

2 Years at 0% RH		13 Years in hermetic Storage		9 Years over silica gel	
Hexanal	100	Carbon disulphide	100	Hexanal	100
Hexanol	18	Acetic Acid (me ester)	90	Pentanal	16
Pentanal	10	Unidentified	59	1.4 Dichlorobenzene	10
1 Pentanol	9.2	Hexanal	68	Butanal	8
2 Pentylfuran	8.4	Pentanal	64	Octanal	7.6
2 Ethyl 4 pentenal	4.2	Pentane	54	2 Methylpropanal	5.9
Nonanal	3	2 Methylpropanal	42	2 Methylbutanal	4
Heptanal	6	1 Hexanol	39.5	5 Methylhexanal	3.6
Pentane	6	2 Pentylfuran	34.4	Methyl ester Hexanoic acid	3.1
Heptanoic Acid Metyl ester	N.D	Methyl ester Hexanoic acid	20.3	Nonanal	2
1,3 Hexadiene 3 ethyl, 2 methyl	0.6	Pentanal	13	2 Ethyl 4 Pentenal	2
Octanal	3	5 Methyl 2 hexanone	11.2	Pentane	2.7
Nonanal	3	Dodecane	7.6	Heptanoic acid Methyl ester	0.4
Methyl ester Hexanoic acid	0.3	Undecane	7.8	Hexanol	0.9
Dodecanal	0.3	1 Pentanol	6.3		
		2 Heptanol	6.6		
		Heptanoic acid Methyl ester	5		
		Nonanal	4.9		
		4,7 Dimethylundecane	4.1		
		Pentyl ester Acetic acid	3.1		
		Octane	3.4		
		1, 2 Dimethylbenzene	3.9		
		Dodecanal	3.5		

(ii) Volatiles derived from the heating dry seeds

It was observed in earlier packed column studies that the rationale behind this technique seemed justified inasmuch as differences were apparent between seeds of different viability. Unfortunately accurate quantification or identification was not possible because of uncertainties with the resolution capabilities of the packed columns, and apparent breakdown products in the aldehyde standards. Because of the limited access to the GC-MS facility, four seed lots were chosen for study which differed widely as regards duration of storage and viability. Details are recorded in Table 7.10. Reconstructed ion chromatograms are presented in Figures 7.16, A - C and 7.17 A. A full chromatogram is not presented in the latter case as a result of operator error in data acquisition.

In Table 7.9 a semi-quantitative measure of the relative abundance of some of the major compounds identified is given. This was obtained using the Finnigan GC-MS computer programme designed for analysis of organic compounds in water, and made use of response factors in the library programme rather than those obtained from known standards. A full printout of all data is given in Tables A - E in the Appendix.

While it might have been expected that differences between samples would be of a quantitative or qualitative nature, certain compounds were apparently unique to certain seed lots. This was the case with carbon disulphide, acetic acid, acetic acid methyl ester and propanone. Pentane was seen to constitute a smaller element of the volatile profile with no immediately obvious relationship to storage or viability. The elution of several other compounds close to pentane, such as 2 methylpropanal, propanone, thiobis methane and acetic acid, further supports the contention that in earlier packed-column studies many compounds may have been co-eluting to give the single pentane-like peak. As was noted earlier, butanal did not seem to constitute a significant element of the volatile profile of most samples, being especially low in seeds of high viability.

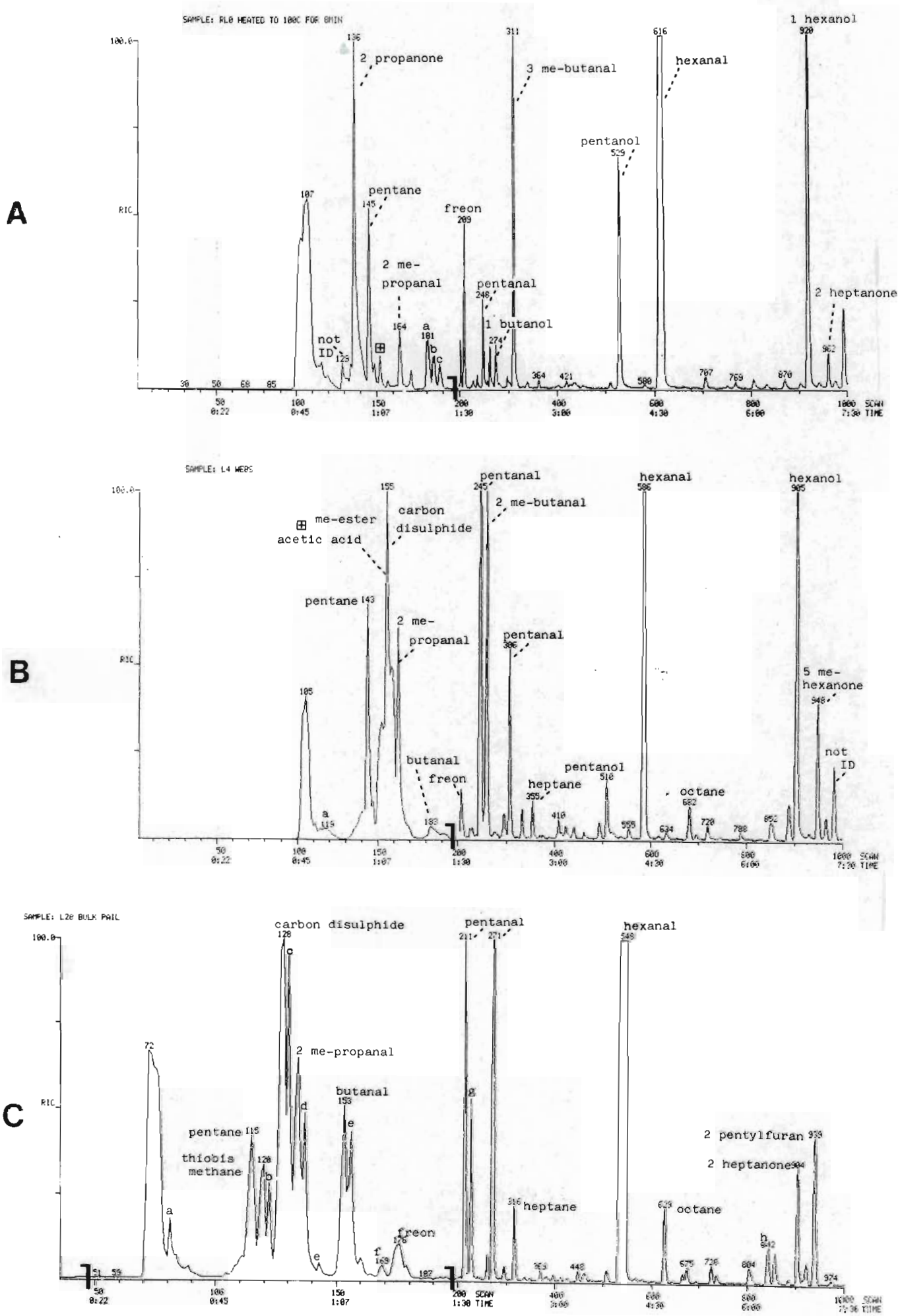

A
SAMPLE: RL0 HEATED TO 100C FOR 8MIN
100.0
RIC
2 propanone
3 me-butanal
hexanal
1 hexanol
pentanol
pentane
freon
2 me-
propanal
pentanal
2 heptanone
1 butanol
not
ID
a
b
c
SCAN
TIME
B
SAMPLE: L4 WEBS
me-ester
acetic acid
carbon
disulphide
pentanal
2 me-butanal
hexanal
hexanol
pentane
2 me-
propanal
pentanal
5 me-
hexanone
not
ID
butanal
freon
heptane
pentanol
octane
C
SAMPLE: L20 BULK PHIL
carbon disulphide
pentanal
hexanal
2 me-propanal
butanal
pentane
thiobis
methane
freon
heptane
octane
2 pentylfuran
2 heptanones

Figure 7.16 OPPOSITE:

Reconstructed ion chromatograms for volatiles derived from two seed lots of cv. Great Lakes (A & C) and of Webb's Wonderful (B) after heating at 100°C for 8 minutes. All major peaks are identified. Some, such as the methylester of acetic acid which was a dominant element in (B), was minor in (A) - denoted by ⊞. Note non-uniform scale of abscissa, denoted by slash bars.

Minor peaks in A were: a. 3 butene 2-one; b. butanal; c. 2 butanone

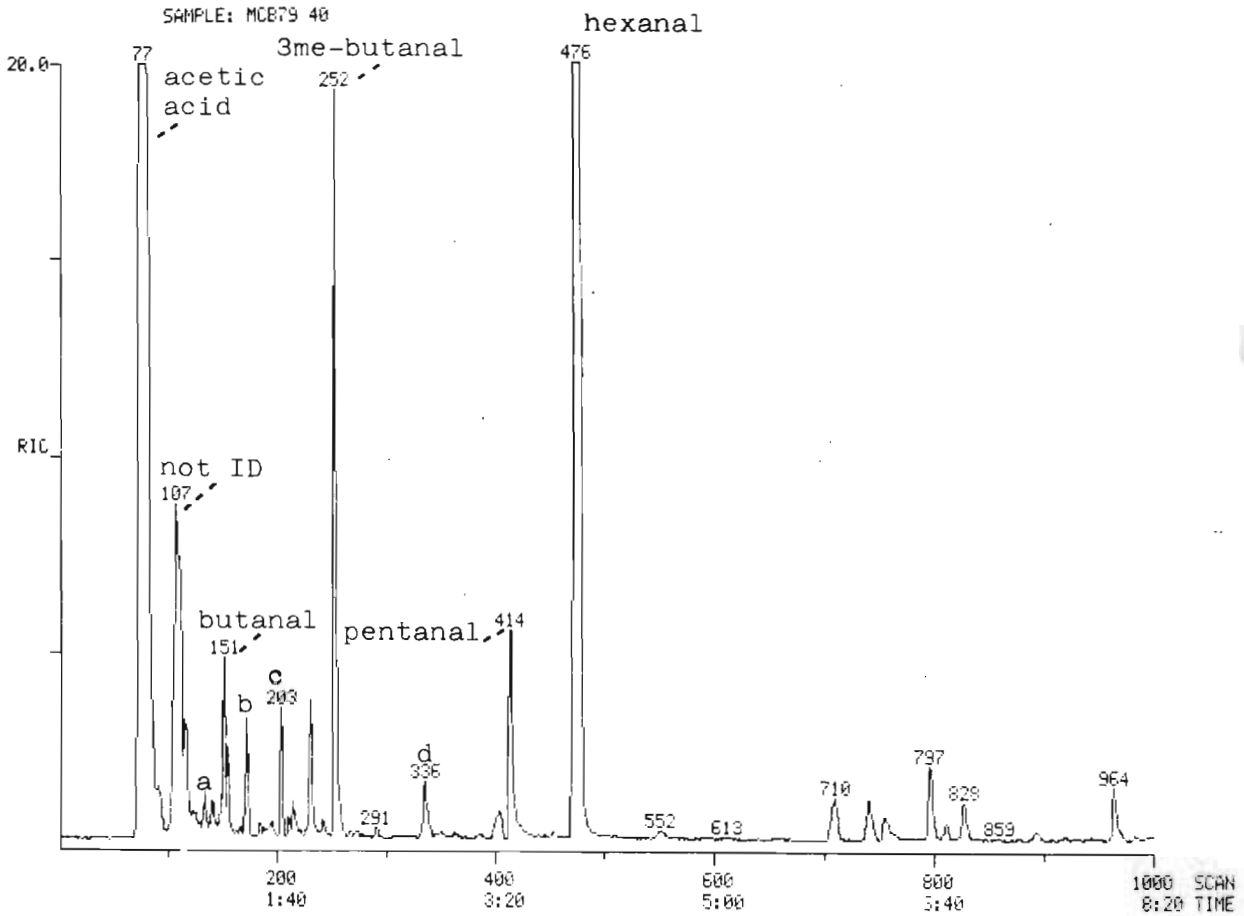
Minor peaks in B & C were: a. difluorodimethylsilane; b. 2 methylpropanal; c. c. carbon disulphide; d. 2 methylpropanal; e. butanal; f. 2 methylfuran g. 2 methylbutanal; h. 1,2 Dimethylbenzene.

Table 7.10

Storage conditions, dates of purchase and percentage germination for four seed lots used in the GC - MS studies on heated seeds. R.I.C. of volatiles identified are presented in Figures 7.16 A-C and 7.17 A.

Storage Conditions	Date of Purchase; Cultivar	Percentage germination at 48 Hours
2 Years at 0% RH, 20°C	1983, Great Lakes R200	97
13 Years at 20°C Hermetically sealed	1972, Webb's Wonderful	89
9 Years at 20°C over silica gel	1976, Great Lakes, R200	50
5 Years at 20°C and 40% RH	1979, Great Lakes	0

A


SAMPLE: MCB79 40
20.0
RIC
77
acetic acid
3me-butanal
252
hexanal
476
not ID
107
butanal
151
pentanal
414
a
b
c
203
d
336
291
552
613
710
797
829
859
964
200 1:40
400 3:20
600 5:00
800 8:20
1000 SCAN TIME

B

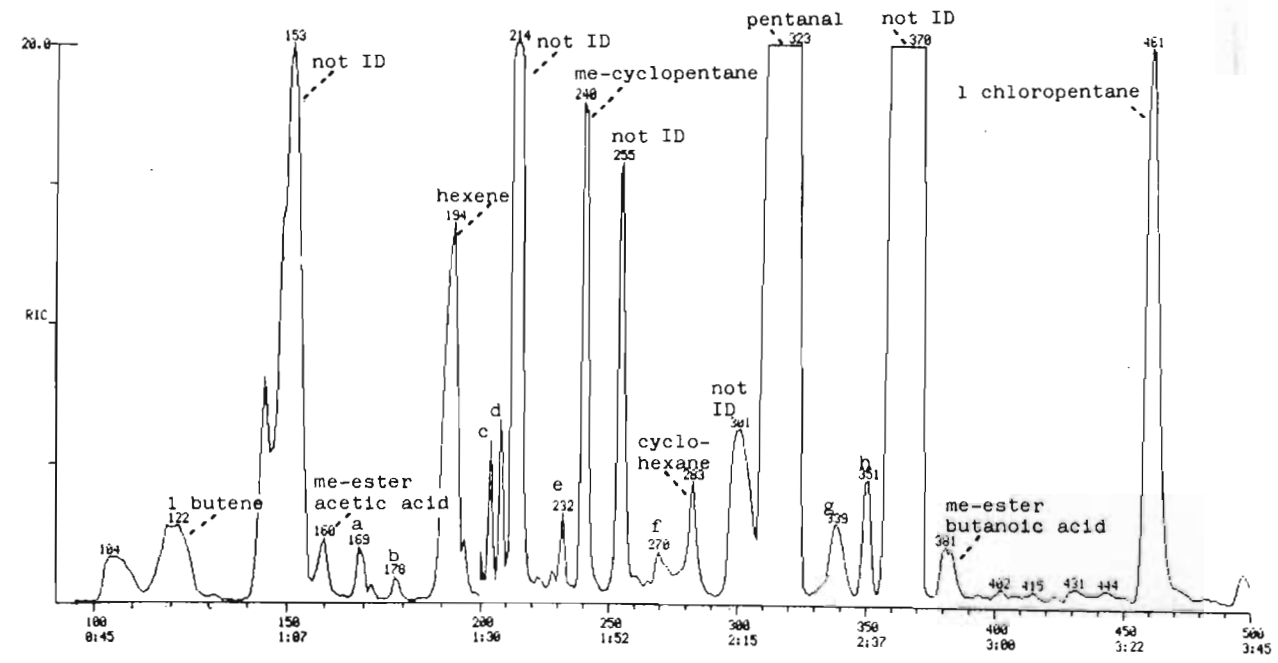

20.0
RIC
153
not ID
214
not ID
me-cyclopentane
240
not ID
255
pentanal
323
not ID
370
461
1 chloropentane
hexene
194
not ID
301
cyclo-hexane
283
1 butene
122
me-ester acetic acid
160
a
169
b
178
c
d
e
232
f
270
g
339
h
351
me-ester butanoic acid
381
100 0:45
150 1:07
200 1:30
250 1:52
300 2:15
350 2:37
400 3:00
450 3:22
500 3:45

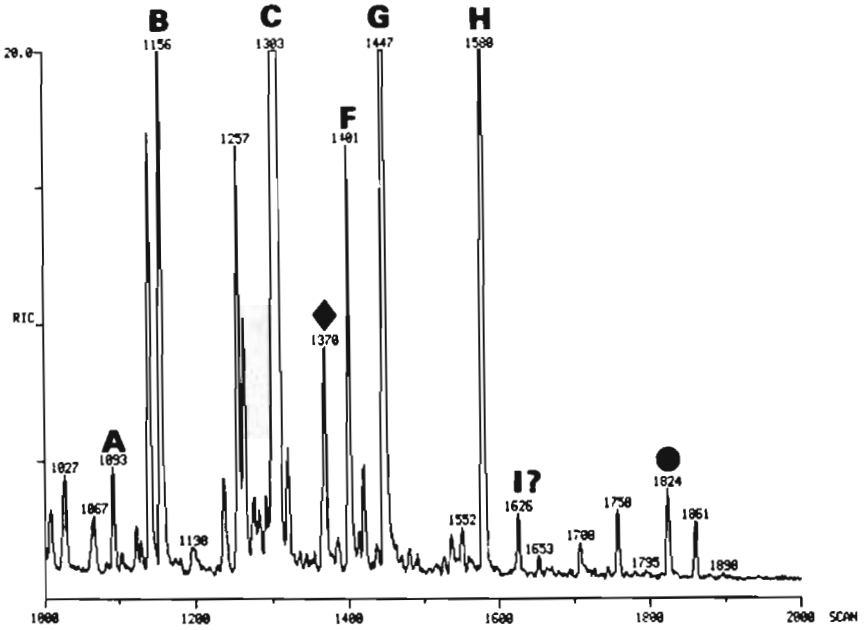
Table 7.11

Relative abundance of volatiles identified by GC-MS on heating the silica gel seed drier to 100°C for 8 minutes. Values are normalized to 100 for the most abundant volatile identified. A full list is given in the Appendix, D.

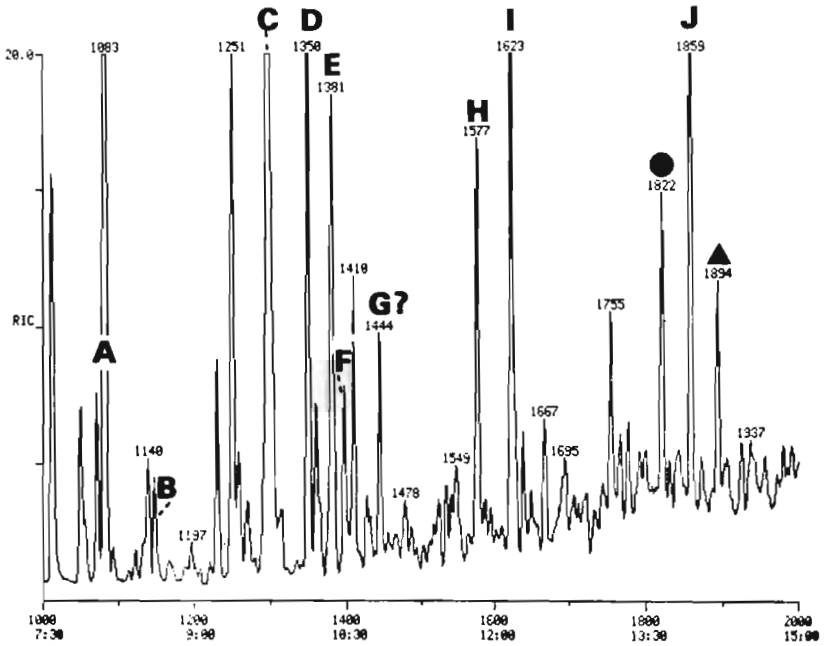
Hexanal	100
Octane	71
Pentanal	44
3 Ethyl 2,3 dimethyl pentane	30
Hexyn 3-ol	22
Nonane	10
Heptanone	10
Methyl ester hexanoic acid	7.7
1,2 Dichloropentane	8.8
1 Chloropentane	6.9
1 Pentanol	4.3
1 Butene	3.5
Chlorobutane	3.1
2 Pentanone	3.1
Pentanol	2.5
Pentamethylheptane	0.7
Undecane	10
Dodecane	12

Hexanal was equally evident in all samples and served more as a reference marker on all chromatograms than an indicator of seed viability. The most conspicuous differences, evident from visual inspection of the reconstructed ion chromatograms and Table 7.9 was the presence of pentanol and hexanol in samples of high viability (Figures 7.16 A & B) compared to negligible levels in the non-viable (Figure 7.17 A) or low viability samples (Figure 7.16 C; Table 7.9, 0.9). The levels of many volatiles were consistently higher for the hermetically sealed sample which may either reflect the length of storage or the possibility that volatiles or their precusor products may have been contained within the sealed tins. An alternative explanation may be sought in the manner of storage at 0% RH, since the storage of seeds over concentrated sulphuric acid might be expected to oxidize any aldehydes, alcohols or unsaturated hydrocarbons. Both these explanations presuppose that these volatile products would be produced in the course of normal storage at ambient conditions without recourse to heating at 90°C. Roberts (1972) has suggested that any temperature other than absolute zero may be regarded as a heat treatment. Evidence for this viewpoint was obtained by examining the volatile profile derived from the heating of the silica gel used as a drier for storage of lettuce seeds. Although the results are probably somewhat extreme in that the sample was long-term post-mortem (estimated at approximtely 11 years), many of the compounds identified by computer search were common to heated seeds. The presence of chlorinated hydrocarbons (chloropentane, chloropropane and chlorobutane) was attributed to some action by the cobalt chloride indicator of the gel (Figure 7.17, B, Table 7.11). These data also suggests that the low pentane values obtained in the seed lot stored for 9 years over silica gel may have been partly the result of selective absorbtion by the silica gel as well as loss to headspace. For the sample stored for two years the only loss would have been to the headspace, since sulphuric acid would not be expected to oxidize this hydrocarbon (Horvat, Lane & Shepherd, 1964). Comparable levels of less volatile aldehydes such as nonanal are thought to be a result of the low heating temperatures or the dynamics of thermal decomposition rather than a measure of deterioration between samples. Likewise

(i)


20.0
RIC
B
1156
C
1303
G
1447
H
1588
F
1401
1257
1370
A
1893
1827
1867
1138
I?
1626
1552
1653
1758
1700
1795
1824
1861
1898
1000
1200
1400
1600
1800
2000
SCAN

(ii)


20.0
RIC
1083
1251
Ç
D
1358
E
1381
I
1623
J
1859
H
1577
1822
1894
1410
G?
1444
1755
A
F
1667
1140
B
1549
1695
1337
1137
1478
1000
7:30
1200
9:00
1400
10:30
1600
12:00
1800
13:30
2000
15:00

(iii)

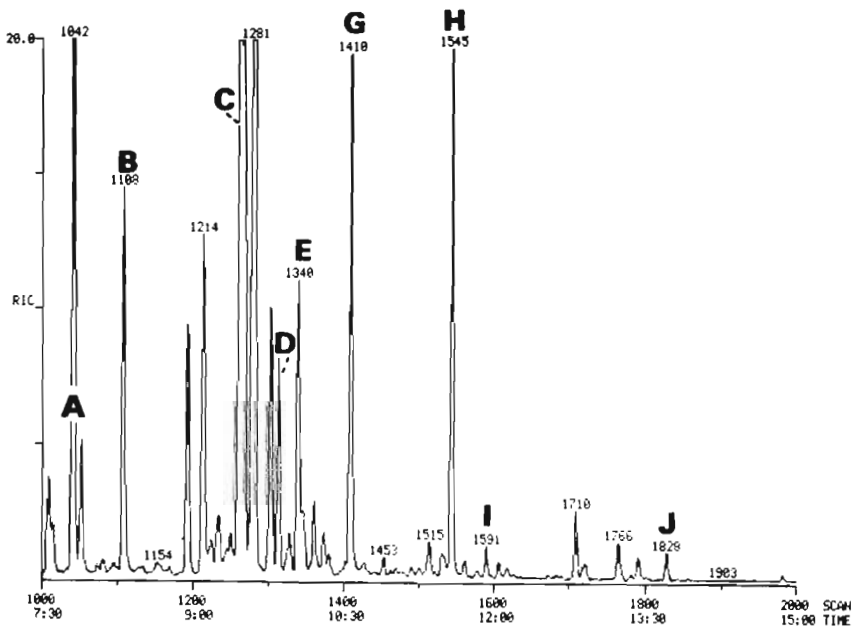

20.0
RIC
1042
1281
G
1410
H
1545
C
B
1108
1214
E
1340
D
A
1710
I
1591
1515
1766
J
1828
1154
1453
1903
1000
7:30
1200
9:00
1400
10:30
1600
12:00
1800
13:30
2000
SCAN
15:00 TIME

Figure 7.18

R.I.C. showing many volatiles identified in the higher mass range by GC - MS for

(i) high viability (97% germination) seed of cv. Great Lakes

(ii) high viability, long term hermetically stored seed of cv. Webb's Wonderful (89% germination)

(iii) seed of low viability stored for 9 years (50% germination)

These are further scans of the samples A,B, & C seen in Figure 7.16 Peaks common to all three samples are:

A. Methyl ester of hexanoic acid

B. 2 ethyl 4 pentenal

C. Pentylfuran

D. Hexyl ester of acetic acid

E. Methyl ester of heptanoic acid

F. 3 octen 2-one

G. 2 ethyl 4 pentenal

H. Nonanal

I. Undecane

J. Dodecane

In addition 3 ethyl, 2 methyl 1, 3 hexadiene (diamond); dodecanal (circle) and 2, 6 dimethylundecane (triangle) were seen in some samples.

relationships between pentanal and viability are apparently not simple, being influenced by both the duration and conditions of storage. There appeared to be little relationship between the preponderance of methyl adducts of the aldehydes and esters of the carboxylic acids (principally acetic and hexanoic) and viability. The unsaturated aldehyde, 2 ethyl 4 pentenal may have possible diagnostic value, although low levels were obtained.

Further, this compound was identified twice in the computer search which raises some uncertainty over the correct identification. Nevertheless, levels were consistently higher for both scans in question (Peaks, B. & G.; Figure 7.18). Many volatiles were evident in the higher mass range and although some volatiles were observed which may have been of diagnostic value, it was concluded that for simplicity of analysis and interpretation that heptanone was an acceptable cut-off point.

Features which may be of significance were identified as:

1. Low levels of the methyl ester of hexanoic acid in high viability samples (A in Figure 7.18; chromatograms i & ii).
2. A more complex spectrum of volatiles in viable seeds stored in hermetically sealed tins(Figure 7.18; chromatogram ii).
   The presence of both undecane (I in Figure) as well as 2,6 dimethyl undecane (indicated by a triangle) either suggests the genesis of the straight chain alkane from the latter, or that further (possibly free radical) reactions lead to the conversion of undecane to the branched, methylated product. The presence of a hexyl ester of acetic acid (D in Figure) in addition to the hexanoic acid (A in Figure) may be seen as further support for this suggestion.
3. The presence of a greater range of unsaturated compounds, including 3-octen-2-one (F in Figure), 2-ethyl-4-pentenal (G in Figure), 3-ethyl, 2 methyl 1,3 Hexadiene (Diamond in Figure) in more viable seed.

It has been noted that volatile compounds probably account for about only 5% of the starting material in autoxidation studies (Lomanno &

Nawar, 1982). Earlier attempts at quantitation on packed column suggested that 830 nl of pentane and 313 nl of pentanal were produced per gm of seed, values which are probably over estimates in view of the likelihood of co-elution. While the headspace sampling technique is simple to execute, quantitation is fraught with many pitfalls which will be briefly considered.

It is well-known that direct injection of headspace gas rarely yields satisfactory results for trace components or higher boiling point compounds (Jennings & Rapp, 1983). Thus, for both imbibed and dry heated seeds the volatiles identified are as much a property of their physical characteristics as representative of their in situ production.

Many potential sources of error have been identified in headspace analysis. Large differences in the rates of diffusion of individual substances into the vapour phase can be a potential source of error, as can the sample : headspace ratio. In addition, absorption of volatiles by rubber septa can be considerable, especially if these are aldehydes (Hackenberg & Schmidt, 1977). These considerations must be thoroughly investigated when undertaking quantitative analysis. Absorbtion by septa would not, of course, be a potential source of error in studies using the automated headspace sampler. The studies thus far have tended to be entirely qualitative with the assumption that any sources of error would not substantially alter overall trends. Critical quantitative analysis will not be without difficulties since standard solutions cannot be readily related to compounds present in the tissues because of vapour pressure interactions between volatiles (Jennings & Rapp, 1983). Nevertheless, comparison between different samples by use of a volatile internal standard might permit a simpler quantitative or semi-quantitative approach. Thus, dry seeds could be given a suitable period of pre-equilibration with such internal standards before being subjected to heating treatments. However, if seeds were to be imbibed it would be necessary to find some compound which would be non-toxic to the tissues and have no effect on metabolism - a formidable challenge. It is perhaps for some of the above difficulties that, some workers have expressed data in terms of ng or nl per sampled

headspace column (del Rosario, de Lumen, Habu, Flath, Mon & Teranishi, 1984). In some instances qualitative and quantitative studies are undertaken together (Fischer, Legendre, Lovgren, Schuller & Wells, 1979).

DISCUSSION

As will become evident below, lipid hydroperoxide breakdown products offer the most logical, although by no means the simplest, explanation for the large range of volatiles produced in both imbibing seeds and those subject to dry heating. In addition, some highly speculative suggestions for a metabolic origin of volatiles in imbibing seeds will also be presented at the conclusion of this thesis (Chapter 8; page *180*).

It has been noted that the lipid peroxidation literature is full of speculative mechanisms explaining the origin of compounds not predicted on the basis of simple cleavage reactions (Frankel, 1984). This comment applies to model studies which are usually conducted on single fatty acid methyl esters. Therefore attempts to explain the origin of the volatiles noted in the present study will be immeasurably complicated by many different lipid classes, fatty acids and proteins, all of which may be interacting in unknown ways. The mechanisms given below are drawn from the extensive literature on lipid oxidation and must of necessity be highly selective. Furthermore, in seeking to make comparisons between the volatile profiles encountered in the present study and those in the published literature it is necessary to consider the following:

1. Analysis of oxidation products is frequently undertaken using single fatty acid methyl esters in homogenous bulk-phase. There are reasons for believing that mechanisms in a non-homogenous system, such as seed, may be very different from peroxidation in bulk phase samples.

Davidovich et al., (1980) have highlighted some differences between homogenous and inhomogenous systems undergoing oxidation which are

considered relevant to the present study:

(i) Unlike homogeneous systems of oil, the process may be heterogeneous as regards phases of lipids, proteins and water.

(ii) Diffusional factors maybe significant since in liquid oil, oxygen levels may be as low as 0.3 ppm.

(iii) Oxygen consumption in the system described by those authors, was 10 times greater than could be accounted for by lipid only, suggesting some other oxygen-consuming reaction(s).

The contention that lipid oxidation in biological membranes is likely to be the result of a complex interplay of many factors, is reinforced by the studies of Mead and his co-workers (see Mead, 1980). That author has investigated the oxidation of various fatty acid monolayers, bound to layers of silica gel. In such a model system, the kinetics of linoleic acid oxidation were found to be quite different from those of bulk phase systems. Whereas the latter characteristically produce hydroperoxides, monolayers yielded epoxides on oxidation.

This was thought to occur as a result of transfer of oxygen from one peroxy radical to an adjacent double bond of a neighbouring molecule. Further investigation showed that the introduction of unsaturated fatty acids into the monolayer of linoleic acid, led to diminution in oxidation of linoleic acid and yielded hydroxy-epoxy fatty acids. These oxidation products were also observed in liposomes, suggesting that intra-molecular oxygen transfer could also prevail in biological membranes.

2. Thermal decomposition of hydroperoxides is frequently undertaken well in excess of 100°C, using an on-column injection method. This method consists of placing the sample directly into the heated injection part of the gas chromatogram. Volatiles produced on decomposition were then swept onto part of the column at room temperature and a

Table 7.12 A

Isomeric distribution of fatty acid hydroperoxides, and the percentages formed by free-radical autoxidation of individual fatty acids in a model system (from Frankel, 1984).

FATTY ACID	ISOMERIC HYDROPEROXIDES FORMED AND PERCENTAGES PRODUCED						
Oleic	8-OOH 27%	9-OOH 23%	10-OOH 23%	11-OOH 27%			
Linoleic		9-OOH 50%				13-OOH 50%	
Linolenic		9-OOH 30%			12-OOH 12%	13-OOH 12%	16-OOH 46%

Table 7.12 B

Isomeric distribution of fatty acid hydroperoxides, and the percentages formed by photosensitized oxidation of individual fatty acids in a model system. Compare with the above.

FATTY ACID	ISOMERIC HYDROPEROXIDE FORMED AND PERCENTAGES PRODUCED							
Oleic		9-OOH 50%	10-OOH 50%					
Linoleic		9-OOH 31%	10-OOH 18%		12-OOH 18%	13-OOH 33%		
Linolenic		9-OOH 21%	10-OOH 13%		12-OOH 13%	13-OOH 14%	15-OOH 13%	16-OOH 25%

temperature programme begun. Trace compounds can be detected at parts per billion levels although the method is not selective for low molecular weight compounds of high volatility, as is the case in headspace sampling techniques (St. Angelo et al., 1980). Furthermore, triglycerides and phospholipids may not undergo decomposition routes directly comparable to those of model systems, especially in view of the lower heating temperatures used in this study.

3. There is a lack of uniformity as regards possible reaction pathways in hydroperoxide decomposition; the free radical mechanisms involved are considered complex with both primary and secondary reaction products (Frankel, 1982). These may be represented as follows:

$$RO^* + ROOH \longrightarrow ROH + ROO^*$$

$$2\,ROO^* \longrightarrow 2\,RO^* + O_2$$

$$2\,ROOH \longrightarrow RO^* + ROO^* + H_2O$$

$$ROO^* + RH \longrightarrow ROOH + R^*$$

$$R^* + O_2 \longrightarrow ROO^*$$

A further mechanism not covered by the above reactions but which has been considered likely in the thermal oxidation of the triglyceride of linolenic acid is the so-called Russell mechanism (Selke & Rohwedder, 1983). Two peroxy radicals react to form a tetroxide dimer, whose decomposition gives singlet oxygen as one of the products. This exceptionally reactive species produces oxidation of unsaturated fatty acids by a different, nonradical mechanism and reacts directly with the carbon-carbon double bonds. Hydroperoxides are formed from such reactions which differ from those of free radical autoxidation, as can be seen in the Table 7.12 (Frankel, 1984).

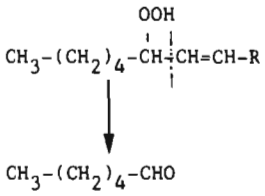
Since carbon-carbon cleavage is frequently seen on either side of the alkoxy radical, it is evident that some breakdown products may be produced which differ from those of autocatalytic lipid autoxidation. Thus a facile origin of hexanal becomes possible by the following

Table 7.13

Suggested aldehydes produced on thermal decomposition of hydroperoxides derived from the autoxidation of the pure fatty acids (from Keeney, 1962).

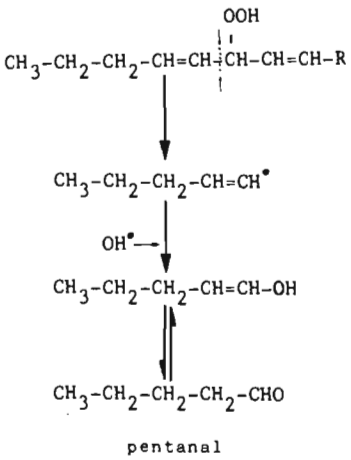
FATTY ACID	INTERMEDIATE	ALDEHYDES PRODUCED
Oleic	11 Hydroperoxy - 9-ene	Octanal
	9 Hydroperoxy - 10-ene	2 Decenal
	8 Hydroperoxy - 9-ene	2 Undecenal
	10 Hydroperoxy - 8-ene	Nonanal
Linoleic	12 Hydroperoxy - 9,11 diene	Hexanal
	11 Hydroperoxy - 9,12 diene	2 Octenal
	9 Hydroperoxy - 10,12 diene	2,4 Decadienal
Linolenic	16 Hydroperoxy - 9,12,14 triene	Propanal
	14 Hydroperoxy - 9,12,15 triene	2 Penenal
	12 Hydroperoxy - 9,13,15 triene	2,4 Heptadienal
	13 Hydroperoxy - 9,11,15 triene	2 Hexanal
	11 Hydroperoxy - 9,12,15 triene	2,5 Octadienal
	9 Hydroperoxy - 10,12,15 triene	2,4,7 Decadienal

reactions of the 12 hydroperoxide:



In this and subsequent illustrations R represents the ester portion of the fatty acid

If a double bond occurs on the other side of the hydroperoxy group, a saturated aldehyde may still be produced according to the following (Frankel, 1984) scheme:



pentanal

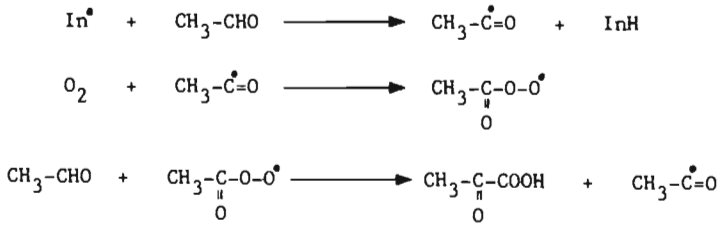
Aldehydes have long been identified as being mainly responsible for the oxidative off-flavours in oils, although ketones, alcohols and acids (by further oxidation of aldehydes) may be formed (Hoffman, 1962). Depending on the site of peroxidation it has further been noted that specific aldehydes may arise from a particular fatty acid in the hydrocarbon chain as is indicated in Table 7.13. (Keeney, 1962).

A more detailed analysis has been provided by Frankel (1982) in which the relative percent of all minor and major breakdown products are listed for oxidized oleate, linoleate, linlenate and soybean oils but this provides little insight into the likely complexities of a mixed system such as a seed with storage triglycerides, phospholipids and proteins. It has been noted that the total number of possible free aldehydes is greater than those theoretically expected, possibly because of cis-trans isomerism and double bond shifts (Hoffman, 1962). Further explanations may be sought in the fact that the aldehydes are regarded as unstable, susceptible to polymerization and condensation reactions, as well as further possible oxidation to carboxylic acids (Keeney, 1962).

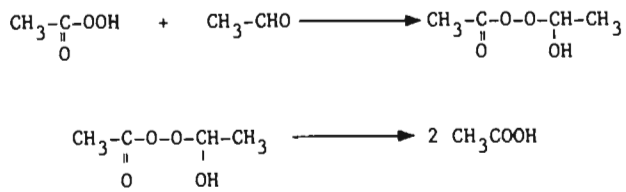
A comparison between the above list and the aldehydes identified from seeds shows remarkably little relationship to the proportions of fatty acids present (18:1 22%; 18:2 66%; 18:3 1.5%). The general absence of unsaturated aldehydes (except ethyl pentenal) was puzzling since these might be expected at the lower heating temperatures employed. This could suggest more complex reactions such as reactions between unsaturated aldehydes and proteins.

Smouse & Chang (1967) collected volatile compounds from reverted off-tasting soybean oil with a low peroxide value (4,3meq/Kg) by vacuum steam distillation and fractionated the products by repeated gas chromtography. The volatile compounds were estimated as being present at 10 ppm level. A range of saturated carboxylic acids were present from acetic (small), propanoic (medium), and butanoic - octanoic (large). Dominant aldehydes were pentanal, hexanal, octanal, nonanal, 2 heptanal, 2 octenal and 2 nonanal. Heptanone and octanone were dominant ketones, whilst ethanol and pentanol were major peaks among the alcohols. Small levels of ethylacetate were present in addition to hexanol and pentenal. The above study is significant from many others, particularly in the low peroxide value of the oil and the collection of breakdown products since it did not rely on the traditional on-column thermal decomposition technique.

Volatile acid components have been ignored in much of the literature or possibly do not develop in model systems employing fatty acid methyl esters. Smouse & Chang offer the following secondary autoxidation of aldehydes as part of the possible mechanism for acid formation



The reaction so far is, in effect, a restatement of the free-radical autocatalytic mechanisms. The formation of acetic acid is suggested to occur via a peracid mechanism involving:



In the above reactions acetaldehyde has been used in order to offer an explanation for the formation of acetic acid methyl ester in some seed samples. The peracid intermediate could serve as a possible precusor for the methylester by reaction with a methyl free radical. Other suggestions for the origin of acidic volatiles have been made including (a) hydrolysis of the ester linkages, (b) oxidation of fatty acids of longer chain length, (c) thermal oxidation of an carbon atom of the acids in triglycerides (Kawada, Krishnamurthy, Mookherjee & Chang, 1967).

More recently attention has been directed at the possible existence of and involvement of other peroxidation products in lipid autoxidation. Mead (1980) has stressed the pitfalls inherent in autoxidation studies using single fatty acid groups. Whereas 9 & 13 hydroperoxides were

predominant species (33 & 45%) produced on oxidation of a phosphatidylcholine monolayer system, the presence of saturated fatty acids led to smaller yields of 9 & 13 hydroperoxides (16 & 10%), and significant rises in trihydroxy- and epoxy-fatty acids (45 & 12%, respectively). Significant progress in structural aspects in this area has only become possible recently with advances in separation techniques such as combined HPLC & GC/MS (Neff, Frankel & Weisleder, 1981; Gardner & Selke, 1984; Coxon, Price & Chan, 1981).

In view of the recent nature of research into these fatty acid species the present author is unable to offer especially meaningful consquences as regards likely thermal decomposition of such products. It could however be expected that the presence of hydroxy groups would lead to an increased incidence of alcohols. However since high levels of hexanol and pentanol were diagnostic of high viability samples only it may be assumed that these were produced in situ by thermal peroxidation reactions. Alcohols may, of course, be oxidized to their corresponding aldehydes, while milder oxidations are capable of producing caboxylic acids.

It is perhaps significant that a number of alcohols were noted in the headspace of ground, but unheated, soybeans using essentially the same headspace concentration techniques used in the present GC-MS study (del Rosario, de Lumen, Habu, Flath, Mon & Teranishi, 1984).

High levels of acetic acid, hexanol, pentanol, 3 methylbutanol, 2 propanol and acetone were detected in the headspace of the seeds although those authors were concerned with flavour studies and presented no data on seed germination. Lower molecular weight hydrocarbons (below hexane) were present in only trace amounts (0.05 ng/750 ml of swept headspace) and no sulphur compounds were reported apart from traces of hydrogen sulphide and dimethyldisulphide. Few aldehydes were detected and, when present, were seen at very low levels. The highest value was 1.6 ng/750ml headspace for hexanal.

It should be noted that high levels of hexanol and pentanol were diagnostic of heated, dry seed of high viability whilst for imbibing seeds high levels of these compounds were indicative of viability decline. A metabolic reduction of aldehydes may be implicated in the latter instance.

Moll, Biermann and Grosch (1979) have noted an increase in trihydroxy acids in ground soybeans held at 20°C and 63% RH for 3 months. Their studies suggested that protein thiols were responsible for the breakdown of hydroperoxides to hydroxy fatty acids. It should be stressed that ground seed material was used in these studies which almost certainly will have altered cell organization; additionally the RH employed may have been sufficiently high to permit some enzymic or fungal activity. Nevertheless it has been known for some time that lipid peroxides and their breakdown products react with either amino acids or proteins. Karel (1980) has drawn attention to the following reactions:

1. Cross linking and inactivation of enzymes.
2. Protein scission.
3. Destruction of amino acids and amino acid residues (histidine, tryptophan and lysine).
4. Production of free radicals in proteins.

The above author further notes that attempts to detect free radicals (by ESR) in peroxidizing lipids were without success, yet on theoretical grounds one would expect them to be present. This may be taken to mean that either radical levels were not sufficiently high enough for detection, or that line widths of signals were so wide that detection was impossible. On the other hand the existence of radicals in low-moisture or aqueous food systems is well-documented. Roubal (1970) has detected the presence of trapped radicals in dry lipid-protein systems undergoing oxidation in air at room temperature (over 12 hours!), and concluded that since free radicals precede aldehydes in the oxidation of lipid, radicals are the principal cause of protein polymerization and amino acid destruction. Although sulphur containing amino acids would be

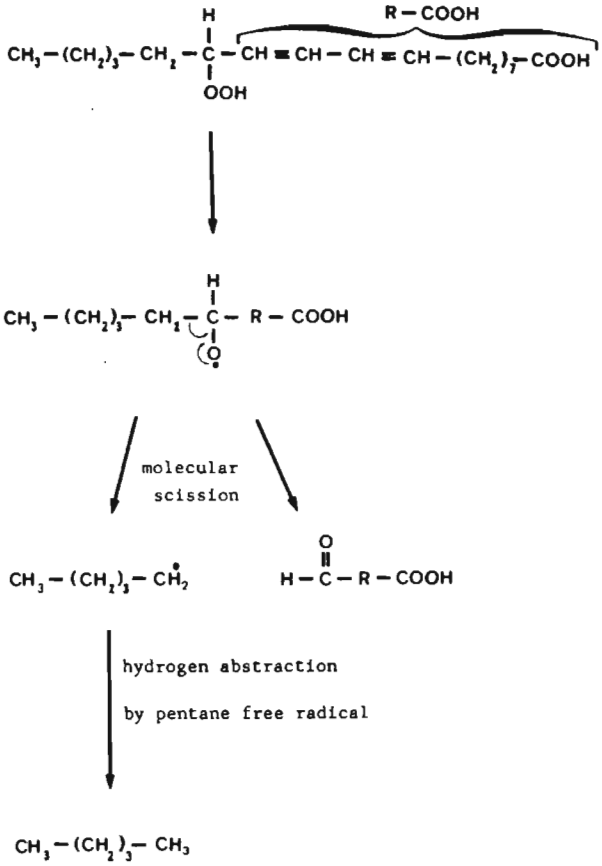
expected to be most susceptible to oxidation, it is notable that methionine, threonine, lysine and histidine were shown to exhibit autoxidant activity in a model system at low water content (Riisom, Sims & Fioriti, 1980).

More recently, it has been shown that peroxidized linoleic acid potentiates lipid peroxidation in erythrocytes, and leads to the formation of cross-linked membrane proteins (Koster, Slee, Rutten, van Beysterveld and Monfoort, 1983). Furthermore the process was independent of malondialdehyde production, serving to highlight potential pitfalls of using this aldehyde to assay the extent of peroxidative damage.

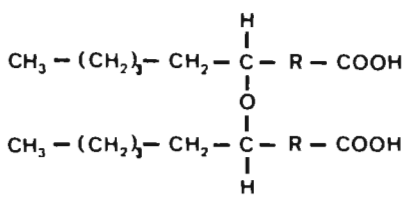
It is clear therefore that while literature from the oil chemists supports the possibility that the volatiles obtained were derived from lipid peroxidation products and further free-radical mechanisms, the involvement of proteins cannot be ignored.

Brief consideration will now be given to the origins of the hydrocarbons and chlorohydrocarbons present in the sample headspace. It is considered significant that chloroethane, dichloroethane, tetrachloroethane and chloropentane were driven off the silica gel drier of long-term post-mortem seeds (Table 7.14; Figure 7.19,B) since this suggests that these shorter chain alkanes are probably produced during deterioration in storage in the long term. Pentane was identified in both imbibing seeds and those subjected to heating in the dry state and has been regarded as an indicator of lipid peroxidation.

Using on-column injection Evans, List, Dolev, McConnell and Hoffman (1967) proposed the production of pentane from the 13 hydroperoxide of linoleic acid by the following mechanism.



In an alternative mechanism, electron donation from the C-R bond (above) and its resultant scission would produce hexanol rather than pentane, and a presumptive 12 carbon caboxylic acid. Peroxy groups, it was suggested could dimerize yielding:



This product too would be thermolabile and on decomposition would yield pentane and oxygenated fatty acids. Further support for the suggestion that pentane may be released from seed tissues at room

temperature is suggested by the results of studies by Horvat, Lane, Ng and Shepherd (1964). A range of saturated hydrocarbons (from methane to pentane) was produced in a model oxidation system comprising methyl linoleate on glass wool at room temperature. Furthermore, these hydrocarbons were detectable well ahead of the appearance of higher molecular weight carbonyls such as hexanal and pentanal. The appearance of relatively large amounts of pentane from seeds stored in hermetically sealed tins after 13 years storage at 20°C (Table 7.9) was seen as support for this argument, while the presence of lower levels in the other samples is thought to reflect both the likely losses from continuous opening of the storage containers or adsorbtion by silica gel drier. The correlation between thermally-derived pentane, pentanal and hexanal and the hydroperoxide level of edible oils has been shown to be high (Evans, List, Hoffmann & Moser, 1969; St. Angelo, Legendre, and Dupuy, 1980). This may be so for seeds in storage also, as the packed column studies suggested (Figure 7.11) if it is assumed that these collectively constitute a considerable element of the volatiles. This is partly supported also by GC-MS studies, also (Table 7.9).

Hexanal is a common breakdown product from autoxidized linoleic acid at low and moderate temperatures and may account for a major proportion (66 mol%) of the volatiles in some food systems (Schieberle & Grosch, 1981). Its ubiquity is such that it has been used as an indicator of off-flavour in low fat food (Fritsch & Gale, 1977). While high levels were found in the GC-MS studies, with these values being normalized to 100 relative to other volatiles, the packed column studies did not suggest that hexanal could serve as an earlier indicator of deterioration. A slow conversion of ^{14}C labelled hexanal to hexanoic acid has been shown in soybean oil oxidized in bulk phase at 25°C (Michalski & Hammond, 1972) and in the present studies the methylester of hexanoic acid may have arisen from hexanal rather than from the hydrolysis of the glycerol-fatty acid ester linkage.

Issues which must still be addressed include:

1. the presence of the alcohols hexanol and pentanol which would

tend to increase the counts for viable material in packed column studies and hence tend to spuriously suggest impaired quality.

2. The loss of certain volatile components such as pentane, and the possible reactivity of aldehydes with tissue proteins. For this reason comparisons between seed lots can presumably only be made if treatment from the time of harvest is equivalent, and if storage conditions are identical.

CHAPTER 8

OVERVIEW AND SYNTHESIS

1. The Status of the Free Radical Theory

While it has been noted that evidence for the free-radical hypothesis is somewhat weak (Halmer & Bewley, 1984) it has been observed that if lipid oxidation is not the cause, evidence that membrane deterioration is an important component of seed deterioration continues to grow (Roberts, 1983). As has been pointed out (Bewley & Black, 1982), variations in ageing conditions and the techniques (apart from any other considerations) yield a highly confused picture in those studies which have sought to find evidence for free-radical involvement in seed deterioration. (Table 6.10). The few attempts which have been made to detect free-radicals directly have yielded contradictory results (Conger & Randolph, 1968; Priestley et al., 1980; Buchvarov & Gantcheff, 1984), while attempts to seek declines in antioxidant levels have been equally unsupportive of free-radical involvement (Priestley et al., 1980, Fielding & Goldsworthy, 1980). There is little doubt that bulk-phase oil oxidation may have influenced thinking to an undue extent, by disregarding factors such as inhomogeneity, interactions with proteins and the water content. No studies, as far as the present writer is aware have used oxidation of anhydrous food systems as the starting point for seed deterioration studies even though the parallels are striking (Karel, 1980).

Since hydration is an inseparable component of seed germination, it is surprising that many ageing studies have not investigated this area, especially in view of the suggestion that peroxidative damage is amplified on imbibition (Villiers, 1973). A major difficulty in the present study has been the inability to interpret ultrastructural events in terms of biochemical function. In the earlier ultrastructural studies (Chapter 5, B) it must be assumed that the root tip cells are essentially at a "point of no return" and that cell

death is inevitable. While characterizing the sequence of events leading to cell death provides possible evidence for sites of lability and resistance to deteriorative events which collectively precipitate cell death (Figure 5.1) it does not provide information on the primary deteriorative event. Biochemical measures under such circumstances would be meaningless. On the other hand there is need to define some biochemical parameters in cells of reduced vigour but high viability. The most notable ultrastructural feature to emerge in this regard (Chapter 5, D) was the presence of plasmalemma irregularities in early imbibition, but it is extremely difficult to interpret such images meaningfully in terms of in vivo cellular events. Ruptures to the plasmalemma may represent the "point of no return" of those cells in the population whose probability of death is greatest, but tells us little of what events may precipitate such damage. Even relatively simple measurements such as ion leakage, which would be expected to assist in interpretation of such ultrastructural events are not without difficulties. It has been argued that high leakage represents a greater proportion of dead cells in the seed, rather than a greater number of "leaky" cells, but provides little insight into the deteriorative changes of the viable population of cells (Powell & Matthews, 1981).

Biochemical evidence exists which suggests repair of damage to the genome as a post-imbibitional event (Osborne, 1980) but comparable evidence is lacking for membranes. Membrane repair and turnover have been inferred from ultrastructural observations (Berjak & Villiers, 1972a; Villiers, 1980). Similarly the improvements in germination obtained when aged seeds are imbibed in osmotica may be interpreted as amelioration of imbibition injury or permitting some (as yet unidentified) membrane restitution (Woodstock & Tao, 1981; Woodstock & Taylorson, 1981).

There is an urgent need to back up the observations of the present study and those of others in terms of specific biochemical changes in phospholipid metabolism. Studies by Cuming & Osborne (1978) have clearly demonstrated how radiotracer studies could provide valuable

insights into membrane synthesis and repair. The observation that embryos of reduced viability appear susceptible to plasmolysis as late as 36 hours post-imbibition may be interpreted as incomplete restitution of membrane defects. This might possibly explain why seeds of reduced vigour are more susceptible to stress in field conditions. The present study has provided evidence for increased peroxidation with ageing but no evidence for loss of lipid unsaturation with viability loss in lettuce seeds. On the other hand, HPLC studies (Chapter 6) suggest marked changes in the U.V. absorbance characteristics of phospholipids, although what this observation means in structural terms was not determined. It has been suggested (Slater, 1984) that HPLC may offer considerable potential in future lipid oxidation studies. A thorough categorization of the lipid oxidation products should be an important first step in understanding the possible involvement of peroxidation in seed ageing. The many diverse findings in this thesis can at present only be linked by speculative suggestions, many of which are freely taken from the results of other research fields, but all may be satisfactorily accommodated within the free-radical theory of ageing.

2. Towards a Working Definition of the Free-radical Theory of Seed Ageing

It is suggested that the current pre-occupations with damage to the genome (*in sensu* Osborne) and with membrane fatty acid changes represents a somewhat simplified and one-sided view of seed ageing. It is perhaps desirable to enunciate the free-radical theory and to consider modifications which should be made to accommodate the special case of the dry seed. This may then form the basis for further, more broadly-based research. "The free-radical theory of ageing states that there is a single basic cause of ageing and postulates that free-radical reactions are involved in ageing and age-related disorders. These reactions arise upon exposure to ionizing radiation, from non-enzymatic and enzymatic reactions, particularly those of photosynthesis and the reduction of oxygen to

water" (Harman, 1981).

It should be noted at the outset that oxygen is seen as the main source of damaging free-radical reactions. Harman notes that over 90% of oxygen consumed by mammals is utilized in the mitochondria, and argues that some of the free-radical generated during the reactions involving oxygen would produce changes in the mitochondria at a rate related to oxygen consumption. One likely alteration would be in the reduction of mitochondrial DNA which would clearly profoundly influence both mitochondrial function and the cell as a whole. The appearance of abnormal mitochondria in seeds in enforced, long-term dormancy may be a case in support of this proposal. While it might be expected that some lesions would be repairable and that grossly defective organelles might be sequestrated and digested, the appearance of abnormal, retained mitochondria (Villiers, 1972) suggests that the machinery for replacement may itself have become defective. While there is at present little evidence that mitochondria are specific targets or initiators of free-radicals in the ageing of dry seeds, Berjak (pers. comm.) has suggested that the mitochondrial compartment may be preferentially hydrated and so serve as a focus for free-radical generation. This suggestion could explain:

1. Respiratory deterioration manifest on imbibition of aged seed.

2. The ultrastructural appearance of mitochondria in imbibed, aged seed (Berjak & Villiers, 1972a; Hallam, 1973; Berjak, Dini & Gevers, 1985).

   In vitro studies have indicated that lipid peroxidation in animal mitochondria causes a rapid disintegration of membrane structure (Shimada & Yasuda, 1979). Thus the appearance of mitochondria in aged seed tissue may be the result of peroxidative damage in the dry state or on imbibition.

3. The relationship between moisture content, temperature and

ageing. The initial hydrogen abstraction step in autoxidation is presumed to be independent of temperature although subsequent reactions are influenced by availability of water and temperature. Peroxidation can take place in protein-lipid food systems (Karel, 1980) from which it might be inferred that the general cytoplasm might be an equally likely site for free-radical autoxidation apart from membrane systems. On the other hand, the redox potentials between individual constituents of the electron transport chain would make the mitochondrial membranes sites of much higher risk. Suggestions have also been made that mitochondrial enzymes may be displaced or lost from the mitochondria (Bewley, 1979), a suggestion supported by the studies of Duke & Kakefuda (1981), which showed that legume seeds without testae lose mitochondrial marker enzymes into the bathing medium. Thus respiratory elements which are normally physically separate may come together, resulting in a large redox potential between the two respiratory carriers. Consequently the potential for producing activated oxygen would be greater.

Since the free-radical theory as defined by Harman specifically relates to hydrated systems it is suggested that the four-step electron reduction of oxygen to water cannot be seen as the only pathway leading to the generation of free-radicals and/or the initiation of lipid peroxidation in seed storage. It is suggested that the definition (above, page *182)* be altered to read:

"These reactions arise upon exposure to oxygen, from enzymatic reactions such as those of lipoxygenase and enzymes of the respiratory chain, as well as non-enzymatically through the action of metal ions both free and sequestered (eg. in phytoferritin)".

It has been proposed that the heightened sensitivity of anhydrobiotic organisms to chilling injury during imbibition is a manifestation of membrane arrangement (Priestley & Leopold, 1980). There are difficulties associated with applying the above arguments to lettuce since Pradet (1982) has shown that respiratory competency is rapidly

achieved during early imbibition in lettuce. While the matter of enzymic activity at low moisture contents will be addressed later, the significance and nature of oxygen toxicity to air-dry seeds should be considered. The damaging effects of oxygen storage to seeds is a documented, if somewhat poorly researched subject. It has been noted (Halliwell & Gutteridge, 1984) that, by definition, molecular oxygen is itself a free radical. However, because of spin restrictions, electrons tend to be accepted singly.

In this regard the findings that higher partial pressures of oxygen bring about more rapid deterioration of seeds, but without concomitant change in PUFA levels (Ohlrogge and Kernan, 1981) is seen as especially significant. These authors suggested, therefore, that oxygen acted differently, and at some other site than lipids, in bringing about deterioration. Later, Ohlrogge and Kernan (1983) extended that work to other systems. They showed that mutants of E. coli, the fatty acid levels of which could be altered by exogenous fatty acid supplements, were all equally susceptible as regards growth in paraquat, H_2O_2, ozone or singlet oxygen. Thus, in that study material, there appeared to be some target(s) other than membrane lipids. However, in such hydrated systems, the levels of free radicals and their sites of action, may well be fundamentally different from those in dry seeds. It has been observed, for instance, that bean leaves exposed to ozone develop necrotic lesions and permeability changes, sooner than detectable changes in phospholipids or their fatty acid composition (Fong and Heath, 1981). If the arguments advanced by Barber and Thompson (1983) are applicable here, then it is possible to explain these observations as a result of some peroxidation product causing phase changes by intercalating with membrane phospholipids. What is perhaps also likely, though largely ignored because of experimental difficulties, are changes to membrane proteins.

Borg et al., (1978) in discussing the cytotoxic reactions of free radical species of oxygen, indicate two possible mechanisms of action which are not necessarily mutually exclusive. The first involves irreversible cross-linking of functionally important proteins by

electrophilic attack of sulphydryl groups, while the other suggests that damaging reactions occur to 'target molecules' by the reactive oxygen products and by autoxidation reactions. Since biological membranes contain target molecules, such as unsaturated fatty acids, and sulphydryl-containing proteins (e.g. membrane ATPase), the membrane is potentially subject to attack on two fronts. That plants and animals are known to possess defence mechanisms against possible attack by free radicals and their by-products is well documented (Halliwell, 1981; Knox & Dodge, 1985). While many aspects of free radical chemistry and biology are known, these are derived mostly from either model non-aqueous solvent systems or hydrated metabolizing cells, mostly animal. Recently, however, these ideas have been increasingly applied to plant systems, especially in leaf and flower senescence (Leshem, 1984).

There are, however, several ways in which dry seeds are likely to be different from hydrated, metabolising tissues:

1. Scavenging and dismutation mechanisms, such as superoxide dismutase (SOD) and catalase, are presumably inoperative and, even if this were not the case, would not be replaceable if inactivated in storage. Indeed, evidence suggests that for soybeans, SOD activity is induced only after a few hours imbibition (Stewart and Bewley, 1980). This may be too late to control free radical levels if these were substantial and damage could attain lethal levels.

2. Those free radicals which might arise as a result of metabolic events, are likely to be generated at a much slower rate in dry seeds than in hydrated tissue, and their build-up or dissipation may be dictated largely by conditions prevailing at their sites of formation.

3. The presence of tightly-bound water (Clegg, 1979) to different cytoplasmic sites and changes with increasing levels of seed moisture content are likely to produce complex interactions between free radical build-up, scavenging antioxidants and

interactions with protein sulphydryl groups.

4. Mitochondrial cytochromes, microsomal proteins like cytochromes b_5 and P_{450}, may be important as sites of slow and uncontrolled initiation of free radicals.

Lipoxygenases have been studied for many years as a factor contributing to the oxidative stability of seed-derived foods. Much of this work has been concerned with minimizing enzyme activity, since it has become evident that this enzyme may function at low moisture contents, producing lipid hydroperoxides which may react with proteins and amino acids (Gardner, 1979) or generate undesirable flavour components.

Brockmann & Acker (1977) have shown that in a model system of sunflower oil on an inert carrier, lipoxygenase activity increased between 45 - 65% RH. It was also shown that phospholipids could serve as a substrate. Whereas maximum hydroperoxide levels from autoxidation (i.e. oil on carrier only) were noted after 300 hours at 15% RH at 25°C, hydroperoxide levels from enzyme activity reached a peak at 100 hours under the same conditions. Although this study may be criticised on the grounds that enzyme levels were not specified, it does confirm the potential for lipoxygenase activity at low moisture contents in seeds.

Edwards (1976) has suggested that metabolism in seeds of Sinapis is only reduced to a low level at moisture contents of 4 - 6%. For lettuce seeds this would correspond to a storage RH below 60%, which is in broad agreement with the values obtained by Brockmann & Acker (1977). Some evidence was obtained to suggest that a fundamentally different pattern of deterioration took place in seeds at 60% RH but this will require confirmation. It has further been found in the present study that carbon dioxide is produced by seeds in storage and that some of this related to decline in the level of oxygen. The production of carbon dioxide by seeds in storage has been well-documented although its origins are not entirely clear (Stanwood & Bass, 1981). Quite apart from the production of carbon

dioxide from low-level cellular metabolism or micro-organisms, it is significant that many lipid autoxidation studies have suggested that carbon dioxide may be produced as a by-product of peroxidation (Loury, 1975, Selke et al., 1970).

3. Free-radicals and Genetic Damage

The delay noted in the germination of aged seed has frequently been attributed to the requirement for repair of damage to the genome and subcellular components. This may take place as a normal part of cellular turnover, or be "unscheduled" as has been suggested for DNA repair (Osborne, 1983). While there is little doubt that DNA repair takes place as an early post-imbibitional event, the causative agent of damage has not yet been identified with certainty.

While endonucleases have been suggested, no studies have been conducted which unequivocably demonstrate such enzyme activity at low moisture contents. Indeed it might even be argued that to reduce such a likelihood special packaging or protective measures may have been selected for. An examination of seeds in the dehydration phase of development and on hydration, with specific reference to DNA packaging and endonuclease activity is urgently needed. Although Osborne has invoked endonucleases as a possible cause of strand breaks, evidence for free-radical initiation of such damage cannot be excluded. It has been observed that H_2O_2 induces single strand breaks in intracellular DNA when included in the culture medium of fibroblasts, although it is unable to do this to isolated DNA in vitro (Hoffman & Meneghini, 1974). In addition linoleic acid hydroperoxide has been shown to cause site-specific cleavage of the guanine nucleotides of DNA (Inouye, 1984).

A new perspective on DNA strand scission in the dehydrated state has been provided by Bucala, Model & Cerami (1984). It is well known that reducing sugars react nonenzymatically with protein amino groups to initiate non-enzymatic browning (Maillard reaction). The above workers have obtained evidence to suggest that glucose-

6-phosphate and glucose (at a much slower rate) form adducts with the primary amino groups of bases that inhibit template function. Later events include depurination, elimination, and strand scission. The physiological significance of these studies has yet to be evaluated for seeds in particular and for ageing in general. The possibility cannot be excluded that an early consequence of imbibition may be uncontrolled increases in peroxidation, superoxide, hydroxyl radicals or hydrogen peroxide, which may damage the DNA. In vitro studies (Lesko, Lorenzen & Tso, 1980) have shown that single strand breaks to the DNA are caused by the generation of the above radicals. It is not necessary therefore to invoke the action of endonucleases, and damage to the genome may be satisfactorily accommodated within the framework of the free-radical theory.

4. The Metabolic Imbalance Concept

Evidence is accumulating to suggest that volatile profiles may provide a new avenue in vigour evaluation (Woodstock & Taylorson, 1981; Fielding & Goldsworthy, 1982; Gorecki, Harman & Mattick, 1985). Furthermore, a combined analysis of volatiles from dry as well as imbibing seeds may prove to be a more satisfactory approach in the evaluation of quality than either method alone. In this regard the suggestion that seed fungi may modify the volatile profiles by metabolic involvement at imbibition (Gorecki et al., 1985) is particularly significant. Heating seeds dry would thus represent a method of evaluating peroxidation without this particular complication. For reasons of simplicity it has been assumed in the present studies that the heating of dry seeds leads to the breakdown of pre-existing lipid hydroperoxides, but this may not be an entirely valid assumption. Evidence has been obtained (Hailstones & Smith, unpublished) that total lipid hydroperoxides decline markedly after such heating treatments in seeds of cabbage and soybean. Inadequate and non-uniform hydration of seed tissue has been identified as a likely cause of variability in volatile studies (Gorecki et al., 1985) and hypochlorite sterilization followed by total immersion of the seeds has been advocated as a solution to the

problem. This approach may induce soaking injury and lead to a somewhat biased profile for deteriorated seeds (see below). It is perhaps not desirable to view deterioration entirely in terms of lipid peroxidation and the volatile by-products but perhaps to take cognisance of a natural element of damage as a consequence of imbibition. Collectively these factors may precipitate cell death. Severe membrane peroxidation leading to osmotic fragility may result in membrane rupture on imbibition with limited potential for membrane restitution. This may be taken as the extreme end-point of membrane deterioration. Impaired ionic regulation, which would not be detectable at the electron-microscope level, may lie somewhat at the other end of the continum of damage, and contribute to impaired synthetic activity but still permit slow germination. In this regard, the enzymes responsible for the removal of the damaging effects of free-radicals are seen as especially important. Since some of the pioneering studies on levels of catalase and peroxidase levels in deteriorating seeds were investigated at a time when the full signficance and role of these enzymes was not fully appreciated (in the context of free-radical damage) it is suggested that these could be profitably re-explored.

Although de novo synthesis as an early post-imbibition event is not precluded, the level of SOD itself may be lowered by activated oxygen species either during dry storage or as an early post-imbibitional event. It has been shown that in a microbial system SOD activity may be lost by the oxidation of a single histidine residue (Bray, Cockle, Fielden, Roberts, Rotilio & Calabrese, 1974).

Woodstock & Taylorson (1981) have suggested that an ethanol and acetaldehyde build-up in soybean seed ageing may be the result of a metabolic imbalance between glycolysis, the TCA cycle, and the electron transport chain. It has been observed that release of radioactivity from ^{14}C glucose as CO_2 is lower than from ethanol in Phaseolus (Morohashi & Shimokariyama, 1975), from which it was suggested that the PPP was less active than glycolysis. Unfortunately meaningful interpretation of such data can be complex, as Bewley & Black (1982) have pointed out in connection with the studies made by Anderson & Abdul-Baki (1971) on patterns of glucose

utilization in barley seeds.

The development of natural anaerobiosis has been investigated in seeds of soybean, maize, pea, bean and lentil, and lactate and ethanol have been shown to accumulate for up to 30 hours after imbibition (Leblova, Sinecka & Vanickova, 1974). This study reported that whereas alcohol dehydrogenase synthesis was induced on imbibition, lactate dehydrogenase was present in dry seeds. It seems reasonable, therefore, to view the imbalance as some perturbation of normally operating pathways, but a full explanation is not yet to hand. It has been cautioned (Woodstock & Taylorson, 1981) that the TCA cycle should not be seen as inoperative during early imbibition. Certainly in the light of studies by Pradet (1982) that oxidative phosphorylation is rapidly established in lettuce seeds after imbibition, reasons for the production of ethanol in aged lettuce seeds (Chapter 7, p. 155) should perhaps be sought elsewhere. In this regard, studies by Fucci, Oliver, Coon & Stadtman (1983) are seen as particularly significant for they suggest a possible free-radical basis for such an imbalance. Those authors demonstrated that a mixed function oxidase system (which produced an unidentified activated oxygen species) was capable of initiating partial($\pm$50%) inactivation of "key" enzymes of metabolism; others were seen to be unaffected. Labile enzymes were alcohol dehydrogenase, lactate dehydrogenase, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, enolase, phosphoglycerate kinase and pyruvate kinase. On the other hand, aldolase, hexokinase, fructose 1 - 6 diphosphatase and malate dehydrogenase were reported to be unaffected. It is suggested that the production of activated oxygen either during seed storage or during imbibition would severely impair operation of the glycolytic pathway between glyceraldehyde-3-phosphate and pyruvate. A reversal of glycolysis, such as might be expected in a fat storing seed, would nevertheless be possible by input of dihydroxyacetone phosphate from lipolysis. It is further suggested that, as a result of input into the TCA cycle from amino acids and possibly fatty acids, a metabolic spillover into pyruvate may occur. Decarboxylation of pyruvate could result in the production of acetaldehyde which, in turn, could be reduced to

ethanol. Impaired activities of alcohol and lactate dehydrogenase might be expected to lead to a delayed clearance of these metabolites, with possible debilitating effects on membranes (as outlined in detail earlier in Chapter 7).

This suggestion may have some relevance to the observation that low vigour seeds have higher levels of ethanol which persist for longer under soaking conditions (Woodstock & Taylorson, 1981). PEG soaking was shown to prevent water injury but also slowed respiratory rates. This may have reduced the possibility of metabolic imbalance. Although evidence implicating free-radicals is highly circumstantial, it is largely unnoticed in studies of this kind that the commonly used osmotica, PEG and mannitol, are used as radical scavengers in radiobiological studies. In this connection, Tien, Svingen & Aust (1982) have shown that in vitro, mannitol was as effective as catalase in controlling peroxidation (as measured by MDA) from hydroxyl and superoxide radicals.

A recent study of some significance is the observation that dehydration injury may be linked to free-radical attack on phospholipids or enhanced phospholipase activity (Senaratna, McKersie & Stinson, 1985). Investigations by Mead (1980) have shown that in vivo phospholipase A_2 removed oxidised fatty acids from rat lung membranes at twice the rate of unoxidised fatty acids. The biochemical effects of aldehyde products of lipid peroxidation have been the subject of intensive study in animal systems. Acetaldehyde is regarded as highly reactive towards tissue components although 4-hydroxy-nonenal has been a compound of particular interest (Dianzani, 1982; Esterbauer, 1982). The incubation of hepatocytes in acetaldehyde produced nearly twice as much malondialdehyde as carbon tetrachloride, at the same concentrations (Stege, 1982). Since carbon tetrachloride induced a two-and-a-half fold increase in peroxidation over control levels, it may be that anaerobiosis in seeds has the potential to produce significant peroxidation by the action of acetaldehyde. A delay in the clearance of this metabolite could be expected to produce profound cellular damage.

Significantly, this latter study showed that viability (as measured by dye exclusion) was not affected by acetaldehyde. Ultrastructural damage is not precluded since the microtubular system and mitochondrial function have been identified as subcellular sites of damage (Lieber, 1980).

5. Membranes and Peroxidation

At the present time it is difficult to identify the primary cause of the metabolic imbalance concept outlined above, but altered membrane properties may be seen as another indirect mechanism whereby such changes may be brought about. From a practical point of view it may also be difficult to identify "target" membranes because of the metabolic interdependence of different cellular compartments. The plasmalemma may be seen as the limiting compartment of the glycolytic pathway (and the pentose phosphate pathway), while the inner mitochondrial membrane may be seen as the limiting element of the Krebs cycle and the electron transport chain. However, damage or changes in the properties of the plasmalemma may be able to amplify damage to mitochondria by changes to cytoplasmic pH or intracellular ion levels (eg. Ca^{++}). For instance a compensatory increase in throughput of the electron transport chain to compensate for such altered cytoplasmic ion levels may lead to enhanced mitochondrial peroxidative damage. Ongoing peroxidative damage is seen as an unavoidable consequence of mitochondrial activity (Vladimirov, Olenev, Suslova & Cheremisina, 1980), consequently sublethal damage to mitochondrial membranes in the dry state may become debilitating as part of an attempt to maintain cellular homeostasis.

A further difficulty is evident in attempting to evaluate the importance of lipid peroxidative damage in dry storage and the consequences of such damage on imbibition. The similar changes in total lipid hydroperoxides levels between aged and unaged seeds (Figure 6.3) may suggest that cellular defences against peroxidative damage may be a factor which distinguishes the viable from the

non-viable seed. Bewley (1979) has indicated that in a desiccation-tolerant moss there is a sharp rise in lipid peroxidation on rehydration with concomitant increases in SOD and catalase, which would be expected to ameliorate any possible damage. On the other hand, in a desiccation intolerant moss these protective enzymes decline precipitously on imbibition and increasing free radical induced peroxidation might be expected to lead to lethal damage. Recent studies implicating free-radicals or phospholipase in the de-esterfication of membrane phospholipids during dehydration injury in soybean axes (Senaratna, McKersie & Stinson, 1985) and the inhibition of catalase activity in chilling injury (Patterson, Payne, Chen & Graham, 1984) serve to highlight the importance of free radicals and defense mechanisms in cell injury.

It has been observed that freeze-dried leaf protein concentrate of 15-20% lipid content is less prone to oxidation than the total lipid isolate. Increasing temperatures and moisture contents were seen to bring about changes in both lipids and proteins (Hudson & Karis, 1976). While it could be argued that this "protective" effect of protein may simply reflect differences in physical disposition of lipid in freeze-dried systems, it may indicate that free-radical chain termination may be effected by hydrogen abstraction from proteins. Evidence for this suggestion in seeds is non-existent, and it is obvious that this reaction must be seen as quite separate from the proposed role of aldehyde breakdown products of lipid peroxidation, such as malondialdehyde. The ability of this aldehyde to inactivate enzymes has been known for some time in animal systems (Chio & Tappel, 1969) although it is only recently that attempts have been made to implicate malondialdehyde in plant senescence (Stewart & Bewley, 1980; Dhindsa et al., 1981). However the assay is not free from difficulties, especially since acetaldehyde and sucrose are reported to interfere with the assay (Beuge & Aust, 1978).

That lipid oxidation in biological membranes is likely to be the result of a complex interplay of many factors is reinforced by the studies of Mead and his co-workers (see Mead, 1980). That author has investigated the oxidation of various fatty acid monolayers

bound to layers of silica gel. In such a model system the kinetics of linoleic acid oxidation were found to be quite different from those of bulk phase systems. Whereas the latter characteristically produce hydroperoxides monolayers yielded epoxides on oxidation.

This was thought to occur as a result of transfer of oxygen from one peroxy radical to the adjacent double bond of a neighbouring molecule. Further investigation showed that the introduction of unsaturated fatty acids into the monolayer of pure linoleic acid, led to diminution in oxidation of linoleic acid and yielded hydroxy-epoxy fatty acids. These oxidation products were also observed in liposomes, suggesting that difficulties in intra-molecular oxygen transfer could also prevail in biological membranes. It is perhaps apposite to consider the results of membrane studies in cotyledonary senescence for possible parallels and contrasts with likely membrane changes in seed ageing, bearing in mind the hydrated and dehydrated nature of the two systems.

Pauls and Thompson (1980) have suggested that membranes of normal cells are extensively liquid crystalline (fluid), but that on deterioration, an increasing proportion of the membrane assumes gel-phase properties and becomes increasingly rigid. Not only are these changes apparent on senescence, but they can also be induced as a result of ozone treatment (Pauls and Thompson, 1980), or by substances such as paraquat, (Chia, Thompson & Dumbroff, 1981). The effects of such altered physical properties on membrane function are known to be profound (Schnitzky & Henkart, 1979). 'Islands' of different fluidity may be produced in the membrane; such lateral phase separation may lead to leakage, and an altered distribution and placement of proteins in the membrane.

It has further been shown in the Phaseolus cotyledon system, that the overall ratio of unsaturates : saturates did not change appreciably during senescence (McKersie et al., 1978). A striking increase in sterols was noted and it was suggested that this could possibly have a rigidifying effect on the membrane.

In a later study, McKersie and Thompson (1979). showed that pure liposomes exhibited similar properties to those shown by intact microsomal membranes, from which it was deduced that proteins, per se, were not the primary determinants of the altered properties. However, addition of a neutral lipid fraction to a phospholipid preparation from young (liquid crystalline) microsomes, induced gel-phase properties. This rigidifying effect was thought to be brought about by some abundant, but as yet unidentified, product of peroxidation, which may act directly with unsaturated fatty acid chains, possibly intercalating between them (Barber and Thompson, 1983).

Recent observations and proposals concerning leaf senescence may provide further additional pointers. Of particular importance is the suggestion that rapid turnover of the 32 K dalton protein of chloroplasts may be the result of free-radical attack (Woolhouse, 1984). It was argued that this renders this extrinsic membrane protein molecule liable to proteolytic attack. Attention has already been drawn to the observation by Mead (see above) that peroxidation may render fatty acids in the membrane liable to increased phospholipase activity. This might suggest that seed membranes may be liable to damage by both autoxidation and phospholipase activity on imbibition while the work of Thomson and co-workers lays emphasis on the physical properties of membrane lipids only. The above serve rather to highlight what is not known as regards seed membranes in ageing but nevertheless implicates free radicals in a variety of damaging or potentially damaging effects.

It has been suggested that if membrane peroxidation is important in seed ageing that it may occur at levels lower than those previously considered significant (Priestley & Leopold, 1979). This was borne out in a later study (Priestley and Leopold, 1983) where changes taking place in soybean seeds stored at 4°C and low humidity for 44 months were examined. For variety Wayne, it was noted that a 12% decline in viability was accompanied by a 2.5% decline in the molar percentage of linolenic acid. In the variety, Chippewa, a 60% decline in viability was accompanied by losses of 3.1% in total seed

fatty acids, a 1.7% loss in total seed polar lipid fatty acids and an 11.5% loss in total axis fatty acids. Significantly, linolenic acid values were nearly 4 times greater in the axis, thus making the axis liable to potentially greater peroxidative damage. This is in keeping with the findings of Buchvarov and Gantcheff (1984), who showed the presence of free radicals in axis, rather than cotyledonary tissue.

Examination of 6 different cultivars of soybean showing a 28% difference in viability (ranges 95 - 67%) revealed a parallel 9.6% decline in linoleic acid levels, and a marked increase in total lipid hydroperoxides (Smith & Blakeway, unpublished results). Stewart and Bewley (1980) found for soybean seeds under an even more extreme ageing regime (100% RH and 45°C) than that used by Priestley and Leopold (1979) that linoleic and linolenic acid levels declined with ageing. However, in this study, seeds were non-viable by the second day and while substantial changes may have taken place subsequently, it is perhaps more pertinent to examine changes occurring only over the first two days. In the first day of ageing, viability declined to 24%, while linolenic acid levels declined by 21% and linoleic acid by some 2% of the control values. It may be tentatively concluded from the above three studies that ageing in the long term or under accelerated conditions may bring about changes which differ in degree rather than kind, although more broadly-based studies which examine other areas of likely damage should also be examined.

Peanut seeds aged at 90% RH and 38°C, showed a decline in the fatty acid levels of the phospholipid fractions (Pearce and Abdel Samad, 1980). On an area percent basis, the change was small (2.6%) but when values were expressed per unit dry weight of the phospholipid and glycolipid fractions, it was seen that these fell to some 50% of the control value by the time viability has fallen to the same extent. Those workers also examined fatty acids of the polar lipid fraction in two cultivars from two different harvests, stored at 30% RH and 5°C. These different lots all presented different viabilities and conductivities, but it could not be said with

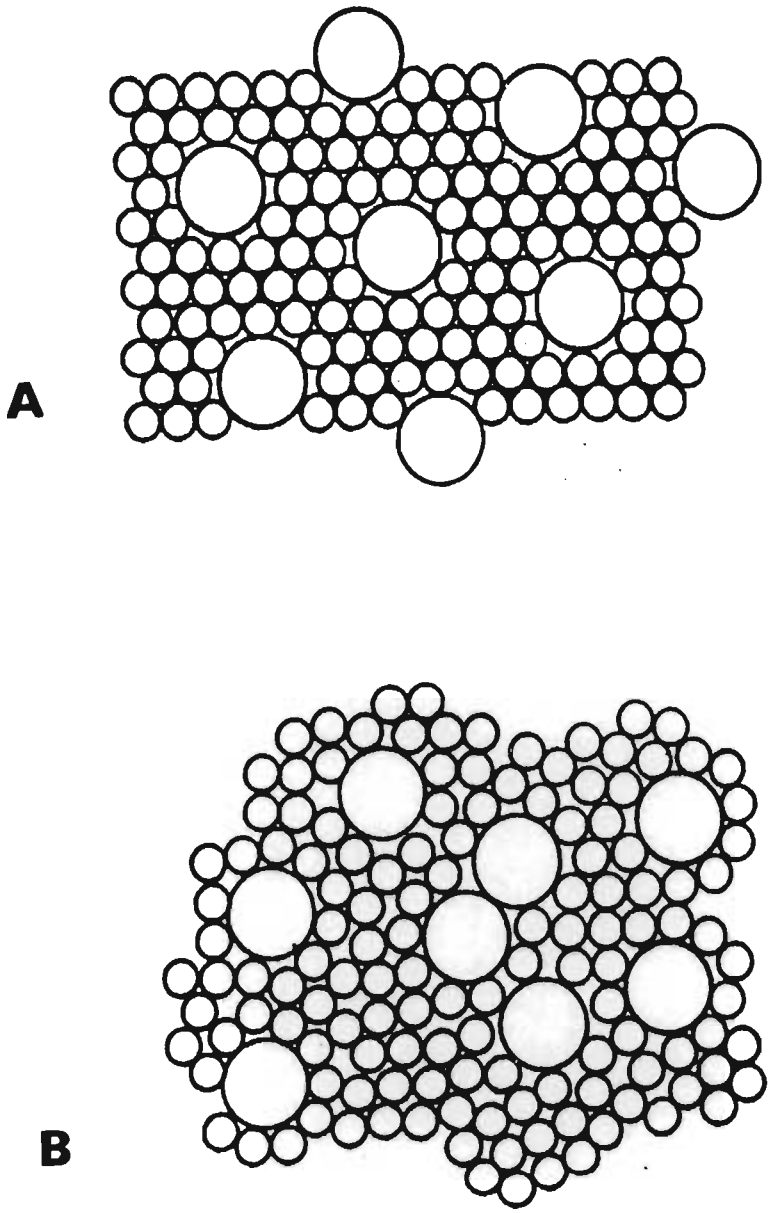


Figure 8.1

Simplified scheme (from Cornell at al., 1979) suggesting how random arrays of close-packed discs, simulating membrane proteins and lipid may interact. Figure A represents the face view of a normal, liquid crystalline membrane, while in Figure B membrane rigidification has taken place as a result of peroxidation. This could include either lipid-lipid interactions of the kind described by Mead (1980) or reactions with integral membrane protein SH groups. The proportion of lipid to protein (small to large circles) corresponds to 10:1. According to this illustration the membrane may be leaky (open spaces) without obvious discontinuity. For simplicity the movement of hydrocarbon fatty acid 'tails' of the phospholipid are ignored.

certainty whether the changes were strictly age-related. Lipid hydroperoxides could not be detected and those authors speculated that this could indicate that significant peroxidation was not occurring, or that the hydroperoxides had broken down, or that antioxidants may be implicated.

Additional support for the suggestion that changes to membrane fatty acids need not be large to bring about significant alterations in membrane properties comes from the studies of Serhan, Anderson, Goodman, Dunham & Weissman (1981). It was shown that 1 and 5 mole % of phosphatidic acid or oxidized trienoic acids, respectively, acted as ionophores in a liposome system. This activity was 2 - 3 orders of magnitude less than the ionophore A23187, but the concentrations employed were low enough to exclude the possibility that the ionophonic properties were caused by disruption of membrane structure through the formation of micelles. In general terms, ionophonic activity is thought to be conferred by the specific structural arrangement of oxygen atoms. The present writer would like to suggest that the presence of hydroperoxy groups in the hydrophobic interior of membranes could provide the necessary environment for movement of ions and molecules which are normally excluded. A simplified representation of how this might take place is presented in Figure 8.1. An element not considered in this illustration is the effect which differing chain lengths or fatty acid unsaturation per se might have within the plane of the bilayer.

6. Some speculative Consequences of Imbalances in Fatty Acid Metabolism

It is unfortunate that nothing is known about the mechanism of lipase activation or its regulation in normal metabolism or seed ageing. Should increased activity of lipases, phospholipases or impaired fatty acid metabolism be one of the consequences of ageing then fatty acids may rise to unphysiologically high levels.

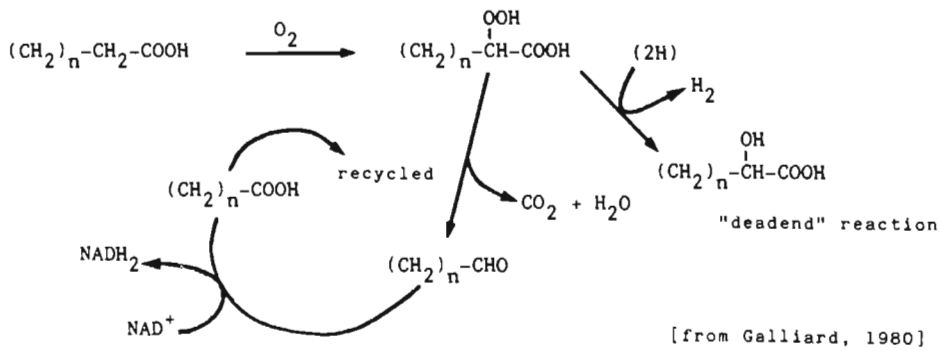
It has been suggested that in addition to a pre-existing acid lipase

present in the membranes of the lipid body, an alkaline lipase activity increases in the glyoxysomes over several days of germination (Beevers, 1975; Bewley & Black, 1982). Since the latter probably arises from de novo synthesis as a "late" post imbibitional event, it can probably be ignored for the purposes of the present argument. The fusion of lipid bodies noted in the present ultrastructural studies presumably reflect altered properties of the limiting membrane which may be related to some form of deviation from the normal pattern of mobilization, possibly as a consequence of peroxidation. Micromolar concentrations of oleic acid have been shown to alter the morphology and effect the fusion of sarcoplasmic reticulum vesicles (Herbette, Favreau, Segalman, Napolitano & Watras, 1984). If the fusogenic ability of this fatty acid is regarded as a general membrane phenomenon, it could be argued that any intracellular perturbation which leads to an increase of oleic acid could lead to fusion of the limiting membrane of lipid bodies.

Fatty acids have additionally been shown to be inhibitory to chloroplast electron transport (McCarthy & Jagendorf, 1965) and also the electron transport system of animal mitochondria (Piper, Sezer, Schwartz, Hutter & Spieckermann, 1983).

It has further been suggested that the inhibition of normal metabolism (glycolysis and the TCA cycle) on tissue slicing may be due to the generation of free fatty acids (Theologies & Laties, 1981). α-oxidation may have been underestimated in the past (Galliard, 1980) because of excessive concern with β-oxidation at the time of its discovery in plants when possible parallels with mammalian systems were of prime concern. Although evidence is lacking for seeds during early imbibition, young leaf tissue has been shown to oxidize fatty acids more rapidly through α-oxidation than the β pathway. Furthermore, since CoA-SH activation is an important initial step in β-oxidation, and -SH containing compounds are potentially at risk as regards reactivity with free radicals (Biaglow, Varnes, Clark & Epp, 1983), α-oxidation may be further favoured until such time as free-radicals decline or the oxidized S-S groups are reduced.

A metabolic imbalance of another kind is thus proposed in which β-oxidation is blocked or limited, and α-oxidation prevalent. In essence, α-oxidation pathways involve oxidation of a C_n fatty acid to C_{n-1} aldehydes with the liberation of CO_2. The initial reaction product is a 2-hydroperoxy acid which is decarboxylated to a C_{n-1} aldehyde as indicated below:



[from Galliard, 1980]

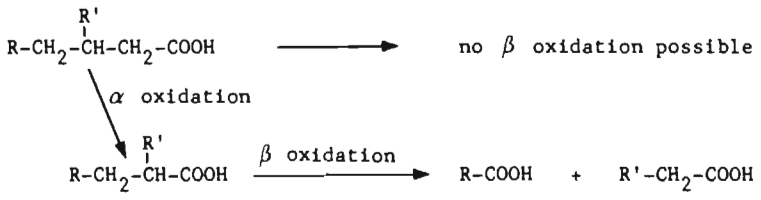
The 2-hydroxy fatty acid is seen as a "deadend" product (Stumpf, 1976). It is thought that the peroxidation step and the subsequent reduction are stereospecific reactions and that saturated and unsaturated fatty acids are equally effective substrates (Galliard, 1980).

Particular aspects of this pathway which are seen as significant in the light of what has been discussed above are:

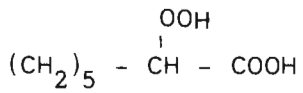
1. Activated molecular oxygen is seen as initiating the attack on the α-carbon of the free fatty acid.

2. GSH and glutathione peroxidase were seen as favouring the production of "deadend" 2-OH fatty acids.

3. The pathway was seen as one in which blocking groups on the fatty acid chain could be eliminated, and the substrate rendered liable for oxidation:



It was earlier suggested (Chapter 7) that alcohols and aldehydes such as hexanol and hexanal may be derived by fatty acid scission involving free-radical mediated processes. It is possible to speculate that α-oxidation may offer an alternative route for the production of short chain aldehydes and alcohols. Lauric acid was suggested as the lower limit of carbon length which may enter the pathway (Stumpf, 1976), but if this is not so, then it may be speculated that the presence of 13 hydroperoxides of linoleic acid (the most abundant fatty acid in lettuce seeds) may, after repeated oxidation cycles, lead to:



This may give rise to hexanal on decarboxylation as indicated in the above cycle, but may also be reduced to the deadend 2-OH heptanoic acid, which on decaboxylation could yield hexanol.

Although the full physiological significance of oxidation is not yet clear, Laties (1978) has shown that the respiratory burst in sliced potato tuber tissue is due mainly to this pathway and that carbohydrate-based respiration is a later event in the "ageing" process. Fatty acids released by the action of acyl hydrolases as a consequence of cutting are seen as a significant element in the initiation of the pathway.

It is also perhaps possible to invoke the action of lipoxygenases in the production of hexanal (and possibly hexanol also) as a product of a putative hydroperoxide lyase on the hydroperoxide of linoleic acid according to the reaction (Galliard & Chan, 1980):

$$CH_3(CH_2)_4\ CH = CH - CH_2 - CH = CH - (CH_2)_7\ COOH$$

$$\downarrow \text{ Lipoxygenase}$$

$$CH_3(CH_2)_4\ \overset{\overset{OOH}{\uparrow}}{CH} - CH = CH - CH = (CH_2)_7\ COOH$$

$$\downarrow \text{ Hydroperoxide Lyase}$$

$$CH_3(CH_2)_4\ CHO + OHC - CH_2 - CH = CH - (CH_2)_7\ COOH$$

Hexanal — 12 Oxo-cis-9-dodecenoic acid

At present no connection is known or has been suggested between the α-oxidation pathway and its potential to produce volatile aldehydes and the above lipoxygenase pathway which may also produce aldehydes. Recent attempts, albeit unsuccessful, to implicate hydroxy fatty acids, lipoxygenase and alternative respiration within a unified framework (Dupont, Rustin & Lance, 1982) serve to highlight the difficulties in attempting to place complex interlocking metabolic pathways into a simple schema. There is little doubt that research in these areas may yield results which will ultimately permit what are apparently disparate processes to be linked within a common framework.

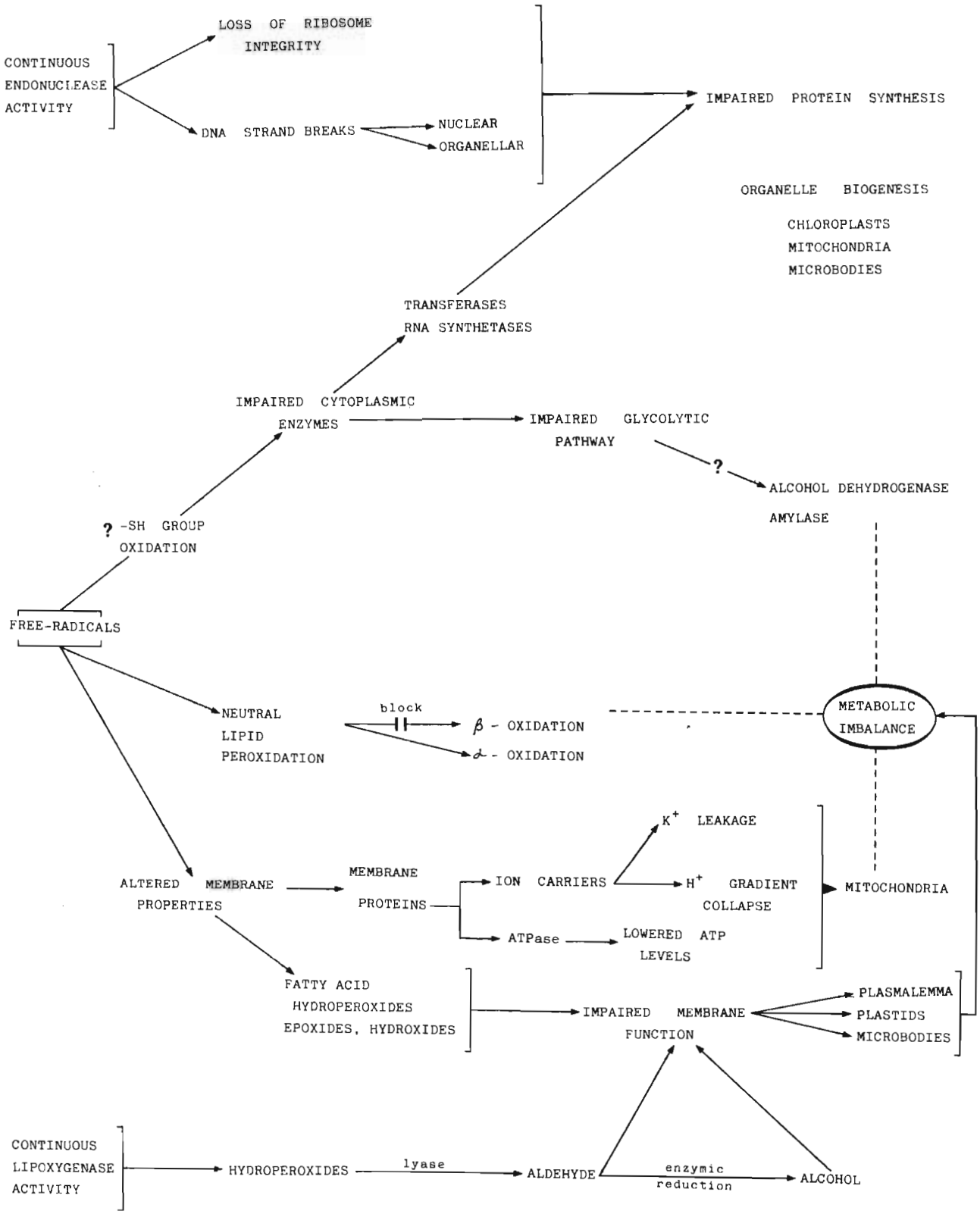

CONTINUOUS ENDONUCLEASE ACTIVITY
LOSS OF RIBOSOME INTEGRITY
DNA STRAND BREAKS
NUCLEAR
ORGANELLAR
IMPAIRED PROTEIN SYNTHESIS
ORGANELLE BIOGENESIS
CHLOROPLASTS
MITOCHONDRIA
MICROBODIES
TRANSFERASES
RNA SYNTHETASES
IMPAIRED CYTOPLASMIC ENZYMES
IMPAIRED GLYCOLYTIC PATHWAY
?
ALCOHOL DEHYDROGENASE
AMYLASE
? -SH GROUP OXIDATION
FREE-RADICALS
NEUTRAL LIPID PEROXIDATION
block
β - OXIDATION
α - OXIDATION
METABOLIC IMBALANCE
ALTERED MEMBRANE PROPERTIES
MEMBRANE PROTEINS
ION CARRIERS
K+ LEAKAGE
H+ GRADIENT COLLAPSE
ATPase
LOWERED ATP LEVELS
MITOCHONDRIA
FATTY ACID HYDROPEROXIDES EPOXIDES, HYDROXIDES
IMPAIRED MEMBRANE FUNCTION
PLASMALEMMA
PLASTIDS
MICROBODIES
CONTINUOUS LIPOXYGENASE ACTIVITY
HYDROPEROXIDES
lyase
ALDEHYDE
enzymic reduction
ALCOHOL

Figure 8.2

Speculative scheme showing possible consequences of free-radical action on various metabolic activities. The pathways outlined are seen as incomplete in seeds in the dry state, the full metabolic consequences becoming manifest only on imbibition. Seed moisture content is postulated as having an important influence on the level and extent of free-radical and enzymatic activity in dry storage.

Free-radicals arising from either mitochondrial activity(albeit limited) or from lipid peroxidation are seen as inactivating -SH groups of important enzymes of the transcription and translation machinery of the cell, as well as some enzymes of the glycolytic pathway, the latter leading to metabolic imbalance. Peroxidation of the neutral lipids may lead to an imbalance between α- and β-oxidation pathways.

Altered membrane properties may occur as a result of peroxidation and lead to:

1. changes in intrinsic membrane proteins of both the plasmalemma and the mitochondria, leading to losses of ion gradients (see also Figure 5.2 and accompanying text, earlier)
2. rigidification or lateral phase separation of membrane phospholipids.

Two examples of continuous enzymatic activity are presented, namely endonucleases(in sensu Osborne) and lipoxygenases(for which only indirect support is available, e.g. Brockmann & Acker, 1977).

It is further speculated that the potentially damaging hydroperoxides which accumulate during dry storage are converted by a hydroperoxide lyase on imbibition to aldehydes. Some of these may be metabolized directly or converted into alcohols. An 'overload' of alcohols and aldehydes may 'amplify' membrane damage.

An outline summarizing the proposals of this Chapter is presented in Figure 8.3,from which it may be seen that free radicals are viewed as a major contributory element to membrane changes and metabolic imbalance. In addition continuous endonuclease and lipoxygenase activity are seen as enzymic components contributing to ageing (as outlined in the working definition in Section 2 of this chapter, page 179). These proposals may serve as a framework for further and more definitive study since much of the supporting evidence for these proposals has been gathered from widely divergent sources.

Note added in proof: During the final stages of assembly of this thesis a review appeared (Wilson & McDonald, Seed Science and Technology, 1986, 14, 269-300) which is entirely supportive of the theoretical arguments developed here. It also lends substantive support to the suggestions contained herein that headspace analysis may offer a new research direction in seed vigour studies, as well as providing further support for the role of free radical peroxidation of lipids as an important determinant in seed ageing.

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Erratum : The following list of references have been omitted. They should appear in the numbered places indicated in the left hand margin of the references list, pages 204-241.

1. Bewley, J.D., Leung, D.W.M. & Ouelette, F.B. (1983). The co-operative role of endo-β-mannase, β-mannosidase and α-galactiosidase in the mobilization of endosperm cell wall hemicelluloses of germinating lettuce seed. In: Mobilization of Reserves in Germination, Eds. Nozzolillo, C., Lea, P.J., Loewus, F.A., Plenum, New York.

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APPENDIX

Finnigan GC-MS computer programme printout for volatiles identified from the heating of dry seeds (A-C) and from heating the silica gel desiccant of hermetically stored, non-viable seeds (D).

Calculated amounts are semi-quantitative, use being made of library programme response factors; values also given as area percentages of total counts obtained.

See text page 164.

FINNIGAN ORGANICS IN WATER ANALYZER
QUANTITATION REPORT FILE: FLSRQ

DATA: SEED12.TI
12/05/84 15:30:00
SAMPLE: RL0
SUBMITTED BY: ANALYST:

A

AMOUNT=AREA(HGHT) * REF. AMNT/(REF. AREA(HGHT)* RESP. FACT)
RESP. FAC. FROM LIBRARY ENTRY

NO	NAME	NO	M/E	SCAN	TIME	REF	RRT	METH	AREA(HGHT)	AMOUNT	%TOT
1	PENTANE	1	TOT	145	1:05	20	0.235	A VV	159122.	6.090	3.12
2	METHANE, THIOBIS-	2	TOT	145	1:05	20	0.235	A VV	9644.	0.369	0.19
3	METHANE, THIOBIS-	3	TOT	149	1:07	20	0.242	A VB	19191.	0.734	0.38
4	ACETICACID, METHYLESTER	4	TOT	152	1:08	20	0.247	A VB	19698.	0.754	0.39
5	PROPANAL, 2-METHYL-	5	TOT	164	1:14	20	0.266	A BV	50972.	1.951	1.00
6	2-PROPENAL, 2-METHYL-	6	TOT	171	1:17	20	0.278	A VB	15119.	0.579	0.30
7	3-BUTEN-2-ONE	7	TOT	180	1:21	20	0.292	A BV	64929.	2.485	1.27
8	3-BUTEN-2-ONE	8	TOT	185	1:23	20	0.300	A VB	55001.	2.105	1.08
9	FURAN, 2-METHYL-	9	TOT	202	1:31	20	0.328	A BV	18448.	0.706	0.36
10	METHANE, TRICHLORO-	10	TOT	210	1:34	20	0.341	A VB	79836.	3.055	1.56
11	NOT IDENTIFIED	11	TOT	236	1:46	20	0.383	A BB	5613.	0.215	0.11
12	PENTANAL	12	TOT	248	1:52	20	0.403	A BV	48704.	1.864	0.95
13	BUTANAL, 2-METHYL-	13	TOT	257	1:56	20	0.417	A VV	4141.	0.158	0.08
14	BUTANAL, 2-METHYL-	14	TOT	262	1:58	20	0.425	A VV	26324.	1.007	0.52
15	BENZENE	15	TOT	262	1:58	20	0.425	A BV	3203.	0.123	0.06
16	BENZENE	16	TOT	267	2:00	20	0.433	A VV	2179.	0.083	0.04
17	PENTANAL	17	TOT	311	2:20	20	0.505	A VB	264940.	10.139	5.19
18	HEPTANE	18	TOT	363	2:43	20	0.589	A BB	8773.	0.336	0.17
19	1-PENTANOL	19	TOT	529	3:58	20	0.859	A BB	241471.	9.241	4.73
20	CYCLOBUTANOL, 2-ETHYL-	20	TOT	616	4:37	20	1.000	A BB	2612970.	100.000	51.22
21	2-HEXENAL	21	TOT	805	6:02	20	1.307	A BB	10696.	0.409	0.21
22	2-CYCLOPENTEN-1-ONE, 3-METHYL-	22	TOT	832	6:14	20	1.351	A BB	4888.	0.187	0.10
23	BENZENE, ETHYL-	23	TOT	871	6:32	20	1.414	A BB	7164.	0.274	0.14
24	1-HEXANOL	24	TOT	920	6:54	20	1.494	A BB	479815.	18.363	9.41
25	NOT IDENTIFIED	25	TOT	962	7:13	20	1.562	A BV	34980.	1.339	0.69
26	HEPTANAL	26	TOT	993	7:27	20	1.612	A BV	81583.	3.122	1.60
27	HEPTANAL	27	TOT	993	7:27	20	1.612	A BV	81583.	3.122	1.60
28	HEXANOICACID, METHYLESTER	28	TOT	1093	8:12	20	1.774	A BB	7946.	0.304	0.16
29	PHENOL, 3, 5-DIMETHYL-	29	TOT	1123	8:25	20	1.823	A BV	2479.	0.095	0.05
30	BENZALDEHYDE	30	TOT	1141	8:33	20	1.852	A BV	34990.	1.339	0.69
31	4-PENTENAL, 2-ETHYL-	31	TOT	1156	8:40	20	1.877	A BV	60887.	2.330	1.19
32	1-DODECENE, 2-ETHYL-	32	TOT	1238	9:17	20	2.010	A BB	10074.	0.386	0.20
33	7-OCTEN-4-OL	33	TOT	1265	9:29	20	2.054	A VV	18299.	0.700	0.36
34	FURAN, 2-PENTYL-	34	TOT	1303	9:46	20	2.115	A VB	218461.	8.361	4.28
35	OCTANAL	35	TOT	1309	9:49	20	2.125	A VB	80665.	3.087	1.58
36	BENZENE, 1, 4-DICHLORO-	36	TOT	1321	9:54	20	2.144	A VB	7826.	0.299	0.15
37	1, 3-HEXADIENE, 3-ETHYL-2-METHYL-	37	TOT	1370	10:16	20	2.224	A BV	17608.	0.674	0.35
38	3-OCTEN-2-ONE	38	TOT	1401	10:30	20	2.274	A BV	29927.	1.145	0.59
39	4-PENTENAL, 2-ETHYL-	39	TOT	1448	10:52	20	2.351	A BB	110937.	4.246	2.17
40	NONANAL	40	TOT	1580	11:51	20	2.565	A BB	78542.	3.006	1.54
41	DODECANAL	41	TOT	1825	13:41	20	2.963	A BB	6901.	0.264	0.14
42	HYDRAZINE, 1, 1-DIPHENYL-	42	TOT	2612	19:35	20	4.240	A BB	5168.	0.198	0.10

FINNIGAN ORGANICS IN WATER ANALYZER
QUANTITATION REPORT FILE: FLSRQ

DATA: SEED11.TI
12/05/84 14:16:00
SAMPLE: L4 WEBS
SUBMITTED BY: ANALYST:

B

AMOUNT=AREA(HGHT) * REF. AMNT/(REF. AREA(HGHT)* RESP. FACT)
RESP. FAC. FROM LIBRARY ENTRY

NO	NAME	NO	M/E	SCAN	TIME	REF	RRT	METH	AREA(HGHT)	AMOUNT	%TOT
1	NOT IDENTIFIED	1	TOT	105	0:47	7	0.677	A BB	980871.	73.529	7.51
2	SILANE, DIFLUORODIMETHYL-	2	TOT	111	0:50	7	0.716	A BB	30163.	2.261	0.23
3	NOT IDENTIFIED	3	TOT	143	1:04	7	0.923	A BV	792106.	59.379	6.07
4	PENTANE	4	TOT	143	1:04	7	0.923	A BB	726551.	54.465	5.57
5	ACETICACID, METHYLESTER	5	TOT	155	1:10	7	1.000	A VV	1207730.	90.536	9.25
6	CARBONDISULFIDE	6	TOT	155	1:10	7	1.000	A VB	1333980.	100.000	10.22
7	CARBONDISULFIDE	7	TOT	155	1:10	7	1.000	A VB	1333980.	100.000	10.22
8	PROPANAL, 2-METHYL-	8	TOT	162	1:13	7	1.045	A VB	568472.	42.614	4.36
9	BUTANAL	9	TOT	183	1:22	7	1.181	A BV	86964.	6.519	0.67
10	FURAN, 2-METHYL-	10	TOT	200	1:30	7	1.290	A BV	8890.	0.666	0.07
11	METHANE, TRICHLORO-	11	TOT	207	1:33	7	1.335	A VB	16118.	1.208	0.12
12	PENTANAL	12	TOT	245	1:50	7	1.581	A BV	857147.	64.254	6.57
13	BUTANAL, 2-METHYL-	13	TOT	258	1:56	7	1.665	A VB	504431.	37.814	3.86
14	PENTANAL	14	TOT	306	2:18	7	1.974	A VB	177565.	13.311	1.36
15	2,4-HEXADIENAL, (E,E)-	15	TOT	334	2:30	7	2.155	A BB	20814.	1.560	0.16
16	HEPTANE	16	TOT	355	2:40	7	2.290	A BB	33481.	2.510	0.26
17	DISULFIDE, DIMETHYL	17	TOT	410	3:04	7	2.645	A BB	16368.	1.227	0.13
18	BENZENE, METHYL-	18	TOT	494	3:42	7	3.187	A BB	16850.	1.263	0.13
19	1-PENTANOL	19	TOT	509	3:49	7	3.284	A BB	83845.	6.285	0.64
20	CYCLOBUTANOL, 2-ETHYL-	20	TOT	586	4:24	7	3.781	A BB	912134.	68.376	6.99
21	OCTANE	21	TOT	682	5:07	7	4.400	A BV	45710.	3.427	0.35
22	PENTANOICACID, METHYLESTER	22	TOT	720	5:24	7	4.645	A BV	18123.	1.359	0.14
23	HEXANENITRILE	23	TOT	851	6:20	7	5.490	A BB	22656.	1.698	0.17
24	BENZENE, ETHYL-	24	TOT	855	6:25	7	5.516	A BB	13990.	1.049	0.11
25	BENZENE, 1,2-DIMETHYL-	25	TOT	888	6:40	7	5.729	A BV	52582.	3.942	0.40
26	1-HEXANOL	26	TOT	905	6:47	7	5.839	A BB	526923.	39.500	4.04
27	2-HEXANONE, 5-METHYL-	27	TOT	948	7:07	7	6.116	A BV	149219.	11.186	1.14
28	NOT IDENTIFIED	28	TOT	966	7:15	7	6.232	A BB	21402.	1.604	0.16
29	NOT IDENTIFIED	29	TOT	966	7:15	7	6.232	A BB	21467.	1.609	0.16
30	NOT IDENTIFIED	30	TOT	982	7:22	7	6.335	A BB	83421.	6.254	0.64
31	2-HEPTANOL	31	TOT	1012	7:35	7	6.529	A BB	87581.	6.565	0.67
32	ACETICACID, PENTYLESTER	32	TOT	1051	7:53	7	6.781	A BB	41976.	3.147	0.32
33	PENTANE, 1-NITRO-	33	TOT	1072	8:02	7	6.916	A BV	31716.	2.378	0.24
34	HEXANOICACID, METHYLESTER	34	TOT	1083	8:07	7	6.987	A VV	270736.	20.295	2.07
35	BICYCLO[3.1.0]HEX-2-ENE, 2-METHYL	35	TOT	1123	8:25	7	7.245	A BB	5045.	0.378	0.04
36	NOT IDENTIFIED	36	TOT	1133	8:30	7	7.310	A BV	7919.	0.594	0.06
37	TRICYCLO[2.2.1.02,6]HEPTANE, 1,7,	37	TOT	1140	8:33	7	7.355	A BV	22177.	1.662	0.17
38	4-PENTENAL, 2-ETHYL-	38	TOT	1148	8:37	7	7.406	A VB	18466.	1.384	0.14
39	BENZENE, 1-ETHYL-2-METHYL-	39	TOT	1197	8:59	7	7.723	A BB	8084.	0.606	0.06
40	1-DODECENE, 2-ETHYL-	40	TOT	1231	9:14	7	7.942	A BB	41342.	3.099	0.32
41	NOT IDENTIFIED	41	TOT	1251	9:23	7	8.071	A BV	88035.	6.599	0.67
42	NOT IDENTIFIED	42	TOT	1259	9:27	7	8.123	A VV	21199.	1.589	0.16
43	BENZENE, 1,2,3-TRIMETHYL-	43	TOT	1291	9:41	7	8.329	A BV	14214.	1.066	0.11
44	FURAN, 2-PENTYL-	44	TOT	1298	9:44	7	8.374	A BB	459645.	34.456	3.52
45	DECANE, 5,6-DIMETHYL-	45	TOT	1298	9:44	7	8.374	A BV	33460.	2.508	0.26
46	DECANE, 5,6-DIMETHYL-	46	TOT	1303	9:46	7	8.406	A VV	36318.	2.723	0.28

NO	NAME	NO	M/E	SCAN	TIME	REF	RRT	METH	AREA(HGHT)	AMOUNT	%TOT
47	HEXANOICACID, ETHYLESTER	47	TOT	1312	9:50	7	8.465	A VB	8154.	0.611	0 06
48	BENZENE, 1, 4-DICHLORO-	48	TOT	1315	9:52	7	8.484	A VB	7360.	0.552	0.06
49	ACETICACID, HEXYLESTER	49	TOT	1350	10:07	7	8.710	A BV	113740.	8.526	0 87
50	DECANE	50	TOT	1360	10:12	7	8.774	A VB	31405.	2.354	0.24
51	NOT IDENTIFIED	51	TOT	1366	10:15	7	8.813	A VB	8492.	0.637	0.07
52	HEPTANOICACID, METHYLESTER	52	TOT	1381	10:21	7	8.910	A BB	66891.	5.014	0 51
53	BENZENE, 1-METHYL-2-(1-METHYLETHY	53	TOT	1383	10:22	7	8.923	A BB	46699.	3.501	0.36
54	3-OCTEN-2-ONE	54	TOT	1397	10:29	7	9.013	A BV	30795.	2.309	0.24
55	CYCLOHEXENE, 1-METHYL-4-(1-METHYL	55	TOT	1410	10:34	7	9.097	A VB	43741.	3.279	0.34
56	DECANE, 4-METHYL-	56	TOT	1427	10:42	7	9.206	A BV	18091.	1.356	0 14
57	2-OCTENAL, (E)-	57	TOT	1444	10:50	7	9.316	A VV	30438.	2.282	0.23
58	NAPHTHALENE, DECAHYDRO-, TRANS-	58	TOT	1479	11:06	7	9.542	A BV	10952.	0.821	0.08
59	NOT IDENTIFIED	59	TOT	1525	11:26	7	9.839	A VB	10452.	0.784	0.08
60	NOT IDENTIFIED	60	TOT	1535	11:31	7	9.903	A BV	12480.	0.936	0 10
61	NOT IDENTIFIED	61	TOT	1549	11:37	7	9.994	A VB	25828.	1.936	0 20
62	DECANE, 3-METHYL-	62	TOT	1550	11:37	7	10.000	A VB	18047.	1.353	0.14
63	NONANAL	63	TOT	1577	11:50	7	10.174	A VV	65945.	4.943	0.51
64	NOT IDENTIFIED	64	TOT	1588	11:55	7	10.245	A VV	6435.	0.482	0.05
65	UNDECANE	65	TOT	1623	12:10	7	10.471	A BB	104055.	7.800	0 80
66	NAPHTHALENE, DECAHYDRO-2-METHYL-	66	TOT	1623	12:10	7	10.471	A VB	41553.	3.115	0.32
67	NOT IDENTIFIED	67	TOT	1628	12:13	7	10.503	A VB	18858.	1.414	0.14
68	OCTANOICACID, METHYLESTER	68	TOT	1639	12:18	7	10.574	A BV	14536.	1.090	0.11
69	NAPHTHALENE, DECAHYDRO-2-METHYL-	69	TOT	1667	12:30	7	10.755	A BB	15261.	1.144	0.12
70	BENZENE, 1-METHYL-2-(2-PROPENYL)-	70	TOT	1690	12:40	7	10.903	A BB	7066.	0.530	0 05
71	NOT IDENTIFIED	71	TOT	1695	12:43	7	10.935	A BV	23237.	1.742	0.18
72	NOT IDENTIFIED	72	TOT	1695	12:43	7	10.935	A VV	16478.	1.235	0.13
73	NOT IDENTIFIED	73	TOT	1713	12:51	7	11.052	A VV	4643.	0.348	0 04
74	NOT IDENTIFIED	74	TOT	1723	12:55	7	11.116	A VB	11280.	0.846	0.09
75	NAPHTHALENE	75	TOT	1755	13:10	7	11.323	A BV	26014.	1.950	0.20
76	NOT IDENTIFIED	76	TOT	1755	13:10	7	11.323	A VV	32164.	2.411	0.25
77	NOT IDENTIFIED	77	TOT	1767	13:15	7	11.400	A VB	15636.	1.172	0.12
78	NOT IDENTIFIED	78	TOT	1778	13:20	7	11.471	A BB	13816.	1.036	0.11
79	DODECANAL	79	TOT	1822	13:40	7	11.755	A BV	46537.	3.489	0.36
80	DODECANE	80	TOT	1859	13:57	7	11.994	A VV	101525.	7.611	0.78
81	UNDECANE, 2, 6-DIMETHYL-	81	TOT	1894	14:12	7	12.219	A BV	36550.	2.740	0 28
82	NOT IDENTIFIED	82	TOT	1956	14:40	7	12.619	A VB	8421.	0.631	0.06
83	NAPHTHALENE, 2-METHYL-	83	TOT	2008	15:04	7	12.955	A VB	13937.	1.045	0.11
84	OCTANE, 2, 3, 7-TRIMETHYL-	84	TOT	2024	15:11	7	13.058	A VV	21465.	1.609	0.16
85	NAPHTHALENE, 2-METHYL-	85	TOT	2040	15:18	7	13.161	A BB	4433.	0.332	0.03
86	UNDECANE, 4, 7-DIMETHYL-	86	TOT	2076	15:34	7	13.394	A BB	54559.	4.090	0.42
87	NOT IDENTIFIED	87	TOT	2237	16:47	7	14.432	A BB	8839.	0.663	0.07
88	HYDRAZINE, TETRAPHENYL-	88	TOT	2609	19:34	7	16.832	A BV	3966.	0.297	0.03

FINNIGAN ORGANI... ... TER ANALYZER
QUANTITATION REPORT FILE: FLSRQ

DATA: BEED10.TI
12/05/84 13:08:00
SAMPLE: L28 BULK PAIL
SUBMITTED BY: ANALYST:

C

AMOUNT=AREA(HGHT) * REF.AMNT/(REF.AREA(HGHT)* RESP.FACT)
RESP. FAC FROM LIBRARY ENTRY

NO	NAME	NO	M/E	SCAN	TIME	REF	RRT	METH	AREA(HGHT)	AMOUNT	%TOT
1	SILANE, DIFLUORODIMETHYL-	1	TOT	81	0:36	17	0.148	A BB	80073.	0.709	0.32
2	PENTANE	2	TOT	115	0:52	17	0.210	A VB	304306.	2.695	1.21
3	PROPANAL, 2-METHYL-	3	TOT	134	1:00	17	0.245	A VV	661796.	5.860	2.62
4	BUTANAL	4	TOT	153	1:09	17	0.279	A BV	484367.	4.289	1.92
5	BUTANAL	5	TOT	153	1:09	17	0.279	A BV	484367.	4.289	1.92
6	FURAN, 2-METHYL-	6	TOT	169	1:16	17	0.308	A VV	20675.	0.183	0.08
7	METHANE, TRICHLORO-	7	TOT	176	1:19	17	0.321	A VB	56630.	0.501	0.22
8	PENTANAL	8	TOT	211	1:35	17	0.385	A BV	819488.	7.256	3.25
9	BUTANAL, 2-METHYL-	9	TOT	224	1:41	17	0.409	A VB	489529.	4.335	1.94
10	PENTANAL	10	TOT	271	2:02	17	0.495	A VB	1827150.	16.179	7.25
11	2,4-HEXADIENAL, (E,E)-	11	TOT	295	2:13	17	0.536	A VB	31585.	0.280	0.13
12	HEPTANE	12	TOT	316	2:22	17	0.577	A BB	203084.	1.798	0.81
13	DISULFIDE, DIMETHYL	13	TOT	369	2:46	17	0.673	A BB	23569.	0.209	0.09
14	BENZENE, METHYL-	14	TOT	448	3:22	17	0.818	A BB	26159.	0.232	0.10
15	1-PENTANOL	15	TOT	462	3:28	17	0.843	A BB	46550.	0.412	0.18
16	2-HEXANONE	16	TOT	507	3:48	17	0.925	A BB	41658.	0.369	0.17
17	CYCLOBUTANOL, 2-ETHYL-	17	TOT	548	4:07	17	1.000	A BB	11293200.	100.000	44.79
18	OCTANE	18	TOT	629	4:43	17	1.148	A BB	262616.	2.325	1.04
19	PENTANOICACID, METHYLESTER	19	TOT	667	5:00	17	1.217	A BV	28588.	0.253	0.11
20	BENZENE, CHLORO-	20	TOT	726	5:27	17	1.325	A BV	50023.	0.443	0.20
21	2-HEXENAL	21	TOT	736	5:31	17	1.343	A BB	27164.	0.241	0.11
22	1-UNDECENE	22	TOT	804	6:02	17	1.467	A BB	41126.	0.364	0.16
23	BENZENE, ETHYL-	23	TOT	806	6:04	17	1.474	A BB	32644.	0.289	0.13
24	BENZENE, 1,2-DIMETHYL-	24	TOT	842	6:19	17	1.536	A BV	133995.	1.187	0.53
25	1-HEXANOL	25	TOT	856	6:25	17	1.562	A BB	110836.	0.981	0.44
26	2-HEXANONE, 5-METHYL-	26	TOT	904	6:47	17	1.650	A BV	367132.	3.251	1.46
27	NOT IDENTIFIED	27	TOT	922	6:55	17	1.682	A VB	60523.	0.536	0.24
28	HEXANAL, 5-METHYL-	28	TOT	938	7:02	17	1.712	A BB	408013.	3.613	1.62
29	NOT IDENTIFIED	29	TOT	939	7:03	17	1.714	A BB	443198.	3.924	1.76
30	ACETICACID, PENTYLESTER	30	TOT	1009	7:34	17	1.841	A BB	75105.	0.665	0.30
31	NONANE	31	TOT	1015	7:37	17	1.852	A VB	22078.	0.195	0.09
32	HEXANOICACID, METHYLESTER	32	TOT	1042	7:49	17	1.901	A BV	352542.	3.122	1.40
33	1-HEXANOL	33	TOT	1053	7:54	17	1.922	A VB	68322.	0.605	0.27
34	BENZENE, 1-METHOXY-4-METHYL-	34	TOT	1074	8:03	17	1.960	A BV	3311.	0.029	0.01
35	4-PENTENAL, 2-ETHYL-	35	TOT	1108	8:19	17	2.022	A BV	171774.	1.521	0.68
36	OXETANE, 3-(1-METHYLETHYL)-	36	TOT	1193	8:57	17	2.177	A BB	119553.	1.059	0.47
37	3-OCTANONE, 2-METHYL-	37	TOT	1214	9:06	17	2.215	A BV	163397.	1.447	0.65
38	3-OCTANONE	38	TOT	1225	9:11	17	2.235	A VV	18211.	0.161	0.07
39	5-HEXEN-2-ONE	39	TOT	1233	9:15	17	2.250	A VV	28226.	0.250	0.11
40	2-OCTANONE	40	TOT	1234	9:15	17	2.252	A VV	40937.	0.362	0.16
41	CYCLOPENTANE, 1-ETHYL-1-METHYL-	41	TOT	1250	9:22	17	2.281	A VB	14398.	0.127	0.06
42	FURAN, 2-PENTYL-	42	TOT	1263	9:28	17	2.305	A BV	916513.	8.116	3.63
43	OCTANAL	43	TOT	1263	9:28	17	2.305	A BV	854244.	7.564	3.39
44	NOT IDENTIFIED	44	TOT	1281	9:36	17	2.338	A VB	1195310.	10.584	4.74
45	BENZENE, 1,4-DICHLORO-	45	TOT	1281	9:36	17	2.338	A VB	1189110.	10.529	4.72
46	HEPTANE, 2,2,4,6,6-PENTAMETHYL-	46	TOT	1303	9:46	17	2.378	A BV	148110.	1.311	0.59
47	ACETICACID, HEXYLESTER	47	TOT	1314	9:51	17	2.398	A VB	98441.	0.872	0.39
48	1,3-HEXADIENE, 3-ETHYL-2-METHYL-	48	TOT	1329	9:58	17	2.425	A BB	18032.	0.160	0.07
49	BENZENE, 1,2-DICHLORO-	49	TOT	1340	10:03	17	2.445	A BB	127831.	1.132	0.51
50	HEPTANOICACID, METHYLESTER	50	TOT	1340	10:03	17	2.445	A BV	28144.	0.249	0.11
51	HEPTANOICACID, METHYLESTER	51	TOT	1345	10:05	17	2.454	A VB	20146.	0.178	0.08
52	BENZENE, 1-METHYL-3-(1-METHYLETHY	52	TOT	1349	10:07	17	2.462	A VV	25044.	0.222	0.10
53	3-OCTEN-2-ONE	53	TOT	1362	10:13	17	2.485	A BV	37271.	0.330	0.15
54	BICYCLO[2.2.1]HEPT-2-ENE, 1,7,7-T	54	TOT	1375	10:19	17	2.509	A VV	19399.	0.172	0.08
55	4-PENTENAL, 2-ETHYL-	55	TOT	1410	10:34	17	2.573	A BB	219787.	1.946	0.87
56	1,4-CYCLOHEXADIENE, 1-METHYL-4-(1	56	TOT	1453	10:54	17	2.651	A BB	7127.	0.063	0.03
57	NOT IDENTIFIED	57	TOT	1515	11:22	17	2.765	A VB	18822.	0.167	0.07
58	NOT IDENTIFIED	58	TOT	1531	11:29	17	2.794	A BV	11965.	0.106	0.05
59	NOT IDENTIFIED	59	TOT	1532	11:29	17	2.796	A BV	12478.	0.110	0.05
60	NONANAL	60	TOT	1545	11:35	17	2.819	A VB	217333.	1.924	0.86
61	4-UNDECENE, (E)-	61	TOT	1562	11:43	17	2.850	A VB	7836.	0.069	0.03
62	UNDECANE	62	TOT	1591	11:56	17	2.903	A VB	14381.	0.127	0.06
63	OCTANOICACID, METHYLESTER	63	TOT	1608	12:04	17	2.934	A BV	6103.	0.054	0.02
64	BENZENE, 1,3,5-TRICHLORO-	64	TOT	1710	12:49	17	3.120	A BV	28856.	0.255	0.11
65	5-DECANONE	65	TOT	1719	12:54	17	3.137	A VB	7753.	0.069	0.03
66	AZULENE	66	TOT	1723	12:55	17	3.144	A VB	5116.	0.045	0.02
67	NOT IDENTIFIED	67	TOT	1766	13:15	17	3.223	A BB	19875.	0.176	0.08
68	DECANAL	68	TOT	1792	13:26	17	3.270	A VV	9346.	0.083	0.04
69	DODECANE	69	TOT	1828	13:43	17	3.336	A BB	13362.	0.118	0.05

FINNIGAN ORGANICS IN WATER ANALYZER
QUANTITATION REPORT FILE: FL8RQ

DATA: SILGEL.TI
12/05/84 10:06:00
SAMPLE: SILICA GEL L23
SUBMITTED BY: ANALYST:

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AMOUNT=AREA(HGHT) * REF. AMNT/(REF. AREA(HGHT)* RESP. FACT)
RESP. FAC. FROM LIBRARY ENTRY

NO	NAME	NO	M/E	SCAN	TIME	REF	RRT	METH	AREA(HGHT)	AMOUNT	%TOT
1	SILANE, DIFLUORODIMETHYL-	1	TOT	111	0:50	49	0.179	A BV	100680.	0.252	0.03
2	1-BUTENE	2	TOT	122	0:55	49	0.196	A VV	1425430.	3.572	0.44
3	ETHANE, CHLORO-	3	TOT	126	0:57	49	0.203	A VV	119921.	0.300	0.04
4	ETHANE, CHLORO-	4	TOT	132	0:59	49	0.213	A VB	101672.	0.255	0.03
5	NOT IDENTIFIED	5	TOT	145	1:05	49	0.233	A BV	3863580.	9.681	1.19
6	NOT IDENTIFIED	6	TOT	154	1:09	49	0.248	A VV	12944300	32.435	3.99
7	NOT IDENTIFIED	7	TOT	153	1:09	49	0.246	A VV	11777600.	29.512	3.63
8	ACETICACID, METHYLESTER	8	TOT	160	1:12	49	0.258	A VB	865600.	2.169	0.27
9	PROPANE, 1-CHLORO-	9	TOT	169	1:16	49	0.272	A BB	790624.	1.981	0.24
10	2-PROPENAL, 2-METHYL-	10	TOT	178	1:20	49	0.287	A BB	318624.	0.798	0.10
11	ETHANE, 1, 1-DICHLORO-	11	TOT	184	1:23	49	0.296	A VV	8813.	0.022	0.00
12	NOT IDENTIFIED	12	TOT	194	1:27	49	0.312	A BV	8252100.	20.928	2.57
13	1-HEXYN-3-OL	13	TOT	194	1:27	49	0.312	A BV	8910410.	22.327	2.74
14	1-HEXENE	14	TOT	204	1:32	49	0.329	A VV	233789.	0.586	0.07
15	1-HEXENE	15	TOT	208	1:34	49	0.335	A VV	156133.	0.391	0.05
16	FURAN, 2-METHYL-	16	TOT	204	1:32	49	0.329	A VV	168752.	0.423	0.05
17	FURAN, 2-METHYL-	17	TOT	208	1:34	49	0.335	A VV	303351.	0.760	0.09
18	NOT IDENTIFIED	18	TOT	214	1:36	49	0.345	A VV	4405880.	11.040	1.36
19	NOT IDENTIFIED	19	TOT	223	1:40	49	0.359	A VB	15105.	0.038	0.00
20	1-PROPENE, 3-CHLORO-2-METHYL-	20	TOT	224	1:41	49	0.361	A VV	12523.	0.031	0.00
21	1-PROPENE, 3-CHLORO-2-METHYL-	21	TOT	232	1:44	49	0.374	A VB	102187.	0.256	0.03
22	CYCLOPENTANE, METHYL-	22	TOT	232	1:44	49	0.374	A BV	172104.	0.431	0.05
23	CYCLOPENTANE, METHYL-	23	TOT	240	1:48	49	0.386	A VB	1137480.	2.850	0.35
24	NOT IDENTIFIED	24	TOT	255	1:55	49	0.411	A BV	744260.	1.865	0.23
25	NOT IDENTIFIED	25	TOT	255	1:55	49	0.411	A BV	1234760.	3.094	0.38
26	BUTANE, 1-CHLORO-	26	TOT	255	1:55	49	0.411	A BV	1265400.	3.171	0.39
27	BENZENE	27	TOT	270	2:01	49	0.435	A BV	136405.	0.342	0.04
28	2-BUTENAL, 2-METHYL-	28	TOT	273	2:03	49	0.440	A VV	113932.	0.285	0.04
29	METHANE, TETRACHLORO-	29	TOT	277	2:05	49	0.446	A VV	50487.	0.127	0.02
30	CYCLOHEXANE	30	TOT	283	2:07	49	0.456	A VB	420309.	1.053	0.13
31	NOT IDENTIFIED	31	TOT	301	2:15	49	0.485	A BV	1267000.	3.175	0.39
32	2-PENTANONE	32	TOT	301	2:15	49	0.485	A BV	1263370.	3.166	0.39
33	PENTANAL	33	TOT	323	2:25	49	0.520	A VB	17580900	44.052	5.41
34	ETHENE, TRICHLORO-	34	TOT	338	2:32	49	0.544	A BB	41185	0.103	0.01
35	1-HEPTENE	35	TOT	339	2:33	49	0.546	A BB	323888	0.810	0.10
36	NOT IDENTIFIED	36	TOT	339	2:33	49	0.546	A BB	865000	0.916	0.11
37	HEPTANE	37	TOT	351	2:38	49	0.565	A BV	262710.	0.909	0.11
38	NOT IDENTIFIED	38	TOT	370	2:46	49	0.596	A VB	16097200	40.115	4.93
39	BUTANOICACID, METHYLESTER	39	TOT	381	2:51	49	0.614	A BB	206406.	0.517	0.06
40	FORMICACID, BUTYLESTER	40	TOT	383	2:52	49	0.617	A BB	223169.	0.559	0.07
41	DISULFIDE, DIMETHYL	41	TOT	415	3:07	49	0.668	A BB	33750.	0.085	0.01
42	FURAN, 2, 3-DIHYDRO-4-METHYL-	42	TOT	431	3:14	49	0.694	A BV	81480.	0.204	0.03
43	PENTANE, 1-CHLORO-	43	TOT	461	3:27	49	0.742	A BB	2780120.	6.966	0.86
44	NOT IDENTIFIED	44	TOT	483	3:37	49	0.778	A BB	16123.	0.040	0.00
45	BENZENE, METHYL-	45	TOT	497	3:44	49	0.800	A BB	162788	0.408	0.05
46	1-PENTANOL	46	TOT	528	3:58	49	0.850	A BB	1713320.	4.293	0.53

NO	NAME	NO	M/E	SCAN	TIME	REF	RRT	METH	AREA(HGHT)	AMOUNT	%TOT
47	HEPTANE, 2, 3-DIMETHYL-	47	TOT	576	4 19	49	0 928	A VB	74852	0 188	0 02
48	UNKNOWN	48	NOT	FOUND							
49	NOT IDENTIFIED	49	TOT	621	4 39	49	1 000	A BB	39968500	100 000	12 29
50	HEXANAL	50	TOT	621	4 39	49	1 000	A BB	39671600	99 407	12 21
51	NOT IDENTIFIED	51	TOT	621	4 39	49	1 000	A BB	35396100	88 669	10 89
52	OCTANE	52	TOT	705	5 17	49	1 135	A BB	28622000	71 720	8 81
53	NOT IDENTIFIED	53	TOT	705	5 17	49	1 135	A BB	28632500	71 745	8 81
54	PENTANOICACID, METHYLESTER	54	TOT	724	5 26	49	1 166	A BV	591766	1 483	0 18
55	1-PENTANOL	55	TOT	732	5 29	49	1 179	A VB	1032370	2 587	0 32
56	2-HEXENAL	56	TOT	786	5 54	49	1 266	A BB	146984	0 368	0 05
57	NOT IDENTIFIED	57	TOT	839	6 18	49	1 351	A BV	76462	0 192	0 02
58	NOT IDENTIFIED	58	TOT	846	6 21	49	1 362	A VV	19424	0 049	0 01
59	NOT IDENTIFIED	59	TOT	853	6 24	49	1 374	A VV	170481	0 427	0 05
60	BENZENE, ETHYL-	60	TOT	856	6 25	49	1 378	A VB	84410	0 212	0 03
61	BENZENE, ETHYL-	61	TOT	890	6 40	49	1 433	A BV	57832	0 145	0 02
62	1-HEXANOL	62	TOT	906	6 48	49	1 459	A BB	538975	1 351	0 17
63	2-HEPTANONE	63	TOT	957	7 11	49	1 541	A BV	4129510	10 348	1 27
64	PENTANE, 1, 2-DICHLORO-	64	TOT	966	7 15	49	1 556	A VB	3535800	8 860	1 09
65	HEPTANAL	65	TOT	983	7 22	49	1 583	A BB	621043	1 556	0 19
66	NOT IDENTIFIED	66	TOT	1009	7 34	49	1 625	A BV	31074	0 078	0 01
67	NOT IDENTIFIED	67	TOT	1034	7 45	49	1 665	A BB	18724	0 047	0 01
68	UNKNOWN	68	NOT	FOUND							
69	NONANE	69	TOT	1060	7 57	49	1 707	A BB	4112070	10 304	1 27
70	HEXANOICACID, METHYLESTER	70	TOT	1086	8 09	49	1 749	A BV	3096670	7 759	0 95
71	1-HEXANOL	71	TOT	1094	8 12	49	1 762	A VB	322736	0 809	0 10
72	NOT IDENTIFIED	72	TOT	1131	8 29	49	1 821	A VB	111783	0 280	0 03
73	CYCLOHEXANE, PROPYL-	73	TOT	1131	8 29	49	1 821	A VB	116523	0 292	0 04
74	1-UNDECENE	74	TOT	1148	8 37	49	1 849	A BV	329682	0 826	0 10
75	4-OCTANONE	75	TOT	1225	9 11	49	1 973	A BV	22757	0 057	0 01
76	OXETANE, 3-(1-METHYLETHYL)-	76	TOT	1232	9 14	49	1 984	A VB	110583	0 277	0 03
77	3-OCTANONE	77	TOT	1263	9 28	49	2 034	A BV	220457	0 552	0 07
78	2-OCTANONE	78	TOT	1273	9 33	49	2 050	A VB	171048	0 429	0 05
79	3-HEXEN-1-OL	79	TOT	1287	9 39	49	2 072	A BB	219065	0 549	0 07
80	UNKNOWN	80	NOT	FOUND							
81	OCTANAL	81	TOT	1305	9 47	49	2 101	A BV	222064	0 556	0 07
82	HEXANOICACID, ETHYLESTER	82	TOT	1315	9 52	49	2 118	A VB	91858	0 230	0 03
83	NOT IDENTIFIED	83	TOT	1315	9 52	49	2 118	A VB	96985	0 243	0 03
84	PENTANE, 3-ETHYL-2, 3-DIMETHYL-	84	TOT	1350	10 07	49	2 174	A BB	12017800	30 112	3 70
85	NOT IDENTIFIED	85	TOT	1350	10 07	49	2 174	A BB	12517200	31 365	3 85
86	DECANE	86	TOT	1363	10 13	49	2 195	A BB	275377	0 690	0 08
87	NOT IDENTIFIED	87	TOT	1363	10 13	49	2 195	A BB	268716	0 673	0 08
88	HEPTANOICACID, METHYLESTER	88	TOT	1382	10 22	49	2 225	A BB	45226	0 113	0 01
89	3-OCTEN-2-ONE	89	TOT	1399	10 30	49	2 253	A BV	117705	0 295	0 04
90	HEPTANE, 2, 2, 4, 6, 6-PENTAMETHYL-	90	TOT	1441	10 48	49	2 320	A BB	1026510	2 572	0 32
91	NOT IDENTIFIED	91	TOT	1441	10 48	49	2 320	A BB	1052950	2 639	0 32
92	UNKNOWN	92	NOT	FOUND							
93	DECANE, 2, 5, 9-TRIMETHYL-	93	TOT	1465	10 59	49	2 359	A BV	346520	0 868	0 11
94	UNDECANE, 2, 5-DIMETHYL-	94	TOT	1499	11 15	49	2 414	A VV	212699	0 533	0 07
95	HEXANE, 2, 2, 4-TRIMETHYL-	95	TOT	1512	11 20	49	2 435	A VV	102403	0 257	0 03
96	DECANE, 3, 3, 4-TRIMETHYL-	96	TOT	1518	11 23	49	2 444	A VB	94810	0 238	0 03
97	NOT IDENTIFIED	97	TOT	1550	11 37	49	2 496	A BB	53386	0 134	0 02
98	NOT IDENTIFIED	98	TOT	1568	11 46	49	2 525	A BV	306491	0 768	0 09
99	NOT IDENTIFIED	99	TOT	1568	11 46	49	2 525	A BV	319212	0 800	0 10
100	NONANAL	100	TOT	1580	11 51	49	2 544	A VB	73072	0 183	0 02
101	UNDECANE	101	TOT	1626	12 12	49	2 618	A VV	274038	0 687	0 08
102	BENZENE, 1, 3, 5-TRICHLORO-	102	TOT	1745	13 05	49	2 810	A BV	6175	0 015	0 00
103	5-DECANONE	103	TOT	1753	13 09	49	2 823	A BV	17645	0 044	0 01
104	5-DECANONE	104	TOT	1759	13 12	49	2 833	A VV	7479	0 019	0 00
105	NAPHTHALENE	105	TOT	1759	13 12	49	2 833	A VB	9427	0 024	0 00
106	NOT IDENTIFIED	106	TOT	1783	13 22	49	2 871	A BV	9181	0 023	0 00
107	NOT IDENTIFIED	107	TOT	1792	13 26	49	2 886	A VV	4611	0 012	0 00
108	NOT IDENTIFIED	108	TOT	1801	13 30	49	2 900	A BB	122152	0 306	0 04
109	NOT IDENTIFIED	109	TOT	1821	13 39	49	2 932	A BV	11993	0 030	0 00
110	NOT IDENTIFIED	110	TOT	1826	13 42	49	2 940	A VB	8677	0 022	0 00
111	DODECANE	111	TOT	1863	13 58	49	3 000	A BB	57192	0 143	0 02
112	OCTANOICACID, 3-METHYLBUTYLESTE	112	TOT	2017	15 08	49	3 248	A VB	11143	0 028	0 00
113	TRIDECANE	113	TOT	2081	15 36	49	3 351	A BB	21105	0 053	0 01
114	HYDRAZINE, TETRAPHENYL-	114	TOT	2601	19 30	49	4 188	A BV	157647	0 395	0 05