A CONTRIBUTION TO THE BIOCHEMISTRY OF <u>ERWINIA CHRYSANTHEMI</u>.

SR [by]

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CHAPTER 1

INTRODUCTION

Phytopathogenic bacteria constitute an important research topic, since these organisms cause enormous crop losses through disease. Past research has largely been "phenomenological" i.e. the disease was studied from a aetiological point of view, with disease symptoms and the pathogen being described and identified, but with little attempt being made to determine the underlying mechanisms involved. In the past decade, this approach has changed, and there is greater emphasis on the biochemical, physiological, and genetic aspects of pathogenesis.

Pathogenesis involves a complex series of interactions between host and pathogen. Not only must the pathogen gain entry into the host, it must, in addition, establish itself by evading the host's defence system and by drawing nourishment from the host's tissues. Because of its complexity, pathogenesis is imperfectly understood and at present, biochemical studies can only cast indirect light on the subject.

In this thesis, some selected characteristics of the soft rot bacterium, Erwinia chrysanthemi pv. zeae are presented. These include an investigation into the lactose metabolism of a mutant isolated by the author as well as a structural study of an extracellular polysaccharide produced by the organism. In addition, the tissue degrading pectolytic enzyme, pectate lyase, elaborated by E. chrysanthemi has received attention.

The precise bearing of these studies on the pathogenicity of E. chrysanthemi must remain a matter for speculation. They do, however, contribute to a broader understanding of the physiology and biochemistry of this organism, which should, ultimately, contribute to an understanding of its pathogenicity.

1.1.   Classification of the genus Erwinia.

The genus Erwinia is a member of the Enterobacteriaceae (Dye, 1968, 1969a,b,c; Cowan, 1974; Starr and Chatterjee, 1972, Chatterjee and Starr, 1980; Starr, 1981), and share many traits in common with Escherichia coli. They are all short Gram-negative rods, and are motile by peritrichous flagella. The Erwinia exist in nature as saprophytes, epiphytes, and pathogens (Cowan, 1974). Members of this genus have also been isolated from man and other animals (Starr and Chatterjee, 1972, Cowan, 1974, Lelliott, 1974, Starr, 1981).

The genus has been divided into four groupings (Dye, 1968, 1969a,b,c; Lelliott, 1974; Starr, 1981) as follows:

i.   "Carotovora group" (Dye, 1969a; Lelliott, 1974; Starr, 1981). This has also been called the "soft-rot group", and the "pectolytic group". Waldee (1945) referred to this group as the genus Pecto- bacterium. Individual species all characteristically produce large amounts of pectolytic enzymes. Erwinia carotovora and Erwinia

chrysanthemi, the dominant members of this group, have been isolated from a wide range of plants, including potato, rice, Begonia spp., Chrysanthemum spp., Dianthus spp., Philodendron spp., Dieffenbachia spp., and many others (Dye, 1969a; Starr and Chatterjee, 1972; Lelliott, 1974; Dickey, 1979; Goto, 1979; Chatterjee and Starr, 1980; Thomson et al., 1981).

ii. "Herbicola group" (Dye, 1969b; Lelliott, 1974; Starr, 1981). This group are also known as the "yellow-pigmented erwinia group", and as the "herbicola-lathyri group" (Dye, 1969b; Lelliott, 1974; Starr, 1981). They exist largely in nature as non-pathogenic saprophytes and epiphytes. Some members of the group have, however, been isolated from human and animal tissue, and named Enterobacter agglomerans by the clinical microbiologists (Starr and Chatterjee, 1972; Starr, 1981).

iii. "Amylovora group" (Dye, 1968; Lelliott, 1974; Starr, 1981). Members of the amylovora group are also called the "white non-pectolytic erwinias", or the "true erwinias, or Erwinia sensu strictu" (Dye, 1968; Lelliott, 1974; Starr, 1981). Many of these strains are phytopathogens, but do not produce either pectolytic enzymes or yellow pigments. They do however, cause a dry necrotic rot or wilt symptom on plants.

iv. "Atypical Erwinias" (Dye, 1969c; Starr, 1981). This is a miscellany of strains that fit into neither of the groups above.

1.2.   Pectic enzymes and their substrates.

Pectic substances occur in nature mainly in the middle lamella of plants
and as components of the primary cell wall where they consist of chains
of α-1,4-linked galacturonic acid (GalA) residues interspersed with
α-1,2-linked rhamnosyl (Rha) units (McClendon, 1964; Bateman and Basham,
1976; Aspinall, 1980; Cooper, 1983; Fogarty and Kelly, 1983; McNeil et
al.; 1984). It is becoming apparent from the work by Albersheim and his
colleagues that the structure of these galacturonans are very complex and
that a number of different rhamnogalacturonans with distinct structures
occur as components of the primary cell wall (McNeil et al., 1984). The
carboxyl groups of the galacturonic acid may be either methylated, or
cross-linked to other carboxyl groups via Ca^{2+} bridges (Cooper, 1983;
Fogarty and Kelly, 1983).

Pectins characteristically have most of their free carboxyl groups
methylated, whereas pectic acids (polygalacturonic acids, PGA) contain
only a few methylated carboxyl groups (Chesson, 1980; Cooper, 1983;
Fogarty and Kelly, 1983). The pectic enzymes are classified according to
the type of reaction they carry out (Chesson, 1980; Collmer and Berman et
al., 1982; Cooper, 1983; Fogarty and Kelly, 1983). Pectin esterase (PE,
EC 3.1.1.11), also called pectin methyl esterase (PME), removes methyl
groups from the carboxyl groups of pectin to form methanol and a free
carboxyl group (Chesson, 1980; Collmer and Berman et al., 1982; Fogarty
and Kelly, 1983). Pectic enzymes have been found to cleave the galact-

uronan backbone in two ways. The first is a hydrolytic cleavage in which the polymer is cleaved by the introduction of a molecule of water, and are called galacturonases (Chesson, 1980; Collmer and Berman et al., 1982; Cooper, 1983; Fogarty and Kelly, 1983). The second type of cleavage involves a β-elimination, resulting in an unsaturated bond between C4 and C5 of the GalA at the non-reducing end of the polymer, and are called lyases or trans-eliminases (Chesson, 1980; Collmer and Berman et al., 1982; Cooper, 1983; Fogarty and Kelly, 1983). The nature of the polymer also determines the classification of the enzyme. Those acting on pectin are called pectin methylgalacturonase (PMG), and pectin methyl lyase (PML), while those more active on polygalacturonic acid are called polygalacturonase (PG), and polygalacturonate lyase or pectate lyase (PL) (Chesson, 1980; Collmer and Berman et al., 1982; Cooper, 1983; Fogarty and Ward, 1983). Finally, each enzyme can be classified as to whether it is a randomly acting enzyme (endoenzyme), or whether it acts non-randomly from the non-reducing end of the polymer (exo-enzyme) (Chesson, 1980; Collmer and Berman et al., 1982; Cooper, 1983; Fogarty and Ward, 1983). In the soft-rot bacteria, the major end products of breakdown are either saturated or unsaturated oligouronides. These compounds are transported into the cell where they are further degraded by intracellular oliguro-nide lyase (OGL) (Moran et al., 1968; Hatanaka and Ozawa, 1970; Collmer and Bateman, 1981a; Collmer and Berman et al., 1982).

The polygalacturonases and the lyases differ from one another in a number of ways. The enzymes differ in their pH optima, about 5,5 for PG and 8,5 for PL (Chesson, 1980; Collmer and Berman et al., 1982; Cooper, 1983;

Fogarty and Ward, 1983). Only the lyases require divalent metal ions, mainly Ca^{2+}, for activity, and are completely inhibited by the presence of a chelating agent such as EDTA (Moran et al., 1968b; Garibaldi and Bateman, 1971; Chesson, 1980; Collmer and Berman et al., 1982; Cooper, 1983; Fogarty and Ward, 1983). This latter property allows the assay of PG in the presence of PL by including EDTA in the assay mixture. Finally, the nature of the products of the two enzymes are different; saturated oligouronides are produced by the PG and PMG enzymes, whereas unsaturated oligouronides are produced by the PL and PML enzymes. The unsaturated bond between C4 and C5 of the oligouronide generated by PL and PML, in conjunction with the carbonyl of the carboxyl group, displays a strong absorption at 230 nm for pectic acids, and 235 nm for the pectin derivatives (Albersheim et al., 1960; Starr and Moran, 1962; Rexova-Bankova and Markovic, 1976; Collmer and Berman et al., 1982; Fogarty and Kelly, 1983). Furthermore, after treatment with acid periodate and thiobarbituric acid, the unsaturated products generate a chromophore with a maximum absorption at 548 nm (Weissbach and Hurwitz, 1959; Starr and Moran, 1962; Rexova-Benkova and Markovic, 1976; Collmer and Berman et al., 1982). Neither GalA nor the saturated oligouronides give rise to this chromophore under the same conditions.

There is some confusion as to the terminology of the different pectic enzymes (cf. nomenclature in Collmer and Berman et al., 1982, and Fogarty and Ward, 1983). The nomenclature proposed by Collmer and Berman et al. (1982) will be used in the rest of this thesis. A list of the different enzymes, their Enzyme Commission (EC) numbers, and their synonyms are

given in Table 1. A diagrammatic representation of the mode of action of representatives of each group are of enzyme is presented in Fig. 1.

Two major roles can be assigned to the pectic enzymes produced by pathogens. Firstly, the enzymes may play an essential role in the pathogenicity of the organism by causing tissue maceration and cell death (Stephens and Woods, 1975; Bateman and Basham, 1976; Collmer and Berman et al, 1982; Cooper, 1983). Secondly, the pectic enzymes may also play a catabolic role, enabling the microorganism to grow by degrading the pectic substances of the plant (Bateman and Basham, 1976; Collmer and Berman et al, 1982; Cooper, 1983). The pectic enzymes have also been shown to stimulate the plant defenses, possibly by producing oligosaccharides from cell walls that invoke the hypersensitive response (HR) (Gardner and Kado, 1976; Azad and Kado, 1984; Davis et al., 1984).

Pectic lyase is the predominant enzyme secreted by E. carotovora and E. chrysanthemi (Basham and Bateman, 1975a,b; Mount et al., 1970; Stephens and Wood, 1975). Both strains also produce PG (Chatterjee and Starr, 1977; Garibaldi and Bateman, 1970). The role of the PG in pathogenicity has not yet been elucidated, although an important role in the induction of PL has been assigned to it (Collmer and Bateman, 1982; Collmer and Berman et al., 1982). E. carotovora and E. chrysanthemi produce at least two different PL isoenzymes (Garibaldi and Bateman, 1970; Mazzuchi et al., 1974; Pupillo et al., 1976). It is not clear if the two PL isoenzymes are coded for by the same gene, although evidence suggests that

Table 1. Pectic enzymes and their substrates.

Substrate	Recommended Name	Abbreviation	EC no.	Synonym
		Extracellular		
PGA	Pectate lyase	PL	4.2.2.2	PATE PGTE PAL
PGA	Polygalacturonase	PG	3.2.1.15	
PGA	Exo-poly-α-D-galacturonosidase	exoPG	3.2.1.82	
		Intracellular		
$(GalA)_n$	Exopolygalact-uronate lyase	exoPL	4.2.2.9	
$u(GalA)_2$ or $(GalA)_2$	Oligogalacturonide lyase	OGL	4.2.2.6	OGTE

Abbreviations:
PGA   – polygalacturonic acid
GalA   – galacturonic acid
$(GalA)_2$ – saturated digalacturonic acid
$u(GalA)_2$ – unsaturated digalacturonic acid
PATE   – polygalacturonic acid trans-eliminase
PGTE   – polygalacturonic acid trans-eliminase
PAL   – pectic acid lyase

Adapted from Collmer and Berman et al. (1982).
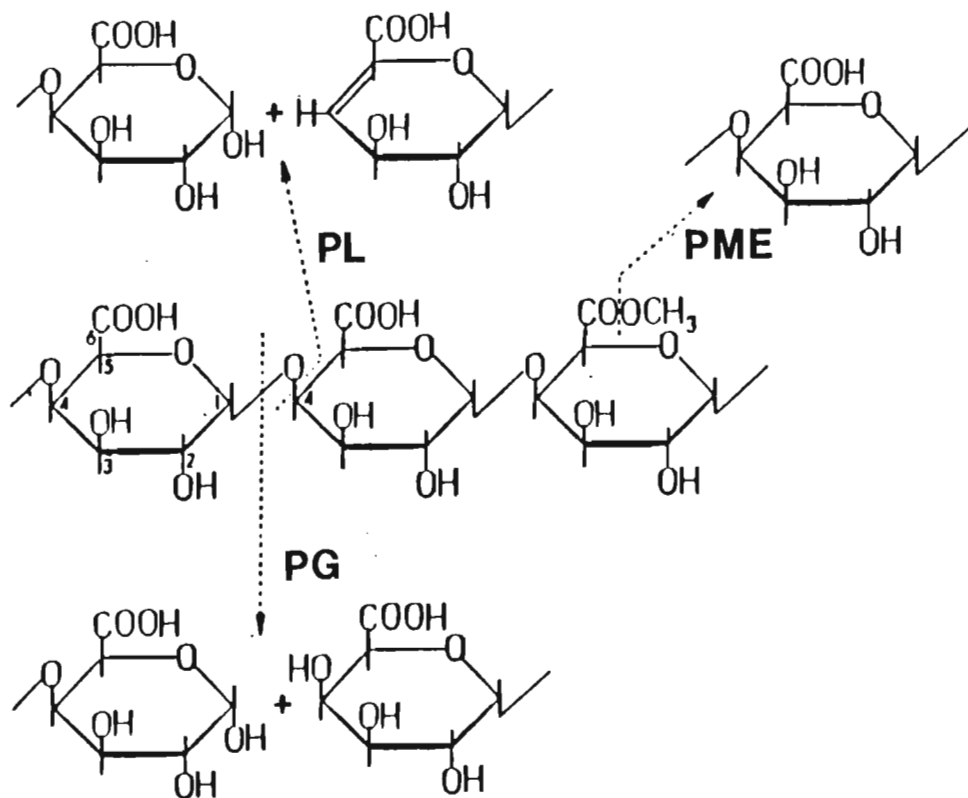
Fig. 1. Mode of action of pectate lyase (PL), polygalacturonase (PG), and pectin methylesterase (PME) on an α-1,4-linked polygalacturonide chain (after Cooper, 1983).

at least two of the PL's in E. chrysanthemi are antigenically distinct, and furthermore, that they differ antigenically from the analogous PL'S in E. carotovora (Mazzuchi et al., 1974). These data argue that these enzymes are coded for by different genes. More recently, using isoelectrofocusing in ultra-thin polyacrylamide gels and detecting the PL isozymes by a sensitive zymogram procedure, Bertheau et al. (1984) and Collmer et al. (1985) have detected 12 isozymes. However, it is not known if these isozymes are coded for by separate genes, or if they are generated as artifacts by post-translational modification.

The pectate lyase enzymes in E. carotovora and E. chrysanthemi have been directly implicated in tissue maceration and cell death (Mount et al., 1970; Garibaldi and Bateman, 1971; Stephens and Wood, 1975; Bateman and Basham, 1976). Pectate lyase negative mutants of E. carotovora neither macerate nor kill plant tissue (Chatterjee and Starr, 1977,1978). Only the endo-PL enzymes appear to be involved in tissue maceration and cell death, due mainly to the fact that relatively few cuts in the interior of a molecule cause major structural changes and properties of the molecule (Garibaldi and Bateman, 1971; Bateman and Basham, 1976; Chesson, 1980; Collmer and Berman et al., 1982). A further factor is that, unlike exo-PL, they do not require a free terminal group for activity, few of which are present in the cell wall. Low concentrations of PL (0,002 U) have been found to cause ultrastructural changes in the cultured red kidney bean cells, although much higher concentrations were required to cause similar damage to rice cells (Baker et al., 1980).

The involvement of endo-PL in tissue maceration and cell death has been shown to involve two separable phases. Tribe (1955), Basham and Bateman (1975), and Stephens and Wood (1975) have all found that plant tissue was macerated by pectic enzymes equally well in both hyper- and hypotonic solution. However, cell death ocurred much more rapidly in hypotonic solution, suggesting that bursting of the osmotically fragile protoplasts may play a major role in cell death (Basham and Bateman, 1975; Stephens and Wood, 1975). Stephens and Wood (1975) have also presented evidence implicating other toxic factors in cell death.

The PL isozymes in both $\underline{E}$. $\underline{carotovora}$ and $\underline{E}$. $\underline{chrysanthemi}$ are inducible by PGA or its degradation products (Tsuyuma, 1977, 1979; Chatterjee $\underline{et}$ $\underline{al}$., 1979; Collmer and Bateman, 1979, 1981, 1982; Ferguson and Chatterjee, 1981, Collmer and Berman $\underline{et}$ $\underline{al}$., 1982; Collmer and Whalen $\underline{et}$ $\underline{al}$., 1982; Cooper, 1983; Chatterjee $\underline{et}$ $\underline{al}$., 1985). The high molecular mass of the PGA molecule precludes its transport into the cell (Collmer and Berman $\underline{et}$ $\underline{al}$., 1982). Both $\underline{E}$. $\underline{carotovora}$ and $\underline{E}$. $\underline{chrysanthemi}$ produce relatively high basal levels of the pectic enzymes which after release into the medium, generate saturated and unsaturated oligouronides (see review by Collmer and Berman $\underline{et}$ $\underline{al}$., 1982). These oligouronides, after transport into the cell, have been postulated to be the inducers of both PG and PL (Tsuyuma, 1978, 1979; Stack $\underline{et}$ $\underline{al}$., 1980). The work by Collmer and Bateman (1982), Collmer and Whalen $\underline{et}$ $\underline{al}$. (1982), and Chatterjee $\underline{et}$ $\underline{al}$. (1981, 1985) however, provides convincing evidence that neither the saturated nor the unsaturated oligouronides are the direct inducers of

PL, but that the inducer is one of the deoxyketuronic acids generated during further metabolism of these oligouronides.

Both PG and PL have been found to be catabolite repressible by a number of sugars, including glucose, sucrose, and fructose (Hubbard et al., 1978; Mount et al., 1979). Furthermore, the catabolite repression brought about by the sugars can be reversed by cyclic 3',5'-adenosine monophosphate (cAMP) (Hubbard et al., 1978; Mount et al., 1979). Pectate lyase synthesis in E. carotovora and E. chrysanthemi is also subject to "self catabolite repression" by saturated digalacturonic and unsaturated digalacturonic acid, the end products of PL and PG digestion of PGA (Tsuyuma, 1977; 1979; Chatterjee et al., 1981, 1985; Collmer and Bateman, 1981, 1982). The nature of the repressor is not known, although Chatterjee et al. (1985) have found that strains of E. chrysanthemi deficient in 2-keto-3-deoxy-gluconate oxidoreductase (ketodeoxyuronate dehydrogenase) produce substantially lower levels of both PL and OGL. They speculated that the intracellular pools of ketodeoxyuronides generated during the catabolism of saturated and unsaturated oligogalacturonides may either rise to unphysiological levels, and therefore inhibit enzyme production, or that these compounds may inhibit PL synthesis by some type of feedback mechanism.

Recently, Keen et al. (1984) and Collmer et al. (1985) cloned the PL genes from E. chrysanthemi into E. coli. Both studies showed that the genes were expressed normally in E. coli, although the enzymes were not secreted into the medium as found for E. chrysanthemi, but were located

in the periplasm. Some of the strains isolated by Keen et al. (1984) and

Collmer et al. (1985) did release about 50% of the PL into the medium,

but the latter workers believed that this was due to a leakiness of the

periplasm, as β-lactamase, a periplasmic protein, was also released under

the same conditions.

This system offers an exciting tool to study the release of extracellular

enzymes from Gram-negative bacteria.

1.3.   Lactose, melibiose, and raffinose metabolism.

The lac operon in Escherichia coli codes for the enzymes involved in

lactose (4-0-β-D-galactopyranosyl-D-glucose) metabolism. The lacZ gene

codes for β-galactosidase, the lacY gene for lactose permease, and the

lacA gene for thiogalactoside transacetylase (Beckwith, 1980; Zabin and

Fowler, 1980). The first two proteins are directly involved in lactose

metabolism; however, the physiological role of thiogalactoside trans-

acetylase is not known, although it has been implicated in the detoxi-

fication and elimination from the cell of non-metabolizable β-galactoside

analogues (Andrews and Lin, 1976). The lac operon is situated on the

chromosome, and maps at about 8 min in a 100 min map (Bachmann, 1983). The

operon is also frequently found associated with various plasmids

(Beckwith, 1980).

Klebsiella aerogenes possesses both a plasmid-borne and a chromosomal lac

operon (Reeve and Braithwaite, 1970; Brenchley and Magasanik, 1972; Reeve

and Braithwaite, 1973; Reeve, 1976). The plasmid-borne, but not the

chromosomal lac operon, is very similar to the E. coli lac operon, and it has been suggested that the Klebsiella plasmid-borne lac operon originated from E. coli (Reeve, 1973; Reeve and Braithwaite, 1972, 1973, 1974). Salmonella typhimurium does not have a chromosomal lac operon (Sanderson and Roth, 1983), although some strains do metabolize lactose via a plasmid borne lac operon (Brenner, 1981).

The observation that E. coli mutants containing a deleted lacZ gene could grow on lactose (Warren, 1972; Campbell et al., 1973) lead to the discovery of a second β-galactosidase (Campbell et al., 1973; Hall and Hartl, 1974, 1975). This enzyme, coded for by the ebgA (evolved β-galactosidase) gene, is more active on aryl-β-D-galactosides than on lactose (Campbell et al., 1973; Hartl and Hall, 1974; Hall, 1976). The ebgA gene, and its adjacent regulatory gene (ebgR), maps at about 68 min on a 100 min map (Bachmann, 1983), is regulated independently of the lac operon (Hall and Hartl, 1974, 1975; Hall and Clarke, 1977). Mutations in both the ebgA gene, and in the ebgR gene are required for growth on lactose (Hartl and Hall, 1974, 1975; Hall and Clarke, 1977; Hall, 1978).

Two types of mutation in the ebg operon are required for lacZ deletion strains to grow on lactose. Firstly, the ebgR gene, which codes for the ebg repressor (Hall and Hartl, 1975), must mutate to either allow lactose to be a more efficient inducer of the operon, or it must mutate to allow constitutive expression of the ebg operon (Hartl and Hall, 1974, 1975; Hall and Clarke, 1977). Secondly, the ebgA gene must mutate to allow higher specificity and activity on lactose in order for lactose to be

hydrolyzed sufficiently rapidly for growth (Hartl and Hall, 1974; Hall, 1976; Hall and Clarke, 1977).

The mel operon of E. coli, which maps at about 93 min on a 100 min map (Bachmann, 1983), contains the A and B genes which code for α-galactosidase and melibiose permease respectively (Schmitt, 1968). Both K. aerogenes and S. typhimurium possess mel operons similar to the E. coli operon (Levinthal, 1971; Reeve and Braithwaite, 1973).

Raffinose is not normally a substrate of E. coli, although certain pathogenic strains of this organism bear a plasmid encoded raf operon (Ørskov and Ørskov, 1973; Schmitt et al., 1973; Schmid and Schmitt, 1976). The operon codes for three structural genes: rafA codes for an α-galactosidase, rafB codes for a raffinose permease, and rafD codes for an invertase (Schmid and Schmitt, 1976). A regulatory gene, rafC is associated with the structural genes (Schmid and Schmitt, 1976). Little is known about raffinose metabolism in other members of the Enterobacteriaceae.

The substrate specificities of the lac and mel permeases differ among the various members of the Enterobacteriaceae. The lac permease of E. coli has wide specificity, and transports a variety of α- and β-galactosides, including lactose, IPTG, TMG, melibiose, and galactose (Rotman et al., 1968). The mel permeases of E. coli and S. typhimurium are similar in specificity, and can transport TMG, melibiose, and galactose, but not lactose or IPTG (Prestidge and Pardee, 1965; Rotman et al., 1968;

Schmitt, 1968; Levinthal, 1971), The mel permease of K. aerogenes differs from the E. coli and S. typhimurium mel permeases in being able to transport lactose in addition to TMG, melibiose, and galactose (Reeve and Braithwaite, 1973; Wilson et al., 1982). Lactose transport by the E. coli raf permease is competitively inhibited by melibiose and raffinose (Schmid and Schmitt, 1976).

The whole question of α- and β-galactoside transport in E. coli is complicated by the multiple permeases involved, many of which exhibit multiple overlapping substrate specificities (Rotman et al., 1968; Wilson et al., 1982; Wilson and Wilson, 1983).

The fermentation of lactose is a widely used taxonomic marker in the classification of the Enterobacteriaceae (see Cowan, 1974), and has been used as one of the characters scored in the classification of the Erwinia (Lelliott, 1974). Dickey (1979) found that all 322 strains of E. chrysanthemi that he studied possessed a β-galactosidase active on β-ONPG despite the fact that most of the strains were classified as Lac⁻. Other workers also classified lactose fermentation by E. chrysanthemi as being either variable or delayed (Lelliot, 1974; Goto, 1979).

There have been few reports on lactose metabolism in E. chrysanthemi. Lac⁻ strains of E. chrysanthemi have been used as acceptors of a F'lac episome derived from E. coli, although no mention was made of any other aspects of lactose metabolism by this organism (Chatterjee and Starr, 1977). Work in our laboratory on a locally isolated strain of E.

chrysanthemi demonstrated that it was normally Lac⁻ but that Lac⁺ strains could be readily isolated from the wild-type parent (Gray et al., 1984). The β-galactosidase from both the Lac⁻ and the Lac⁺ strains exhibited similar activity on β-ONPG and lactose, indicating that the Lac⁻ phenotype could not be explained by a low activity or specificity of this enzyme for lactose (Gray et al., 1984). An extension of these studies demonstrated that both the Lac⁻ and the Lac⁺ strains of E. chrysanthemi did not possess a lac operon analogous to the E. coli lac operon, and that neither a lactose permease nor a thiogalactoside transacetylase were co-ordinately induced with the β-galactosidase (Gray et al., 1985). It was found that a mutation allowing constitutive synthesis of the mel operon was responsible for the Lac⁺ phenotype of E. chrysanthemi (Gray et al., 1985). During the course of writing this thesis, a report appeared confirming some of the results presented above was published (Hugouvieux-Cotte-Pattat and Robert-Baudouy, 1985).

1.4. Bacterial cell surfaces, extracellular polysaccharides, and pathogenicity.

The demonstrable role of extracellular polysaccharides (EPS) in pathogenesis prompted the author to determine the structure of the EPS elaborated by E. chrysanthemi.

Many Gram-negative bacteria produce EPS, either tightly bound to the cell in the form of a bacterial capsule, or loosely associated in the form of a slime (Sutherland, 1977; Troy, 1979). A wide variety of sugars are

present in EPS, including hexoses, methylpentoses, uronic acids, and amino sugars. Pentoses are not as common in EPS as they are in lipopolysaccharides (LPS). Non-carbohydrate molecules such as formate, acetate, pyruvate, succinate, phosphate, and sulphate may also form part of the EPS molecule (Sutherland, 1977; Troy, 1979).

Extracellular polysaccharides can be classified as either homopolysaccharides or as heteropolysaccharides (Sutherland, 1977). Homopolysaccharides, such as cellulose, dextran, and levan, consist of a single carbohydrate moiety, whereas heteropolysaccharides are composed of a repeating unit composed of a number of different sugars (Sutherland, 1977; Troy, 1979). Many of the heteropolysaccharides are acidic due to the presence of an uronic acid (Sutherland, 1977; Troy, 1979).

Extracellular polysaccharides and LPS, a component of the outer membrane of Gram-negative bacteria, appear to play a complex interrelated role in pathogenesis. Therefore in order to gain some insight into the role of EPS in pathogenesis, it is first necessary to discuss briefly the cell envelope of Gram-negative bacteria, and in particular, the LPS.

The Gram-negative cell is enclosed by a complex structure composed of a plasma membrane, a peptidoglycan layer, and an outer membrane (Rogers et al., 1980). Imbedded in, and associated with the outer membrane are proteins, phospholipids, lipopolysaccharide (LPS), and polysaccharides. The LPS consists of a hydrophobic part, lipid A, which is part of the

outer membrane, and a hydrophilic polysaccharide which is orientated externally toward the outside of the cell.

Lipid A usually consists of a $\beta(1,6)$-linked disaccharide, normally composed of glucosamine, to which are attached fatty acids (Wilkinson, 1977; Luderitz et al., 1982). The polysaccharide portion of the LPS is composed of two regions; the core region and the outer- or O-chain (Wilkinson, 1977; Luderitz et al., 1982). The core characteristically contains 3-deoxy-D-manno-octulosonic acid (KDO), a heptose, usually L-glycero-D-manno-heptose, and a variety of other hexoses and amino sugars (Wilkinson, 1977; Luderitz et al., 1982). Both the core region and the lipid A region are phosphorylated. The O-chain, the serologically dominant portion of the LPS molecule, is made up of a repeating unit composed of a number of different sugars including hexoses, methyl-pentoses, pentoses, uronic acids, and amino sugars (Wilkinson, 1977; Luderitz et al., 1982). Many rare sugars occur in the O-chain of the LPS.

The exposed position of the LPS on the outer surface of the cell also results in many important biological functions for the molecule. In addition to their involvement as endotoxins and as serological deter-minants (Wilkinson, 1977; Jann and Jann, 1977; Ørskov et al., 1977; Luderitz et al., 1982), they are important physiologically to the bacterial cell, and act as a barrier to the passive diffusion of many compounds of high molecular weight (e.g. some antibiotics and other poisons) into the cell (Luderitz et al., 1982). Their presence is essen-tial for growth of the cell, and cells that do not contain LPS stop

growing and die (Luderitz et al., 1982). Lipopolysaccharides are also central components in many host-symbiont and host-pathogen interactions in both animals and plants (Wilkinson, 1977; Corpe, 1980; Bauer, 1981; Pistole, 1981; Keen and Holliday, 1982; Luderitz et al., 1982; Sequeira, 1982, 1983,1984a,b; Sparling, 1983; Vance, 1983). Finally, many bacterio-phage receptors on the Gram-negative cell surface are also LPS (Lindberg, 1977).

Compatible bacteria multiply and establish themselves in the host, whereas incompatible bacteria are immobilized and eliminated from the host. In plants, this is accomplished by the hypersensitive response (HR), which is characterized by the collapse and desiccation of the infected area, and a decline in bacterial numbers (Keen and Holliday, 1982; Sequeira, 1982, 1983,1984a,b). Some compatible reactions e.g. the establishment of various Rhizobium spp. in the roots of their hosts are beneficial to the plant (Bauer, 1981; Vance, 1983), whereas others e.g. the infection of dicotyledons by Agrobacterium tumefaciens, or the infection of potatoes by Pseudomonas solanacearum are damaging (Keen and Holliday, 1982; 1982; Sequeira, 1982, 1983,1984a,b). Extracellular polysaccharides appear to play a major role in determining host speci-ficity in Rhizobium-host interactions (Bauer, 1981; Vance, 1983), whereas LPS appears to be important in Agrobacterium tumefaciens-host specificity (Keen and Holliday, 1982; Sequeira, 1982, 1983,1984a,b).

One of the best documented examples of the involvement of polysaccharides in inducing the HR is the interaction of Pseudomonas solanacearum with

tobacco or potato cells. However, the role of EPS and LPS in the patho-
genicity of P. solanacearum is not clear although Kelman (1954) demon-
strated that EPS is essential for pathogenicity.

Sequeira et al. (1977) found that incompatible strains of P. solanacearum
induced a host response within 12-24 h after infusion into tobacco
leaves, whereas there was no detectable response in 48 h or more with the
compatible strain. Electron microscopic examination of the infected area
revealed that the incompatible strains were attached to the tobacco
cells, whereas the compatible strain was free to move and multiply in the
intercellular spaces (Sequeira et al., 1977). A hydroxyproline rich
glycoprotein (HPRG) isolated from potato tubers, strongly agglutinated
the avirulent, but not virulent strain of P. solanacearum (Sequeira and
Graham, 1977). The agglutination could be prevented by addition of EPS to
the assay mixture, and it was concluded that the agglutination reaction
was due to the interaction of LPS and HPRG (Sequeira and Graham, 1977).
This conclusion was supported by the finding that virulent cells, in
which the EPS had been removed by careful washing, were agglutinated as
well (Sequeira and Graham, 1977). It thus appeared as if the EPS played
a protective role either by masking the LPS binding sites on the bacter-
ial cell surface, or by neutralizing HPRG by binding to it. Differences
in the binding of virulent and avirulent P. solanacearum cells to
suspension-cultured tobacco cells, and tobacco leaf cell walls in vitro,
have been found, with the virulent K60 cells binding less strongly than
the avirulent B1 cells to both the tobacco cells and to the cell walls
(Duvick and Sequeira, 1984a). The binding appeared to be non-specific,

and the authors concluded that ionic interactions between the cell wall components and the bacterial LPS were possibly the most important factors in binding (Duvick and Sequeira, 1984a). The role of the EPS in planta has also been questioned, as recent work has shown that the EPS only binds to HPRG at low ionic strengths, whereas LPS binds to HPRG under both low and high ionic strengths (Duvick and Sequeira, 1984b). As there is evidence that the ionic strength of the intercellular fluid may be as high as 0,1 M, the importance of the EPS-HPRG interaction is not clear (Duvick and Sequeira, 1984b). The role of the EPS may not be involved in determining specificity and binding, but may rather be involved in ensuring an environment conducive to multiplication of the pathogen e.g. by inducing water-soaking of the infected tissue (Sequeira, 1984).

Some avirulent mutants of P. solanacearum induce the HR, while others do not. Whatley et al. (1980) isolated a number of avirulent mutants from virulent P. solanacearum K60, and the ability of the avirulent strains to induce a HR was found to correlate with the structure of the LPS (Whatley et al., 1980). The lipopolysaccharides isolated from the non-HR-inducing strains were similar to the LPS from the virulent parent strain in their slower migration on SDS-gel electrophoresis, whereas the LPS from the HR-inducing strains migrated more rapidly (Whatley et al., 1980). The interpretation was that the LPS from the non-HR-inducing strain contained a complete O-antigen, whereas the LPS from the HR-inducing strain did not (Whatley et al., 1980). Chemical analyses of the two types of LPS confirmed the interpretation as did the similar susceptibility of the parent K60 strain, and the avirulent smooth LPS strains to lysis by

bacteriophage CH154 (Whatley et al., 1980). Whatley et al. (1980) concluded that rough LPS may be necessary for initial recognition of the bacterium by the host and that the O-antigen was involved in masking some portion of the LPS responsible for this recognition.

More recently, Hendrick and Sequeira (1984) isolated a large number of mutants from P. solanacearum resistant to lysis by bacteriophages Psso 154 and Psso NCL (equivalent to CH 154 and NC-L in previous studies). All of the mutants isolated were avirulent and non-HR-inducing. Furthermore, most were found to have incomplete LPS by SDS-gel electrophoresis and chemical analysis (Hendrick and Sequeira, 1984). All of the isolates produced EPS, although in reduced amounts (Hendrick and Sequeira, 1984). The presence of EPS did not appear to be responsible for the non-HR-inducing character of the mutants, as EPS$^-$ mutants of the rough LPS strains were still non-HR-inducing (Hendrick and Sequeira, 1984). Thus, the non-HRinducing character of the rough-LPS EPS$^+$ mutants is not due to the masking of the LPS by EPS, and Hendrick and Sequeira (1984) concluded that neither the loss of the O-antigen nor the loss of EPS was sufficient to convert P. solanacearum K60 into a HR-inducing strain. However, EPS is important in pathogenicity, as two of the non-HR-inducing rough LPS mutants that produced sufficient EPS were also pathogenic to tobacco seedlings (Hendrick and Sequeira, 1984).

The problem is complex, and although EPS and LPS are both involved as pathogenic determinants in P. solanacearum, their contribution and function in pathogenicity is not known. Although it was first thought

that a specific interaction of EPS and LPS with components of the cell wall (e.g. HPRG) occurred, this no longer appears to be the case. A knowledge of the structures of the EPS and LPS from different mutants, and their relationship may provide some clue as to their involvement in pathogenicity. Currently, little is known about the structures of the EPS and LPS from P. solanacearum, and only a preliminary report on the structure of the O-antigen from the LPS from P. solanacearum strain K60 (virulent non-HR-inducing) has appeared (Baker et al., 1984).

Virulent strains of Erwinia amylovora, the causal agent of fire blight in rosaceous plants, all produce an EPS called amylovorin, whereas avirulent strains do not (Eden-Green, 1974; Goodman et al., 1974; Huang et al., 1975; Ritchie and Klos, 1977; Ayers et al., 1979). The EPS was originally thought to be host specific (Goodman et al., 1974), but Beer et al. (1983) dispute this. Cut apple shoots placed in dilute solutions of the EPS show wilt symptoms within a few hours (Eden-Green, 1974; Goodman et al., 1974), and this is thought to be due to the occlusion of the vascular system of the plant by the EPS (Suhayda and Goodman, 1981). Observations by Huang et al. (1975) and Suhayda and Goodman (1981) suggest that the EPS is also plays an important role in pathogenicity by protecting the virulent cells from the host defenses. Thus, avirulent cells are immobilized by the host, whereas the virulent cells are free to move and multiply in the host (cf. P. solanacearum above). Romeiro et al. (1981a) isolated an agglutinating factor (AF) from apple tissues which agglutinated avirulent but not virulent cells of E. amylovora. The AF did not appear to be a lectin, and the binding of AF to E. amylovora appeared

to be due to ionic forces between the basic AF and the acidic (anionic) surface of the bacterial cells (Romeiro et al., 1981a). Agglutination of the bacterial cells by AF was not inhibited by any specific sugar, although agglutination was inhibited by the presence of polyanionic compounds such as amylovorin, sodium polygalacturonic acid and carboxy-methyl cellulose (Romeiro et al., 1981a). The AF also bound to the core region of LPS extracted from E. amylovora, and the agglutination was inhibited by amylovorin (Romeiro et al., 1981b). The role of the EPS in the virulence of E. amylovora therefore appears to reside in its ability to prevent agglutination by host factors such as AF.

These examples illustrate the importance of EPS and other cell surface carbohydrates in determining compatibility and incompatibility of bacteria in plants. The actual role of the carbohydrates are difficult to evaluate due to a lack of knowledge of the structure of the polysacchar-ides, and the specificity of their receptors. This will provide a rich area for future research.

1.5. Scope of thesis.

The thesis is divided into 3 sections. The first section (Chapter 2) covers studies on PL production and regulation. The isolation of a Lac$^+$ mutant is also described in this chapter. Lactose metabolism by the Lac$^-$ and Lac$^+$ mutants is described in Chapter 3, and the two strains are compared. Finally, Chapter 4 describes the production, purification, and

partial structure of an EPS produced by E. chrysanthemi growing on lactose.

CHAPTER 2

PECTATE LYASE PRODUCTION BY ERWINIA CHRYSANTHEMI.

The essential role of pectate lyase (PL) in the pathogenicity of E. chrysanthemi makes it an important enzyme of study. In this chapter, the production of PL by a locally isolated strain of E. chrysanthemi is investigated, and the effects of different media, and medium components on PL production determined. The PL isozyme spectrum of the organism is also determined, and compared to other strains of E. chrysanthemi reported in the literature. The isolation and characterization of a lactose utilizing mutant is described, and compared to the parent strain in terms of its pathogenicity and general physiological characteristics. Finally, some aspects of the regulation of PL synthesis are investigated, with particular reference to the simultaneous induction of PL and β-galactosidase.

2.1.   MATERIALS AND METHODS.

2.1.1.   Organism.

A strain of E. chrysanthemi (strain FH1) isolated locally by Mildenhall (1973) was used in these studies. It has been deposited, as strain SR 260, in Professor A Kelman's collection of soft-rot bacteria held in the Department of Plant Pathology, University of Wisconsin, Madison, USA.

The organism was maintained on nutrient agar at 20°C, as suspensions in distilled water at room temperature, and as lyophilized cultures.

2.1.2. Media.

The following media were used in the studies reported in this chapter. All media were sterilized by autoclaving at 100 kPa (121°C) for 15 min.

A.   Nutrient Agar (NA) and Nutrient Broth (NB).

Nutrient agar and nutrient broth (Difco Laboratories, Detroit, Michigan) were rehydrated before autoclaving, according to the manufacturer's instructions.

B.   Xanthomonas campestris (XC) medium.

The yeast-extract-salts medium used by Nasuno and Starr (1967) for the screening of PL activity in X. campestris contained:

Yeast Extract (YE, Difco)	4,0 g
$(NH_4)_2SO_4$	0,5 g
KH_2PO_4	1,0 g
$MgSO_4.7H_2O$	0,5 g
$CaCl_2.2H_2O$	0,1 g
Carbon Source	8,0 g
H_2O	1000 ml
pH	7,3

Monosaccharides and disaccharides were filter sterilized (0,45 m HAWP filter, Swinnex Holder, Millipore, Bedford, Massachusetts), and poly-saccharides were autoclaved before being added aseptically to the sterile basal medium. Pectin, when used as a carbon source, was autoclaved for 5 min at 100 kPa (121°C) in order to minimize degradation.

The calcium salts were made up and autoclaved separately, and added aseptically to the sterile basal medium.

A version of this medium containing 1,11 g $CaCl_2.2H_2O$ l^{-1} (7,55 mM) was also used, and called HXC-medium (High calcium XC-medium).

C.   Husain-Kelman medium.

The medium devised by Husain and Kelman (1958) contained:

$(NH_4)_2SO_4$	1,32 g
$MgSO_4.7H_2O$	0,08 g
$ZnSO_4.H_2O$	0,008 g
YE	1,0 g
K_2HPO_4	2,5 g
Casamino acids (Difco)	2,5 g
Carbon Source	8,0 g
H_2O	1000 ml
pH	7,3

The carbon source was made up separately in H_2O, autoclaved, and added aseptically to the basal medium. The volume of H_2O in the basal medium was decreased proportionately so that the final volume was maintained. The $ZnSO_4.7H_2O$ was made up and autoclaved separately as a 100-fold concentrated solution (0,8 g per 100 ml H_2O), and 1 ml added aseptically per 100 ml of dispensed medium.

D.   Garibaldi-Bateman (GB) medium.

The medium devised by Garibaldi and Bateman (1971) contained:

K_2HPO_4	13,2 g
$(NH_4)_2SO_4$	2,0 g
$MgSO_4.7H_2O$	0,2 g
YE	1,0 g
Carbon Source	8,0 g
H_2O	1000 ml
pH	7,5

The carbon source was made up and sterilized separately, and added to the cooled medium as described above.

E.   Yeast-salts (YS) medium.

YS-medium was prepared according to Scott (1953) as modified by Mildenhall et al. (1981), and contained:

$Na_2HPO_4.2H_2O$	1,77 g
KH_2PO_4	0,27 g
NH_4NO_3	0,40 g
H_2O	950 ml

The pH was adjusted to 7,3-7,4, the medium dispensed, and after autoclaving, the following sterile supplements were added per 950 ml medium:

$MgSO_4$	1 ml of a 1 M solution of $MgSO_4$
Yeast extract	10 ml of 9% (w/v) Difco yeast extract
Carbon source	25 ml of a 7,2% (w/v) solution (any modifications are mentioned in the text)
H_2O	to 1 litre

This medium was also solidified by the addition of 15 g agar l^{-1} prior to sterilization.

F.   Eosin-Methylene Blue (EMB) agar.

EMB agar was prepared according to Miller (1972, pp. 47-55), and contained:

Distilled H_2O	930 ml
Bacto tryptone (Difco)	10,0 g
Yeast extract (Difco)	1,0 g
NaCl	5,0 g
Agar (Difco)	15,0 g
KH_2PO_4	2,0 g

The medium was sterilized by autoclaving, and the following autoclaved supplements added aseptically to each batch of medium immediately prior to dispensing into Petri dishes (about 15 ml medium per Petri dish).

Eosin Yellow	10 ml of a 4% (w/v) solution
Methylene Blue	10 ml of a 0,65% (w/v) solution
Sugar	50 ml of a 20% (w/v) solution

The plates were stored in plastic bags to prevent dehydration.

G.   MacConkey agar.

MacConkey agar (Difco) was rehydrated according to the manufacturer's instructions by dissolving 50 g of the dry medium in 1 litre of H_2O. The

medium was dispensed into smaller batches, and autoclaved. The medium was dispensed into Petri dishes (about 15 ml per dish) at least 24 h before use to ensure a dry surface (Difco Manual, Difco Laboratories, 9th ed., 1972), or allowed to stand at room temperature in the laminar flow hood with their lids ajar for a few hours. The plates were stored in plastic bags to prevent dehydration of the medium.

H.   Tetrazolium (TCZ) agar.

TCZ agar was prepared according to Miller (1972, pp. 47-55), and contained:

Nutrient agar (Difco)	23,0 g
NaCl	1,0 g
H_2O	950 ml

The agar was melted by steaming in a bench-top autoclave, and 50 mg of 2,3,5-triphenyltetrazolium chloride (TCZ, Sigma, St. Louis. Missouri) was added. The TCZ must be added to the medium before autoclaving (Miller 1972, pp. 47-55). Heating was continued until the TCZ had completely dissolved, after which the medium was sterilized by autoclaving. The medium was allowed to cool slightly, and 50 ml of a 20% (w/v) sugar solution (either glucose, galactose, or lactose) was added. The medium was poured into Petri dishes (about 15 ml per dish), and stored in plastic bags in the dark.

I.   <u>5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)agar.</u>

X-Gal agar was prepared by adding X-Gal (Sigma, 40 mg in 1 ml dimethyl formamide) to 1 litre of sterile YS-agar prepared as above. The medium, after supplementation with 0,2% (w/v) glycerol, 0,2% (w/v) lactose, singly or in combination, and/or 0,5 mM IPTG, was dispensed into Petri dishes and stored in the dark in plastic bags.

J.   <u>Crystal-Violet-Pectate (CVP) medium.</u>

CVP medium was prepared as described by Schaad (1980). An MSE Atomixer was preheated with hot water, and 500 ml of boiling water was added. The blender was started on low speed with 1-2 sec bursts, and the following added:

> 1,0 ml 0,075 (w/v) crystal violet solution
>
> 4,5 ml 1 M NaOH
>
> 3,0 ml 10% (w/v) freshly prepared $CaCl_2.2H_2O$
>
> 2,0 g Difco agar
>
> 1,0 g $NaNO_3$

The solution was blended at high speed for 15 sec, and 9,0 g of sodium polypectate (NaPP, Ral Tech Scientific Services, Inc., 3301 Kingman Blvd., P.O. Box 7545, Madison, Wisconsin, 53707) was added with blending to ensure that the NaPP did not clump. An aliquot (0,5 ml) of a 10% (w/v)

solution of sodium dodecyl sulphate (SDS, Pierce) was added, and the medium transferred to an aluminium foil capped 2 1 Erlenmeyer flask. The medium was autoclaved at 100 kPa, 121ºC, 25 min, and the pressure allowed to drop slowly to prevent bubble formation. The medium was dispensed into Petri dishes as soon as possible, and allowed to dry for at least 2 days at room temperature before use, as no surface water should be present on the medium for effective use (Schaad, 1980).

2.1.3. Physiological Tests.

The API 20 and API 50 CH Enterobacteriaceae (Apparaeils et Procedes d'Identificion, API Systeme, Montalieu-vercieu, France) were used to check the identity of the various strains of E. chrysanthemi. These kits consist of a series of cupules containing different carbon sources to which a suspension of bacteria is added. An indicator in the cupules is used to determine if the carbon source is being metabolized or not.

Fresh overnight cultures of bacteria grown on NA plates were used as inocula for the API tests, which were performed according to the manufacturer's instructions. All the API 50 tests were carried out anaerobically by covering the cupules with sterile liquid paraffin.

2.1.4. Pathogenicity trials.

The pathogenicity of E. chrysanthemi was checked on maize seedlings

(Hartman and Kelman, 1973), and by potato tissue maceration (Kelman and Dickey, 1980).

A.   Infection of maize plants.

$\underline{E}$. chrysanthemi was grown overnight in NB with shaking at 30°C, sediment-ed by centrifugation (10 000 X g, 4°C, 20 min), and suspended in physio-logical saline (0,9% (w/v) NaCl). The cells were recovered by centri-fugation, and suspended in 0,1% (v/v) Tween-80 (Sigma), and their pathogenicity towards 2 week old seedlings of maize (Zeae mays L., cv. SSM$_{442}$) determined by the method of Hartman and Kelman (1973). The whorls of the plants were filled with bacterial cells, and the plants incubated at 33°C and 99-100% relative humidity. Control plants, inoculated with sterile 0,1% (v/v) Tween-80 were prepared in parallel. After 24 h, the relative humidity was decreased to 65-70%, and the plants checked for wilting and soft rot. The plants were checked again for wilting and soft rot at 48 h. Any plants that wilted and rotted within 48 h were consider-ed to be infected with virulent bacteria.

Pieces of rotted plants were placed on NA plates, where the motile $\underline{E}$. chrysanthemi moved onto the surface during growth. Colonies were purified by repeated streaking on NA.

B.   Maceration of potato slices.

The ability of E. chrysanthemi to macerate potato tissue was checked by
the method described by Kelman and Dickey (1980). Thick slices were cut
from washed, alcohol flamed, peeled potato slices, and placed in sterile
Petri dishes containing 3-4 mm of sterile distilled water. A shallow
ditch was dug into the centre of the potato slice, which was heavily
inoculated with cells from a 24 h NA slant culture. A non-inoculated
slice was included for each tuber used. Rotting was checked for by
drawing a sterile inoculating loop across the point of inoculation to
determine if the rot had spread beyond the point of inoculation. Slight
rotting at the point of inoculation was scored as negative. Virulent
strains of Erwinia spp. caused the tissue to rot and go black within 6 h.

2.1.5. Buffers.

All buffers not explicitly mentioned in the methods and materials section
were made up by dissolving the required amount of the acidic or basic
form of the buffer salt in 90% of the final volume of water, titrating
the buffer to the correct pH with the appropriate acid or base and making
up to volume with water.

2.1.6. Protein Assay.

Protein was assayed by the colorimetric method of Lowry et al. (1951).

A.   Reagents

Reagent A: 5% (w/v) Na_2CO_3 in H_2O.

Reagent B: 0,5% (w/v) $CuSO_4.5H_2O$ in 1% (w/v) sodium citrate
                          solution.

Reagent C: Immediately before use, 50 ml of reagent A was mixed
                          with 20 ml of reagent B.

Reagent D: Folin-Ciocalteau reagents (BDH) was diluted to 1 M in
                          acid (determined by titrating an aliquot of the
                          reagent with 0,1 M NaOH to a phenolphthalein end-
                          point).

B.   Standard

A bovine serum albumin Fraction V (BSA) stock solution was prepared by
dissolving BSA (Sigma, 100 mg) in H_2O (25 ml). Aliquots (1 ml) were
stored frozen at -20^oC.   Appropriate dilutions in H_2O of this stock
solution were prepared as required.

C.   Method.

(i).   Extraction of protein from cells.   Bacterial cells were resus-
            pended in 0,5 M NaOH to a protein concentration of about 4 mg

ml^{-1} (about 8 mg (dry weight) of cells ml^{-1}), and the protein extracted by heating in a boiling water bath for 10 min. The standards were treated in the same way, as it has been found that heating of protein in NaOH solution reduces the intensity of the developed colour. (Lowry et al, 1951).

(ii).   Assay.   Samples (0,05 ml) containing up to 0,2 mg protein were added to 0,5 M NaOH (1,0 ml), and reagent C (2,5 ml) was added with mixing (Whirlymixer, Fisons Scientific Apparatus, Loughborough, UK). After standing for 10 min, reagent D (0.5 ml) was added with rapid mixing (Whirlymixer) and the absorbance of the samples determined 30-120 min later in a Spectronic-88 Spectrophotometer (Bausch and Lomb, Rochester, N.Y., U.S.A.).

   2.1.7. Assay of uronic acid.

Uronic acids were assayed by the carbazole method of Dische (1947) as described by Keleti and Lederer (1974).

A.   Reagents.

(i).   Carbazole (0,1% w/v).   Carbazole (0,1% w/v) was prepared by dissolving 10 mg carbazole (Merck) in 10 ml of 95% ethanol. The reagent was prepared fresh monthly and stored in a dark bottle at $4^{o}C$.

(ii).   <u>Glucuronolactone standard</u>.   A fresh glucuronolactone standard
(1 mg ml^{-1} in H$_2$O, Sigma) was prepared before each experiment.
Suitable dilutions were used to construct a standard curve.

B.   <u>Procedure</u>.

Samples (0,2 ml containing up to 0,01 mg uronic acid) were pipetted into
100 X 13 mm test-tubes, and the tubes were cooled to 4°C in an ice-bath.
Ice cold concentrated sulphuric acid (1,2 ml) was added slowly to each
tube, and after mixing, the tubes were heated in a boiling water bath for
20 min. After cooling to room temperature, carbazole reagent (0,04 ml)
was added with immediate mixing and the absorbances of the tubes were
determined at 535 nm against a reagent blank after standing at room
temperature for 2 h.

It was found that an unknown compound in the growth medium interfered
with the carbazole assay, producing a green colour rather than the pink
colour typical of the carbazole assay. Spectra (400-700 nm) of the
chromophore produced by the interfering compound (i.e. medium in which
the glucuronic acid was depleted), standard glucuronic acid, and a
mixture of the two (i.e. medium in which glucuronic acid was not deple-
ted), were run on a Beckman 35 (Beckman Instruments, Fullerton, Cali-
fornia) scanning spectrophotometer. The interfering compound was found to
have an absorbance maximum at 700 nm, a wavelength at which the
chromophore from glucuronic did not absorb. The ratio of the absorbance

at 535 nm to that at 700 nm for the interfering compound was found to be 0,392. A rough correction could therefore be made for the interfering compound by determining the absorbance of the samples at two wavelengths, 535 and 700 nm, and by subtracting 0,392 X A_{700} from the 535 nm reading. The results for a glucuronolactone standard curve, treated in this way, was linear and passed through the origin.

2.1.8. Assay of reducing sugar.

Reducing sugar was assayed by the 3,5-dinitrosalicylic acid (DNS) method described by Miller (1959).

A.   Reagents.

(i).   DNS Reagent. The DNS reagent was prepared as follows:

NaOH	10,00 g
DNS	10,00 g
Phenol	2,00 g
Sodium potassium tartrate	200,00 g
Sodium sulphite	0,05 g
H_2O	to 1 litre

(ii).   Glucose standard.   A stock glucose solution was prepared by dissolving 100 mg glucose in 100 ml of 0,2% (w/v) aqueous benzoic acid, and stored in the refrigerator at 4oC. Solutions

were stable for at least 6 months. Dilutions from this stock in water were prepared as required.

B.   Procedure.

DNS solution (5 ml) was added to samples (1 ml containing up to 1 mg of reducing sugar) in 150 X 16 mm test tubes. After mixing, the tubes were capped with glass marbles, and heated in a boiling water bath ($98^{o}C$) for 5 min. After cooling, the absorbances of the solutions were determined at 575 nm against a reagent blank in a Spectronic-88 spectrophotometer.

The glucose standard curve did not pass through the origin due to the destruction of about 0,15 mg of reducing sugar by oxidation. The upper limit of the assay is 1 mg of glucose, and the lower limit about 0,2 mg of glucose. The range of the method could be extended to a lower limit of about 0,025 mg if 0,2 mg glucose was added to both the sample and the blank. This procedure had the effect of correcting the standard curve so that it passed through the origin.

2.1.9. Isoelectrofocusing (IEF).

Isoelectrofocusing was carried out in a 110 ml LKB model 8100-10. IEF column (LKB-Produkter, AB, Bromma, Sweden) according to the manufacturer's instructions. Additional information was obtained from the article by Haglund (1971).

A.   Preparation of samples for IEF.

All operations were performed at 4°C in a cold room. Cell-free culture filtrates were adjusted to pH 8,0, and protein precipitated by the slow addition of solid $(NH_4)_2SO_4$ to 90% saturation (660 g 1^{-1}). During this procedure, the pH was maintained at 8,0. The precipitate was allowed to stand for 2h, collected by centrifugation (30 000 x g, 4°C, 60 min), and redissolved in a minimal volume of 1% (w/v) glycine. The sample was applied to a column (2,5 x 40 cm) of Sephadex G-75, and eluted with 1% (w/v) glycine at a flow rate of 50 ml h^{-1}. Fractions (5 ml) were collected, and assayed for PL activity. Active fractions were pooled, and applied to the IEF column in the light gradient solution as described below.

B.   Reagents for IEF.

(i).   Dense anode solution. This contained:

c.H_2SO_4	0,2 ml
H_2O	14,0 ml
Sucrose	12,0 ml

(ii).   Light cathode solution. This contained:

Ethanolamine	0,2 ml
H_2O	10,0 ml

(iii).   Dense gradient solution. This contained:

Ampholine (pH range 3-10)	2,25 ml
H_2O	39,75 ml
Sucrose	28,0 ml

(iv).   Light gradient solution. This contained:

Ampholine (LKB, pH 3-10)	0,75 ml
H_2O + sample	59,25 ml

C.   Preparation of IEF column. The column was assembled, and the dense anode solution introduced as described in the manufacturer's instructions.   A density gradient prepared from solutions (iii) and (iv) by an LKB model 8121 gradient mixer, was pumped into the column, followed by the light anode solution.   Electrofocusing was carried out for 48 h at 4°C until a constant power consumption was observed. Maximum power was maintained below 3W.

The central electrode compartment was emptied, and the column drained from the bottom by pumping H_2O into the top of the column.   The absorbance of the effluent was continuously monitored at 280 nm by an LKB Uvicord II. Fractions (2 ml) were collected, assayed for PL activity, and the pH measured at 4°C. Active fractions were pooled, and stored at 4°C.

In some experiments, the active fractions in the pI range of 7-9,5 were pooled, and applied to the column (2,5 cm x 40 cm) of Sephadex G-25 equilibrated with 1% (w/v) glycine. The column was eluted with 1% (w/v) glycine at a flow rate of 50 ml h^{-1} and fractions (5 ml) were assayed for PL activity. Active fractions were pooled, and focused on a narrower range ampholine (pH 6-10) gradient as described above.

2.1.10. PL assays.

A modification of the method described by Dave and Vaughn (1971) was used to measure PL activity. Assays were performed at 30°C with 0,4% (w/v) sodium polygalacturonate (NaPP) in 0,05 M Tris-HCl, pH 8,5, as substrate. The increase in absorbance at 230 nm due to the production of 4,5-unsaturated uronides by the action of PL on NaPP was continuously recorded, and the initial linear slope used to calculate enzyme activity as described below.

A.   Substrate (0,4% (w/v) NaPP in 0,05 M Tris-HCl, pH 8,5).

NaPP (Merck, Darmstadt, W. Germany, or Nutritional Biochemical Company, ICN Pharmaceuticals Inc., Cleveland, USA) (1,0 g) was dispersed in H_2O (200 ml) with rapid stirring, and 12,5 ml of 0,1 M Trizma base (Sigma, 12,114 g per 100 ml H_2O) was added, followed by the addition of 2,5 ml of 0,1 M $CaCl_2$ (1,470 g $CaCl_2.2H_2O$ per 100 ml H_2O). The pH was adjusted to 8,5 with 2 M HCl and stirring continued after making up to 250 ml with H_2O, until all the NaPP (and its calcium salt) had dissolved. The pH was

rechecked, and the solution was filtered through Whatman (Whatman Ltd., Springfield Mill, Kent, UK) GFA glass-fibre filter paper. Fresh substrate was prepared weekly, and stored at 4°C.

B.   Method.

Substrate (2,95 ml) was pipetted into a quartz cuvette, and equilibrated at 30°C for 5 min. After the addition of enzyme solution (0,05 ml), the increase in absorbance at 230 nm was continuously recorded on a Beckman Acta III spectrophotometer for 3-5 min. The initial linear slope of the trace was used to calculate enzyme activity, defined as the production of 1 mole of unsaturated uronide per minute. A molar extinction coefficient of 4 600 M^{-1} cm^{-1} (Nagel and Anderson, 1965) was used to calculate enzyme activity.

2.1.11. Assay of β-galactosidase activity in permeabilized cells with o-nitrophenol-β-D-Galactopyranoside as substrate.

Intracellular levels of β-galactoside were assayed, after toluene permeabilization, by the method of Miller (1972, pp. 352-355).

A.   Reagents.

(i).   o-Nitrophenyl-β-D-galactopyranoside (β-ONPG). β-ONPG (100 mg) was dissolved in H_2O (25 ml) to give a 13,2 mM solution. The solution, prepared weekly, was stored at 4°C.

(ii).   Buffer.   Z-buffer was prepared according to Miller (1972, pp.

352 -355) and contained:

Na_2HPO_4	16,10 g	(0,06 mole)
NaH_2PO_4	5,50 g	(0,04 mole)
KCl	0,75 g	(0,01 mole)
$MgSO_4.7H_2O$	0,246 g	(0,001 mole)
2-ME	2,7 ml	(0,05 mole)

The ingredients were dissolved in H_2O (900 ml) and adjusted to pH 7,0

with 5 M NaOH before being made up to 1 l with water.

(iii).   Sodium carbonate solution.   A 1 M solution of Na_2CO_3 was

prepared by dissolving 132 g of the salt in H_2O (1 l).

B.   Procedure.

Bacterial cell suspension (0,1-0,5 ml, depending on activity) was

pipetted into a 25 ml conical flask, made up to 1 ml with Z-buffer, and

one drop of toluene was added. The toluene was removed from the samples

in a shaking water bath at 37°C for 40 min. After equilibration for 5 min

by incubating at 30°C, the reaction was begun by the addition of β-ONPG

(0,20 ml) and the samples were incubated at 30°C until the development of

a definite yellow colour. The reaction was stopped by the addition of 1 M

Na_2CO_3 (1 ml), and after dilution with H_2O (3 ml), their absorbances at

420 and 550 nm were determined. Correction for turbidity was made by subtracting $1.711 \times A_{550}$ (a factor representing the ratio of absorbances at 420 and 550 nm) from the absorbance at 420 nm (Miller, 1972, pp. 352-355). The absorbance at 550 nm is due to the cells as o-nitrophenol (ONP) has negligible absorption at this wavelength.

2.1.12. Thiobarbituric acid Assay.

Formyl pyruvate is produced by periodate oxidation of 4,5-unsaturated uronides, which reacts with acid 2-thiobarbituric acid (TBA) to form a red chromogen with a maximum absorbance at 548 nm (Weissbach and Hurwitz, 1959; Preiss and Ashwell, 1963). Neither NaPP nor galacturonic give a positive TBA test (Preiss and Ashwell, 1963).

A.   Reagents.

(i).   0.025 M HIO_4 in 0.0625 M H_2SO_4. H_5IO_6 (BDH, 0.570 g) was dissolved in 0.0625 M H_2SO_4 (100 ml).

(ii).   2% (w/v) Na_2AsO_2 in 0.5 M HCl. Na_2AsO_2 (2 g) was dissolved in 0.5 N HCl (100 ml).

(iii).   0.3% (w/v) TBA (pH 2). TBA (0.3 g) was added to H_2O (90 ml), titrated to pH 2.0 with 1.0 M NaOH, made up to 100 ml with H_2O and filtered.

B.   Method.

Sample (0,20 ml) containing up to 0,05 umole unsaturated product, was added to 0,25 ml of acid HIO_4 reagent and mixed. After standing at room temperature (22°C) for 20 min, the oxidation was stopped by the addition of 0,50 ml Na_2AsO_2 solution. Two minutes later, 0,3% TBA reagent (0,5 ml) was added with mixing, and the solution heated in a boiling water-bath for 10 min. After cooling to room temperature, the absorbance spectrum of the red chromophore was scanned between 400 and 700 nm in a Beckman Acta III recording spectrophotometer.

2.1.13. Determination of the pH optimum of PL.

A.   Reagents.

Substrate (0,4% (w/v) NaPP was made up in the following buffers containing 1 mM $CaCl_2$.

pH 4-6	0,05 M acetate.
pH 7-8	0,05 M phosphate.
pH 8,4-8,8	0,05 M Tris-HCl.
pH 9,0-10,2	0,05 M Gly-NaOH.

The pH of each substrate was checked at 22°C before us.

2.1.14. Paper chromatography of culture supernatants.

The presence of specific sugars in culture supernatants was determined by descending paper chromatography as described by Smith (1960).

A.   Reagents.

(i).   Solvent.   The mobile phase contained.

        Ethyl acetate   120 ml
        Pyridine   50 ml
        H_2O 40 ml

(ii).   Visualization reagent.   The spray reagent contained.

        Phthalic acid   1,70 g
        Aniline   1,0 ml
        Glacial acetic acid   5,0 ml
        Trichloroacetic acid (40% (w/v))   5,0 ml
        Absolute ethanol   90,0 ml

B.   Method.

Cell free culture supernatants (0,01 ml) were spotted onto Whatman No. 1 chromatography paper, and developed by descending chromatography for 18 h. The papers were dried, and the sugars visualized by spraying with

the phthalic acid reagent, drying, and heating at 105°C for 10 min. All the sugars appeared as dark spots on a light background.

2.1.15. Studies on PL and β-galactosidase induction.

These studies were performed in 250 ml side-arm flasks containing either YS-medium (35 ml). The inoculum consisted of cells passed through two log phases of growth; firstly in NB, and secondly, in YS medium supplemented with 0.18% (w/v) glycerol. In some experiments, YS medium was further supplemented with 0.18% (w/v) lactose. Additional details are given in the results section.

2.2.   RESULTS.

2.2.1. PL reaction products.

The local isolate of E. chrysanthemi secreted an extracellular pectic enzyme active on NaPP. The products of NaPP degradation exhibited a absorption maximum at 230 nm, and after reaction with acid-periodate and TBA, at 548 nm.

Preliminary experiments on the crude enzyme gave a pH optimum of activity of 8,5. The time course of the PL assay (Section 2.1.10) at 230 nm was linear over the first 5 min at 30°C up to an absorbance of 1,5.

2.2.2. Isoelectrofocusing of PL isoenzymes.

Two clearly separated peaks of PL activity were observed after IEF of crude culture supernatants (Fig. 2). The larger peak had a pI of about 9,2, and the smaller peak a pI of about 5,0 (Fig. 2). The pI 9,2 peak appeared to consist of at least two isozymes, as a shoulder of PL activity was observed at about pI 8,4 (Fig. 2). Efforts, however, to resolve the larger peak into further PL isozymes were not successful.
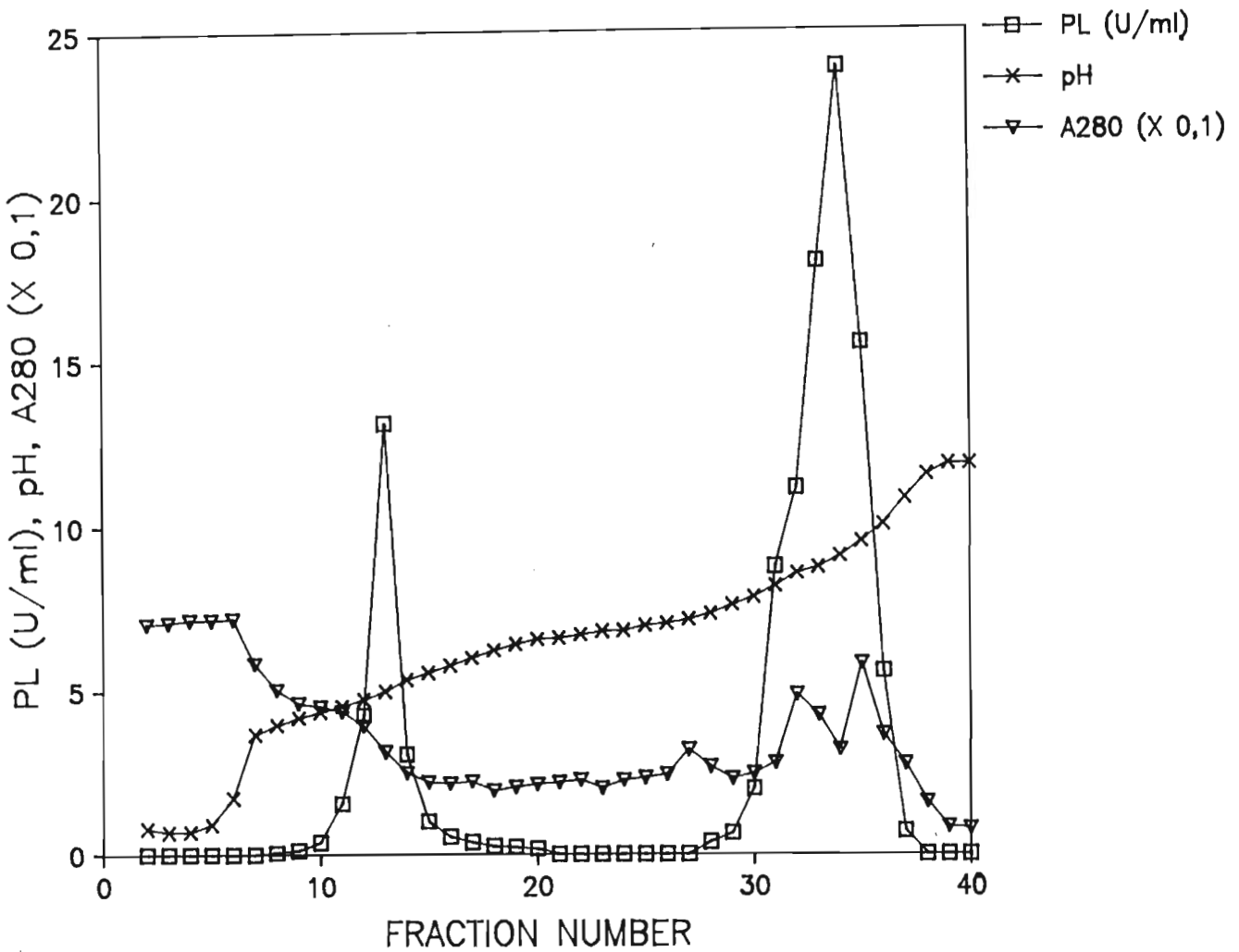
Fig. 2. PL isozyme pattern from E. chrysanthemi. Crude culture super-
natant, after $(NH_4)_2SO_4$ precipitation and chromatography on
Sephadex G-75, was electrofocused on a pH 3-10 gradient, and
analyzed as described in Section 2.1.7.

During isoelectrofocusing, a heavy precipitate formed near the anode. This precipitate fell through the gradient, disrupting it. A prior chromatography of the 90% ammonium sulphate precipitate dissolved in 1% (w/v) glycine on Sephadex G-75, pre-equilibrated with 1% (w/v) glycine, reduced the formation of the precipitate. Placing the anode at the bottom of the column in the densest part of the gradient also helped minimize the effect of the precipitate.

2.2.3. pH optima of the PL isoenzymes.

The pH optima of the different pI isoenzymes from the IEF column and of the unfractionated (UF) ammonium sulphate precipitate, after Sephadex G-75 chromatography, were determined on both NaPP and pectin (Figs. 3 and 4). All the fractions exhibited a pH optimum of about pH 8,5 on NaPP, and about 9,0 on pectin.

The PL isoenzymes all exhibited a requirement for Ca^{2+} for activity. The addition of 1 mM EDTA to the assay mixture completely inhibited enzyme activity, which was restored by the addition of Ca^{2+} to the assay mixture.

2.2.4. PL production by E. chrysanthemi.

The composition of the growth medium exerted an important effect on PL production by E. chrysanthemi. As preliminary experiments showed that
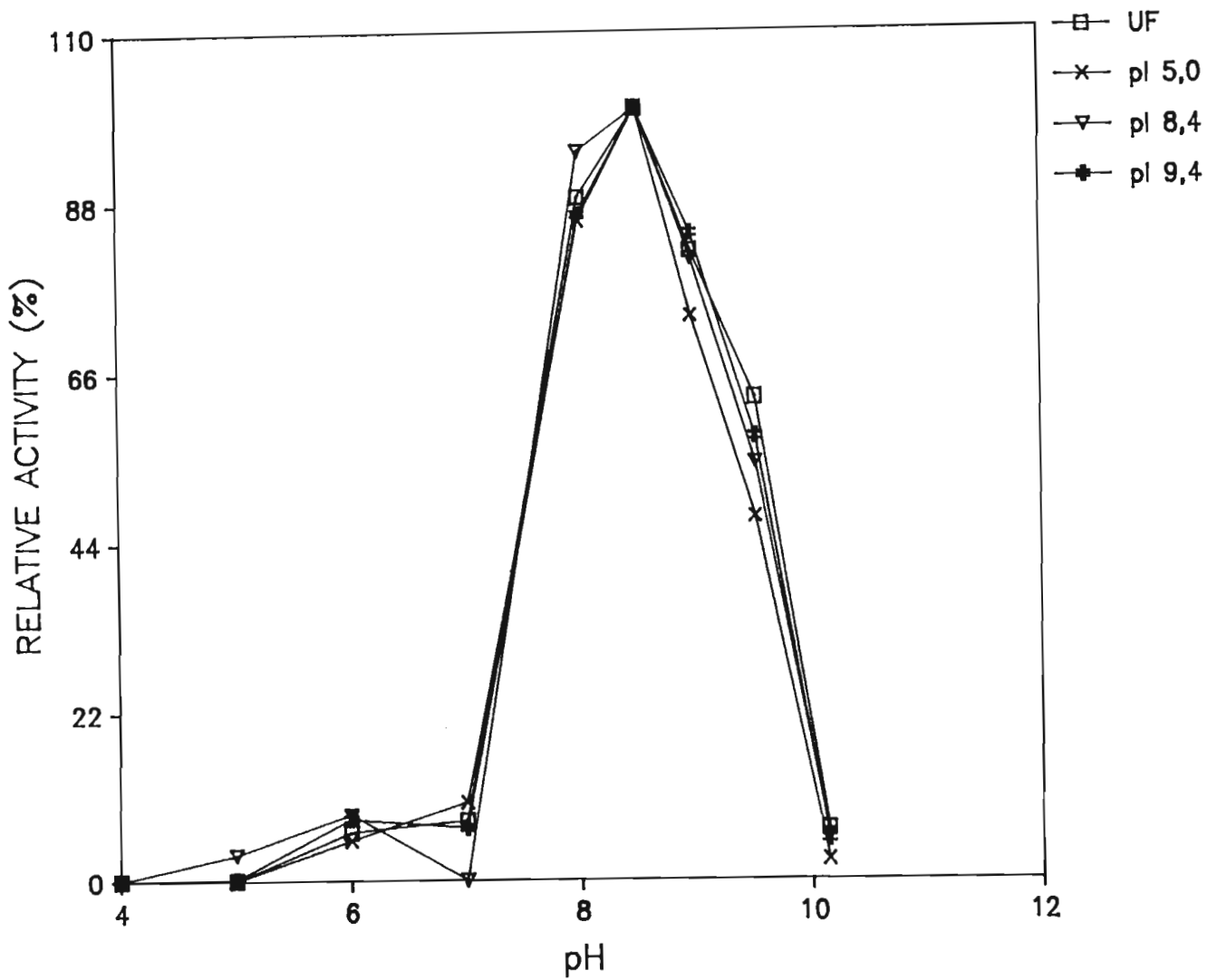
Fig. 3. pH profiles of PL isozymes on NaPP. The major isozymes from IEF and unfractionated (UF) culture supernatants after $(NH_4)_2SO_4$ precipitation were used. The pI 8,4 peak represents a fraction from the shoulder of the pI 9,4 isozyme peak (see Fig. 2).
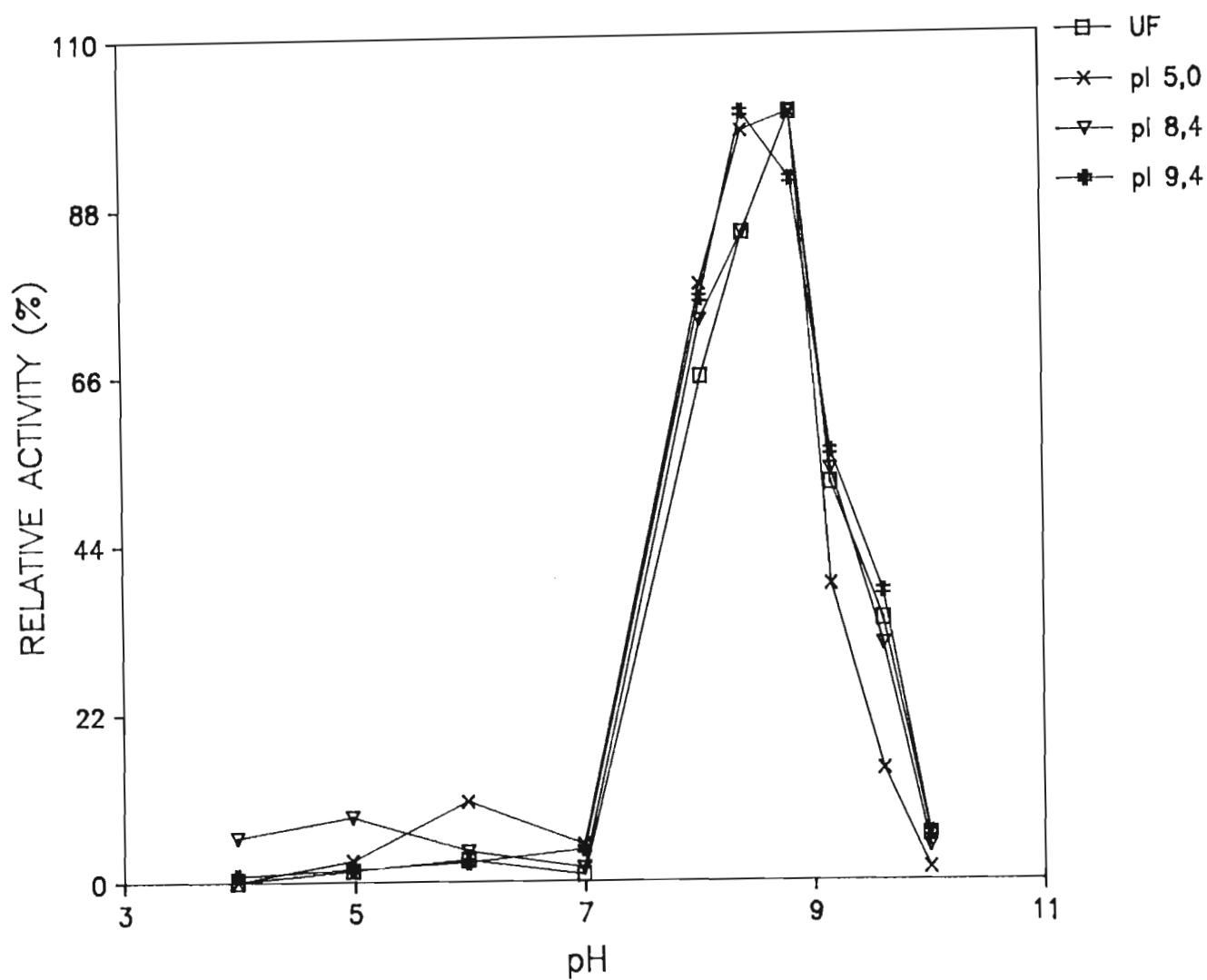
Fig. 4.   pH profiles of PL isozymes on Pectin. The major isozymes from IEF and unfractionated (UF) culture supernatants after $(NH_4)_2SO_4$ precipitation were used. The pI 8,4 peak represents a fraction from the shoulder of the pI 9,4 isozyme peak (see Fig. 2).

only low levels of PL were produced on HK-medium supplemented with 0,8%
(w/v) NaPP as carbon source, this medium was not investigated further.
Growth of E. chrysanthemi on XC- and GB-medium supplemented with 0,8%
(w/v) NaPP as carbon source produced significant amounts of PL. Only low
basal levels of PL were produced in the absence of NaPP.

Due to the high concentration of Ca^{2+} associated with the pectic sub-
stances in the middle lamella, its effect on PL synthesis in XC-medium
was determined (Fig. 5). A 4-fold increase in PL production was observed
on increasing the Ca^{2+} concentration from 0 to 7,55 mM (Fig. 5). Although
the data suggested that concentrations of Ca^{2+} higher than 7,55 mM would
lead to further slight increases in PL levels, this was not tested, as
the formation of an insoluble calcium pectate gel in the medium made it
difficult to handle.

GB-medium was not used to investigate the effect of Ca^{2+} on PL production
due to the high concentration of phosphate in the medium (0,1 M), and the
low solubility product of calcium phosphate ($S = 1 \times 10^{-25}$; Vogel, 1959).

PL production and growth of E. chrysanthemi reached a maximum at 9 h on
GB-, XC-, and HXC-media (Figs. 6 and 7). The best medium for PL product-
ion was HXC-medium, followed by GB- and XC-medium. HXC- and XC-media
supported better growth of E. chrysanthemi than did GB-medium (Fig. 7).
HXC-medium was chosen for the following experiments in which the effects
of different sugars on PL synthesis were measured.
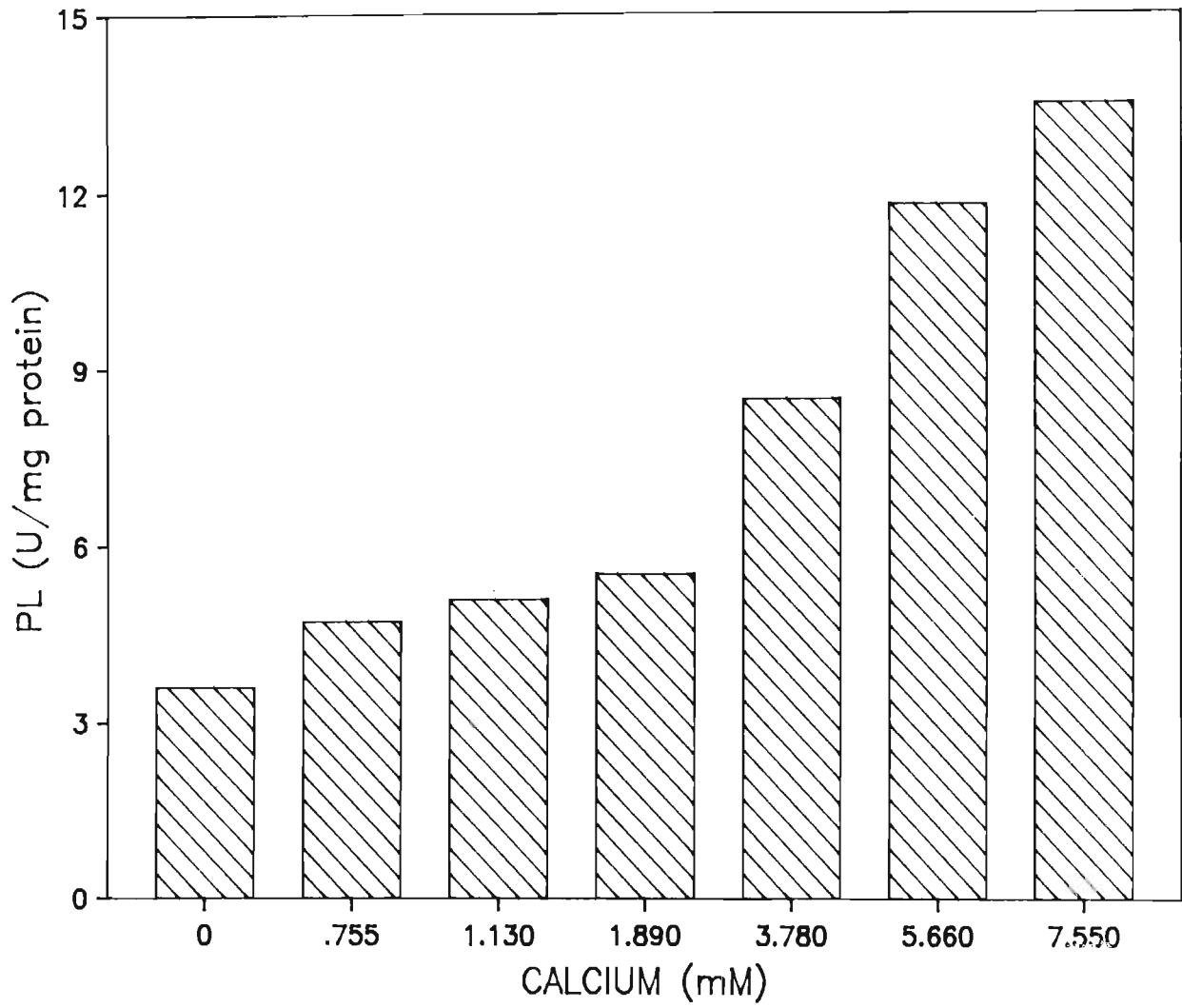
Fig. 5.   Effect of different concentrations of $CaCl_2$ on PL production by
E. chrysanthemi grown on XC-medium supplemented with 1,0% (w/v)
NaPP.
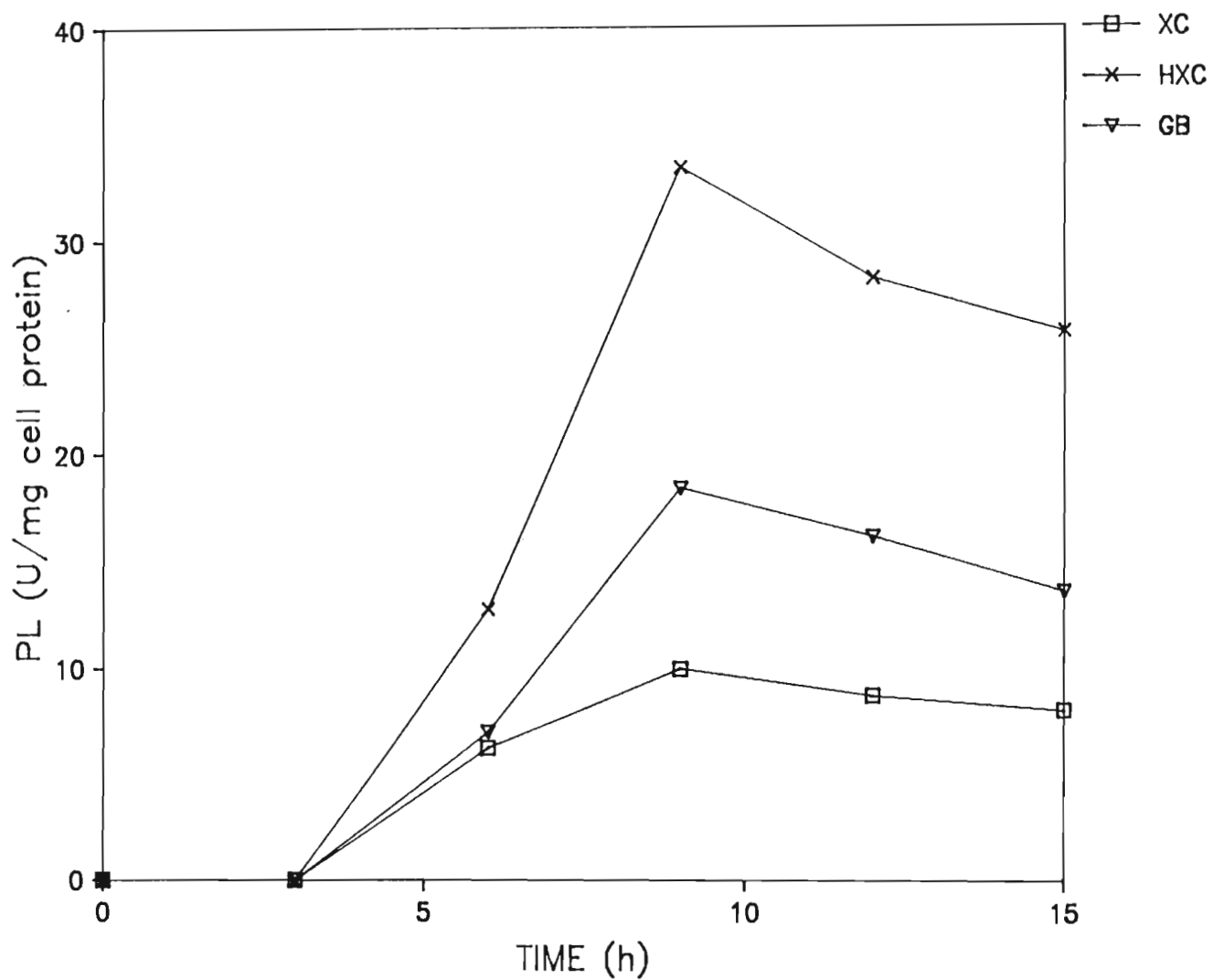
Fig. 6. Comparison of PL production by E. chrysanthemi on Garibaldi-
Bateman (GB) medium, X. campestris (XC) medium, and high
calcium X. campestris (HXC) medium supplemented with 1,0% (w/v)
NaPP.
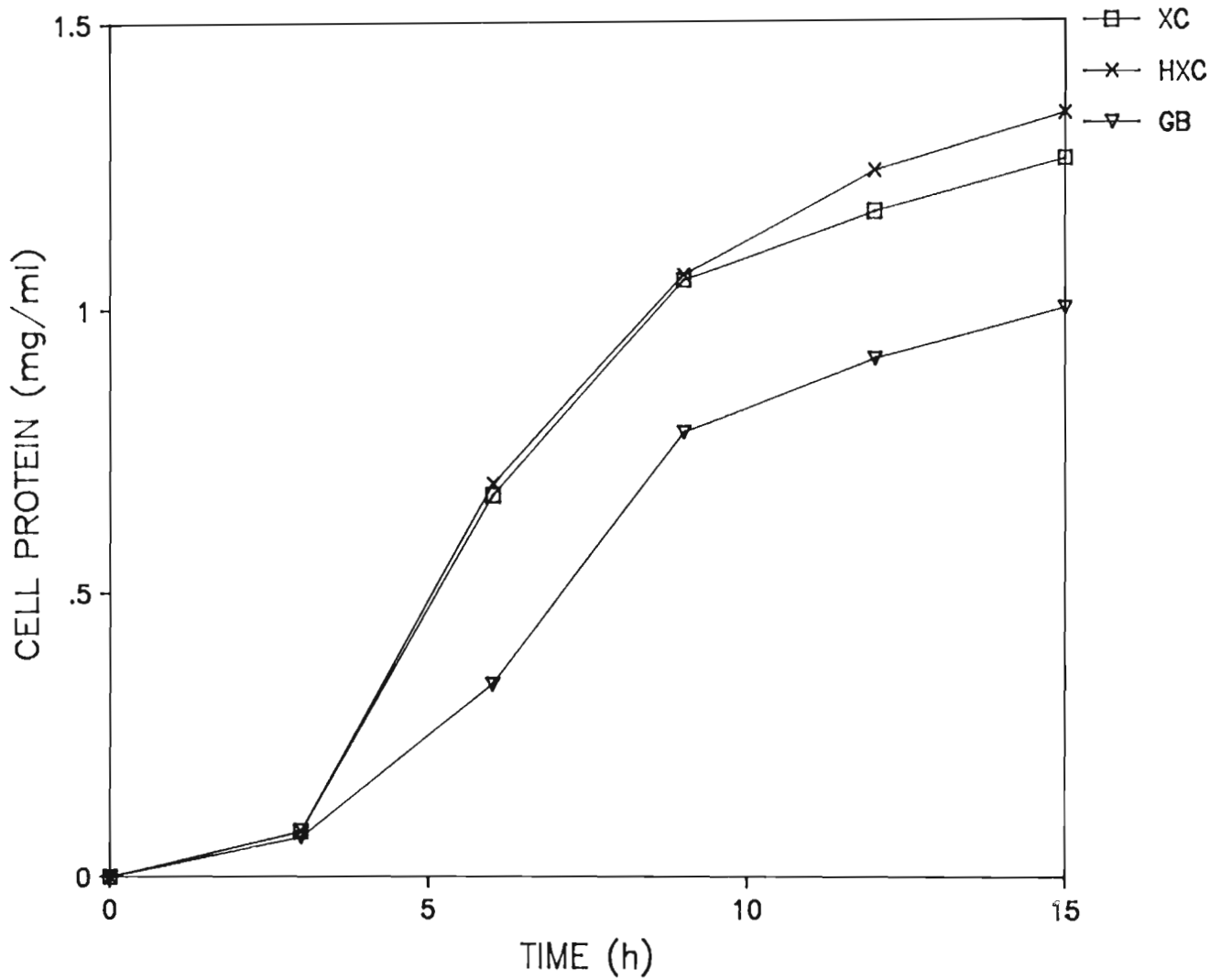
Fig. 7.   Growth of <u>E</u>. <u>chrysanthemi</u> on Garibaldi–Bateman (GB) medium, <u>X</u>. <u>campestris</u> (XC) medium, and high calcium <u>X</u>. <u>campestris</u> (HXC) medium supplemented with 1,0% (w/v) NaPP.

PL production by E. chrysanthemi, in the presence of a number of diffe-
rent carbohydrates, was investigated in HXC-medium supplemented with
either 1,0% (w/v) carbohydrate or 0,5% (w/v) carbohydrate + 0,5% (w/v)
NaPP. After inoculation, the cultures were grown for 9 h on an orbital
shaker at 30°C, centrifuged (50 000 X g, 4°C, 30 min), and assayed for PL
and cell protein as described in Materials and Methods.

E. chrysanthemi grown on NaPP produced high levels of PL (Table 2). No
other carbohydrate tested supported a PL level of more than 8% of that
produced on NaPP (Table 2).

The carbohydrates could be divided into three categories depending on
their effect on PL production. The first category repressed PL synthesis
severely (> 90%), and included D-glucose, sucrose, D-fructose, and D-
mannitol (Table 2). The second group repressed PL production to an
intermediate degree (30-80%), and included D-galacturonic acid, L-arabin-
ose, D-xylose, cellobiose, and D-galactose (Table 2). The last group had
little or no effect on PL production by E. chrysanthemi (< 30%), and
included D-arabinose, L-rhamnose, lactose, D-mannose, and xylan (Table
2).

All the carbon sources tested supported growth of E. chrysanthemi except
for D-xylose, lactose and xylan (Table 2).

Table 2. Growth and PL production by E. chrysanthemi grown on HXC-medium
with different carbon sources.

Carbon Source[1]	Without NaPP[2] Growth (mg ml^{-1})	S.A.[4]	With NaPP[3] Growth (mg ml^{-1})	S.A.
Glucose	0,93	0,28	1,02	0,29
Sucrose	0,93	0,53	1,12	0,87
Mannitol	0,47	0,47	0,93	1,23
Fructose	0,54	0,24	1,18	3,41
Gal Acid	0,47	2,77	1,07	7,16
L-Arabinose	0,54	0,25	1,11	11,95
D-Xylose	0,29	0,00	0,83	12,91
Cellobiose	0,49	0,00	0,89	15,89
Galactose	0,59	0,66	1,11	16,80
Glycerol	0,40	0,88	0,79	20,74
D-Arabinose	0,45	0,28	0,99	25,00
Rhamnose	0,24	0,53	0,96	25,08
Lactose	0,28	0,89	0,96	26,70
Mannose	0,62	1,14	0,83	37,33
Xylan	0,40	1,87	0,79	37,58
NaPP	−	−	1,03	34,86

1. All D-sugars unless otherwise stated.
2. Concentration of carbon source: 1,0%.
3. Concentration of carbon source: 0,5% NaPP + 0,5% carbohydrate.
4. S.A. = Specific Activity (U per mg cell protein).

The effects of glucose, galacturonic acid, and lactose on PL production by $\underline{E}$. $\underline{chrysanthemi}$ during growth in HXC-medium were investigated (Figs. 8-10). Glucose repressed enzyme synthesis severely over the first 9 h of growth, during which period, free glucose was detected in the medium by paper chromatography (Fig. 8). Thereafter, the rate of PL synthesis approached that on NaPP alone (Fig. 8).

The pattern of PL synthesis in the presence of galacturonic acid differed from that in the presence of glucose (Fig. 9). Galacturonic acid decreased the rate of PL synthesis in the NaPP + galacturonic acid cultures over the whole growth period (Fig. 9), despite its complete consumption by 12 h.

Although lactose did not support good growth of $\underline{E}$. $\underline{chrysanthemi}$, its presence in the medium together with NaPP gave rise to much higher levels of PL than did NaPP alone (Fig. 10). Lactose was detected by paper chromatography in all lactose-containing media at the end of the growth curves at 15 h.

2.2.5. Isolation of Lac+ Mutant.

The previous experiments suggested that the wild-type strain of $\underline{E}$. $\underline{chrysanthemi}$ (FH1) was Lac-. This was confirmed by the poor growth
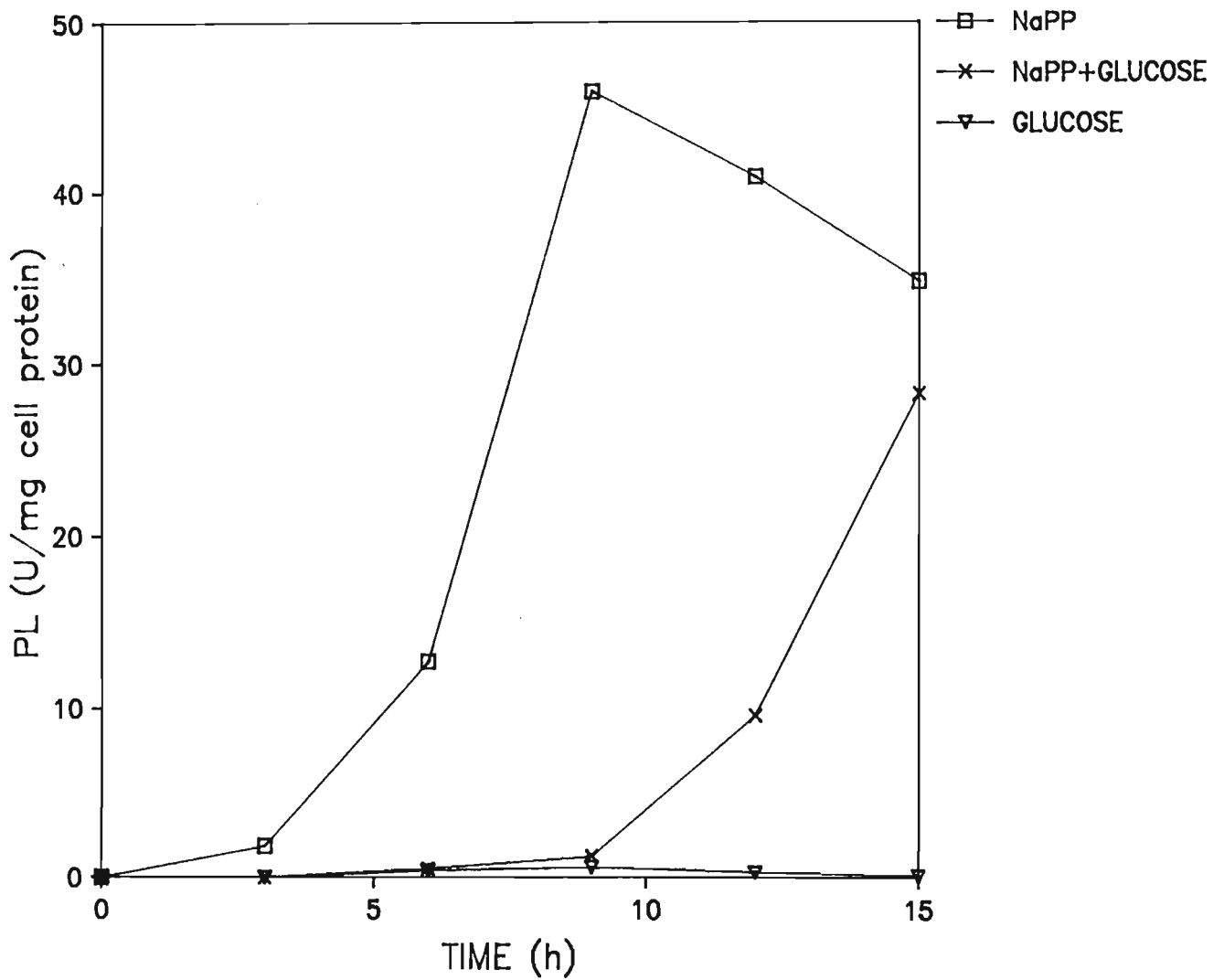
Fig. 8.   PL production by E. chrysanthemi on HXC–medium supplemented with either 1,0% (w/v) NaPP, 0,5% (w/v) NaPP + 0,5% (w/v) glucose, or 1,0 (w/v) glucose.
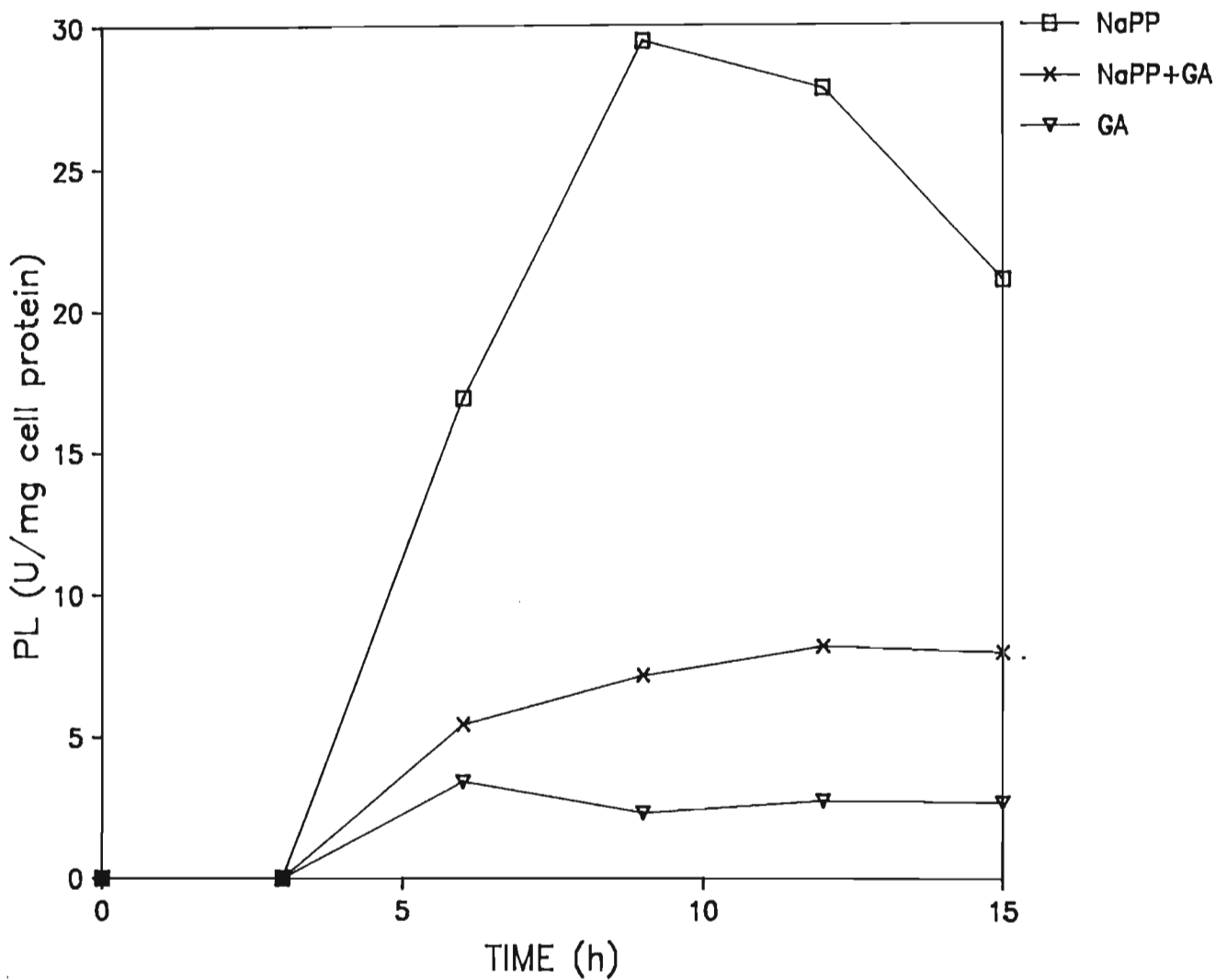
Fig. 9. PL production by $\underline{E}$. chrysanthemi on HXC-medium supplemented with either 1,0% (w/v) NaPP, 0,5% (w/v) NaPP + 0,5% (w/v) galacturonic acid (GA), or 1,0 (w/v) galacturonic acid (GA).
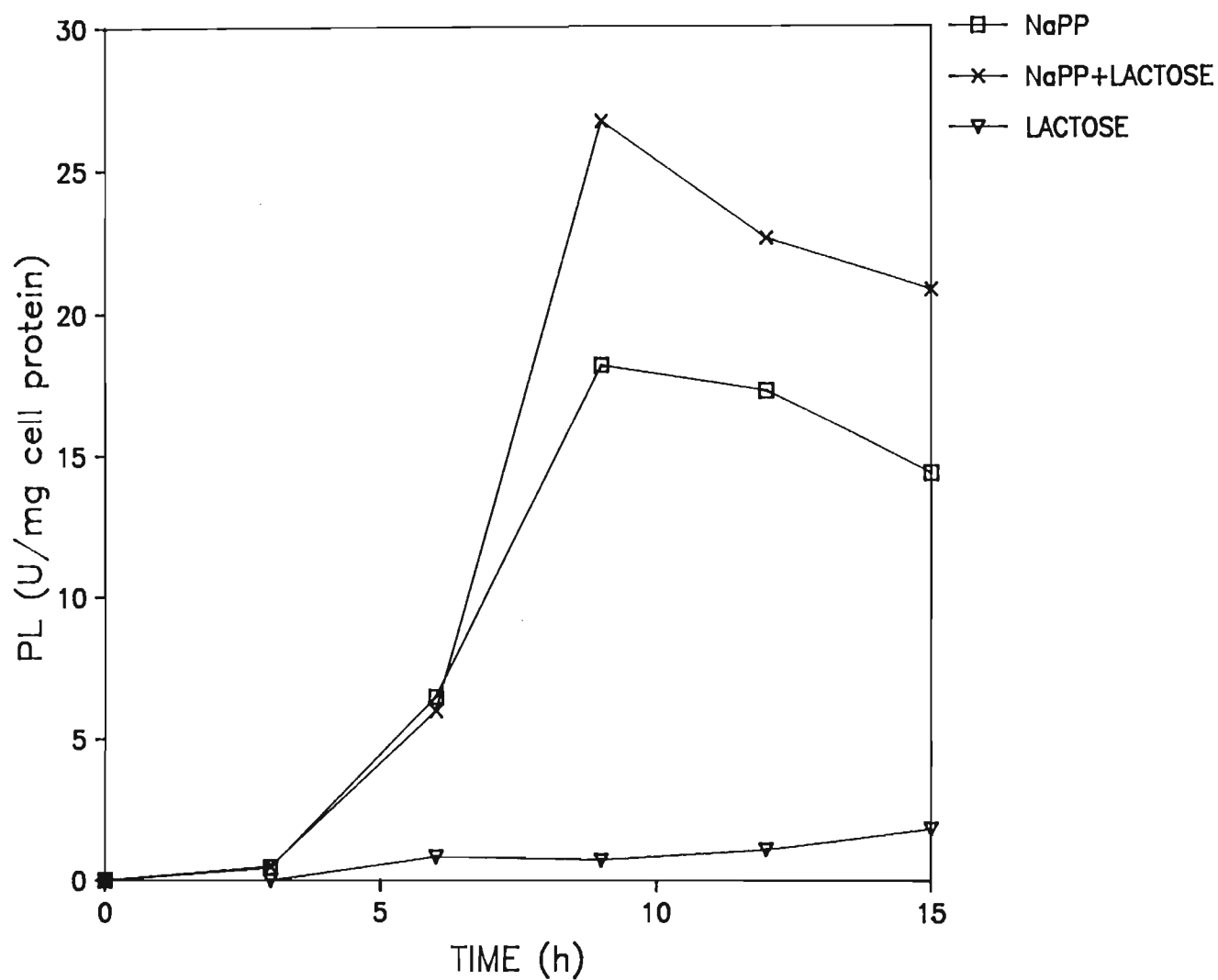
Fig. 10. PL production by E. chrysanthemi on HXC-medium supplemented with either 1,0% (w/v) NaPP, 0,5% (w/v) NaPP + 0,5% (w/v) lactose, or 1,0 (w/v) lactose.

of the organism on YS-lactose agar, where it grew as small clear colonies on the YE in the medium (no growth was observed in the absence of the YE), and by its behaviour on a variety of indicator media (Table 3). After about three days of incubation on the YS-lactose agar, however, some of the clear colonies segregated and gave rise to slimy papillae, which, after purification by repeated streaking on YS-lactose agar, consistently gave rise to slimy colonies. The appearance of the clear parent strain and the slimy derived strain on YS-lactose agar, lead to the two strains being called EC-C (E. chrysanthemi-clear), and EC-S (E. chrysanthemi-slimy).

Slime is produced by EC-C and EC-S from glucose and galactose (Table 3). Thus, it appears as if slime production on lactose indicates the Lac$^+$ phenotype of EC-S rather than any newly acquired ability to synthesize slime. This interpretation is confirmed by the behaviour of EC-C and EC-S, where EC-S is shown to be consistently Lac$^+$, and EC-C, Lac$^-$, on all the media tested (Table 3). Both strains, however, produced low constitutive levels of a β-galactosidase capable of hydrolyzing X-Gal (Table 3).

The Lac$^+$ character of EC-S appears to be stable as no Lac$^-$ revertants were detected after 15 weekly transfers on NA. Furthermore, both strains have been maintained in 20% glycerol at -20°C, as suspensions in distilled water at room temperature, and as NA cultures for over 3 years without any loss in their phenotypical behaviour.

Table 3. Reactions of EC-C and EC-S on Indicator media.

MEDIUM	COLONY TYPE	
	EC-C	EC-S
YS-lactose	small, clear colonies	slimy, spreading colonies
YS-glucose	slimy, spreading colonies	slimy, spreading colonies
YS-galactose	slimy, spreading colonies	slimy, spreading colonies
EMB-lactose	light colonies, no dye change	dark colonies, green sheen, dye change
EMB-glucose	dark colonies, green sheen,	dark colonies, green sheen, dye change
MacConkey agar	colourless colonies	red colonies surrounded by precipitated bile salts
X-Gal-glycerol	blue colonies	blue colonies
X-Gal-glycerol + IPTG	blue colonies	blue colonies
X-Gal-glycerol + lactose	blue colonies	blue colonies
TCZ-lactose	dark red colonies, thin red border	light, spreading colonies
TCZ-glucose	light, spreading colonies	light, spreading colonies
TCZ-galactose	light, spreading colonies	light, spreading colonies

2.2.6. Physiological Tests on EC-C and EC-S.

EC-C and EC-S both gave identical results when subjected to the API 20E test system (Table 4). Both strains were oxidase negative, could ferment D-glucose, D-mannose, inositol, L-rhamnose, sucrose, melibiose, amygdalin, and L-arabinose (Table 4). A β-galactosidase capable of hydrolyzing β-ONPG was present in both EC-C and EC-S, confirming the X-Gal results (Tables 3 and 4). Gelatin was hydrolyzed, and citrate metabolized by both strains.

When the strains were subjected to the API 50 test system, the major difference between the two strains was that EC-S could metabolize lactose, whereas EC-C could not (Table 5). Furthermore, EC-S fermented melibiose and raffinose more rapidly than EC-C, both carbohydrates giving a positive test in three hours in strain EC-S, whereas they were fully positive in EC-C only after 24 h in strain EC-C (Table 5). Both strains grew on and hydrolyzed pectate in CVP medium indicating the presence of pectate hydrolyzing enzymes.

2.2.7. Comparison of pathogenicity of EC-C and EC-S.

Wilt symptoms appeared in both EC-C and EC-S inoculated maize plants within 24 h, and plants rotted within 48 h. E. chrysanthemi could be re-isolated from the rotten tissue, and it was significant that EC-S was isolated from EC-S inoculated plants, and EC-C was re-isolated from EC-C

Table 4. API 20E tests on EC-C and EC-S. Overnight cultures grown on NA
plates were resuspended in distilled water, and used in the API
20 test according to the manufacturer's instructions.

TEST	EC-C[1]	EC-S
ONPG	+	+
ARGININE DIHYDROLASE	−	−
LYSINE DECARBOXYLASE	−	−
ORNITHINE DECARBOXYLASE	−	−
CITRATE UTILIZATION	+	+
HYDROGEN SULPHIDE PRODUCTION	−	−
UREASE	−	−
TYROSINE DEAMINASE	−	−
INDOLE PRODUCTION	+	+
VOGES-PROSKAUER	+	+
GELATIN HYDROLYSIS	+	+
GLUCOSE UTILIZATION	+(g)[2]	+(g)
MANNOSE UTILATION	+(g)	+(g)
INOSITOL UTILIZATION	+	+
SORBITOL UTILIZATION	−	−
RHAMNOSE UTILIZATION	+	+
SUCROSE UTILIZATION	+	+
MELIBIOSE UTILIZATION	+	+
AMYGDALIN UTILIZATION	+	+
L-ARABINOSE UTILIZATION	+	+
OXIDASE	−	−
NITRATE REDUCTION	+	+
NITROGEN PRODUCTION	+	+
CATALASE	+	+

1 (−) Negative reaction, (+) positive reaction.
2 gas

Table 5. API 50 CH tests on EC-C and EC-S. Overnight cultures of EC-C
and EC-S from NA plates were resuspended in the API50E medium,
dispensed in the API 50 galleries, covered with sterile mineral
oil, incubated at 30ºC, and the dye colour change noted after
various times.

SUGAR	EC-C					EC-S				
	3h	6h	24h	48h	72h	3h	6h	24h	48h	72h
CONTROL	0	0	0	0	0	0	0	0	0	0
GLYCEROL	0	0	3	4	5	0	0	3	4	5
ERYTHRITOL	0	0	0	0	0	0	0	0	0	0
D-ARABINOSE	0	3	5	5	5	0	1	5	5	5
L-ARABINOSE	3	5	5	5	5	2	5	5	5	5
RIBOSE	2	4	5	5	5	1	4	5	5	5
D-XYLOSE	0	3	5	5	5	0	3	5	5	5
L-XYLOSE	0	0	0	0	0	0	0	0	0	0
ADONITOL	0	0	1	1	1	0	0	0	0	1
β-METHYL-XYLOSIDE	0	0	0	0	0	0	0	0	0	0
GALACTOSE	4	4	5	5	5	5	5	5	5	5
D-GLUCOSE	5	5	5	5	5	5	5	5	5	5
D-FRUCTOSE	5	5	5	5	5	5	5	5	5	5
D-MANNOSE	5	5	5	5	5	5	5	5	5	5
L-SORBOSE	0	0	0	1	1	0	0	0	0	0
RHAMNOSE	1	3	5	5	5	0	3	4	5	5
DULCITOL	0	0	0	0	0	0	0	0	0	0
INOSITOL	0	0	3	4	5	0	0	2	4	4
MANNITOL	3	5	5	5	5	3	5	5	5	5
SORBITOL	2	3	3	3	3	2	3	3	3	3

Table 5 continued.

SUGAR	EC-C					EC-S				
	3h	6h	24h	48h	72h	3h	6h	24h	48h	72h
α-METHYL-D-MANNOSIDE	0	0	0	0	0	0	0	0	0	0
α-METHYL-D-GLUCOSIDE	0	0	0	0	0	0	0	0	0	0
N-ACETYL-GLUCOSAMINE	4	5	5	5	5	3	5	5	5	5
AMYGDALINE	0	0	0	2	4	0	0	0	0	0
ARBUTINE	2	5	5	5	5	2	5	5	5	5
ESCULINE	0	5	5	5	5	0	5	5	5	5
SALICINE	3	5	5	5	5	3	5	5	5	5
CELLOBIOSE	0	1	4	5	5	0	0	4	5	5
MALTOSE	0	0	0	0	0	0	0	1	1	2
LACTOSE	0	0	2	3	3	0	4	5	5	5
MELIBIOSE	2	4	5	5	5	5	5	5	5	5
SUCROSE	5	5	5	5	5	5	5	5	5	5
TREHALOSE	0	0	0	0	0	0	0	0	1	3
INULIN	0	0	0	0	0	0	0	0	0	0
MELIZITOSE	0	0	1	1	1	0	0	0	0	0
RAFFINOSE	0	1	5	5	5	5	5	5	5	5
AMIDON	0	0	0	0	0	0	0	0	0	0
GLYCOGEN	0	0	1	1	1	0	1	1	1	1
XYLITOL	0	0	1	1	1	0	0	1	1	1
β-GENTIOBIOSE	0	0	1	1	1	0	0	1	1	1
D-TURANOSE	0	0	0	0	0	0	0	0	0	0
D-LYXOSE	0	0	0	0	0	0	0	0	0	0
D-TAGATOSE	0	0	0	0	0	0	0	0	0	0
D-FUCOSE	0	0	0	0	0	0	0	0	0	0
L-FUCOSE	0	0	0	0	0	0	0	0	0	0
D-ARABITOL	0	0	0	0	0	0	0	0	0	0
L-ARABITOL	0	0	0	0	0	0	0	0	0	0
GLUCONATE	0	0	1	2	4	0	1	1	2	4
2-KETO-GLUCONATE	0	0	0	0	2	0	0	0	0	2
5-KETO-GLUCONATE	0	0	1	2	3	0	1	1	2	3

0 = Negative reaction, 1 = Doubtfully weak, 2 = doubtfully positive,
3 = weakly positive but indisputable, 4 = positive, 5 = very positive

inoculated plants i.e. the ability of EC-S to metabolize lactose was retained after passage through the plant.

Both strains were pathogenic as measured by the potato slice method, the slices rotting and turning black within 6h.

2.2.8. Studies on PL and β-galactosidase induction.

Rapid sampling from HXC-medium was difficult because of the presence of the calcium pectate gel. Furthermore, the medium could not be used in side-arm flasks for the rapid monitoring of growth by absorbance readings at 600 nm. The use of YS-medium overcame these problems, and was used in the following experiments, despite the low levels of PL produced on this medium.

The effect of lactose on PL synthesis in EC-S was investigated further on YS-medium supplemented with NaPP, lactose, and a mixture of both lactose and NaPP. The complementary experiment involving the effect of NaPP on β-galactosidase activity was also performed.

In the first experiment, an overnight culture of EC-S grown on YS-glycerol was used as inoculum. It was found that the differential rates of PL synthesis in the NaPP and lactose + NaPP cultures were similar, confirming the previous results that lactose did not repress PL synthesis (data not shown). The reciprocal experiment, in which the effect of NaPP on β-galactosidase was investigated, showed that the presence of NaPP in the

medium lead to much lower differential rates of β-galactosidase synthesis. This result suggested that NaPP could be a repressor of β-galactosidase activity in EC-S (data not shown).

These experiments were repeated, except that the inoculum consisted of lactose grown cells in order to ensure full induction of β-galactosidase at the beginning of the experiment. Again, it was observed that lactose had little effect on PL synthesis, which commenced after a short lag phase in both the NaPP and the NaPP + lactose supplemented cultures (Fig. 11). The differential rate of PL synthesis in the NaPP + lactose supplemented medium was lower than in the lactose supplemented medium up to an A_{600} of about 0,5, after which it increased to a rate comparable to that in the lactose supplemented culture (Fig. 12). The point at which the differential rate of β-galactosidase synthesis increased in the NaPP+lactose culture, corresponded to the point where the NaPP in the medium was completely depleted (Fig. 13). An increase in the reducing sugar levels was observed in both the NaPP and the NaPP+lactose supplemented cultures during growth, but not in the lactose supplemented culture (Fig. 14).

During the previous experiment, a slight lag was observed between the addition of the NaPP and the induction of PL. This lag period probably corresponds to the interval required for inducer generation by the basal levels of PL released into the medium by the organism during growth on non-pectic substances (Tsuyuma, 1977; Collmer and Bateman, 1982).
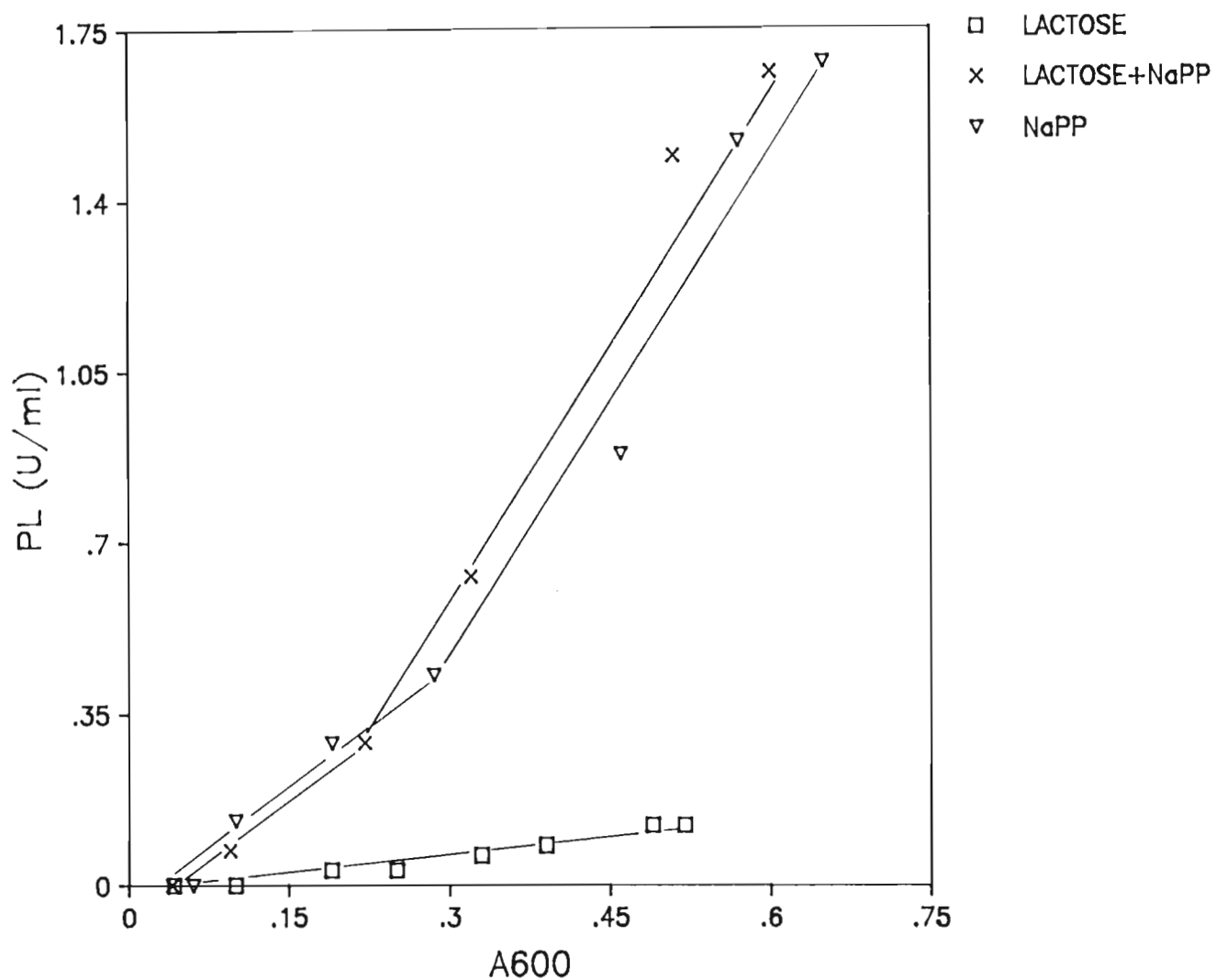
Fig. 11. Differential rate of PL synthesis by E. chrysanthemi strain EC-S in YS-medium supplemented with either 0.18% (w/v) NaPP, 0.09% (w/v) NaPP + 0.09% (w/v) lactose, or 0.18% (w/v) lactose. Growth was followed by measuring the absorbance at 600 nm.
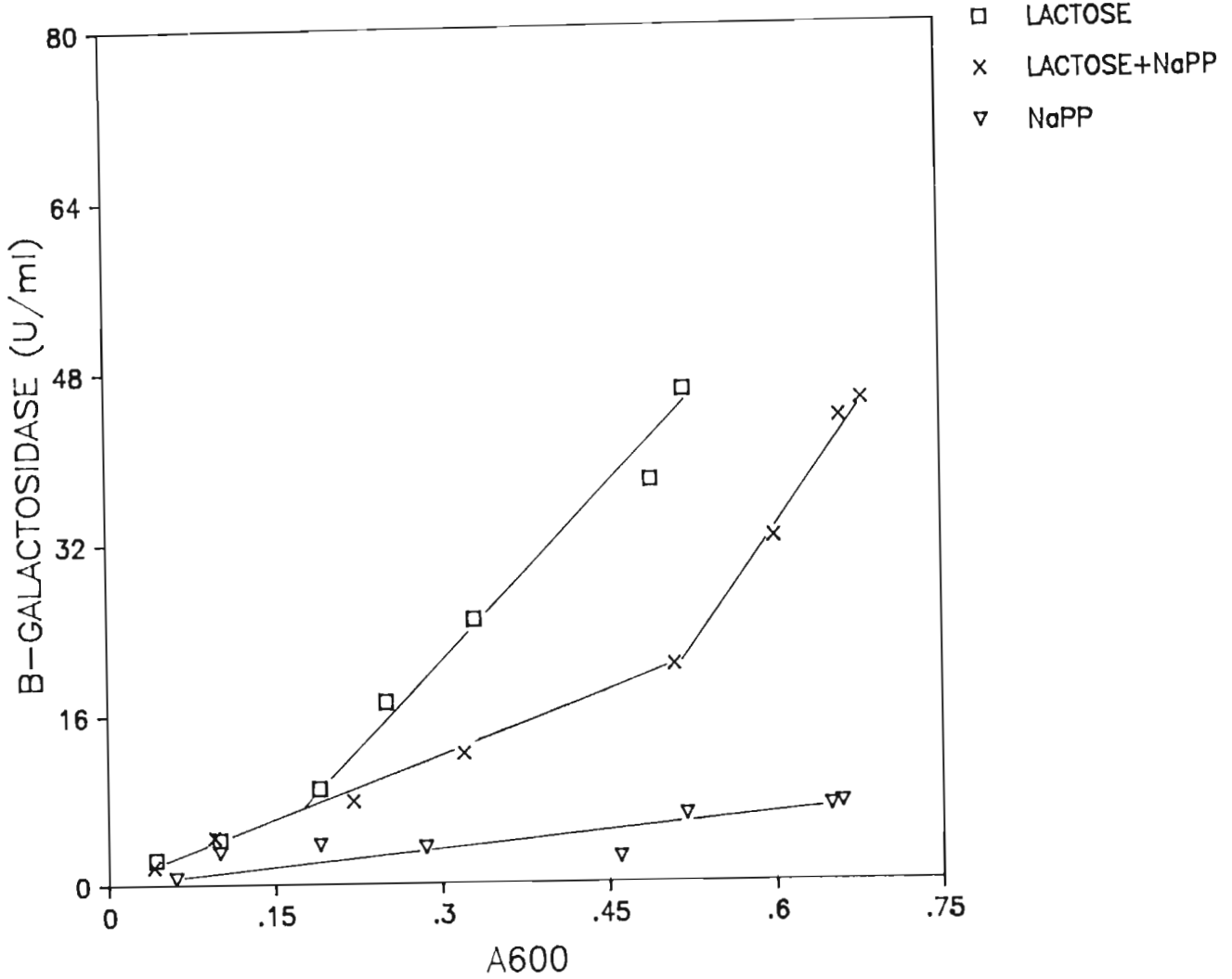
Fig. 12. Differential rate of β-galactosidase synthesis by E. chrysanthemi strain EC-S in YS-medium supplemented with either 0,18% (w/v) NaPP, 0,09% (w/v) NaPP + 0,09% (w/v) lactose, or 0,18% (w/v) lactose.
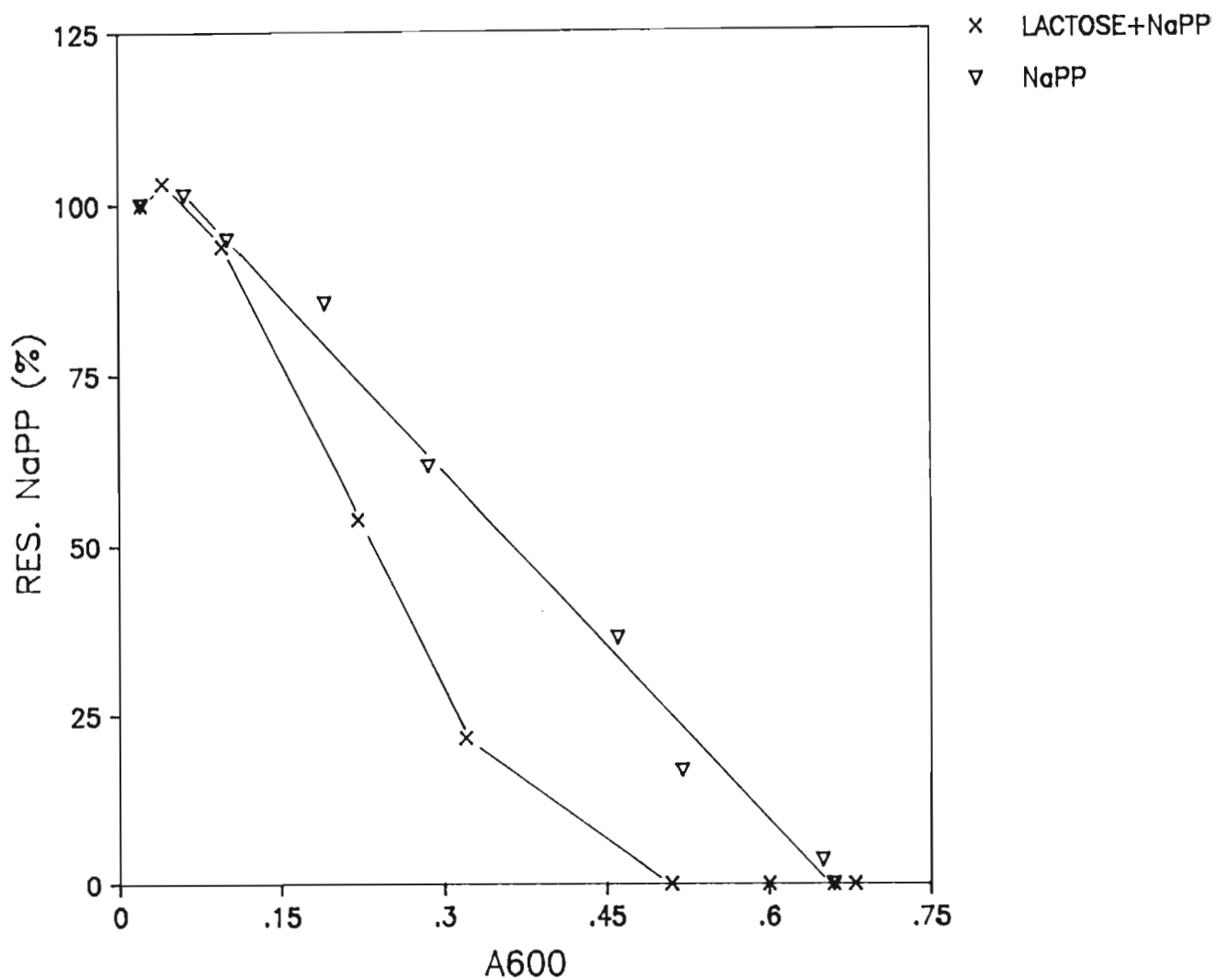
Fig. 13. Residual NaPP during growth of <u>E. chrysanthemi</u> strain EC-S on YS-medium supplemented with either 0,18% (w/v) NaPP, 0,09% (w/v) NaPP + 0,09% (w/v) lactose, or 0,18% (w/v) lactose.
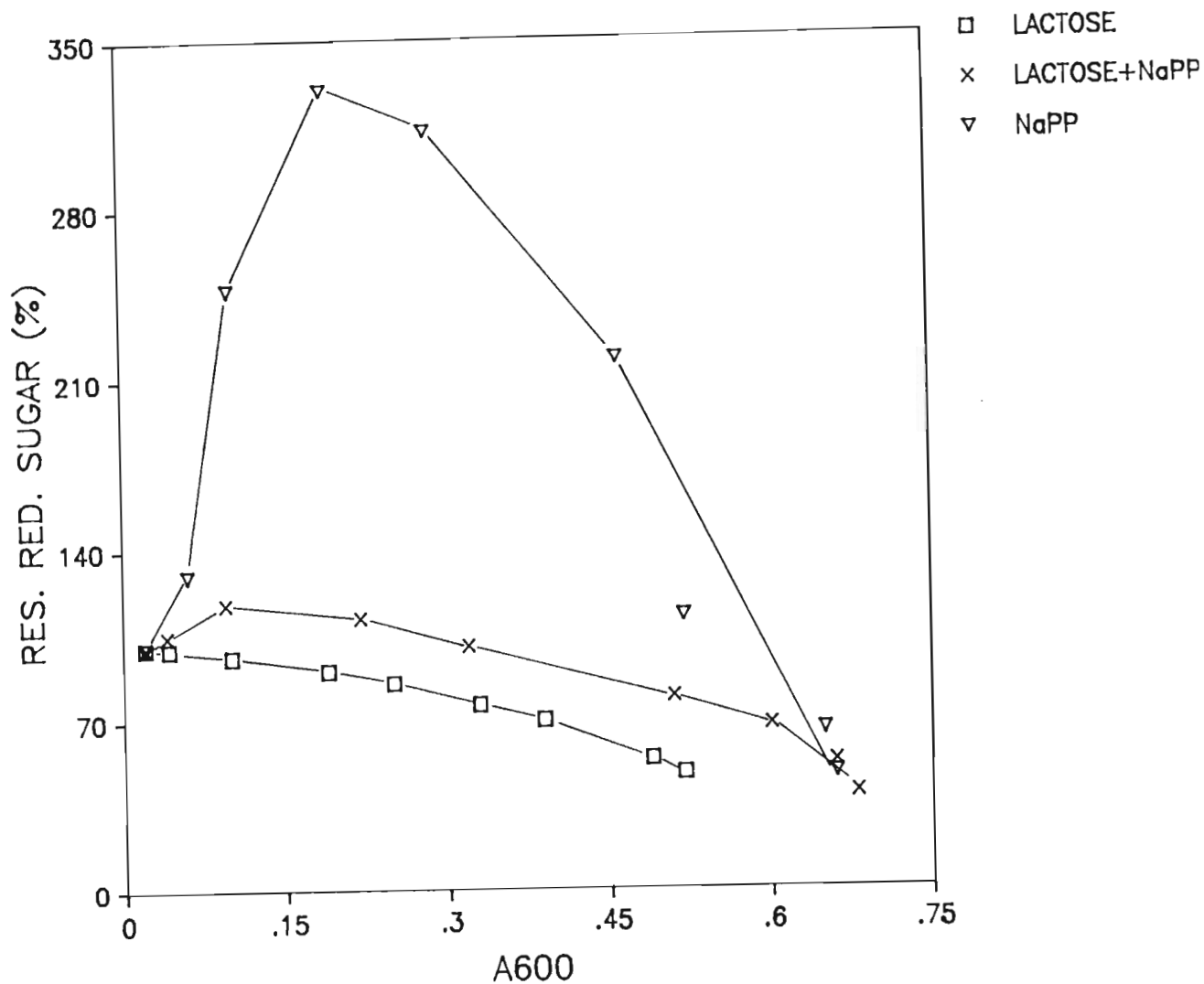
Fig. 14. Residual reducing sugar during growth of E. chrysanthemi strain EC-S on YS-medium supplemented with either 0,18% (w/v) NaPP, 0,09% (w/v) NaPP + 0,09% (w/v) lactose, or 0,18% (w/v) lactose.

The following experiment required that inducer production not be a limiting factor. Three preparations of partially degraded NaPP were tested for their ability to induce PL without a lag phase. Extensively degraded NaPP, while being a good substrate for growth, did not induce any PL in EC-S when present at 0.18%(w/v), and was not investigated further. Both a 15 and a 24 h PL-degraded NaPP, when added to a final concentration of 0.18% (w/v) to EC-S cells growing on glycerol, were equally effective as inducers of PL with no lag phase (Fig. 15). In the following experiments, a 15 h partial digest of NaPP was used.

Overnight cultures of EC-S grown on YS-medium supplemented with lactose were diluted into fresh medium containing lactose, and grown to an A_{600} of about 0.4. Partially degraded NaPP, 0.18% (w/v) final concentration, was added to the flasks, and growth, and PL and β-galactosidase synthesis measured at various intervals. Synthesis of PL commenced within 30 minutes of adding NaPP degradation products (Fig. 16) whereas the differential rate of β-galactosidase synthesis decreased over the same period (Fig. 17).

A similar experiment, in which IPTG replaced lactose as the inducer of β-galactosidase, gave similar results (data not shown).
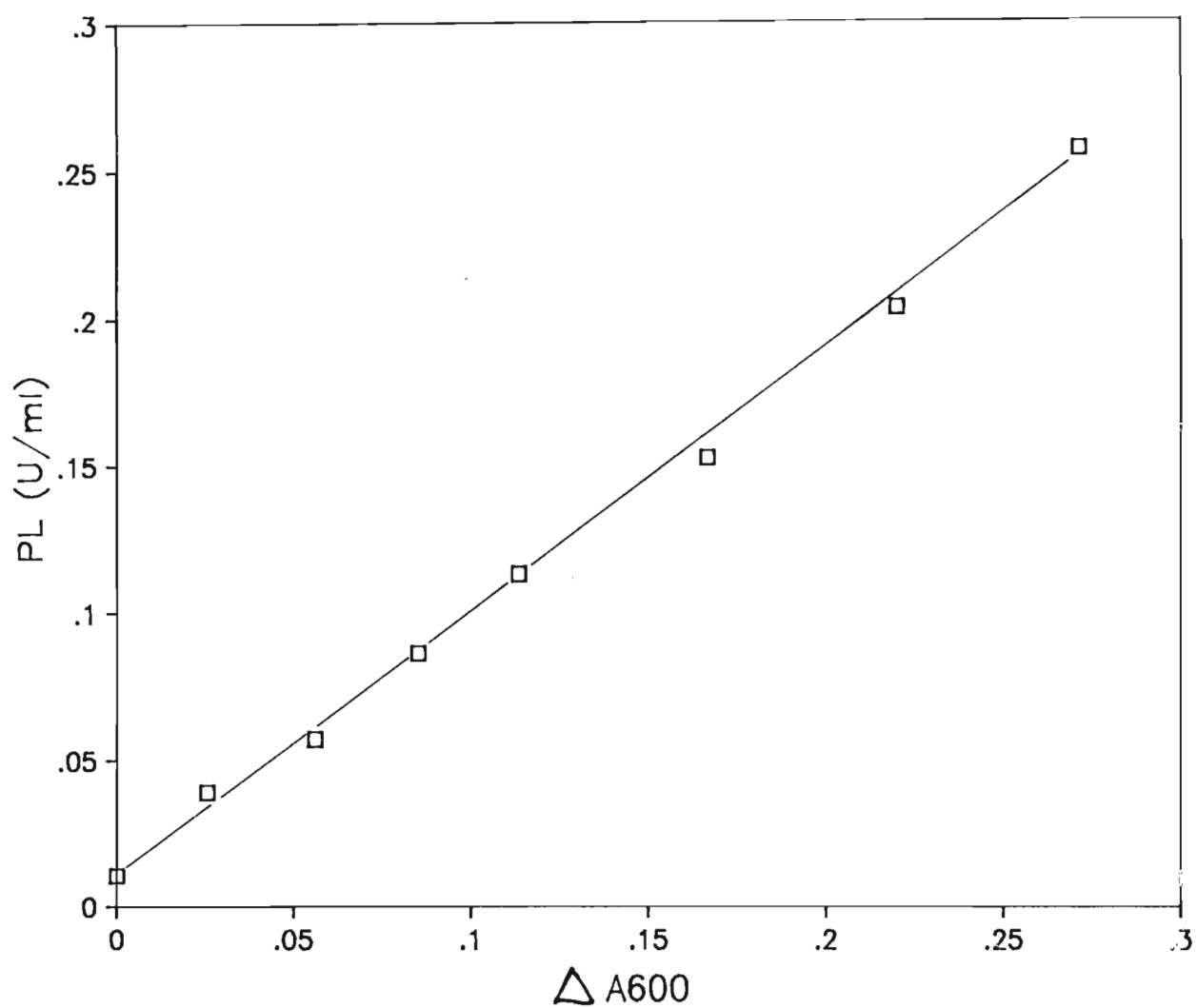
Fig. 15. Induction of PL by partially degraded NaPP. The X-axis represents the difference in A_{600} after addition of the partially degraded NaPP. The initial A_{600} reading of the culture was 0,4.
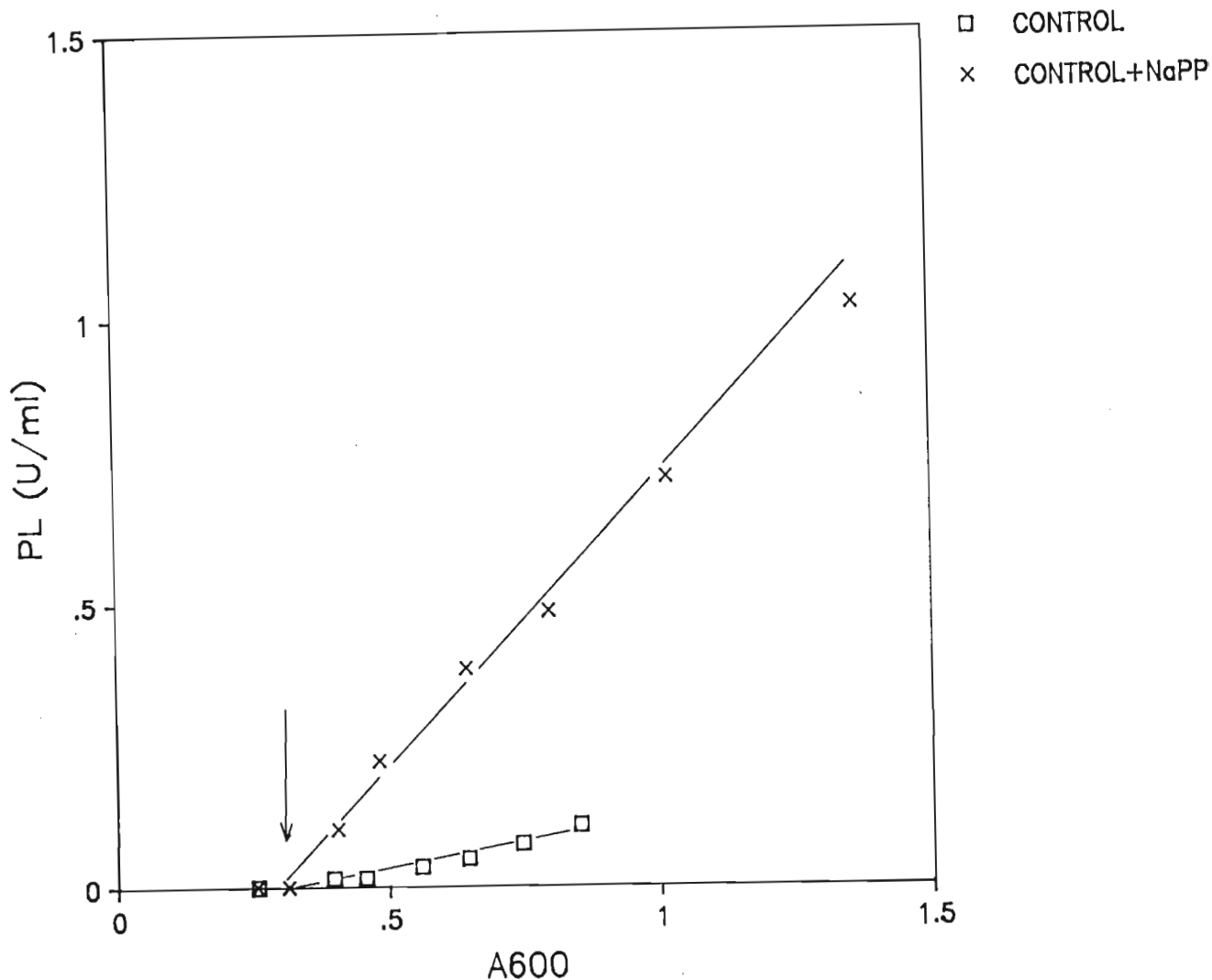
Fig. 16. Effect of NaPP on the differential rate of PL synthesis by $\underline{E}$. $\underline{chrysanthemi}$ strain EC-S growing on YS-medium supplemented with 0,18% (w/v) lactose. An overnight culture of $\underline{E}$. $\underline{chrysanthemi}$ strain EC-S grown on YS-medium was diluted into fresh medium supplemented with lactose, and grown to an A_{600} of about 0,1. At the arrow, partially degraded NaPP (final concentration 0,18% (w/v)) was added, and growth and PL synthesis followed.
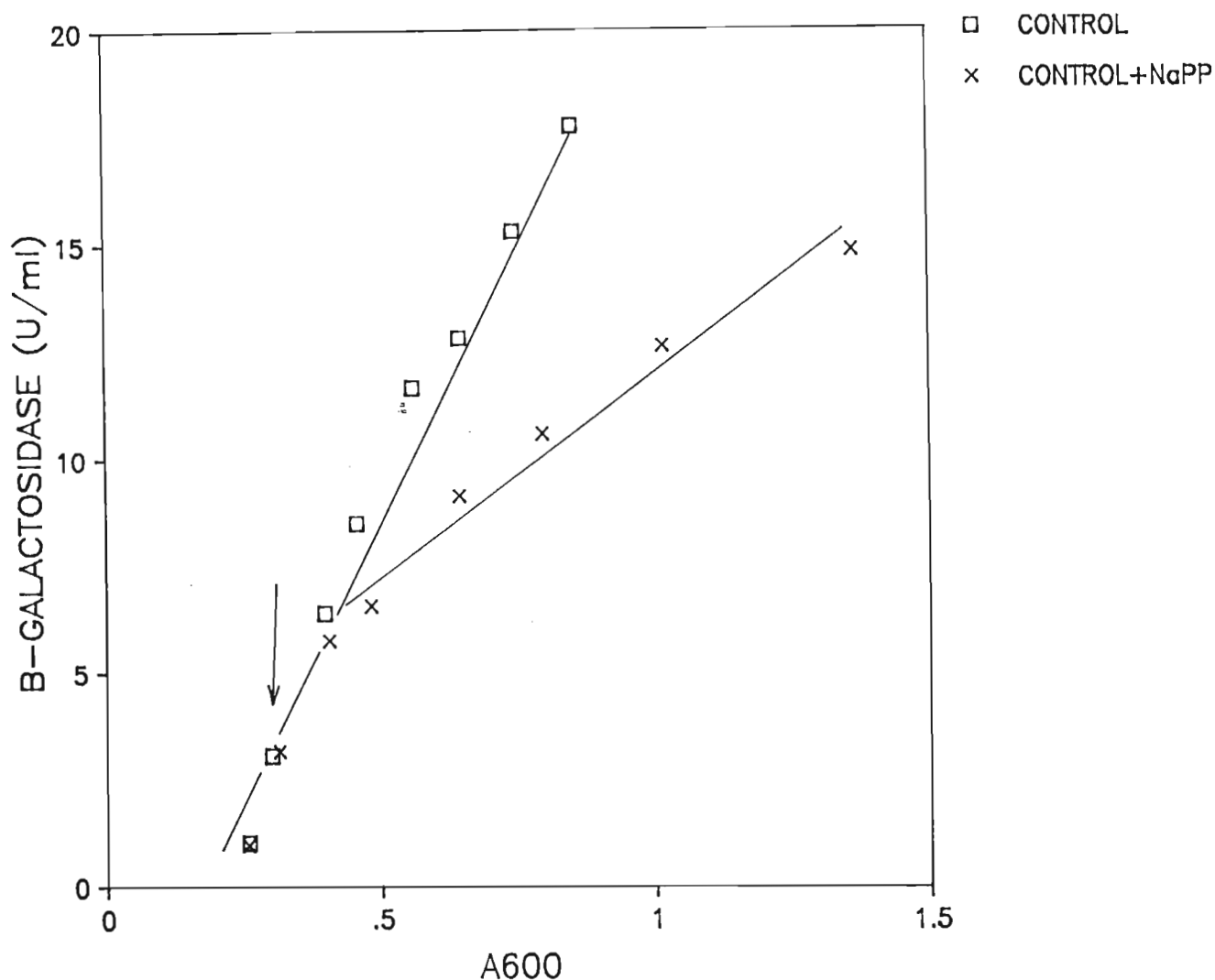
Fig. 17. Effect of NaPP on the differential rate of β-galactosidase synthesis by E. chrysanthemi strain EC-S growing on YS-medium supplemented with 0,18% (w/v) lactose. An overnight culture of E. chrysanthemi strain EC-S grown on YS-medium was diluted into fresh medium supplemented with lactose, and grown to an A_{600} of about 0,1. At the arrow, partially degraded NaPP (final concentration 0,18% (w/v)) was added, and growth and β-galacto-sidase synthesis followed.

Both β-galactosidase (Fig. 18) and PL synthesis (Fig. 19) were subject to catabolite repression by glucose. Glucose (1mM) repressed PL synthesis completely (Fig. 18), whereas β-galactosidase activity, although reduced substantially, was not completely inhibited (Fig. 19).
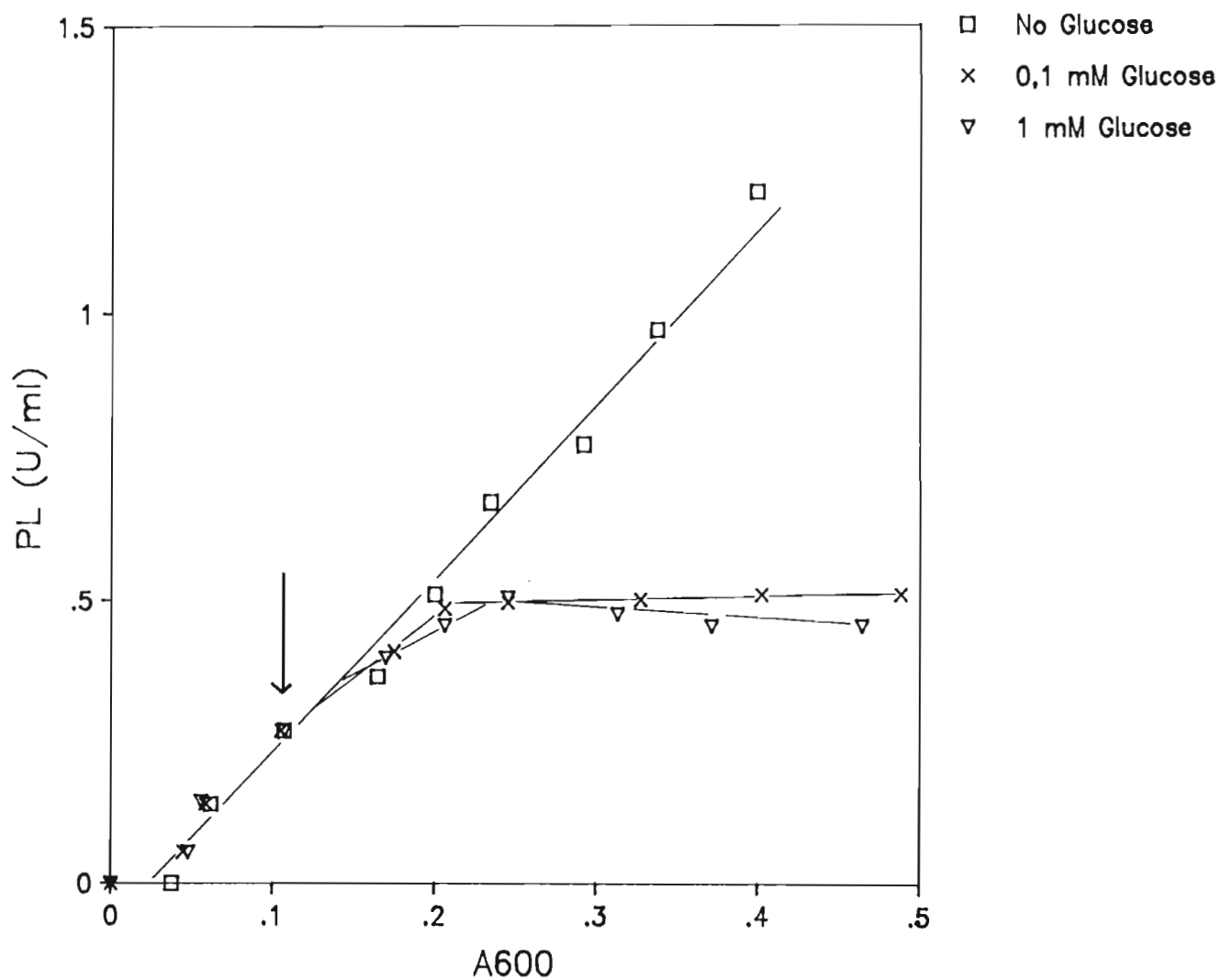
Fig. 18. Effect of 0,1 mM and 1,0 mM glucose on the differential rate of PL synthesis in E. chrysanthemi strain EC-S. Glucose was added at the arrow.
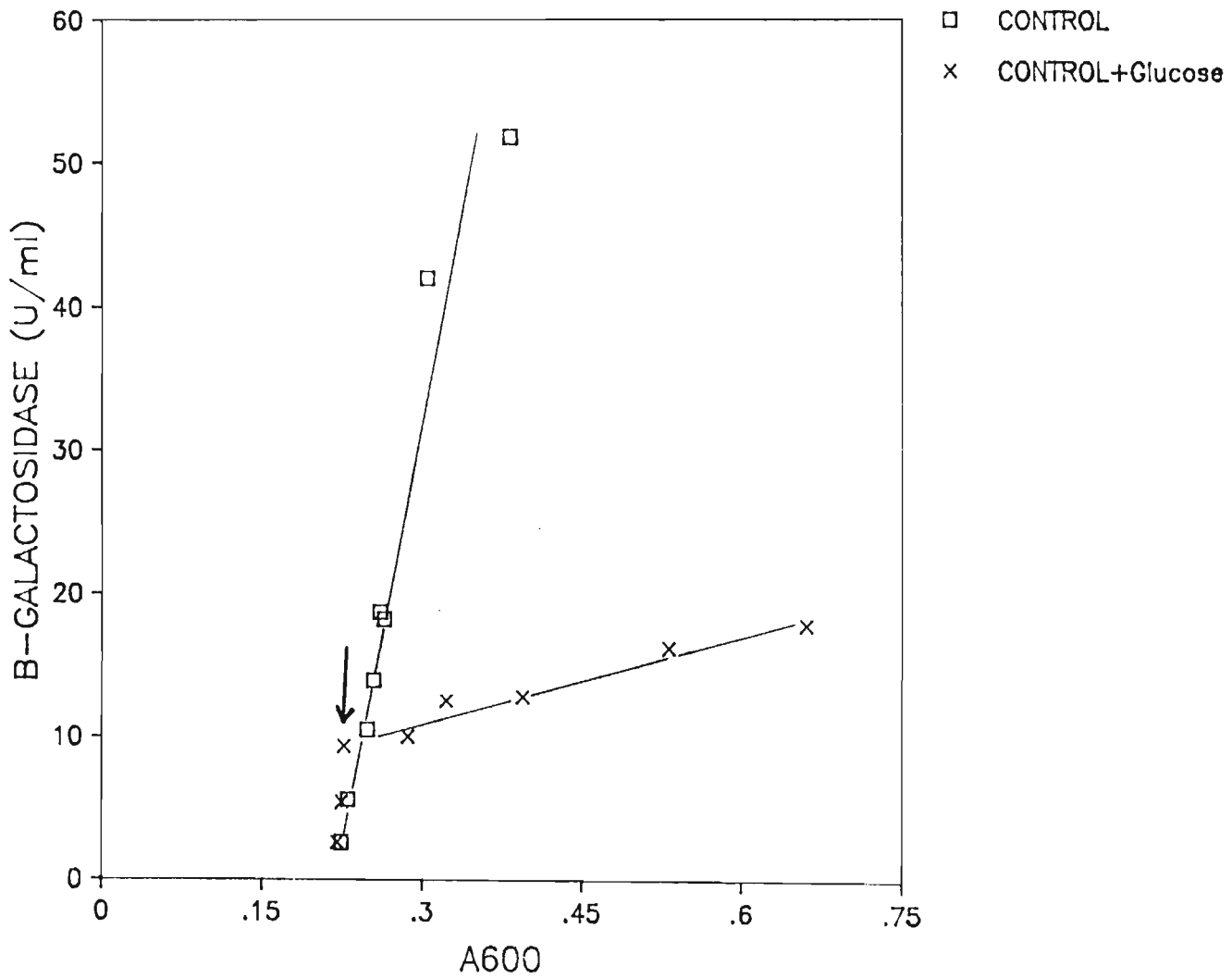
Fig. 19. Effect of 1.0 mM glucose on the differential rate of β-galacto-
sidase synthesis by E. chrysanthemi strain EC-S. Glucose was
added at the arrow.

2.3.   DISCUSSION.

The isolate of E. chrysanthemi used in this study secreted a PL into the medium. The identity of the enzyme was confirmed by the following observations.

(i).   The degradation products from NaPP absorb light maximally at 230 nm, which is characteristic of the unsaturated oligo-uronides generated by PL (Starr and Moran, 1962)

(ii).   The products from the degradation of NaPP generate a red chromophore after reaction with acid-periodate and thiobarbi-turic acid, again characteristic of unsaturated oligouronides (Weissbach and Hurwitz, 1959; Starr and Moran, 1962; Ashwell and Preiss, 1963).

(iii). The enzymes generating these chromophores all exhibited a pH optimum of 8,5 on NaPP, which is characteristic of bacterial PL (Starr and Moran, 1962; Fogarty and Kelly, 1983).

(iv).   The enzymes generating these chromophores are all dependent on Ca^{2+} for activity, and were completely inhibited by the addition of EDTA to the reaction mixture. Enzyme activity could be restored by the addition of Ca^{2+} to the reaction mixture. These results are typical of PL (Starr and Moran, 1962; Fogarty and Kelly, 1983).

At least two PL isozymes are produced by E. chrysanthemi (FH1), one with a pI of about 5,0, and the other with a pI of about 9,2. Isozymes with similar pI's have been reported for other strains of E. chrysanthemi (Garibaldi and Bateman, 1971, Pupillo et al., 1976; Collmer and Whalen et al., 1982). The pI 9,2 enzyme peak was broad, and exhibited a shoulder with an estimated pI of about 8,4. However, attempts to resolve this peak into additional PL isozymes on a narrower range ampholine gradient were unsuccessful, and resulted in the peak becoming broader rather than being resolved into individual isozymes. The failure to detect further isozymes in the pI 9,2 peak may be due to limitations of IEF in columns, as Bertheau et al. (1984) and Collmer et al. (1984), using analytical IEF in ultrathin flat polyacrylamide gels, detected 12 isozymes in E. chrysanthemi.

PL is an inducible enzyme in the local strain of E. chrysanthemi, resembling other strains of E. chrysanthemi (Chatterjee et al., 1978, 1979, 1985; Collmer and Bateman, 1979, 1981, 1982; Ferguson and Chatterjee, 1981; Collmer and Berman et al., 1982; Collmer and Whalen et al., 1982), E. carotovora (Tsuyumu, 1977, 1979; Moran and Starr, 1979; Mount et al., 1979), and Pseudomonas fluorescens (Zucker and Hankin, 1971).

The composition of the growth medium had a major effect on PL production. PL synthesis in HK-medium supplemented with 1,0% (w/v) was low despite good growth of E. chrysanthemi on the medium. Therefore, medium compon-

ents other than NaPP are required for high levels of PL synthesis. Although growth was lower on GB-medium than on XC-medium, PL production on this medium was higher than on XC-medium. The major difference between GB-medium and XC-medium is the presence of higher phosphate levels in GB-medium (0.1 M) as compared to XC-medium (0.008M). The greater buffering capacity of this medium may therefore play a role in the increased level of PL production. However, HXC-medium, which is similar in composition to XC-medium except for the increased levels of Ca^{2+}, gives rise to a four-fold increase in PL levels as compared to XC-medium, and a two-fold increase as compared to GB-medium. These data suggest that factors other than pure buffering capacity are involved in regulating PL synthesis. The results obtained with the increased Ca^{2+} levels are not readily explicable without further research. It has been demonstrated that the products of NaPP degradation are involved in both induction and "self-catabolite" repression of PL synthesis (Tsuyumu, 1977, 1979; Chatterjee et al., 1978, 1979, 1985; Collmer and Bateman, 1981, 1982). It is possible that the addition of excess Ca^{2+} to the medium with the formation of the insoluble calcium pectate gel, results in lower PL activity on the substrate, leading to a slower release of oligouronides, and hence a lower degree of "self-catabolite" repression. This possibility still has to be tested experimentally. The stimulatory effect of Ca^{2+} on PL production may play a significant physiological role, as the middle lamella contains calcium associated with pectic substances (Cooper, 1983).

Two major sugar transport systems exist in facultative anaerobes. The first, a group translocation, involves the coupled transport and phosphorylation of the sugar by the phosphoenolpyruvate:sugar phosphotransferase system (PEP:sugar PTS)(Saier, 1977, 1982; Dills et al., 1977). The second system involves a cation-coupled transport of sugars (Wilson et al., 1982; Wilson and Wilson, 1983).

It has been observed that the PEP:sugar PTS is involved in both catabolite repression and inducer exclusion (Saier, 1977, 1982; Dills et al., 1980; Botsford, 1981; Ullmann and Danchin, 1982). Catabolite repression, the process whereby glucose or some other readily utilizable sugar inhibits the metabolism of a compound requiring an inducible enzyme system, is mediated via a cyclic 3',5'-adenosine monophosphate (cAMP)-cAMP protein receptor (CRP) complex (Pastan and Adhya, 1976; Botsford, 1981; Ullmann and Danchin, 1982). The cAMP-CRP complex, on binding to the promoter region of the operon, increases the frequency of initiation of transcription, resulting in a higher level of operon expression (Botsford, 1981; Ullmann and Danchin, 1982). Factors that decrease the intracellular cAMP levels result in lowered levels of the cAMP-CRP complex, and a subsequent diminution of operon expression. The PEP:sugar PTS has also been implicated in the regulation cAMP levels in the cell by modulating the activity of adenyl cyclase (Saier, 1977, 1982; Dills et al., 1977, Ullmann and Danchin, 1982). Enzyme III^{Glc}, one of the components of the PTS, has been shown to play a crucial role in the activation of adenyl cyclase, although the exact mechanism is obscure (Daniel, 1984).

Inducer exclusion involves the inhibition of transport of the inducer into the cell, again resulting in lowered operon expression (Saier, 1977, 1982; Dills et al., 1980; Botsford, 1981; Ullmann and Danchin, 1982). Enzyme III^{Glc} has been shown to inhibit the both the lactose permease of E. coli (Osumi and Saier, 1982; Nelson et al., 1983, 1984; Saier et al., 1983), and the melibiose permease of S. typhimurium (Saier et al., 1983). Thus, the regulation of operon expression can occur in the absence of any direct effects of cAMP.

Little is known about sugar transport in E. chrysanthemi. However, the pattern of repression exerted by D-glucose, D-fructose, and D-mannitol suggest that they are, as in E. coli, PTS sugars (Dills et al., 1980). The most likely explanation for the strong repressive effect of sucrose on PL synthesis involves the hydrolysis of sucrose in the periplasmic space, and subsequent repression on transport of the glucose and fructose into the cell. This hypothesis would have to be proved by further experimental work.

The second group of sugars exerted an intermediate repressive effect on PL synthesis, and included a number of common components found in plant cells such as D-galacturonic acid, L-arabinose, cellobiose, and D-galactose. The effect of D-xylose appeared to be mediated without the sugar supporting good growth of the cells. The mechanism of repression of these sugars is not clear.

The effect of galacturonic acid on PL synthesis bears additional comment. It behaved both as a poor inducer and as a poor repressor of PL synthesis by E. chrysanthemi. Neither saturated nor the unsaturated digalacturonic acid is thought to be the real inducer of PL synthesis in E. chrysanthemi (Collmer and Bateman, 1981; Chatterjee et al. 1985). These two groups of workers have produced results indicating that the real inducer of PL is 4-deoxy-L-threo-5-hexulose uronic acid and/or 3-deoxy-D-glycero-2,5-hexo-diulosonic acid. Both of these deoxyketuronic acids arise as a result of the further metabolism of the OGL degradation products of intracellular saturated and unsaturated oligogalacturonides (Collmer and Bateman, 1981; Chatterjee et al., 1985) One of the intermediates in the metabolism of galacturonic acid is 2-keto-3-deoxy gluconic acid (Kilgore and Starr, 1959; Preiss and Ashwell, 1963a,b; Collmer and Bateman, 1981), whose structure is similar to the structure of these two compounds.

The mechanism of "self catabolite" repression is not known. The PTS does not seem to be directly involved, as neither the saturated nor the unsaturated oligogalacturonides appear to be a substrate for this transport system. However, cAMP does appear to be involved in "self catabolite" repression in both E. carotovora (Tsuyumu, 1979; Hubbard et al., 1978) and E. chrysanthemi (Collmer and Bateman, 1981). A factor, called the catabolite modulating factor (CMF), in conjunction with cAMP, may also play a role in catabolite repression (Ullmann et al., 1976; Dessein, Schwartz, and Ullmann, 1978; Dessein, Tillier, and Ullmann, 1978; Ullmann and Danchin, 1982). As CMF may be an intermediate of the metabolic pathway that is being repressed, it confers a specificity on

repression not found with a general compound such as cAMP. It appears from the work by Chatterjee et al. (1985) that one of the deoxyketuronic acids mentioned above may play a role in "self catabolite" repression, and may represent an example of a CMF.

The third group of sugars had little effect on PL production by E. chrysanthemi. The lack of effect of at least two of this group, lactose and L-rhamnose, may be best explained by the fact that they supported poor growth of E. chrysanthemi. The result with L-rhamnose is surprising, as E. chrysanthemi can use this sugar as a carbon source (see Table 5). Furthermore, as L-rhamnose is a component of the rhamnogalacturonan found in plant tissue, it may function as a nutrient during the disease process. The poor growth on rhamnose may, however, reflect a lag period during which the enzymes required for rhamnose metabolism are induced.

The effect of D-glucose, D-galacturonic acid, and lactose on PL production was investigated further as a function of the growth of E. chrysanthemi. These data confirmed the results from the previous experiment. The difference between the effect of D-glucose and D-galacturonic acid on PL synthesis again suggested that different mechanisms of repression are implicated with these two sugars. This will be discussed further below. The lack of effect of lactose on PL synthesis appears to be due to it not being metabolized by E. chrysanthemi.

The wild-type isolate of E. chrysanthemi (FH1) is Lac⁻. Growth on a poor medium containing lactose facilitated the isolation of Lac⁺ mutants. The

Lac$^+$ mutant isolated from the wild type strain of E. chrysanthemi closely resembled the parent in term of pathogenicity and general physiological characteristics. The two strains therefore appear to be identical except for the ability of the Lac$^+$ strain to metabolize lactose. Furthermore, melibiose and raffinose metabolism was initiated more rapidly in strain EC-S as compared to strain EC-C, suggesting the possible involvement of these two operons lactose metabolism.

The biochemical changes accompanying the Lac$^-$ to Lac$^+$ phenotype are described more fully in the next chapter. The rest of this chapter will deal with the data obtained during the simultaneous induction of PL and β-galactosidase in the Lac$^+$ mutant (E. chrysanthemi strain EC-S).

During invasion of the host, the pathogen is exposed to a variety of different nutrients, including small molecules such as melibiose, sucrose, and raffinose; structural carbohydrates such as the pectic polysaccharides, hemicelluloses, and cellulose, and proteins and lipids. Many of these compound require the induction of specific enzymes for their metabolism. The pathogen is therefore exposed to a mixture of inducers and possible catabolite repressors. How does the pathogen react to these often conflicting signals?

Little is known about these interactions in phytopathogenic bacteria, although some work has been performed on the sequential induction of cell wall degrading enzymes in phytopathogenic fungi (English et al., 1971; Jones et al., 1972). These workers found that isolated cell walls induced

the pectic enzymes in preference to the any other cell wall degrading enzymes (English et al., 1971; Jones et al., 1972). The ready accessibility of the pectic substances, as compared to the other cell wall components to enzymic degradation may explain, in part, this preferential production of the pectic enzymes. Little other work has been done on this aspect of enzyme induction in fungi.

Although no work of this nature on the sequential production of cell wall degrading enzymes has been done on phytopathogenic bacteria, some work on the simultaneous induction of a number of operons coding for intra-cellular enzymes (lac, ara, and trp operons) has been performed in E. coli (Lis and Schleif, 1973; Piovant and Lazdunski, 1975; Pavlasova et al., 1976,1977, 1980). It was found that the lac operon was always induced in preference to either the ara or trp operons. The conclusion reached by these workers was that the preferential order of induction of the different operons reflected their requirement for different cAMP levels for maximum expression (Lis and Schleif, 1973; Piovant and Lazdunski, 1975; Pavlasova et al., 1976,1977, 1980). Thus, the lac operon could be induced at lower concentrations of cAMP than either the ara (Lis and Schleif, 1973), or trp operon (Piovant and Lazdunski, 1975; Pavlasova et al., 1976,1977, 1980).

In E. chrysanthemi, PL is an extracellular enzyme and β-galactosidase an intracellular enzyme. Contrary to expectations, NaPP was always the preferred substrate when EC-S was grown on a mixture of NaPP and lactose. This occurred even when the cells were pre-induced on lactose, and resus-

pended in YS medium supplemented with both NaPP and lactose. Lactose neither induced PL nor did it inhibit its synthesis. In contrast, NaPP did not induce β-galactosidase, although it did repress β-galactosidase synthesis. The synthesis of PL commenced shortly after the addition of NaPP to E. chrysanthemi strain EC-S growing in YS-lactose medium, with a concomitant repression of β-galactosidase synthesis. The observation that β-galactosidase synthesis only commenced once the NaPP in a medium containing both NaPP and lactose was depleted suggests that this polysaccharide, or its degradation products, was directly involved in the repression of β-galactosidase. Inducer exclusion does not appear to be involved, as similar results were obtained with IPTG, a compound that can enter the cell in the absence of a permease.

If the above effects are mediated via a cAMP-CRP mechanism, then the data suggests that PL synthesis requires a lower cAMP level than is required for expression of the lac operon. However, the data obtained by glucose inhibition of PL and β-galactosidase synthesis does not support this interpretation, as PL synthesis was equally, or more sensitive than β-galactosidase to catabolite repression by glucose. This dichotomy remains unresolved, and may reflect the involvement of different mechanisms of catabolite repression associated with NaPP and glucose.

The problem was not studied further, as the cAMP deficient mutants were no available for the study of different concentrations of cAMP on PL synthesis. Furthermore, it was felt that a study of the basic aspects of lactose metabolism by E. chrysanthemi warranted immediate attention. The

mechanism involved in "self catabolite" repression, however, would seem to be a fruitful future research area, and well worth pursuing.

PL is also an extracellular enzyme, and the picture may be further complicated by additional regulatory mechanisms associated with secretion of the enzyme. The complete cessation of PL synthesis/secretion into the medium after addition of glucose to the medium is another difference observed between the effect of glucose on PL and β-galactosidase synthesis, and suggests that glucose may influence both synthesis and secretion of PL into the medium. The question of regulation of protein secretion in prokaryotes has not received much attention to date, and should prove a challenging problem.

There are obvious biological advantages to the organism in being able to regulate the release of extracellular enzymes. Foremost is the conservation of relatively unique carbon sources in the environment that enhances the competitiveness of the organism in nature. Many organism are able to use glucose in nature, whereas relatively few are able to use pectic compounds. Another possibility to be considered results from the observation by Davis et al. (1984) that endo-PL can release oligogalacturonides from soy bean cell walls that are phytoalexin elicitors. Although similar work has not been done on maize infected with E. chrysanthemi, the possibility remains that expression of the PL enzymes at the wrong stage during infection may result in the production of oligogalacturonides, and subsequent induction of phytoalexin synthesis.

The results presented in this chapter were not intended as a comprehensive study, but rather as part of the characterization of the locally isolated strain of E. chrysanthemi. This strain of E. chrysanthemi appears similar to other strains of E. chrysanthemi described in the literature in terms of the properties of its PL, its isozyme spectrum, the requirement for NaPP or its degradation products for PL induction, and the sensitivity of PL to catabolite repression.

Some aspects of the work described in this chapter could be profitably extended, particularly the role of calcium in PL induction and its involvement in the disease process. Furthermore, the sequential production of enzymes involved in cell wall degradation, and their role in pathogenicity remains a challenging question.

CHAPTER 3

LACTOSE METABOLISM BY ERWINIA CHRYSANTHEMI

In chapter 2, it was shown that a local strain of E. chrysanthemi (FH1) was found to be Lac$^-$ (EC-C) but that a Lac$^+$ (EC-S) strain could be isolated readily after extended growth on a poor medium containing lactose as the major carbon source. In this chapter studies on lactose metabolism in strains EC-C and EC-S are continued and the underlying biochemical changes associated with the Lac$^-$ to Lac$^+$ mutation are investigated.

Both E. chrysanthemi and E. coli are members of the Enterobacteriaceae moreover, the lac operon of E. coli has been well characterised. Therefore it seemed natural to enquire whether E. chrysanthemi possessed a similar genetic apparatus. The lac operon in E. coli codes for three well-defined activities namely, lactose transport (the lacY gene), lactose hydrolysis (the lacZ gene) and thiogalactoside transacetylase activity (the lacA gene), and it was therefore necessary to try to confirm the presence of these activities in E. chrysanthemi. Eventually, it became clear that a lac operon analogous to that in E. coli was not functional in E. chrysanthemi. Differences in lactose metabolism between EC-C and EC-S could not, therefore, be ascribed to mutations within a lac operon. A remaining problem was the nature of the constitutive lactose transporter in EC-S. It seemed plausible to assume that this permease

normally transports some other sugar encountered in the organism's natural environment but, coincidentally, transports lactose as well. Consequently, a search was undertaken for other sugars that might compete with lactose for the EC-S permease. Greatest competition was observed with either melibiose or raffinose. Furthermore, each of these sugars induced a distinct lactose permease in EC-C, one of which appears to be associated with the mel operon, and the other with the raf operon. As is shown below, constitutive expression of the the melibiose permease is probably responsible for the observed lactose permease activity of strain EC-S. It is concluded that the components required for lactose metabolism in E. chrysanthemi are not coded for by a single operon, but by at least two independently regulated operons.

3.1. MATERIALS AND METHODS.

3.1.1. Organism.

The E. chrysanthemi (FH1) strains EC-C and EC-S described in Chapter 2 were used in the studies reported in this chapter.

3.1.2. Media.

The following medium was used in addition to those described in Chapter 2.

A.   Modified M-63 medium.

M-63 medium was prepared as described by Miller (1972, pp. 431-435), and modified according to Robbie and Wilson (1979), and contained:

Distilled H_2O	1 l
KH_2PO_4	13,6 g
$(NH_4)_2SO_4$	2,0 g
$FeSO_4.7H_2O$	0,5 mg
NaCl	2,9 g

The pH was adjusted to pH 7,0 with 10 N NaOH, dispensed as required, and sterilized by autoclaving. Each litre of medium was supplemented as follows:

Mg^{2+}	1 ml of a 1 M solution of $MgSO_4$
Yeast extract (Difco)	10 ml of a 9% (w/v) solution
Sugar/carbon source	50 ml, as specified in the text

The Mg^{2+}, and YE were autoclaved. The sugar, or carbon source was either autoclaved or filter sterilized (0,22 um Millex filter, Millipore).

3.1.3. Glucose Analysis by the Glucose Oxidase-Peroxidase (GOD-POD) Procedure.

Glucose was assayed using a commercial single reagent kit (Merckotest Glucose, GOD-PAP method, E Merck, Darmstadt). Glucose oxidase (β-D-glucose:oxygen-1-oxidoreductase, EC 1.1.3.4) catalyses the oxidation of β-D-glucose to gluconic acid and H_2O_2. The H_2O_2, in the presence of peroxidase (POD, Donor:hydrogen peroxide-oxidoreductase, EC 1.11.1.7), reacts with 4-aminophenazone and 2,4-dichlorophenol to form the dye, antipyrylchloroquinone imine with an absorption maximum at 510 nm. The amount of dye formed is proportional to the amount of glucose oxidized.

A.   Reagents.

(i).   Reaction Solution. The reaction solution contained:

        0,1 M phosphate-0,1 M Tris buffer, pH 8,0

        6 kU 1^{-1} GOD

        2,2 kU 1^{-1} POD

        0,25 mM 4-aminophenazone

        0,30 mM 2,4-dichlorophenol

(ii).   Glucose Standard. The glucose standard contained 10 mg glucose ml^{-1}.

B.   Procedure.

Samples (0,1 ml) were mixed with reaction mixture (2 ml), and allowed to stand at room temperature (20-22°C) for between 30 min and 3 h before reading absorbance at 510 nm against a reagent blank in a Beckman Model 35 Spectrophotometer. A standard curve containing between 0 and 0,06 mg ml^{-1} glucose was prepared at the same time.

3.1.4.   Glucose Analysis by the Hexokinase/Glucose-6-phosphate Dehydrogenase Method.

Glucose was assayed by measuring the reduction of NADP$^+$ in the coupled hexokinase/glucose-6-phosphate dehydrogenase (HK/G6P-DH) reaction, using the method published by Boehringer Mannheim (1977). This method is based on the procedure described by Bergmeyer et al. (1974).

A.   Reagents.

(i).   Buffer (0,75 M Triethanolamine hydrochloride-10 mM Mg^{2+}).
          Triethanolamine hydrochloride (14,0 g) and $MgSO_4.7H_2O$ (0,25 g)
          were dissolved in H_2O (80 ml), titrated to pH 7,6 with 5 N
          NaOH and made up to 100 ml with distilled water. The buffer
          was stored at 4°C, and was stable for 1 month.

(ii).   <u>NADP</u>. NADP solution (11,5 mM) was prepared by dissolving NADP-Na$_2$H (60 mg) in H$_2$O (6 ml), and stored at 4°C under which conditions it was stable for 1 month.

(iii).   <u>ATP</u>. ATP solution (81 mM) was prepared by dissolving ATP-Na$_2$H$_2$ (300 mg) and NaHCO$_3$ in H$_2$O (6 ml). The solution was stable at 4°C for 1 month.

(iv).   <u>HK/G6P-DH</u>. The HK/G6P-DH suspension in 3,2 M (NH$_4$)$_2$SO$_4$, purchased from Boehringer Mannheim, was used undiluted and contained HK (EC 2.7.1.1, 2 mg ml^{-1}) and G6P-DH (EC 1.1.1.4, 1 mg ml^{-1}). Both enzymes had a specific activity of 140 U mg^{-1} protein.

(v).   <u>Glucose Standard</u>. The glucose standard used in the GOD-POD glucose assay (Section 3.1.3) was used to prepare a standard curve.

B.   <u>Procedure.</u>

Samples (up to 1 ml) were pipetted either into disposable plastic

cuvettes or into matched 13 X 100 mm test-tubes (Kimble), and made up to 2 ml with H_2O. The following solutions were added to the samples:

Buffer	1,00 ml
NADP	0,10 ml
ATP	0,10 ml

The solutions were mixed, and after 5 min, their absorbances were determined at 340 nm in a Beckman Model 35 Spectrophotometer (plastic cuvettes) or in a Bausch and Lomb Model 88 Spectrophotometer (13 mm test tubes). HK/G6P-DH suspension (0,02 ml) was added, the solutions mixed, and the reaction allowed to proceed to completion. Preliminary experiments showed that the reaction was complete within 12 min, and that there was no change in A_{340} after a further 18 min incubation at room temperature. The absorbances at 340 nm were determined against a reagent blank, and corrected for any absorbance found before the addition of the HK/G6P-DH suspension. The linearity of the assay was checked by running a standard curve (0–0,1 mg ml^{-1}), and found to be linear up to 0,08 mg of glucose. A molar extinction coefficient for NADPH of 6.22×10^3 M^{-1} cm^{-1} was also used to calculate the glucose concentration of unknowns.

3.1.5.   Preparation of cell-free extracts.

Bacteria were recovered by centrifugation (10 000 X g, 4°C, 20 min),

resuspended in 10 mM Tris-acetate buffer, pH 7,5 containing 10 mM NaCl, and recentrifuged as described above. After resuspension in the same buffer, supplemented with 10 mM 2-mercaptoethanol (2-ME), the cells were disrupted by subjecting them to three 30 sec bursts in a MSE 150 W sonicator at 4°C with 24-26 micron amplitude with a two minute cooling period between each burst. The cell debris was removed by centrifugation (48 000 X g, 4°C, 15 min), and the supernatants stored at 4°C until use.

The β-galactosidase was found to be stable for at least 2 weeks if stored at this stage. For some experiments, aliquots of the supernatant were dialyzed against 50 mM imidazole buffer, pH 7,0 at 4°C (2 X 100 volumes). The enzyme under these conditions was found to be relatively unstable and lost between 25 and 50% of activity in 24 h, therefore fresh enzyme was prepared for each experiment.

3.1.6.   Protein assay.

Protein was assayed by the Lowry procedure (Lowry et al., 1951) as described in Chapter 2 (Section 2.1.6).

3.1.7. Assay of β-galactosidase activity in permeabilized cells with o-nitrophenol-β-D-galactopyranoside as substrate.

A.   Reagents

(i).   o-Nitrophenyl-β-D-galactopyranoside (β-ONPG). β-ONPG (100 mg) was dissolved in H_2O (25 ml) to give a 13,2 mM solution. The solution, prepared fresh weekly, was stored at 4°C.

(ii).   Buffer. Z-buffer was prepared as described in Chapter 2, Section 2.1.11.

(iii).   Sodium carbonate solution.   A 1 M solution of Na_2CO_3 was prepared as described in Chapter 2, Section 2.1.11.

(iv).   Lysis Mixture. The lysis mixture (LM) of Putnam and Koch (1975) was modified by replacing the sodium dodecyl sulphate (SDS) with an equivalent amount of sodium deoxycholate (DOC). The lysis mixture contained:

10% DOC	1 part
0,02 M $MnSO_4$	1 part
Toluene	1 part
2–ME	5 parts

B.   Procedure.

(i).   Toluene as permeabilizing agent (Miller, 1972, pp. 352-355).
          The procedure described in Chapter 2 (Section 2.1.11) was
          followed.

(ii).   Chloroform-SDS as permeabilizing agent (Miller, 1972, pp.
          352-355). The assay described in section (i) was repeated,
          except that 1 drop of chloroform and 1 drop of 0,1% SDS was
          added to the flasks to permeabilize the cells. The flasks were
          not shaken at 37°C for 40 min, but were assayed immediately at
          30°C.

(iii).   Toluene-deoxycholate as permeabilizing agent (Revel et al.,
          1961). The assay described in section (i) was repeated except
          that one drop of toluene and one drop of 1% (w/v) DOC was
          added. After shaking at 37°C for 40 min, the cells were assayed
          at 30°C as described above.

(iv).   LM1 as permeabilizing agent (Putnam and Koch, 1975). Bacterial
          cell suspension (0,1-0,5 ml, depending on activity) was
          pipetted into a 100 X 13 mm test tube, made up to 1 ml with
          Z-buffer, 0,025 ml LM1 added, and the tube immediately vor-
          texed. After equilibration at 30°C, the reaction was started by
          the addition of 0,2 ml β-ONPG, and the assay completed as
          described in section (i) above.

(v).   <u>LM2 as permeabilizing agent</u>. The assay in (iv) was repeated except for the replacement of LM1 by LM2.

3.1.8.   Assay of β-galactosidase in cell-free extracts with β-ONPG as substrate.

A.   <u>Reagents</u>

The same reagents reported in section 3.1.7 were used except that the Z-buffer was replaced by 50 mM imidazole hydrochloride, pH 7,0 containing 10 mM NaCl, 1,0 mM $MgSO_4$ and 100 mM 2-ME. This buffer was prepared by dissolving imidazole (3,404 g), NaCl (0,584 g), $MgSO_4.7H_2O$ (0,246 g), and 2-ME (7,0 ml) in 900 ml H_2O and titrating to pH 7,0 with 2 N HCl before making up to 1 l with H_2O.

B.   <u>Procedure</u>

The reaction mixture contained 0,95 ml buffer, and 0,2 ml substrate (β-ONPG). After temperature equilibration (30°C, 5 min), the reaction was started by the addition of 0,05 ml of suitably diluted enzyme. The reaction was stopped by the addition of 1 ml of 1 M Na_2CO_3 after the appearance of a definite yellow colour. The absorbance of the solution were read at 420 nm against a blank prepared by adding the enzyme to the substrate after addition of the 1 M $NaCO_3$.

3.1.9.   Assay of β-galactosidase on lactose.

A.   <u>Reagents</u>

(i).   <u>Buffer</u>.   100 mM imadazole buffer, pH 7,2, containing 20 mM
NaCl and 13,4 mM $MgSO_4$. Imadazole (6,808 g), NaCl (1,168 g)
and $MgSO_4.7H_2O$ (3,300 g) were dissolved in 900 ml of distilled
water, the pH adjusted to 7,2 with 2 N HCl, and the volume made
up to 1 l with water.

(ii).   <u>100 mM Lactose</u>.   Lactose (3,423 g) was dissolved in H_2O (100
ml), and stored in the refrigerator. Fresh solutions were
prepared weekly.

(iii).   <u>5 M Potassium carbonate</u>.   A 5 M solution of potassium carbon-
ate was prepared by making 6.911 g of the anhydrous salt
up to 10 ml with water.

B.   <u>Procedure</u>

The reaction mixture contained:

Buffer	0,75 ml
Lactose	0,15 ml
H_2O	0,50 ml

After temperature equilibration at 30°C for 5 min, the reaction was started by the addition of 0.1 ml of suitably diluted enzyme. The reaction was stopped by the addition of 0.15 ml 33% (w/v) perchloric acid, and kept at 4°C for 10 min to allow precipitation of protein, which was removed by centrifugation in a Beckman Microfuge (9 800 X g, 2 min). The supernatant was neutralized with a predetermined volume of K_2CO_3 (using phenol red as an indicator), and the insoluble $KClO_4$ allowed to precipitate at 4°C for 10 min. Aliquots of the supernatants were assayed for glucose by the HK/G6P-DH method (Section 3.1.4).

Trichloroacetic acid (5% (w/v) final concentration) replaced the perchloric acid as protein precipitant when the GOD-POD glucose assay (Section 3.2.5) was used. The TCA supernatants were not neutralized before the assay of glucose by the GOD-POD method, as the enzymes used in this assay are not affected by 5% TCA (manufacturer's instructions and personal observation).

3.1.10. α-Galactosidase activity in permeabilized cells with o-nitro-phenyl-α-D-galactopyranoside (α-ONPG) as substrate.

A.   Reagents

(i).   o-Nitrophenyl-α-D-galactopyranoside (α-ONPG).   A 20 mM solution of α-ONPG was prepared by dissolving α-ONPG (120.52 mg, Sigma) in H_2O (20 ml). Fresh solutions were prepared weekly, and stored at 4°C.

(ii).   100 mM Tris-HCl buffer, pH 7,5. Tris (6,056 g, Trizma base, Sigma) was dissolved in H_2O (450 ml), titrated to pH 7,5 with 2 N HCl, made up to 500 ml, and the pH readjusted. Buffer preparation and pH adjustment were done at $22^{o}C$.

(iii).   Sodium carbonate-10 mM EDTA. One millilitre of a 1 M EDTA solution (3,722 g per 10 ml H_2O, titrated with 10 N NaOH to dissolve the EDTA) was added to 100 ml of a 1 M Na_2CO_3 solution.

(iv).   50 mM Dithiothreitol (DTT). DTT (79,47 mg, Boehringer Mannheim) was dissolved in H_2O (10 ml), and stored frozen.

(v).   50 mM Manganous chloride. $MnCl_2.4H_2O$ (0,990 g) was dissolved in H_2O (100 ml) immediately before use.

B.   Procedure.

Cell suspension (0,1 ml), buffer (0,5 ml), H_2O (0,3 ml), and toluene (0,025 ml) were pipetted into a 13 X 100 mm test tube, vortexed, and incubated at $30^{o}C$ for 5 min. The reaction was started by the addition of substrate (0,1 ml), and terminated by the addition of 1 ml of 1 M Na_2CO_3 after the appearance of a definite yellow colour. After suitable dilution with H_2O, the absorbance at 420 and 550 nm was determined in a Spectro-

nic 88 spectrophotometer (Bausch and Lomb). Further calculations were carried out as described in Section 3.2.8.

When the effects of DTT and Mn^{2+} on enzyme activity were tested, they were added to a final concentration of 5 mM without changing the volume of the reaction mixture. The reaction was terminated by the addition of 1 ml of 1 M Na_2CO_3–0,01 M EDTA when DTT and Mn^{2+} were present in the assay, as the coloured Mn^{2+}-DTT complex, formed under alkaline conditions in the absence of EDTA, leads to high blanks (Schmitt and Rotman, 1966).

3.1.11.   Thiogalactoside Transacetylase Assay.

Thiogalactoside transacetylase (acetyl–CoA:galactoside–6-O-acetyltrans-ferse, E.C. 2.3.1.18) transfers the acetyl group from acetyl coenzyme A (acetyl–CoA) to an acceptor such as IPTG (Musso and Zabin, 1973; Zabin and Fowler, 1980) according to the reaction.

Acetyl–CoA + IPTG $\longrightarrow$ CoA + acetylated IPTG
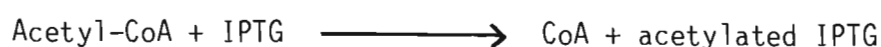
Although acetyl coenzyme A appears to be the natural substrate of the enzyme (Km, 1.8 X 10^{-3} M), the same is not thought of IPTG because of its high Km (0,77 M, Musso and Zabin, 1973). The natural acceptor of the enzyme is not known as no compounds have been found that saturate the enzyme at low concentrations (Musso and Zabin, 1973).

The enzyme was assayed spectrophotometrically, as described by Miller (1972, pp. 371-373), by measuring the amount of free CoA formed during the reaction using the reagent 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB). The reaction involves a disulphide exchange producing thionitrobenzoic acid with an absorption maximum at 412 nm.

A.   Reagents.

(i).   Buffers.

   a.   0,05 M Tris-0,01 M EDTA, pH 7,9. This was prepared by dissolving EDTA ($Na_2EDTA.2H_2O$, 0,372 g) in H_2O (80 ml), and adding 5 ml of 1 M Tris base (Trizma, Sigma, 12,11 g 100 ml^{-1} H_2O). The pH was titrated to 7,9 with 2 N HCl, and the buffer made up to 100 ml after which the pH was readjusted if necessary.

   b.   0,05 M Tris-HCl, pH 7,9. This was made up as above with the omission of the EDTA.

(ii).   Assay medium.   The assay medium consisted of Acetyl-CoA (Boehringer-Mannheim, 5 mg) and IPTG (Sigma, 250 mg) made up to 1 ml with 0,05 M Tris-0,01 M EDTA buffer, pH 7,9.

(iii).   DTNB. DTNB (Boehringer-Mannheim, 25 mg) was made up to 100 ml with 0,05 M Tris-HCl buffer, pH7,9.

B.   Preparation of Cell Free Extracts.

Overnight cultures of EC-C and EC-S as well as a Lac^+ and a Lac^- strain of E. coli K12 were grown at 30°C in an orbital shaker in M-63 medium supplemented with 0,4% (w/v) glycerol and 0,09% (w/v) YE. After diluting (1:25) into fresh M-63 medium supplemented as above, with and without 1 mM IPTG, growth was allowed to continue to an A_{600} of about 0,6. The cells were harvested by centrifugation (10 000 g, 4° C, 10 min), and resuspended in one-tenth of the original culture volume of 0,05 M Tris-0,01 M EDTA, pH 7,9. The cells were sonicated in an MSE 150 W sonicator (22 micron amplitude) for 2 X 30 sec bursts at 4°C, with a 5 min break on ice between each burst of sonication. Each sample was divided into two equal portions. One portion was kept on ice, while the other portion was heated at 70°C for 5 min, and cooled on ice. Both portions were assayed for β-galactosidase and thiogalactosidase activity after removal of cell debris by centrifugation (59 000 g, 4°C, 10 min).

C. Thiogalactoside transacetylase assay.

An aliquot (0,05 ml) of each sample was incubated with assay medium (0,05 ml) for 1 h at 30°C. The reaction was stopped by the addition of DTNB solution (3,0 ml), and the absorbance determined at 412 nm against a water blank in a Spectronic 88 spectrophotometer.

3.1.12.   Hydrolysis of α- and β-ONPG by intact cells of EC-C and EC-S.

A.   Reagents.

The reagents described in Sections 3.1.7 and 3.1.10 were used.

B.   Preparation of cells.

Flasks of NB were inoculated with a loopful of EC-C or EC-S from a NA plate and growth was allowed to proceed on an orbital shaker (200 rpm, 30°C) to late log phase or early stationary phase (10-12 h). Following growth, cells were diluted (1:50) into modified M-63 medium containing 0,4% (w/v) glycerol together with various sugars (0,4% w/v), TMG (0,5 mM) or IPTG (0,5 mM), and grown for a further 8 h under the same conditions. The cells were harvested by centrifugation, (10 000 X g, 4°C, 20 min), resuspended in wash medium (WM), which consisted of modified M-63 medium without YE or glycerol, but containing chloramphenicol (0,15 mg ml^{-1}), and repelleted by centrifugation. Pellets were resuspended in WM to an A_{600} of about 4,0 and stored on ice.

C. Assays.

(i). α-ONPG hydrolysis. The reaction medium in 13 X 100 mm test
tubes contained:

Cells   0,1 ml

100 mM Tris-HCl buffer, pH 7,5   0,5 ml

H_2O 0,3 ml

After equilibration at 30°C for 5 min, the reaction was started
by the addition of α-ONPG (0,1 ml), and stopped after 10 min by
the addition of 1 ml 1 M Na_2CO_3. After dilution with H_2O, the
absorbances at 420 and 550 nm were determined in a Spectronic
88 spectrophotometer. The absorbance at 420 nm was corrected
for turbidity as described in Section 3.1.7.

The effect of different compounds on α-ONPG hydrolysis by
intact cells was tested by adding the compound to the reaction
mixture together with the substrate. Sodium azide (20 mM) was
added to the cells 5 min before assay when the effect of this
compound on α-ONPG hydrolysis by intact cells of EC-C and EC-S
was tested.

(ii). β-ONPG hydrolysis. These assays were performed as above,
except that 0,2 ml substrate was used instead of 0,1 ml.
The volume of water in the reaction mixture was decreased

proportionately to account for this. Other conditions were as described above.

3.1.13. Uptake of ^{14}C-labelled IPTG, TMG, and Lactose by EC-C and EC-S.

A. Reagents.

(i). ^{14}C-IPTG. [Isopropyl-^{14}C]-IPTG (Service des Molecules Marquées, France, 28 mCi mmole^{-1}), purchased from Research Products International Corp. (Mount Prospect, Il, U.S.A.), was diluted with unlabelled IPTG to a specific activity of 0,25 mCi mmole^{-1}.

(ii). ^{14}C-TMG. [Methyl-^{14}C]-TMG (Schwartz-Mann, Orangeburg, N.Y., U.S.A.. 35 mCi mmole^{-1}) was diluted to a specific activity of 0,5 mCi mmole^{-1} with unlabelled TMG.

(iii). ^{14}C-lactose. [D-Glucose-1-^{14}C]-lactose (Amersham International, Buckinghamshire, England, 57,6 mCi mmole^{-1}) was diluted to a specific activity of either 0,125 mCi mmole^{-1} or 0,5 mCi mmole^{-1} with unlabelled lactose as specified in the results.

B.   Preparation of Cells.

Washed cells were prepared as described in Section 3.1.12.

C.   Assays

All assays were performed in 16 X 100 mm test tubes containing:

Cells   0,4 ml

Wash solution   0,5 ml

After equilibration at 30°C for 5 min, uptake was initiated by the addition of substrate (0,1 ml), and aliquots (0,5 ml) were removed at stated time intervals, rapidly filtered through a 0,45 um HAWP Millipore filter on a Millipore filtration manifold and washed with WM (5 X 0,2 ml) at room temperature. The filtration procedure and washing took less than 15 sec. The filters were counted in 10 ml EP liquid scintillation fluid (Beckman Instruments) in a Beckman LS 8100 liquid scintillation counter to a 2σ error of 2% or less. All data are reported as cpm, with no account being taken of quenching, as it was found to be constant within a particular experiment.

The effects of different sugars on substrate uptake were determined by adding the sugar together with the radioactive substrate. The effect of DNP (2 mM) and NaN_3 (20 mM) on substrate uptake was determined after pre-incubation of the cells with these compounds for 30 min before assay.

The effect of formaldehyde (70 mM final concentration) on substrate uptake was determined by adding the compound to the cells 5 min before the addition of substrate.

Blanks were determined by immediately removing a sample after addition of substrate, washing, and counting as above. Alternatively, formaldehyde treated cells were used as blanks, with samples being removed within 15 sec of adding the substrate/formaldehyde.

3.2. RESULTS.

3.2.1. Comparison of different cell permeabilization methods.

The efficiency of cell permeabilization was measured by incubating permeabilized whole cells with extracellular β-ONPG and measuring the rate of β-ONPG hydrolysis by intracellular β-galactosidase. A high rate of hydrolysis indicates a high degree of permeability, whereas a low rate of hydrolysis may indicate a low degree of permeability or inhibition of enzyme activity by components of the permeabilizing agent.

The efficiency of a number of methods used to permeabilize bacterial cells, using the β-galactosidase assay, is presented in Table 6. The most effective permeabilizing agent was the lysis mixture of Putnam and Koch (1975) as modified in this study i.e. LM2. This was followed by $CHCl_3$ + SDS, toluene, and toluene + DOC. The least effective method was the original lysis mixture described by Putnam and Koch (1975).

3.2.2. Effect of SDS, DOC, Mg^{2+}, Mn^{2+}, Na^+, and 2-ME on β-galactosidase activity.

Each of the components of LM1 and LM2 were tested for their effect on β-galactosidase activity in cell-free extracts of EC-C and EC-S. SDS at concentrations above 0,02% were found to inhibit the activity of β-gal-

Table 6. A comparison of some published cell permeabilization methods used in the assay of β-galactosidase. An overnight culture of EC-S grown on YS-medium (0,09% YE + 0,5% glycerol + 0,2% lactose) was centrifuged (10 000 X g, 4°C, 10 min), washed by resuspension and centrifugation from YS medium (0,09% YE), and resuspended in YS medium supplemented with 0,05 mg ml^{-1} chloramphenicol for assay.

Permeabilization Method	A_{420} min^{-1}	Reference
Toluene	0,490	Miller, 1972
CHCl$_3$ + SDS	0,505	Miller, 1972
Toluene + DOC	0,362	Revel et al., 1961
LM1	0,079	Putnam and Koch, 1975
LM2	0,713	This thesis

actosidase from both EC-C and EC-S, whereas concentrations of DOC up to 0,05% were without effect on enzyme from either source.

Maximal activation of the β-galactosidase from both EC-C and EC-S was observed at 1 mM Mg^+ (Fig. 20). The effect of Na^+ on β-galactosidase activity was more complex, and depended on the concentration of Mg^{2+} in the assay mixture (Fig. 21). In the absence of Mg^{2+}, a maximal activation of about 250% ocurred at Na^+ concentrations between 0,025 and 0,2 M, whereas in the presence of 1 mM Mg^{2+}, only a 10% activation was observed (Fig. 21). The activation of β-galactosidase by Mn^{2+} was also dependent on the concentration of Mg^{2+} in the assay mixture, since the presence of 0,02 M Mn^{2+} resulted in a 28% activation in the absence of Mg^{2+}, but only a 5% activation in the presence of 1 mM Mg^{2+}.

The effect of 2-ME on β-galactosidase activity was measured in the presence of 25 mM Na^+ and 1 mM Mg^{2+} (Fig. 22). Optimum β-galactosidase activity was observed over a relatively broad range of 2-ME concentrations (0,1-0,25 M), while concentrations of 2-ME above 0,25 M decreased enzyme activity (Fig. 22).

3.2.3. pH profiles of β-galactosidase from EC-C and EC-S.

The pH profiles of the β-galactosidase from EC-C and EC-S were determined using McIlvaine buffers over the pH range 4 to 7, phosphate buffers
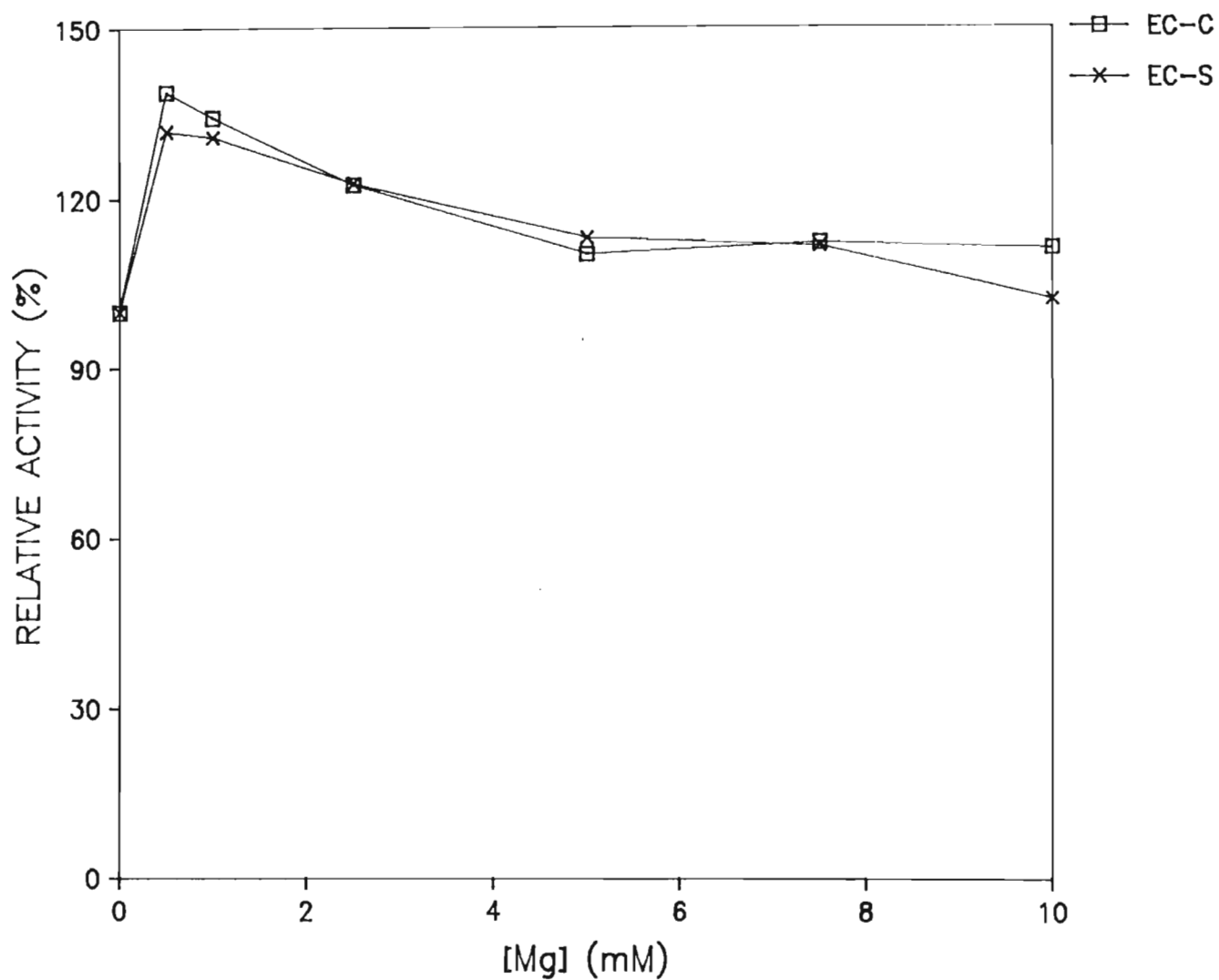
Fig. 20. Effect of Mg^{2+} on β-galactosidase activity in cell-free extracts from strain EC-C and EC-S induced by IPTG.
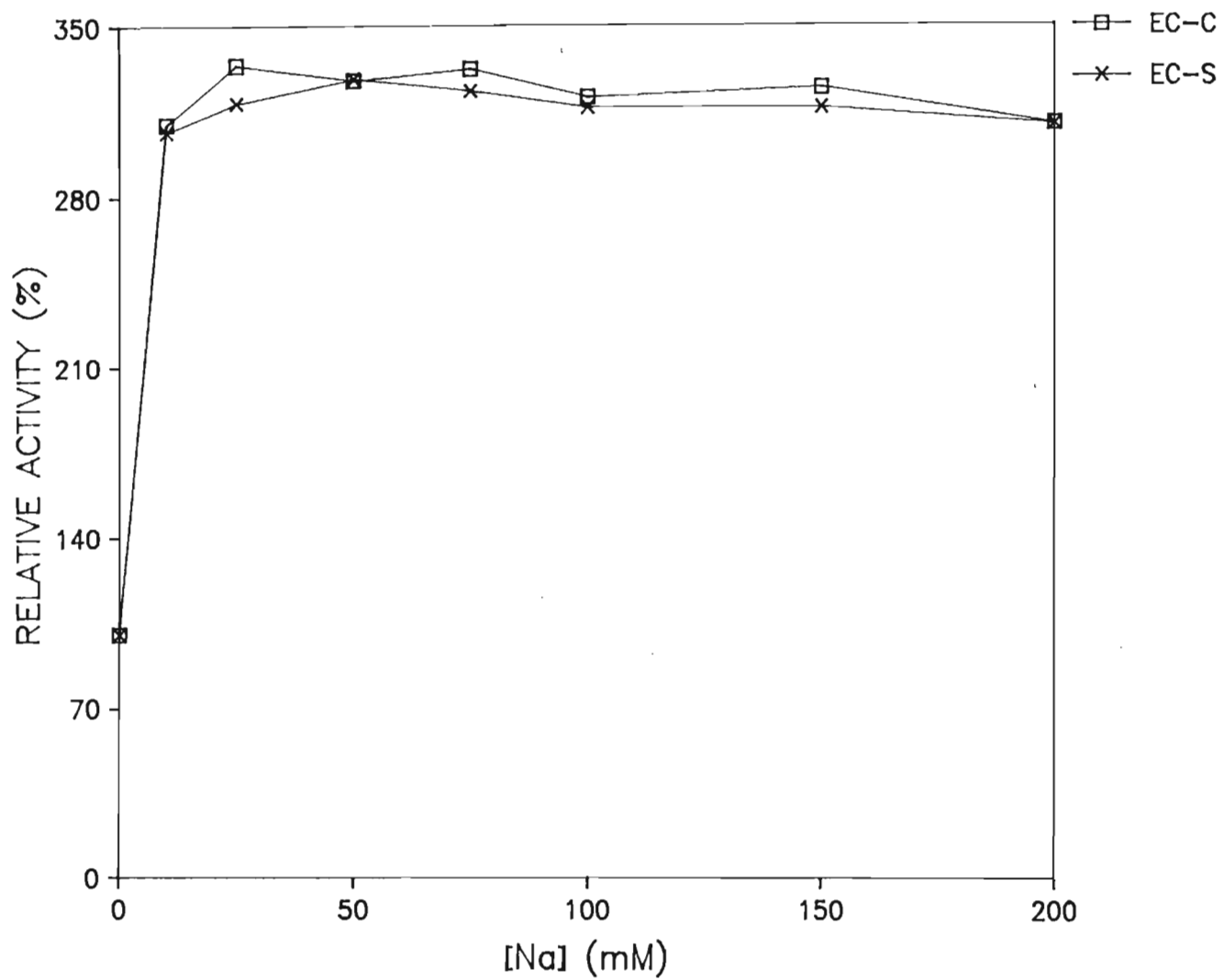
Fig. 21. Effect of Na$^+$ on β-galactosidase activity in cell-free extracts obtained from strains EC-C and EC-S induced by IPTG.
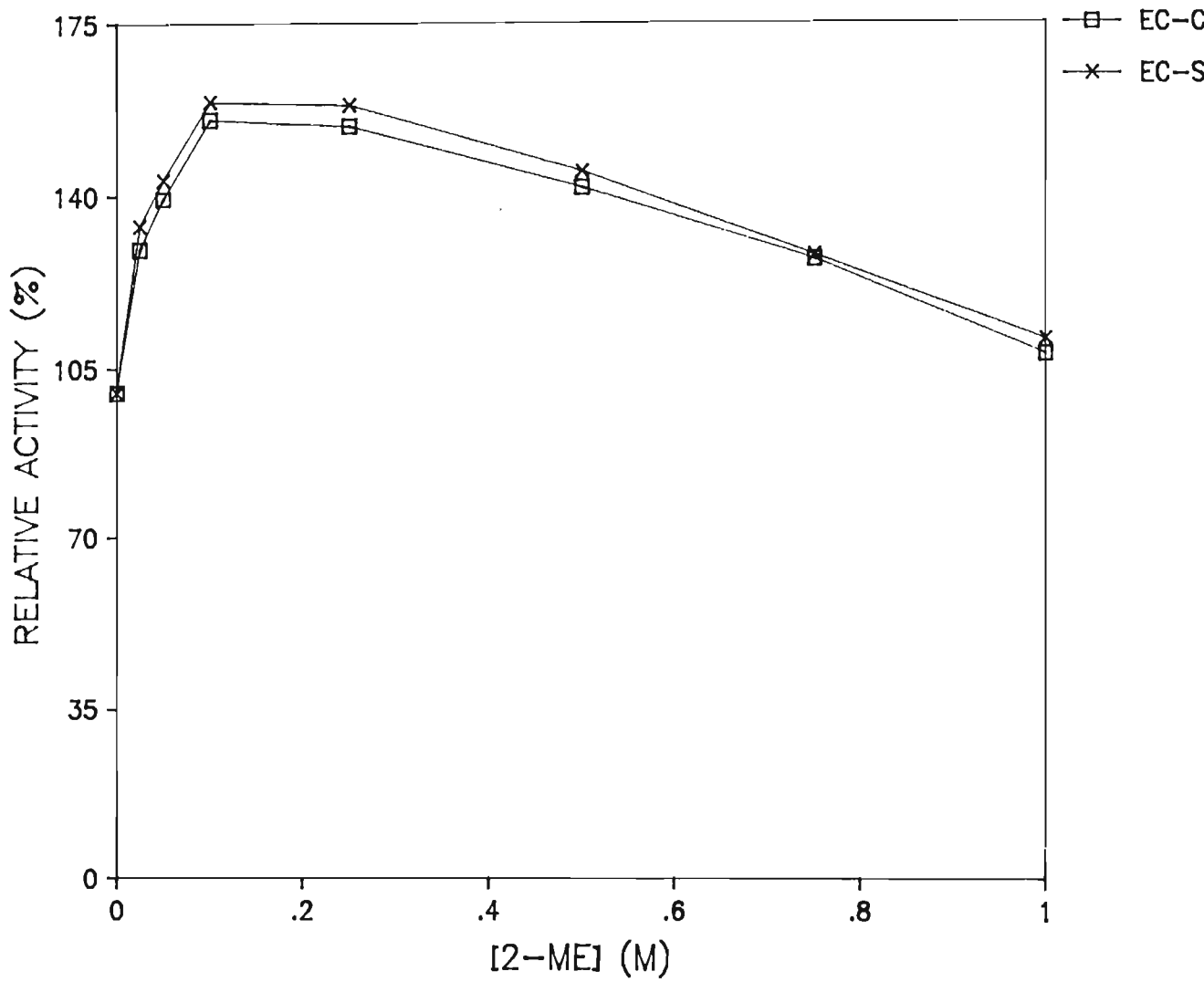
Fig. 22. The effect of different concentrations of 2-ME on β-galactosidase activity in cell-free extracts of IPTG-induced strains EC-C and EC-S.

over the pH range 7 to 8, and NaOH-gly buffers between pH 9 and 10. The pH optimum of both enzymes was found to be about 9,0 (Fig. 23), a value very different from that published for E. coli (about pH 7, Wallenfels and Weil, 1972). This anomalous result was thought to be due to citrate, a compound known to chelate Mg^{2+}, being present in the McIlvaine buffers. To test this idea, the pH profile was redetermined in the pH 5 to 9 range after replacing the McIlvaine buffers with phosphate buffers. The optimum pH under these conditions was found to be about 7,2 (Fig. 24).

Citrate was found to inhibit the -galactosidase activity in cell-free extracts from both strain EC-C and strain EC-S (Fig. 25), with lithium citrate inhibiting β-galactosidase more severely than an equivalent concentration of sodium citrate. Citrate inhibition could be reversed by the addition of Mg^{2+} to the assay medium (Table 7).

3.2.4.   Km for the β-galactosidases from EC-C and EC-S.

The Km's of the β-galactosidases in cell-free extracts of IPTG-induced EC-C and EC-S, and lactose-induced EC-S, were determined in the presence or absence of 100 mM 2-ME. A computer program written in Fortran was used to fit a line to the data by the linear least squares method, using either unweighted data, or data weighted according to the reciprocal of the fourth power of the velocity (Cleland, 1979). The Km values of the
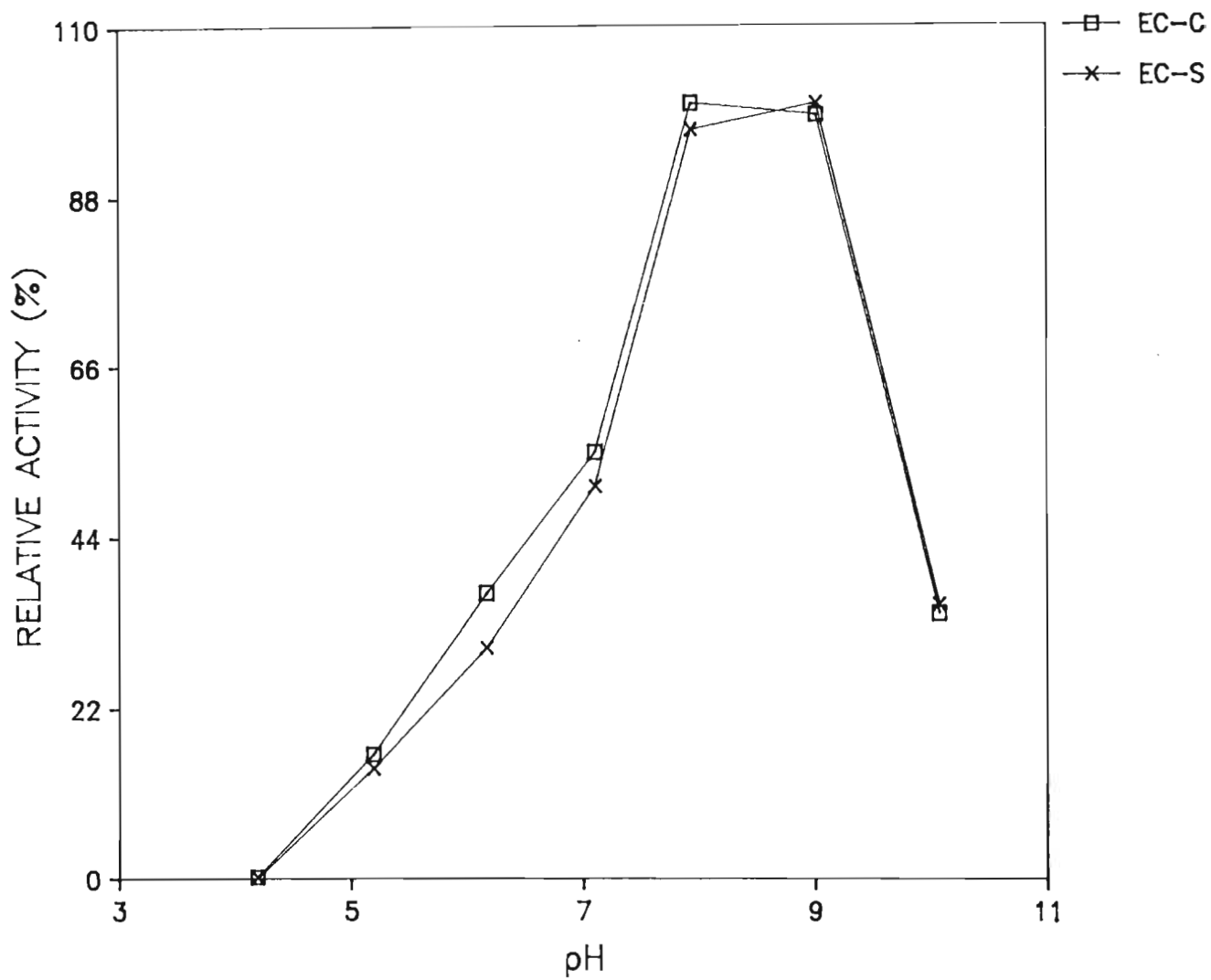
Fig. 23. pH optima for β-galactosidase in cell-free extracts from IPTG-induced strains EC-C and EC-S assayed in the presence of McIlvaine buffers.
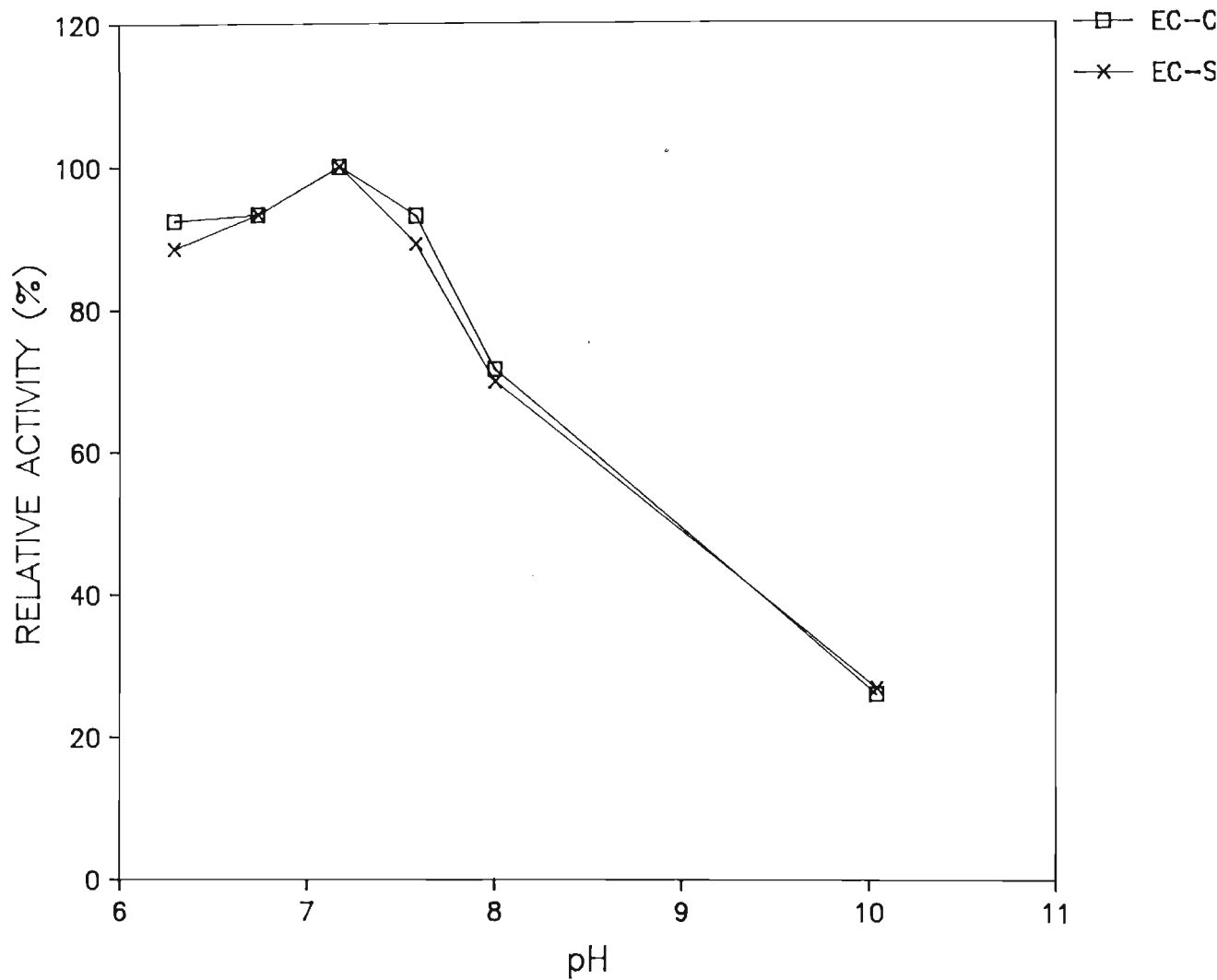
Fig. 24. pH optima for β-galactosidase in cell-free extracts from IPTG-induced strains of EC-C and EC-S assayed in the presence of phosphate buffers.
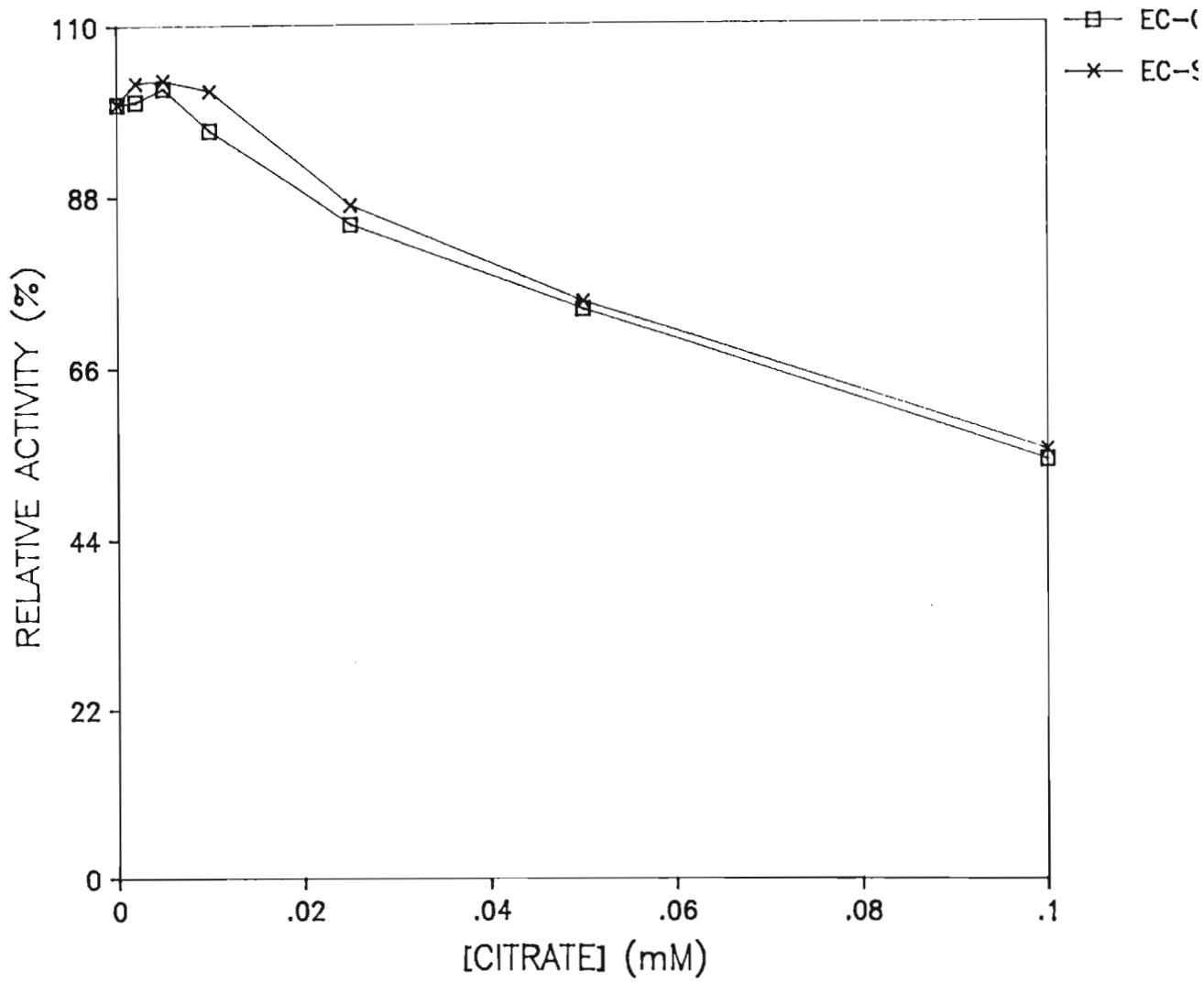
Fig. 25. Inhibition of the β-galactosidase in cell-free extracts of IPTG-induced strains EC-C and EC-S by lithium citrate.

Table 7. Reversal of citrate inhibition of β-galactosidase by the addition of Mg^{2+}. Cell-free extracts from EC-C and EC-S, induced by IPTG, were prepared, and assayed for β-galactosidase in the presence and absence of 10 mM Mg^{2+}.

Buffer	A_{420} 15 min^{-1}	
	EC-C	EC-S
Imidazole	0,891	0,893
Imidazole + 10 mM Mg^{2+}	0,918	0,928
McIlvaine	0,734	0,719
McIlvaine + 10 mM Mg^{2+}	1,061	1,079

Imidazole = 50 mM imidazole buffer, pH 7,0

McIlvaine = McIlvaine buffer, pH 7,0

values of the β-galactosidases from both strain EC-S and strain EC-C, grown on either lactose or IPTG, were similar on β-ONPG, being about 0,3 mM in the absence of 2-ME, and about 0,6 mM in the presence of 100 mM 2-ME (Table 8).

3.2.5.   Assay of β-galactosidase on lactose.

The activity of β-galactosidase in cell-free extracts of IPTG-induced EC-C and EC-S, and lactose-induced EC-S was also assayed using lactose as substrate. Problems with the linearity of the assay were experienced when assaying the liberated glucose by the glucose oxidase method (Fig. 26). The non-linearity of the assay was not due to the presence of either Mg^{2+} or Na^+, as assays performed in the absence of these ions were still non-linear (Fig. 26). In addition, as 2-ME was not present in the assay medium, it could not have been an inhibitory factor. A commercial E. coli (Boehringer Mannheim) preparation gave similar results when assayed on lactose suggesting that the non-linearity was not due to some unknown factor in the cell-free extract.

The failure to obtain linearity prompted the investigation of an alternative glucose assay method. When the assays were performed using the HK/G6P-DH method to determine the liberated glucose, the time course curves for both the EC-S β-galactosidase and the commercial E. coli β-galactosidase, were linear (Fig. 27). This method, therefore, was used to assay the liberated glucose in the following experiment.

Table 8. Km values of β-galactosidases in cell-free extracts of
IPTG-induced EC-C and EC-S, and lactose-induced EC-S. Cell-free
extracts were prepared from overnight cultures of EC-C and EC-S
grown on YS medium (0,09% YE, 0,5% glycerol, and either 0,2%
lactose, or 0,5 mM IPTG), and the Km for δ-ONPG in the presence
and absence of 2-ME was determined for each extract. A least
squares fit using either weighted or unweighted data was fitted
as described in the text.

Enzyme Source	Km (mM)			
	Minus 2-ME		Plus 2-ME	
	Weighted	Unweighted	Weighted	Unweighted
EC-C (I)[a]	0,61	0,78	0,30	0,42
EC-S (I)[b]	0,68	0,87	0,31	0,47
EC-S (L)[c]	0,64	0,74	0,28	0,45

[a]EC-C (I) = EC-C induced by IPTG.

[b]EC-S (I) = EC-S induced by IPTG.

[c]EC-S (L) = EC-S induced by lactose.
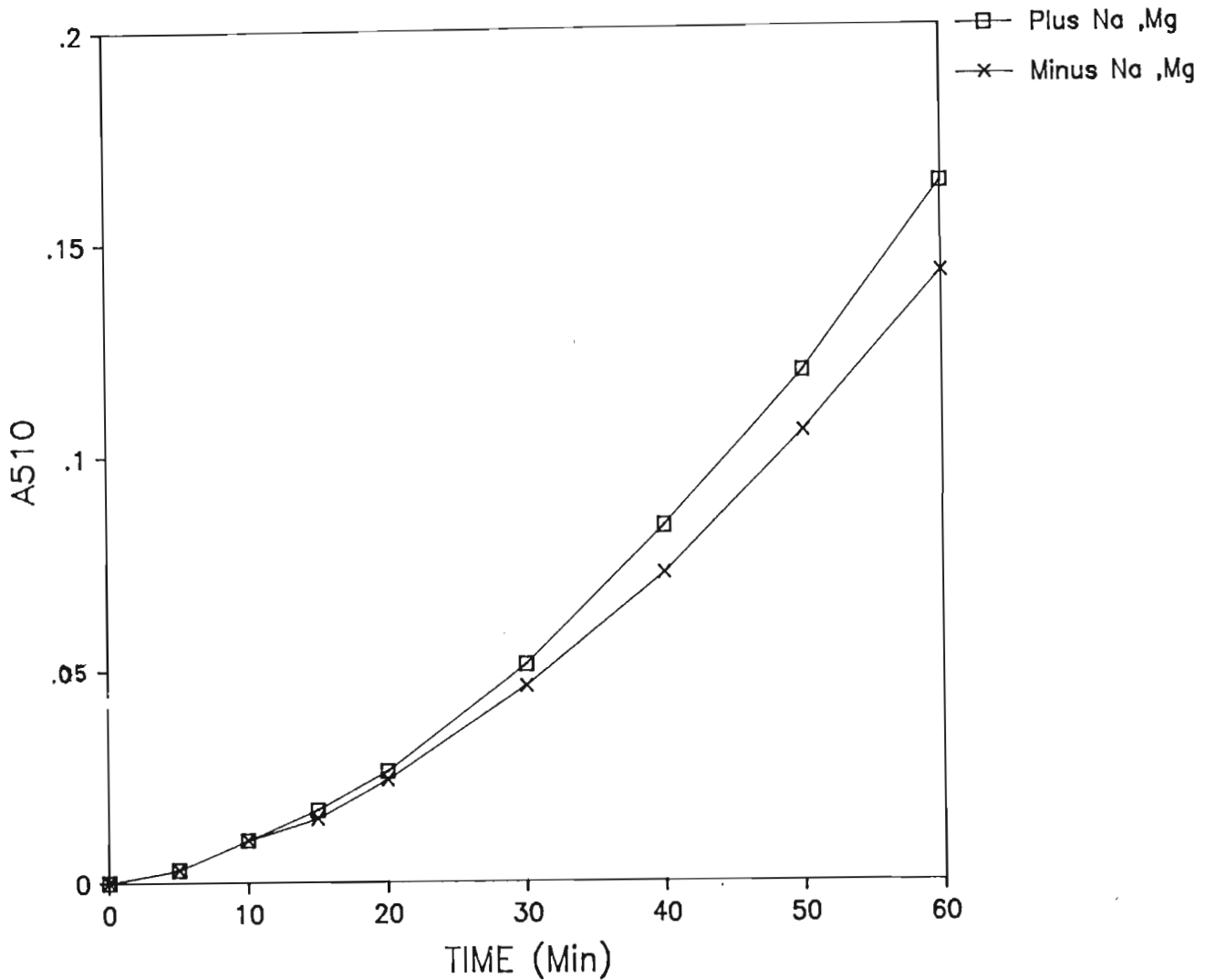
Fig. 26. Time course assay on lactose of β-galactosidase in a cell-free extract from lactose-induced strain EC-S using the GOD-POD glucose assay to measure the liberated glucose. A cell-free extract prepared from an overnight culture of IPTG-induced strain EC-S was used as source of enzyme. The assay was performed in 50 mM imidazole buffer, pH 7,2 supplemented with, and without 6,7 mM Mg^{2+} and 10,0 mM Na^+ as described in Section 3.1.9.
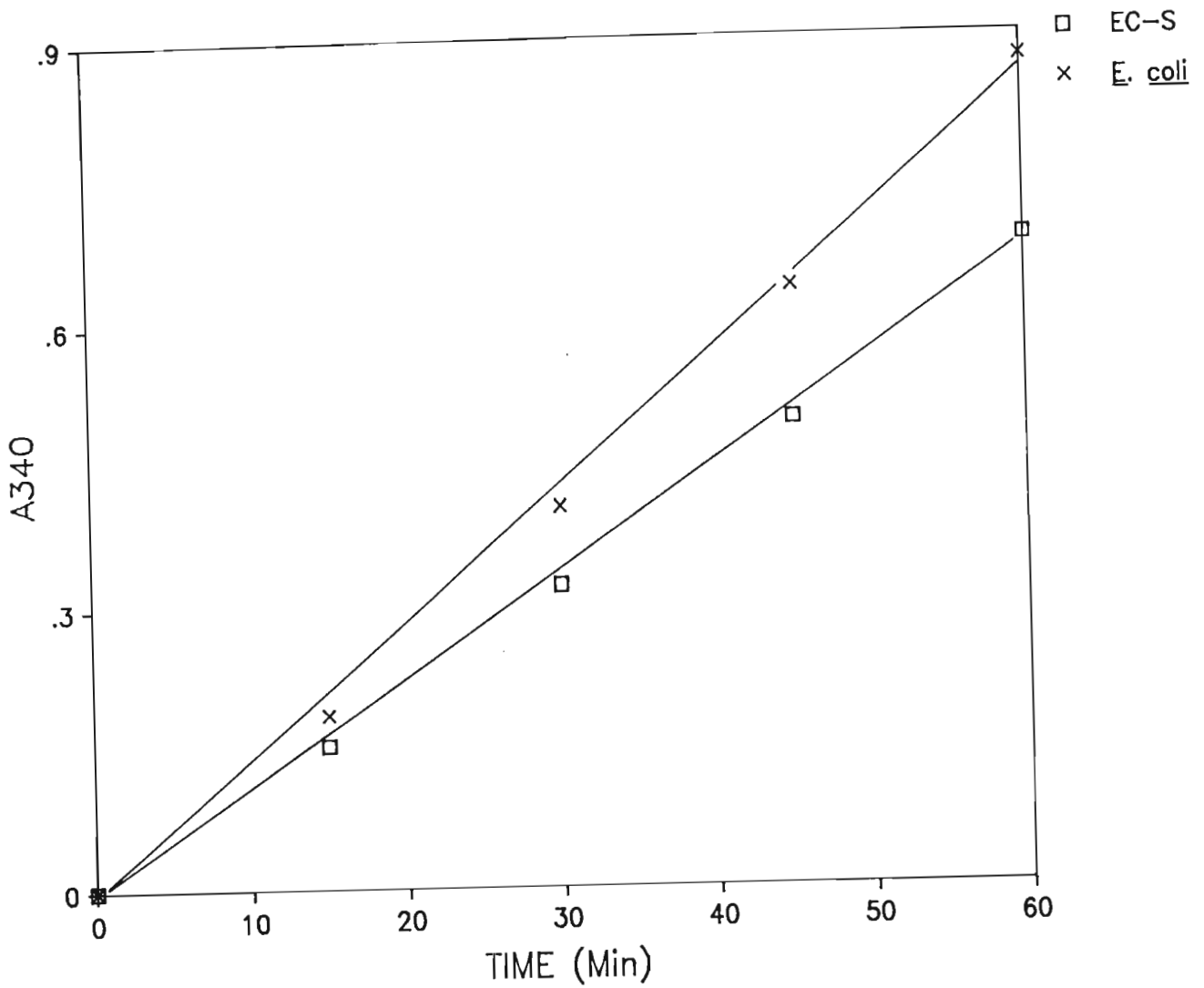
Fig. 27. Time course assay on lactose of β-galactosidase in a cell-free extract from lactose-induced strain EC-S using the HK/G6P-DH method to measure the liberated glucose. A commercial sample of E. coli β-galactosidase was assayed in parallel as a control. See Fig. 26 for additional details.

3.2.6. Comparison of the activity of the β-galactosidases from EC-C and EC-S on lactose and β-ONPG.

The activity of the β-galactosidases in cell-free extracts from IPTG-induced EC-C and EC-S, and lactose-induced EC-S were compared on β-ONPG and lactose (Table 9). The ratio of activity on β-ONPG to that on lactose for the β-galactosidases from EC-C and EC-S were similar, irrespective of the inducer used, with about 18-fold greater activity on β-ONPG than on lactose. In contrast, a purified β-galactosidase from E. coli was only about 9-times as active on β-ONPG as on lactose (Table 9).

3.2.7. Induction of β-galactosidase in EC-C and EC-S by different carbon sources.

Relatively high basal levels of β-galactosidase were produced by both EC-C and EC-S after growth on glycerol (Table 10). The β-galactosidase level in both strains was increased a further 10-fold by growth in the presence of either IPTG or TMG, but lactose was effective as an inducer only in EC-S (Table 10). Neither melibiose nor galactose induced β-galactosidase activity in either strain (Table 10).

Table 9. Comparison of the activity of the β-galactosidases from IPTG-induced EC-C and EC-S, and lactose-induced EC-S on β-ONPG and lactose. Cell-free extracts were prepared from overnight cultures of EC-C and EC-S grown on YS-medium (0,09% YE, 0,5% glycerol, and either 0,5 mM IPTG, or 0,2% lactose) as described previously, and the activity of the β-galactosidase measured on β-ONPG and lactose. A commercial (Boehringer-Mannheim) preparation of E. coli β-galactosidase was included as a control.

Enzyme Source	Ratio (β-ONPG/Lactose) (A_{420}/A_{340})
EC-C (I)[a]	18,7
EC-S (I)[b]	17,2
EC-S (L)[c]	18,6
E. coli	8,7

[a]EC-C (I) = EC-C induced by IPTG.

[b]EC-S (I) = EC-S induced by IPTG.

[c]EC-S (L) = EC-S induced by lactose.

Table 10. β-galactosidase activity of EC-C and EC-S grown on different carbon sources.

Carbon[a] Source	β-galactosidase Activity (U ml^{-1} A$_{600}^{-1}$)	
	EC-C	EC-S
Glycerol	3.5	3.6
Glycerol + raffinose	3.4	2.2
Glycerol + melibiose	5.5	4.3
Glycerol + galactose	7.0	5.1
Glycerol + lactose	8.2	38.8
Glycerol + IPTG	39.1	42.5
Glycerol + TMG	38.1	43.4

[a]Glycerol, melibiose, galactose, and lactose were present at 0.4% (w/v), whereas IPTG and TMG were present at 0.5 mM.

3.2.8. Uptake of ^{14}C-lactose, ^{14}C-IPTG, and ^{14}C-TMG by EC-C and EC-S grown on different carbon sources.

Lactose transport was found to be constitutive in EC-S, but inducible by melibiose and raffinose in EC-C (Table 11). In contrast, galactose was a poor inducer and neither IPTG, TMG or lactose induced any lactose transport at all in EC-C (Table 11).

Melibiose, galactose, 2-deoxyglucose, and glucose (all at 1 mM) inhibited lactose transport in EC-S grown on glycerol + lactose (Table 12, Experiment 1), whereas little or no inhibition of lactose uptake was observed by D-(+)-arabinose, L-(-)-arabinose, α-L-(-)-fucose, L-rhamnose, mannitol, or D-mannose. Lactose transport by EC-S grown in glycerol + lactose was also strongly inhibited by thiodigalactoside (TDG, 2 mM) and IPTG (2 mM), whereas raffinose (2 mM) and TMG (2 mM) were less inhibitory (Table 12, Experiment 2). A similar pattern of inhibition of lactose transport was observed in melibiose-grown EC-C (Table 12, Experiment 3). Raffinose was a potent inhibitor of lactose transport by raffinose-grown EC-C and EC-S (Table 12, Experiments 4 and 5), whereas it was a poor inhibitor of lactose transport by EC-S grown on glycerol (Table 12, Experiment 6). Melibiose, irrespective of the growth conditions of the cells, strongly inhibited lactose transport in both EC-C and EC-S.

Table 11. Uptake of ^{14}C-lactose by EC-C and EC-S grown on different carbon sources.

Carbon Source[a]	EC-C	EC-S
	Lactose uptake[a] (cpm ml^{-1} A$_{600}^{-1}$)	
Glycerol	113	6492
Glycerol + IPTG	280	10784
Glycerol + TMG	189	10550
Glycerol + lactose	110	6930
Glycerol + melibiose	4247	4463
Glycerol + raffinose	1380	4955
Glycerol + galactose	619	5212

[a]See Table 10 for details.

[b]Specific activity of ^{14}C-lactose was 0.5 mCi mmole^{-1}.

Table 12. The effect of different sugars on the uptake of ^{14}C-lactose by EC-C and EC-S grown on different carbon sources.

Competing Sugar (concentration)	Lactose Uptake[a] (cpm ml^{-1} A$_{600}^{-1}$)	% Inhibition
Experiment 1. EC-S grown on glycerol + lactose.		
None	2418	0,0
2-deoxy-glucose (1 mM)	1890	21,8
Galactose (1 mM)	1344	44,4
Glucose (1 mM)	1067	55,8
Melibiose (1mM)	805	66,7
Experiment 2. EC-S grown on glycerol + lactose.		
None	2166	0,0
TMG (2 mM)	1601	26,1
IPTG (2 mM)	287	87,7
TDG (2 mM)	198	90,9
Melibiose (2 mM)	59	97,3
Experiment 3. EC-C grown on glycerol + melibiose.		
None	1400	0,0
TMG (2 mM)	1400	0,0
IPTG (2 mM)	1150	17,8
TDG (2 mM)	147	89,5
Melibiose (2 mM)	22	98,4
Raffinose (2 mM)	714	49,0
Experiment 4. EC-C grown on raffinose.		
None	1065	0,0
Melibiose (2 mM)	9	99,1
Raffinose (2 mM)	159	85,0
Experiment 5. EC-S grown on raffinose.		
None	2084	0,0
Melibiose (2 mM)	77	96,3
Raffinose (2 mM)	275	86,8
Experiment 6. EC-S grown on glycerol.		
None	2403	0,0
Melibiose (2 mM)	43	98,2
Raffinose (2 mM)	1948	18,9

[a]Specific activity of ^{14}C-lactose was 0,125 mCi mmole^{-1}.

Although it is known from the literature that $\underline{E.}$ $\underline{coli}$ is able to transport IPTG, no evidence of ^{14}C-IPTG transport by either EC-C or EC-S could be found, irrespective of the growth conditions. The purity of the IPTG was found to be greater than 90% pure by TLC, and therefore was probably not to at fault. In addition, it was confirmed experimentally that the same ^{14}C-IPTG was transported by $\underline{E.}$ $\underline{coli}$ following growth in the presence of either lactose or IPTG. These observations support the conclusion that neither EC-C nor EC-S was able to transport IPTG.

No conclusive evidence for ^{14}C-TMG transport by either strain EC-C or Strain EC-S could be found irrespective of the growth conditions of the cells. Although the amount of ^{14}C-TMG associated with the cells was quite high, the assay controls were also high. Similar levels of ^{14}C-TMG were also found in cells treated with formaldehyde, a compound known to inhibit carrier mediated transport of β-galactosides in $\underline{E.}$ $\underline{coli}$ (Koch, 1964).

Lactose transport was inhibited by more than 90% in the presence of 2 mM DNP or 20 mM sodium azide, suggesting that lactose accumulation was due to some form of active transport. Formaldehyde was also found to inhibit lactose transport by more than 90%.

3.2.9.   Hydrolysis of α- and β-ONPG by intact cells of EC-C and EC-S.

The rates of α- and β-ONPG hydrolysis by intact cells depend both on the rate at which these substances are transported into the cell, and the level of the galactosidase inside the cell. Thus, a comparison between the hydrolysis rates catalyzed by intact and permeabilized cells shows whether transport or hydrolysis is rate limiting. Similarly, if the galactosidase level is constant, any observed increase in the ONPG hydrolytic activity catalyzed by the intact cells must reflect a change in the transport rate only. Measurements of the hydrolysis of ONPG by intact cells therefore supplements transport data obtained using radioactive tracers.

Low basal levels of β-ONPG hydrolysis occurred in intact cells of EC-C and EC-S grown on glycerol (Table 13). These levels were not further increased in EC-C after induction by either IPTG or TMG despite the high levels of β-galactosidase detected in these cells (Tables 10 and 13). In contrast, there was a 2- to 3-fold increase in β-galactosidase hydrolysis by intact cells of EC-S after induction by either IPTG, TMG or lactose (Table 13).

The basal levels of α-ONPG hydrolysis by intact cells of EC-C were very low, and could be increased after induction by melibiose, and to a lesser extent, by galactose (Table 13). Neither IPTG, TMG nor lactose increased

Table 13. Hydrolysis of α- and β-ONPG by intact cells of EC-C and EC-S.

| Carbon Source | α - or β-ONPG Uptake (nmole α- or β-ONPG hydrolysed min^{-1} ml^{-1} A_{600}^{-1}) | | | |
| | α-ONPG | | β-ONPG | |
	EC-C	EC-S	EC-C	EC-S
Glycerol	0,0	15,6	4,3	5,0
Glycerol + melibiose	10,9	9,1	4,8	4,1
Glycerol + galactose	1,8	11,0	5,5	4,0
Glycerol + IPTG	0,0	15,1	6,0	12,7
Glycerol + TMG	0,0	14,8	5,0	12,4
Glycerol + lactose	0,0	7,8	5,1	7,1

the rate of α-ONPG hydrolysis by intact cells of EC-C (Table 13). In contrast, α-ONPG was hydrolyzed constitutively by intact cells of EC-S (Table 13). The rate of α-ONPG hydrolysis by intact cells of EC-S grown on either melibiose, lactose or galactose was about 35% lower than the levels observed in glycerol grown cells (Table 13).

Neither melibiose (5 mM) nor TDG (2 mM) decreased the basal rate of β-ONPG hydrolysis by EC-C or EC-S grown on glycerol, but did inhibit β-ONPG hydrolysis by IPTG or lactose induced EC-S (Table 14). In addition, neither compound inhibited β-galactosidase in permeabilized cells. Formaldehyde (70 mM) also inhibited the hydrolysis of β-ONPG by IPTG and lactose induced cells of EC-S to basal levels, without significantly inhibiting β-galactosidase activity in permeabilized cells.

Lactose was found to inhibit α-ONPG hydrolysis by intact cells of both EC-C and EC-S, although relatively high concentrations (10mM) were required (Table 15). TDG (2 mM) and formaldehyde (70 mM) were also potent inhibitors of α-ONPG hydrolysis by intact cells of EC-C and EC-S.

3.2.10. α-Galactosidase activity in permeabilized cells of EC-C and EC-S grown on different carbon sources.

No α-galactosidase activity could be detected in cells of EC-S permeabilized by LM2. As the α-galactosidase has been reported to be very

Table 14. Inhibition by melibiose and TDG of β-ONPG uptake and hydrolysis by intact cells of EC-C and EC-S.

Carbon Source	β-ONPG Uptake					
	(nmole β-ONPG hydrolyzed min^{-1} ml^{-1} A$_{600}^{-1}$)					
	EC-C			EC-S		
	Control	+mel[a]	+TDG[b]	Control	+mel[a]	+TDG[b]
Glycerol	2,3	2,2	2,5	2,9	2,3	2,3
Glycerol + melibiose	3,0	2,1	2,2	2,5	2,7	1,9
Glycerol + IPTG	3,6	3,5	3,8	9,8	2,4	4,2
Glycerol +lactose	3,0	2,8	2,7	8,0	3,0	3,6

[a]Melibiose (5 mM)

[b]Thiodigalactoside (2 mM)

Table 15. Inhibition by lactose of α-ONPG uptake by intact cells of EC-C and EC-S.

Carbon Source	α-ONPG Uptake					
	(nmole α-ONPG hydrolyzed min^{-1} ml^{-1} A$_{600}^{-1}$)					
	EC-C			EC-S		
	Control	+lac[a]	+lac[b]	Control	+lac[a]	+lac[b]
Glycerol	–	–	–	12,3	11,4	7,4
Glycerol + melibiose	7,7	6,5	4,5	7,1	6,6	4,9
Glycerol + IPTG	–	–	–	12,4	12,3	9,7
Glycerol + lactose	–	–	–	6,9	6,8	7,2

[a]Lactose (1 mM)

[b]Lactose (10 mM)

sensitive to inhibition by toluene, but stabilized by Mn^{2+} and DTT (Schmitt and Rotman, 1966), the effects of these compounds on the activity of α-galactosidase in EC-S grown on glycerol were investigated. Toluene treated cells exhibited about 60% higher α-galactosidase activity than did the non-permeabilized cells, and this activity not further increased by either Mn^{2+} or DTT (Table 16). It was therefore decided to use toluene to permeabilize cells for all subsequent α-galactosidase assays.

The α-galactosidase levels of EC-C and EC-S grown on different carbon sources followed the pattern observed for intact cells, except that they were about 60% higher than comparable data from intact cells (Table 17). Again it was found that melibiose, and to a lesser extent, galactose, induced α-galactosidase in strain EC-C, whereas the enzyme was expressed constitutively in EC-S (Table 17). As was found for α-ONPG hydrolysis by intact cells of EC-S, lower α-galactosidase levels were observed in EC-S grown on melibiose, lactose or galactose (Table 17). However, the stimulation of ^{14}C-lactose transport observed after induction by IPTG or TMG was not observed for α-ONPG hydrolysis by intact cells of EC-S- (Tables 11, 13, and 17).

Table 16. Effect of Mn^{2+} and DTT on α-galactosidase activity.

Addition	α-galactosidase activity $(U\ ml^{-1}\ A_{600}^{-1})$
Buffer	12,1
Buffer + Toluene	15,5
Buffer + 5 mM Mn^{2+} + Toluene	16,8
Buffer + 5 mM DTT + Toluene	15,0
Buffer + 5 mM Mn^{2+} + 5 mM DTT + Toluene	17,6

Table 17. α-Galactosidase activity of EC-C and EC-S grown on different carbon sources.

Carbon Source	α-galactosidase Activity (U ml^{-1} A$_{600}^{-1}$)	
	EC-C	EC-S
Glycerol	0,2	19,9
Glycerol + IPTG	0,3	18,5
Glycerol + lactose	0,1	17,4
Glycerol + melibiose	10,4	10,4
Glycerol + raffinose	1,8	9,9
Glycerol + galactose	1,1	11,9

3.2.11.   Thiogalactoside transacetylase levels in EC-C and EC-S.

No thiogalactoside transacetylase was found in either EC-C or EC-S, although the β-galactosidase levels found after IPTG induction were elevated to similar levels in both strains (Table 18). The β-galactosidase levels were assayed in non-heated samples, as heating denatured the enzyme. Thiogalactoside transacetylase was also assayed in non-heated samples of E. chrysanthemi in case it was heat labile in this organism. No IPTG induced activity was observed in either EC-C or EC-S. High thiogalactoside transacetylase levels were detected in induced but not in uninduced extracts of E. coli (Table 18).

Table 18. Thiogalactoside transacetylase and β-galactosidase activity in EC-C, EC-S, and a Lac$^+$ and Lac$^-$ strain of E. coli.

Strain	Thiogalactoside transacetylase (A_{412} h^{-1})		β-galactosidase (U ml^{-1})	
	Induced	Uninduced	Induced	Uninduced
EC-C	<0,013	<0,015	610,0	63,1
EC-S	<0,013	<0,015	537,7	40,8
E. coli Lac$^+$	1,010	<0,014	2042,8	13,1
E. coli Lac$^-$	0,045	<0,012	1006,9	56,5

3.3.   DISCUSSION.

Results have been presented of relevance to both the organism and to the methods of analysis. Before investigating lactose metabolism in E. chrysanthemi, consideration was given to the methods used in obtaining the results. For example, it was necessary to take care in choosing a permeabilizing agent for cells. Although toluene by itself is an effective permeabilizing agent, it suffers from the disadvantage that it must be removed from the cells before assay, otherwise the slowly dispersed, fine suspension formed during the mixing of the cells interferes with subsequent spectrophotometric readings. Due to the instability of the suspension, its composition changes continuously, and therefore a correction factor such as that for cell-turbidity cannot be applied. The removal of the toluene is time consuming, requiring shaking in a water bath at 37°C for 40 minutes. Furthermore, as a large surface area is required for removal of the toluene, the permeabilization and assay are performed in 25 millilitre conical flasks, limiting the number of samples that can be assayed in a single shaking water bath.

Several toluene and other organic solvent-based lysis mixtures have been developed to overcome the problems associated with using toluene by itself. However, not all of these were suitable for the present investigation. The lysis mixture devised by Putnam and Koch (1975) inhibited β-galactosidase by about 80%. As Putnam and Koch (1975) used a sensitive fluorimetric β-galactosidase assay, the lysis mixture reagents could be

diluted to levels no longer inhibitory to the assay. This was not possible with β-ONPG as substrate due to a lack of sensitivity. However, the problem of the inhibition of β-galactosidase by the original lysis mixture was eventually solved by replacing the SDS with DOC.

The activity of β-galactosidase in crude extracts from both EC-C and EC-S were similarly affected by SDS, DOC, Mg^{2+}, Mn^{2+}, Na^+, and 2-ME. Although the effects of these compounds were tested on crude enzyme mixtures, all subsequent assays were also done in crude mixtures. Therefore, the effects of these compounds on enzyme activity were determined under conditions used in this study.

The β-galactosidase from E. coli has been highly purified, and intensively studied. Both Na^+ and Mg^{2+} have been found to stimulate the activity of the E. coli β-galactosidase (Hill and Huber, 1971; Wallenfels and Weil, 1972; Huber et al., 1979). The interdependence of the effects of Na^+ and Mg^{2+} for the E. chrysanthemi β-galactosidase are also similar to those observed for the E. coli enzyme (Hill and Huber, 1971). The stimulation of the E. chrysanthemi β-galactosidase by 2-ME, thought to be due to 2-ME being a better galactosyl acceptor than water, is similar to that found for the E. coli enzyme (Wallenfels and Weil, 1972).

The importance of assaying the effects of activators and inhibitors under realistic conditions is further illustrated by the effect of the choice of buffer on the pH optima of β-galactosidases from strains EC-C and EC-S. McIlvaine buffers, due to citrate inhibiting the activity

of the β-galactosidase, apparently shifts the pH optima of the enzymes to higher values. In the absence of citrate, the pH optima for the β-galactosidases from E. chrysanthemi strains EC-C and EC-S were found to be similar to that observed for the E. coli enzyme (Wallenfels and Weil, 1972). Thus, the choice of conditions (in this case buffer) is critical if realistic data are to be obtained in the laboratory.

A problem was experienced with the linearity of the β-galactosidase assay when the liberated glucose was determined by the GOD-POD method. The GOD-POD glucose standard curves are linear under these conditions, and therefore, an unknown factor in the assay medium appears to be responsible for this result. Neither the presence or absence of Na^+ or Mg^+, nor the presence of 2-ME is responsible for this anomalous result. There is no explanation for the non-linearity of the β-galactosidase assay on lactose when the samples are assayed by the GOD-POD method, as the same samples, when assayed by the HK/G6P-DH method, give rise to linear reaction curves.

The Lac$^-$ phenotype of the parent EC-C could result from any of the following, either singly or in combination:

a. the absence of a β-galactosidase with sufficiently high activity on lactose to promote growth. This was a distinct possibility even though the β-galactosidases from EC-C and EC-S showed similar activity towards β-ONPG.

b. the failure of lactose to induce β-galactosidase.

c. the absence of a lactose transport system.

The possibility that the β-galactosidases from strains EC-C and EC-S have different activities on lactose was discounted by the finding that the ratio of the rate of hydrolysis of β-ONPG to that of lactose by both β-galactosidases was similar. In addition, the β-galactosidases from both strains also have similar Km values for β-ONPG, which again points to their basic similarity. It was therefore concluded that the Lac⁻ phenotype of EC-C could not be accounted for by the inability to hydrolyse lactose sufficiently rapidly for growth.

In this connection, it is worth noting that a mutant of E. coli has been found to possess a β-galactosidase with only weak activity on lactose. E. coli possesses two operons coding for a β-galactosidase. The lac operon codes for the β-galactosidase involved in lactose metabolism (Zabin and Fowler, 1980), and the ebg operon codes for a β-galactosidase with low activity on lactose, and no known physiological function (Hall, 1982, 1983). The two operons are distinct, and their products are different in a number of ways. Firstly, as indicated previously, the lac operon possesses three structural genes, one coding for a β-galactosidase (lacZ), one coding for lactose permease (lacY), and one coding for thiogalactoside transacetylase (lacA), whereas the ebg operon codes for two genes, one coding for a β-galactosidase (ebgA) with only weak activity on lactose, and the other coding for a protein of unknown

function ($\underline{ebg}$B). The two operons are also independently regulated, and inducers of the $\underline{lac}$ operon include lactose, IPTG, TMG, and melibiose (Miller, 1980), whereas only lactose induces the $\underline{ebg}$ operon.

Lac$^+$ mutants of $\underline{E}$. $\underline{coli}$ in which the $\underline{lacZ}$ gene has been deleted (Δ $\underline{lacZ}$) have been isolated, and have been shown to arise from a mutation resulting in increased $\underline{ebg}$A β-galactosidase activity on lactose (Campbell, 1973; Hall and Hartl, 1974; Hall and Clarke, 1977). A second mutation allowing constitutive expression of the $\underline{ebg}$ operon was also found to be necessary for the Lac$^+$ phenotype of these Δ $\underline{lacZ}$ mutants (Hall and Clarke, 1977).

The second possibility i.e. that the Lac$^-$ phenotype of EC-C was due solely to the failure of lactose to induce β-galactosidase was also discounted by the observation that EC-C failed to grow on lactose even when IPTG was present to induce the β-galactosidase. Therefore it was concluded that the Lac$^-$ strain (EC-C) did not possess a lactose transport system inducible by lactose, but that this ability was acquired by mutation to the Lac$^+$ strain (EC-S).

These findings, together with the failure to detect thiogalactoside transacetylase activity, suggest that the strains of $\underline{E}$. $\underline{chrysanthemi}$ (FH1) used in this study (EC-C and EC-S) do not have a $\underline{lac}$ operon analogous to that found in $\underline{E}$. $\underline{coli}$. In $\underline{E}$. $\underline{coli}$, the $\underline{lacZ}$, $\underline{lacY}$ and $\underline{lacA}$ genes are expressed coordinately (Zabin and Fowler, 1980). In contrast, β-galactosidase in $\underline{E}$. $\underline{chrysanthemi}$ can be induced in EC-C without

coordinate induction of either a lactose permease or a thiogalactoside transacetylase. Furthermore, in EC-S, a lactose permease is expressed constitutively whereas the β-galactosidase remains inducible. Lastly, melibiose, an inducer of the lac operon in E. coli (Miller, 1980), does not induce β-galactosidase in E. chrysanthemi although it does induce a permease able to transport lactose. It is concluded that lactose transport and hydrolysis are not coordinately regulated in EC-C and EC-S, and that they are probably situated on different operons.

Melibiose and raffinose both induce a lactose transport system in EC-C. The raffinose induced lactose transport system in EC-C differs from the constitutively expressed lactose transport system in EC-S by its increased sensitivity to inhibition by raffinose. Therefore it appears as if the two lactose transport systems are different, and that E. chrysanthemi may possess two independently regulated permeases capable of lactose transport. It seems likely that one of these is associated with the raf operon, and the other with the mel operon. In addition, the competition data also shows that both the melibiose and the raffinose induced permeases have high affinity for melibiose, whereas only the raffinose induced permease has high affinity for raffinose.

The results obtained in this thesis suggest that it is the mel operon which is expressed constitutively in EC-S. Thus, high levels of α-galactosidase and lactose transport are coordinately expressed in EC-S, whereas only low levels of α-galactosidase are induced by raffinose in EC-C as compared to lactose transport. If the α-galactosidase and the

lactose permease are coordinately synthesized from a single operon, the ratio of lactose permease:α-galactosidase activity (cpm/U α-galactosidase) in glycerol-grown EC-S, melibiose-induced EC-C, and raffinose-induced EC-C should be similar. This is not the case, and calculation reveals that it is lowest in the case of glycerol grown EC-S (134.2), of intermediate value in melibiose-induced EC-C (216.8), and highest in raffinose-induced EC-C (591.2) i.e. proportionately higher levels of α-galactosidase are synthesized in glycerol-grown EC-S than in either melibiose- or raffinose-induced EC-C. These data therefore indicate that the raf operon is expressed predominantly in raffinose-induced EC-C, but that both the mel and raf operons are expressed in melibiose-induced EC-C. If this is true, it follows that raffinose is a good inducer of the raf operon, but a poor inducer of the mel operon, and that melibiose is a good inducer of both the mel and raf operons in E. chrysanthemi. This interpretation, however, requires additional experimental verification.

Briefly stated, the available evidence favours the following interpretation. In EC-C, lactose transport can occur via an inducible mel permease and an inducible raf permease, and that the Lac[+] phenotype of EC-S is due to a regulatory mutation allowing constitutive expression of the mel operon.

Although the E. chrysanthemi mel permease resembles those of E. coli, S. typhimurium, and K. aerogenes in not transporting IPTG, it appears to be different from these permeases in not being able to transport TMG (Prestidge and Pardee, 1965; Rotman et al., 1968; Schmitt, 1968; Wilson

et al., 1982) and S. typhimurium (Levinthal, 1971). However, the E. chrysanthemi mel permease resembles the K. aerogenes mel permease in its ability to transport lactose (Reeve and Braithwaite, 1973a; Wilson et al., 1982). In addition, the transport of lactose by the raffinose induced permease in EC-C, and its inhibition by melibiose and raffinose resembles the behaviour of the raf permease in E. coli (Schmid and Schmitt, 1976)

Indirect evidence suggests that the mel permease in E. chrysanthemi can transport melibiose, raffinose, and TDG in addition to lactose. However, transport data obtained only from competition experiments cannot be used as direct evidence that a compound itself is transported by an organism, as it is clear that although IPTG is a powerful inhibitor of lactose transport in E. chrysanthemi, it is not accumulated by the organism. The ability of E. chrysanthemi to grow on melibiose and raffinose, however, is indicative that these two sugars are transported into the cell.

Recently, Hugouvieux-Cotte-Pattat and Robert-Baudoy (1985) isolated Lac[+] mutants from Lac[-] strains of E. chrysanthemi that are similar to the E. chrysanthemi strain EC-S used in this study. Lactose transport by their Lac[+] strains was constitutive, and could be induced by melibiose, raffinose, and galactose in the Lac[-] strains (Hugouvieux-Cotte-Pattat and Robert-Baudoy, 1985). As was found for the strains used in this study, no thiogalactoside transacetylase activity was detected in either the Lac[-] or the Lac[+] strains. Lactose transport by the E. chrysanthemi was assigned to a transport system coded for by the lmrT gene. However, the

results reported in the present study suggest that the lmrT gene may in fact be the melB gene. Hugouvieux-Cotte-Pattat and Robert-Baudoy (1985), did not investigate the transport of any other β-galactosides by their Lac$^+$ mutants, nor did they look at the coordinate expression of a lactose transport system and an α-galactosidase. Furthermore, no differences were sought in substrate specificity between the melibiose and raffinose induced transport system, making a direct comparison of the lmrT permease and the lactose permease of strain EC-S difficult.

The whole question of galactoside transport in E. coli is complex, with at least four permeases capable of α- and β-galactoside transport (Rotman et al., 1968). The problem is further complicated by the multiple common inducers and substrates of these permeases (Rotman et al., 1968). The unravelling of galactoside transport in E. coli required the isolation of the appropriate mutants (Rotman et al., 1968; Schmitt, 1968), and it is apparent that a similar approach is required in E. chrysanthemi. Thus, future studies on the transport of lactose, melibiose, and raffinose will require the isolation of Lac$^-$, Mel$^-$, and Raf$^-$ mutants, as well as combinations of these mutants. Valid kinetic studies will also require mutants lacking the hydrolytic enzymes, as the continual hydrolysis of substrate and efflux of the products from the cell, make the measurement of initial rates of transport difficult.

Substrates of the phosphoenolpyruvate:sugar phosphotransferase system (PTS) such as glucose, 2-deoxyglucose, and mannose, have been found to inhibit both the lac and mel permeases in E. coli (Saier, 1977, 1982;

Dills et al., 1980). The interaction of components of the PTS sugar transport system with the lac and mel permeases has been shown to involve some form of allosteric interaction between the components of the PTS system and the mel or lac permease (Saier et al., 1983; Daniel, 1984). A similar mechanism may be involved in the inhibition by glucose and 2-deoxyglucose of lactose transport in EC-S.

No explanation is immediately forthcoming about the decreased rate of lactose transport after growth on melibiose. This may be due to catabolite repression, but further work is required to establish this. There is evidence that the products of lactose hydrolysis (i.e. glucose and galactose) appear in the medium during growth of E. coli on lactose (Huber et al., 1980; Huber and Hurlburt, 1984). Therefore, it is conceivable that recapture of the glucose by the phosphoenolpyruvate:sugar phosphotransferase in E. coli regulates the rate of lactose metabolism, both by catabolite repression, and by inducer exclusion. A similar mechanism may be operative in E. chrysanthemi during growth on lactose, melibiose, or raffinose. However, as little is known about the metabolism of these sugars in E. chrysanthemi, additional studies are required before any definite conclusions are formed.

It is not clear why IPTG and TMG in the growth medium should enhance lactose transport in strain EC-S, without enhancing the transport of α- and β-ONPG. The fact that this only occurs in EC-S and not in EC-C, and that neither of these two compounds are inducers or substrates of the mel permease, makes interpretation of this phenomenon difficult.

The resistance of the α-galactosidase from E. chrysanthemi to toluene is similar to that of the raf α-galactosidase from E. coli (Schmid and Schmitt, 1976), but is different to the mel α-galactosidases of E. coli and S. typhimurium, both of which are sensitive to toluene inactivation (Hogness and Battley, 1957; Sheinin and Crocker, 1961; Schmitt and Rotman, 1968; Levinthal, 1971; Burstein and Kepes, 1971). Furthermore, neither Mn^{2+} nor DTT, both of which stabilize α-galactosidase activity in cell free extracts of E. coli (Schmitt and Rotman, 1966; Burstein and Kepes, 1971), activated the Erwinia enzyme. The α-galactosidase in EC-C and EC-S also differs from the E. coli enzyme in that a cofactor is not required for activity (Burstein and Kepes, 1971).

Many members of the Enterobacteriaceae have been found to acquire new metabolic functions by mutations that allow the enzymes of different pathways to be used for new functions (Mortlock, 1982; Saier, 1982). Two types of mutations have been found. Regulatory mutations may occur which allow enzymes not inducible by the substrate, to be expressed constitutively, thus allowing metabolism of the substrate (Mortlock, 1982; Saier, 1982). A second type of mutation may change the substrate specificity of an enzyme, allowing metabolism of a compound which is not normally a substrate of the enzyme (Mortlock, 1982; Saier, 1982).

The β-galactosidase in EC-C and EC-S has been shown to have similar activity on β-ONPG and lactose, suggesting that a mutation has not affected substrate specificity of the enzyme. Therefore an altered

specificity of β-galactosidase cannot explain the difference in lactose metabolism between EC-C and EC-S. The results presented in this chapter suggest that EC-S becomes phenotypically Lac[+] due to a mutation allowing constitutive expression of the mel operon, i.e. a regulatory mutation. Little or no work has been reported on lactose metabolism in E. chrysanthemi, and it is not clear if a lac operon exists in any other strains of this organism.

In this chapter, results have been presented that throw light on the processes involved in lactose metabolism in E. chrysanthemi. Major differences have been found in the mechanism and regulation of lactose transport and metabolism in E. chrysanthemi and E. coli. Furthermore, the data may explain why so many strains of E. chrysanthemi are reported to have either a variable or delayed ability to ferment lactose (Dye, 1969a; Lelliot, 1974; Goto, 1979; Dickey, 1979). Therefore, the findings in this chapter can be regarded as having made a definite contribution to the biochemistry of E. chrysanthemi.

CHAPTER 4

POLYSACCHARIDE STRUCTURE

In Chapter 2 it was reported that E. chrysanthemi (FH1) strain EC-S produced copious amounts of an extracellular polysaccharide (EPS) when grown on lactose. This was of interest because polysaccharides have been found to play a major role in the interactions of bacteria with their environment. For example, both the lipopolysaccharides (LPS) and the extracellular polysaccharides (EPS) have been implicated in the pathogenicity of certain animal and plant pathogens (Wilkinson, 1977; Corpe, 1980; Bauer, 1981; Pistole, 1981; Keen and Holliday, 1982; Luderitz et al., 1982; Sequeira, 1982, 1983, 1984a,b; Sparling, 1983; Vance, 1983). At present, little is known about the polysaccharides produced by phytopathogens. Neither the factors that distinguish the surface carbohydrates of virulent and avirulent cells nor their involvement in pathogenicity are known. This is due in part to a lack of knowledge of the structures of the polysaccharides produced by phytopathogens. The determination of the structures of these polysaccharides therefore constitute an important area of research. The possible role of the EPS produced by E. chrysanthemi in pathogenicity together with the scarcity of knowledge of the surface carbohydrates of this species prompted the structural study of the EPS reported in this chapter.

In this chapter, the production, purification, and partial structure of an EPS produced by strain E. chrysanthemi (FH1) strain EC-S during growth on lactose is described using a combination of selective degradative methods, methylation analysis, and NMR spectroscopy.

4.1. MATERIALS AND METHODS.

4.1.1. Organism.

The Lac$^+$ (EC-S) strain of E. chrysanthemi (FH1) described in Chapter 2, was used for polysaccharide production.

4.1.2. Polysaccharide Production.

A. Medium.

YS-agar (described in Chapter 2, Section 2.1.2), supplemented with 0.5% (w/v) lactose was used for polysaccharide production. The yeast extract was filtered through an ultrafilter (type PSAC, molecular mass cut-off 1000, Millipore Corporation, Bedford, Massachusetts) to remove any high molecular mass carbohydrates present before use.

B. Procedure.

Plates of YS-lactose agar were densely streaked in a cross-hatched pattern with E. chrysanthemi strain EC-S, and incubated for 18 h at 30°C,

followed by 3-4 days at room temperature (20-22°C). The plates were incubated lid side up otherwise the EPS/bacterial mixture dripped from the agar surface, resulting in lower EPS yields due to drying out of the agar surface.

The EPS/bacterial mixture was carefully scraped off the agar surface with a bent glass "hockey" stick. Care was taken not to contaminate the EPS with agar fragments from which soluble agar polysaccharides might be extracted during subsequent washing. The viscous mixture was diluted three to four fold and the cells sedimented by centrifugation in a Beckman J2-21 centrifuge (30 000 X g, 4°C, 90 min). The clear, slightly brownish supernatant was decanted and, after resuspension in 0,25 volumes of cold distilled water (4°C), and cells were repelleted as described above. The pooled supernatants were lyophilized, and the dry polysaccharide stored in air-tight jars until further purification.
Approximately 2 g of crude polysaccharide was obtained per three litres of solid medium (about 140 plates).

4.1.3. Purification of EPS.

Crude EPS in water (5 mg ml^{-1}) was clarified by centrifugation (27 000 X g, 20°C, 30 min), and precipitated, with continuous stirring, by the slow addition of 3 volumes of ethanol (95% v/v) containing 50 mM NaOH (preliminary experiments showed that the precipitation of the EPS in the presence of NaOH required a far lower volume of ethanol). A crystal of NaI was added to aid flocculation, and the precipitate allowed to settle

at room temperature for 30 min. Most of the supernatant was decanted off, and the EPS recovered by low speed centrifugation (450 X g, room temperature, 5 min). After dissolution of the EPS in the initial volume of water, the precipitation was repeated twice more. The EPS was dried by suspending it in absolute ethanol (3 X 25 ml), and recovering the suspension by low speed centrifugation each time. The ethanol was removed by suspending the EPS in dry diethyl ether (3 X 25 ml), and recovering the suspension by low speed centrifugation each time. Most of the residual ether was allowed to evaporate at room temperature, the final traces being removed by rotary evaporation at 40°C under vacuum (water pump). The EPS was stored desiccated at room temperature. The final yield of EPS varied between 36% and 50%.

4.1.4. Electrodialysis.

Electrodialysis of the EPS (10 mg ml^{-1}) was carried out to remove charged low molecular-mass contaminants. The EPS, in a dialysis bag, was electrodialyzed at 4°C until the current had stabilized. The water in the electrode and sample compartments was replaced frequently during the dialysis period. The initial voltage and current was 400 V, and 20 mA; the final voltage was 1000 V and current about 3 mA. The yield of EPS after freeze drying was about 80%.

4.1.5. Assay for Neutral Sugars.

The phenol-sulphuric method of Dubois et al. (1956) was used to quantify neutral sugars.

A.   Reagents.

(i).   Phenol (80% w/w).   Phenol (80 g) was added to distilled water (20 g) with gentle warming, and stored in an amber glass bottle at room temperature. This reagent is stable for several months.

(ii).   Sugar Standards.   Standard solutions of glucose, mannose, or rhamnose (1 mg ml^{-1}) were made up in water and stored at -20°C.

B.   Procedure.

A set of matched 150 X 18 mm test tubes were used for all assays. Samples containing 0.01-0.05 mg of sugar were pipetted into the test tubes and made up to 2 ml with water. Phenol (80% w/w, 0.1 ml) was added to each tube followed by the rapid addition of 5 ml of concentrated sulphuric acid from a dispenser. Rapid addition of the acid is necessary as the heat generated by dilution of the acid plays an important role in the development of the chromophore. The absorbance was determined after 30 min against a reagent blank using either a Perkin-Elmer model 35 (Perkin Elmer Corporation, Norwalk, Connecticut, U.S.A.) or a Spectronic 88

spectrophotometer at 490 nm. Standard curves (0,01-0,05 mg) for glucose, mannose, rhamnose, and glucuronic acid were prepared in parallel when required.

4.1.6. Paper and thin layer chromatography.

Paper and thin layer chromatography (TLC) were used, in addition to gas liquid chromatography (GLC), to identify the monosaccharides released by acid (trifluoroacetic) hydrolysis from the native and carboxyl-reduced EPS.

A.   Reagents.

The following reagents were used in addition to those described in Chapter 2 (Section 2.1.14). The solvent described in Section 2.1.14 will be called solvent I, and the solvent used for TLC, solvent II.

(i).   Solvent II.   The mobile phase for TLC contained.

Formic acid	150 ml
Butanone	300 ml
t-Butanol	400 ml
H_2O	150 ml

(ii).   Alkaline silver nitrate visualization reagent.

Reagent 1. A saturated aqueous silver nitrate solution (1 ml) was diluted to 200 ml with acetone, and the precipitate redissolved by the careful addition of water (5-10 ml).

Reagent 2. An aqueous ethanolic sodium hydroxide solution was prepared by dissolving sodium hydroxide (20 g) in a minimum volume of water, and making up to 1 litre with 95% ethanol.

B.   Procedure.

After removal of the TFA from the acid hydrolyzates by rotary evaporation at 40°C, the residue was dissolved in water to a concentration of about 5 mg ml^{-1}, and stored at -20°C.

Samples were analyzed by descending paper chromatography on Whatman No. 1 chromatography paper using solvent I, or by TLC on 0,1 mm cellulose coated plates (Merck) using solvent II. A set of standards containing glucose, mannose, rhamnose, and glucuronic acid were run together with the samples.

After chromatography, the paper and thin layer chromatograms were allowed to dry at room temperature. Thereafter, the paper chromatograms were dipped, and the thin layer chromatograms sprayed, with reagent 1 and allowed to dry. This was followed by dipping (Paper) or spraying (thin

layer) the chromatograms in reagent 2. After drying, the chromatograms were heated at 100°C for 1-2 min.

The background was decolourized by dipping (Paper) or spraying (thin layer) the chromatograms with 5% sodium thiosulphate.

The sugars appeared as dark spots on a light background.

4.1.7. Lowry Protein Assay.

The protein content of the EPS was determined by the Lowry method (described in Chapter 2, Section 2.1.6).

4.1.8. Coomassie Blue Dye-Binding Protein Assay.

In addition to the Lowry procedure, protein was also assayed using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Richmond, California, U.S.A). The method, developed by Bradford (1976), relies on the shift in maximum absorbance from 465 to 595 nm when Coomassie Brilliant Blue G-250 binds to protein in acid solution.

A.   Reagents.

(i).   Dye Reagent. The reagent supplied with the kit was used undiluted.

(ii).   Protein Standard. The standard bovine gamma globulin (1 mg ml^{-1}), supplied with the kit, was used to prepare a standard curve.

(iii).   Phosphate Buffered Saline,(PBS). PBS contained.

NaCl	8,0 g
KH$_2$PO$_4$	0,2 g
KCl	0,2 g
NaH$_2$PO$_4$.2H$_2$O	2,30 g
H$_2$O	to 1 litre
pH	7,3

B.   Procedure.

The micro-assay procedure, as described in the manufacturer's instructions, was used without modification. Polysaccharide (1 mg ml^{-1}, 0,2 ml) was made up to 0,8 ml with PBS, pH 7,3, and undiluted dye reagent (0,2 ml) added with mixing. After standing for 10 minutes at room temperature, the absorbances of the solutions was read against a reagent blank at 595 nm. A protein standard curve was prepared in parallel.

  4.1.9. Assay for Uronic Acids.

The carbazole assay as modified by Bitter and Muir (1962) was to quantify uronic acids.

A. Reagents.

(i).   Borate-Sulphuric Acid Reagent. Sodium borate ($Na_2B_4O_7.10H_2O$, 2.32 g) was dissolved in water (10 ml), and ice-cold concentrated sulphuric acid (390 ml) added with stirring. The reagent was stored in a glass bottle in the refrigerator.

(ii).   Carbazole Reagent. Carbazole (100 mg) was dissolved in absolute ethanol (50 ml), and stored in an amber bottle in the refrigerator at $4^{\circ}C$.

(iii).   Glucuronolactone Standard. Glucuronolactone (17.6 mg) in water (100 ml) was made up fresh before each experiment. The final concentration of glucuronolactone in the standard was 1 μmole ml^{-1}.

B.   Procedure.

A sample (0.2 ml) containing up to 0.2 μmole uronic acid was made up to 0.5 ml with water in matched 100 X 13 mm test tubes, and cold borate-H_2SO_4 reagent (3.0 ml) added with immediate mixing. After heating in a boiling water bath for 20 minutes, the tubes were cooled in ice water to $0^{\circ}C$, and carbazole reagent (0.1 ml) added with mixing. The tubes were heated for a further 10 minutes in a boiling water bath, and allowed to stand at room temperature for 15 minutes, before reading absorbance at

530 nm a reagent blank. A standards curve (0,04-0,2 μmole uronic acid) was prepared and assayed at the same time.

Glucose, mannose, and rhamnose also produce colour with the carbazole reagent, and this contribution to the absorbance has to be corrected for when assaying heteropolymers. This was done by assaying samples containing 0,05 mg of each of these sugars and correcting for their contribution to the absorbance at 530 nm by using a simple mathematical procedure described in the results section (Section 4.2.2).

4.1.10. Assay for 2-keto-3-deoxyoctonic acid (KDO).

KDO was assayed by the procedure of Karkhanis et al. (1973).

A. Reagents.

(i). $0,1\ M\ H_2SO_4$ was prepared by diluting 0,98 g c.H_2SO_4 to 100 ml with H_2O.

(ii). $0,04\ M\ HIO_4$ in $0,0625\ M\ H_2SO_4$ was prepared by dissolving periodic acid, H_5IO_6 (0,911 g) in 100 ml of 0,0625 M H_2SO_4 (0,613 g c.H_2SO_4 per 100 ml H_2O).

(iv). $2,6\%\ (w/v)\ NaAsO_2$ in $0,5\ M\ HCl$ was prepared by dissolving 2,6 g $NaAsO_2$ in 0,5 ml HCl (5 ml c.HCl, SG 1,16, made up to 100 ml with H_2O).

(v).   0,6% (w/v) Thiobarbituric acid (TBA) was prepared by dissolving TBA (0,6 g) in H_2O to a final volume of 100 ml.

(vi).   Standard. A standard KDO solution was prepared by dissolving 100 g KDO (Sigma) in H_2O (1 ml).

B.   Procedure.

Electrodialyzed EPS (2 mg) was weighed into a test tube, and 0,1 M H_2SO_4 (1,0 ml) added. After heating for 30 min in a boiling water bath, the tube was cooled, and centrifuged at top speed for 5 min in a bench top clinical centrifuge to remove any precipitate. A sample (0,5 ml) was removed to a clean test tube, and after the addition, with mixing (vortex mixer), of acid periodate (0,25 ml), was allowed to stand at room temperature for 20 min. Thereafter, acid $NaAsO_2$ solution was added with mixing and after the brown colour had cleared, TBA reagent (0,5 ml) was added. The resulting mixture was heated in a boiling water bath for 15 min, after which dimethylsulphoxide (1,0 ml) was added to the still hot solution. After cooling to room temperature, absorbance was determined at 548 against a reagent blank prepared in parallel, and in which the EPS was omitted.

A standard curve for KDO (1-10 μg) was prepared together with each batch of unknowns.

4.1.11. Analysis of Periodate by Avigad's Method.

Periodate consumption during periodate oxidation was assayed either by the colorimetric method developed by Avigad (1969), or by the absorbance of the periodate ion at 260 nm as described by Grado and Ballou (1961). The method developed by Avigad depends on the decolourization of a violet ferrous 2,4,6-tri-2-pyridylyl-S-triazine (TPTZ) complex by periodate.

A. Reagents.

(i).   Sodium Periodate. A stock solution of 0,1 M sodium periodate (2,1389 g per 100 ml H$_2$O) was prepared fresh weekly, and stored in a dark bottle at 4°C. Appropriate dilutions of this stock solution were made for the preparation of a standard curve before each analysis.

(ii).   TPTZ Reagent. TPTZ (75 mg, 0,24 mmole) was dissolved in glacial acetic acid (46 ml), and 1 M sodium acetate (20,503 g per 250 ml, 210 ml) added with mixing. Freshly prepared ferrous ammonium sulphate (31,4 mg in 100 ml H$_2$O) was added, and the resulting solution made up to 1 litre with water. The pH of the violet solution was 4,0-4,2. The reagent is stable at room temperature for about 1 month. (The initial absorbance of the reagent is about 1,8 at 593 nm, but decreases slightly on storage. Although this does not affect the slope of the standard curve it does decrease the capacity of the reagent for

periodate, i.e. smaller amounts of periodate decolourize the stored reagent than the fresh reagent. The periodate determination is unaffected provided the capacity of the reagent is not exceeded).

B.   Method.

A sample (0,5 ml) containing periodate (< 200 nmole) was added to TPTZ reagent (4,5 ml) in matched 100 X 13 mm test tubes, mixed, and the absorbance determined at 593 nm.

4.1.12. Analysis of Periodate at 260 nm.

Alternatively, periodate consumption was also measured at 260 nm during oxidation studies (Grado and Ballou, 1961).

Procedure.

Samples from the oxidation mixtures were removed, appropriately diluted in water, and the absorbance at 260 nm determined in a Beckman Model 35 spectrophotometer. A 0,9901 X 10^{-6} M solution has an absorbance of 1.0 (Grado and Ballou, 1961).

4.1.13. Molecular Exclusion Chromatography.

A.   Bio-Gel A-1,5m Molecular Exclusion Chromatography.

A column (1,5 X 82 cm) of Bio-Gel A-1,5m (200-400 mesh, Bio-Rad Laborat-
ories) was prepared according to the manufacturer's instructions. The
pre-swollen gel was suspended in 10 volumes of distilled, deionized water
in a measuring cylinder, and allowed to stand until more than 95% of the
gel had settled. The supernatant, including the fines, was siphoned off,
and the procedure repeated twice more. The sized gel was suspended in
water to form a thin slurry, and after de-aeration at room temperature
under vacuum (water pump) for 30 minutes, was packed in a column (1,5 X
82 cm). On completion of packing, two bed volumes of water were pumped
through the column at a flow rate of about 14 ml h^{-1}.

When the column was operated with a different mobile phase, it was re-
equilibrated by passing at least two column volumes of the new mobile
phase through the column.

The packing of the column was checked by chromatography of a mixture (2
ml) of rabbit liver glycogen (4 mg ml^{-1}) and glucose (4 mg ml^{-1}). The
molecular mass of the rabbit glycogen is greater than 5 000 000 (Profes-
sor Rex Montgomery, personal communication), and eluted with the void
volume (V_o) of the column. The elution volume of glucose was used to
estimate the total volume (V_t) of the column. Collected fractions were
analyzed for total carbohydrate by the phenol-sulphuric procedure.

B.   Bio-Gel P-2 Column Chromatography.

Bio-Gel P-2 (200-400, or -400 mesh) was allowed to swell for at least 24 hours in distilled water, after which the fines were removed and a column (1,5 X 62 cm) packed in a similar manner to the Bio-Gel A-1,5m column described above. The column was equilibrated with at least two bed volumes of water, or appropriate eluant, at a flow rate of about 15 ml h^{-1}.

Chromatography of a mixture (2 ml) of laminarin (molecular mass, 7800), stachyose (molecular mass, 660), maltose (molecular mass, 320), and glucose (molecular mass, 180) was used to check the column packing. The concentration of each carbohydrate was 4 mg ml^{-1}.

4.1.14. Anion Exchange Chromatography.

A.   Preparation of DEAE-Cellulose Ion Exchanger.

The ion exchanger(DE-52) is supplied by the manufacturer (Whatman, W and R Balston Ltd., Springfield Mill, Maidstone, Kent, England) pre-swollen, and therefore does not have to be taken through a swelling procedure. A sufficient quantity of the pre-swollen exchanger was suspended in 1 M KCl overnight, and the supernatant decanted. The exchanger was suspended in 10 mM KCl, allowed to settle, and resuspended in a fresh amount of 10 mM KCl. Fines were removed from the DEAE-cellulose by a method similar to

that described above for the molecular exclusion gels, and the exchanger deaerated prior to use.

B. Determination of EPS Absorption and Elution Conditions.

Aliquots of the prepared exchanger, suspended in an equal volume of 10 mM KCl, were pipetted into a series of eight glass centrifuge tubes, and centrifuged at low speed (500 X g, room temperature, 5 minutes). Each pellet was resuspended in one of eight concentrations of KCl, and the exchanger allowed to equilibrate at the new KCl concentrations for one hour. The exchanger was recovered by centrifugation as described above, and the procedure repeated twice more. The contents of each tube were finally resuspended in 1 ml of the equilibrating concentration of KCl. A solution of EPS in water (4 mg ml^{-1}) was clarified by centrifugation in a Beckman J-21 centrifuge (27 000 X g, 4°C, 30 minutes), and diluted two-fold into appropriate concentrations of KCl so that the final KCl concentrations were equal to those in which the exchanger was equili-brated. Aliquots (1 ml) of these EPS solutions were added to the relevant tubes of exchanger and allowed to adsorb for 30 minutes. Samples were removed from the tubes, clarified by centrifugation in a Beckman Micro-fuge (9 800 X g, room temperature, 2 min), and the supernatants assayed for residual EPS by the phenol-sulphuric method.

C.   Column Packing and Equilibration.

A column (2,5 X 33 cm) was packed with DE-52 pre-equilibrated in 10 mM KCl, and a minimum of four bed volumes of 10 mM KCl passed through it at a flow rate of approximately 120 ml h^{-1}.

D.   Chromatography.

Details regarding sample and elution conditions are given, where appropriate, in the results section.

4.1.15. High Pressure Liquid Chromatography (HPLC).

Molecular Exclusion Chromatography.

Homogeneity of the EPS was also examined by HPLC (Altex HPLC, Beckman) using a molecular exclusion column. Polysaccharide (2,4 mg ml^{-1}) dissolved in 0,15 M NaCl was clarified by filtration through a Millex 0,45 μm filter (HAWP, Millipore) and chromatographed at room temperature on a TSK PW 5 000 (Beckman Instruments) exclusion column (7.5 X 300 mm) using 0,15 M NaCl as eluant (0,6 ml min^{-1}). The effluent from the column was continuously monitored using a Refractive Index (RI) detector (Refracto-Monitor, Laboratory Data Control, Riviera Beach, Florida, U.S.A.). A mixture of rabbit glycogen (molecular mass about 5 000 000) and ethylene glycol (3 mg ml^{-1} of each in 0,15 M NaCl) was used to determine the V_o and V_t of the column.

4.1.16.   Determination of the equivalent weight of the uronic acid.

The equivalent weight of the uronic acid was determined by titrating a sample of the electrodialyzed EPS (2,754 mg) with 0,01 M NaOH (5 ml of 0,1 M NaOH, Fisher certified reagent, diluted to 50 ml with H_2O) using a syringe burette (Micro-Metric Instrument Co., Cleveland, Ohio, USA). The pH of the solution was continuously monitored during addition of the base, and the end point determined graphically.

4.1.17.   Ultracentrifugation.

Ultracentrifugation analysis of the EPS was carried out on a Beckman Model E ultracentrifuge fitted with Schlieren optics.

Method.

Polysaccharide (1 mg ml^{-1}) was dialyzed against PBS, pH 7.3, for 24 hours, and clarified by filtration through a Millex 0,45 um filter (Millipore) before analysis in an AN-H head at 60 000 rpm. Photographs were taken every four minutes after the rotor had reached 54 800 rpm. Distances on the photographic plates were measured after the run by a Nikon Shadowgraph model 6C.

4.1.18. Monosaccharide Composition.

A. Reagents.

(i).   Acetic anhydride. Analytical grade acetic anhydride was refluxed over anhydrous sodium sulphate for 30 minutes and distilled. The first fraction was discarded, and the rest collected in an amber bottle and stored over a 4 A^o molecular sieve (Merck).

(ii).   Pyridine. Pyridine (analytical grade) was dried by storage over KOH pellets.

(iii).   Trifluoroacetic Acid (TFA). A 2 M solution of TFA was prepared by diluting 2,28 g of the acid to 10 ml.

B. Hydrolysis of EPS.

Polysaccharide (0,1-1,0 mg) in water (1 ml) was transferred to a 10 ml glass ampoule, and an equivalent volume of 2 M TFA added. The ampoules were sealed in a flame, heated at 98°C for various periods of time (30 min to 18 h), opened, and the contents quantitatively transferred to 25 ml pear-shaped Quickfit flasks. The TFA was removed from the released monosaccharides by rotary evaporation, final traces of TFA being removed by repeated evaporation from water.

When inositol was used as an internal standard, it was added to the ampoule before transferring the contents to the pear-shaped flask. When 2-deoxy-D-galactose was used as an internal standard it was added to the sample after removal of the TFA as it is acid labile (Grimes and Gregor, 1976).

C.   Preparation of Alditol Acetates.

The released monosaccharides were dissolved in water (1 ml), and reduced by the addition of solid sodium borohydride (10 mg). After two hours, the excess borohydride was destroyed by the addition of glacial acetic acid to a final pH of 3.5. The samples were dried by rotary evaporation, and the borate removed, as the volatile methyl borate, by co-distillation with 0.5% (v/v) acetic acid in methanol.

The alditols were converted to the acetates by adding dry pyridine (1ml) and acetic anhydride (1 ml), and heating the flasks at 90-100°C for one hour. Excess reagents were removed by rotary evaporation, the acetic anhydride being removed as the azeotrope by co-distillation with toluene (3 X 5 ml). The acetates were quantitatively removed by dissolving in chloroform (5 X 1 ml) and filtered through a glass wool plugged Pasteur pipette into a glass vial. The chloroform was removed under a stream of nitrogen, and the alditol acetates dissolved in an appropriate volume of chloroform for gas liquid chromatography (GLC).

4.1.19. Amino Sugars.

Amino Sugars were determined on an amino acid analyzer.

Procedure.

Polysaccharide (0,693 mg) was placed in an acid cleaned hydrolysis vial, 4 M HCl (1 ml) added, and the vial sealed in a flame. After heating at 100 °C for 4 hours, the vial was opened and the HCl removed by rotary evaporation. The residue was dissolved in 0,2 ml standing buffer (sodium citrate, pH 2,2 containing 0,1 mM norleucine as internal standard), and an aliquot (0,05 ml) assayed on a Beckman 121 MB Amino Acid Analyzer. A standard sodium buffer, single column, protein hydrolyzate analytical procedure was used. Standards containing glucosamine, galactosamine, or mannosamine were also chromatographed under the same conditions.

4.1.20. Amino Acid Analysis.

The protein content of the EPS was also monitored by amino acid analysis following hydrolysis.

Procedure.

Polysaccharide (0,693 mg) was placed in an acid washed hydrolysis vial and 6 M HCl (1 ml) added. The vial was sealed in a flame, heated at

110°C in an oil-bath for 24 hours, opened, and the acid removed by rotary evaporation. The sample was analyzed as described above.

4.1.21. Uronic Acid Reduction.

The procedure of Taylor and Conrad (1972) was used to reduce the uronic acid in the polysaccharide.

Procedure.

Polysaccharide (39,89 mg, 29,5 μequivalents) was dissolved in water (5 ml), clarified by filtration through a 0,45 um Millex filter (Millipore), and the pH adjusted to 4,75. A tenfold excess of solid 1-ethyl-3-(3-di-methylaminopropyl)-carbodiimide (EDC, 56,6 mg, 295 μmoles) was added, and the reaction allowed to proceed for 2 h during which time the pH was maintained at 4,75 by titration with 0,1 M HCl (Model TTT1/SBR-2 Titrimeter, Radiometer, Copenhagen, Denmark).

After the consumption of acid had ceased, aqueous NaBH$_4$ (2 M, 1,514 g per 20 ml) was added slowly over a one hour period, the pH being maintained at 7,0 with 4 M HCl. A few drops of octyl alcohol were added to control foaming. The initial addition of the borohydride was found to be critical. If it was added too rapidly, the pH of the solution could not be maintained below 7,0, resulting in lowered efficiency of reduction.

After reduction, the polysaccharide was filtered through Whatman No.1 filter paper to remove any precipitate, dialyzed against running water for 48 h, and finally against distilled water for 24 h. The reduced polysaccharide was recovered by freeze drying and stored desiccated at room temperature.

The efficiency of reduction was determined by uronic acid analyses and by DEAE-cellulose chromatography. If necessary, the reduced polysaccharide was subjected to a second reduction.

4.1.22. Isolation of the Aldobiouronic Acid.

The glycosiduronic acid linkage is more resistant to acid hydrolysis than the glycosidic bond (Percival, 1963), allowing the isolation of the uronic acid, still attached to the adjacent neutral sugar (the aldobio-uronic acid), after acid hydrolysis. Both the neutral sugar and the anomeric linkage in the aldobiouronic acid can then be determined.

Procedure.

A.   Conversion of Bio-Rad AG1-X8 (Cl$^-$) to the Acetate Form.

Conversion of the resin to the acetate form was carried out according to the manufacturer's instructions. The resin was first converted to the —OH$^-$ form by packing a glass column with an appropriate amount of resin

which had been pre-washed with water, and passing 1 M NaOH through the column until the effluent was free of chloride (AgNO$_3$ test). The column was washed with water until the effluent had a pH of less than 9.0, and thereafter with 1 M acetic acid until the pH of the effluent was less than 2. Lastly, water was passed through the column until the pH was greater than 4.8. After removal from the glass column, the resin was repacked in a column (1.0 cm X 11 cm) for isolation of the aldobiouronic acid.

B.   Hydrolysis and Isolation of the Aldobiouronic Acid.

Native EPS (175 mg) was dissolved in water (30 ml), clarified by centrifugation (27 000 X g, 20°C, 30 min), and made up to 1 M with respect to TFA by adding an appropriate volume of 4 M TFA. After heating the solution on a water bath (98°C, 6h), the TFA was removed by rotary evaporation, and the resulting yellowish syrup, dissolved in water (3 ml), applied to a column (1 X 11 cm) of Bio-Rad AG1-X8 cation exchange resin (acetate form). The column was washed with 2 bed volumes of water, followed by a linear gradient (200 ml, 0-3 M acetic acid). Aliquots of the fractions were assayed for neutral sugars by the phenol-sulphuric acid method, and for uronic acid by the carbazole method.

   4.1.23.   Gas-Liquid Chromatography.

All volatile carbohydrate derivatives were analyzed by GLC.

The following gas-liquid chromatographic systems were used:

A. Hewlett-Packard 5710A (Avondale, Pennsylvania, U.S.A.) gas chromatograph and a Hewlett-Packard 3380A integrator.

B. Perkin-Elmer Sigma 1 gas chromatograph (Perkin-Elmer Corporation, Norwalk, Connecticut, USA).

C. Varian 3700 gas chromatograph (Varian Associates, Walnut creek, California, USA) and a Hewlett-Packard 3390A integrator.

The following chromatography columns were used in the analyses:

A. OV225 packed column (2 m X 3 mm, Supelco, Inc., Bellefonte, Pennsylvania, U.S.A.).

B. SE-54 fused silica capillary column (25 m X 0,21 mm, Hewlett-Packard).

C. BP5 (equivalent to SE-52/54) fused silica column (25 m X 0,2 mm, Scientific Glass Equipment, SGE, Victoria, Australia).

Analytical conditions are given, where appropriate, in the text.

4.1.24. Gas Chromatography-Mass Spectrometry (GC-MS).

GC-MS analyses were carried out on a Girdel Series 32 GC and a Riebermag R10-10C MS (Paris, France). All data were collected and analyzed using a Digital PDP 8m computer.

The following analytical conditions were used:

Column: SE-52 (25 m X 0,2 mm) fused silica.

Carrier Gas: Helium

Linear Velocity: 30 cm sec^{-1}.

Column Temperature: 160 $^{\circ}$C isothermal.

EI Voltage: 70 eV.

4.1.25. Nuclear Magnetic Resonance (NMR) Spectroscopy.

Proton and carbon-13 NMR spectra were recorded on a Bruker WP 80 SY Fourier Transform Spectrometer (West Germany Bruker Analytische Messtechnik GMBH, Rheinstetten, W-Germany). The instrument was operated at 80,14 MHz for proton and at 20,15 MHz for carbon-13 spectra.

Procedure.

All samples were dissolved in D_2O (99,75%, Merck) and their spectra recorded at 30-35°C in 5 mm tubes. More detailed conditions are given, where necessary, in the text.

4.1.26.   Methylation Analysis.

Methylation analysis was carried out according to the method of Jansson et al. (1976).

A.   Dry Reagents.

(i).   Hexane. Dry hexane was prepared by refluxing analytical grade hexane over calcium hydride for 30 minutes, followed by distillation. The first fraction of the distillate was discarded, and the remainder collected and stored over a 4 A^o molecular sieve (Merck).

(ii).   DMSO. Dry DMSO was prepared from analytical grade DMSO by storing over a 4 A^o molecular sieve.

B.   Preparation of Dimsyl Sodium (Sodium Methyl Sulphinyl Methanide).

Sodium hydride (50% suspension in oil, 2,5 g) was weighed into a 60 ml serum vial fitted with a rubber septum and containing dry hexane (25 ml). The sodium hydride/oil mixture was suspended by swirling, the sodium hydride allowed to settle, and the hexane/oil layer carefully decanted into a dry beaker. This washing procedure was repeated twice. Two hypodermic syringe needles were passed through the rubber septum, and the sodium hydride was dried by removing the hexane under a stream of

nitrogen. Removal of the hexane was speeded up by breaking up any lumps in the vial with a spatula during this procedure. Following this procedure, the sodium hydride became a free flowing powder, and care had to be taken to contain this extremely reactive material in the vial.

The dimsyl sodium was formed by adding dry DMSO (25 ml) to the sodium hydride, and sonicating the solution in Bransonic water bath at 50-55°C until the evolution of hydrogen gas had ceased. During this period, normally about two hours, the colour of the solution changed from a light grey to an opalescent greenish-grey.

The vial of dimsyl sodium was stored in the refrigerator (4°C) in a bottle over a layer of anhydrous Drierite (anhydrous calcium sulphate). The preparation was stable for at least six weeks when stored under these conditions.

Note. Sodium hydride reacts violently with water, including atmospheric moisture, and care was taken in the handling of this reagent. All apparatus was thoroughly dried, and all manipulations were carried out in the fume-hood. Any sodium hydride in the hexane washings was destroyed by the careful addition of ethanol. Dimsyl sodium is also very reactive, and no more than 50 ml was prepared at any one time.

Procedure.

Polysaccharide (0,5-3,0 mg) was dissolved in dry DMSO (0,5-3,0 ml) in a 25 ml Quickfit pear-shaped flask fitted with a rubber septum (sonication of the samples at room temperature was found to help dissolution). The flask was flushed with N_2 via two syringe needles pushed through the rubber septum, the N_2 from the outlet needle being bubbled through light liquid paraffin, which acted as an air trap.

Dimsyl sodium (0,5-1,5 ml) was added dropwise to the flask via a glass syringe, and the resulting mixture, after sonication at room temperature for 30 minutes, was kept at room temperature overnight (16-18 hours) for the polyanion to form. To ensure the completion of reaction, dimsyl sodium must be present in excess. This was determined by adding a drop of the reaction mixture to dry triphenyl methane. Development of a red colour indicated the presence of excess dimsyl sodium (Rauvala, 1979). The solution was cooled to 0^oC, methyl iodide (0,5-1,5 ml) added dropwise to the solidified solution, and the solution was sonicated in a Bransonic water bath for one hour at room temperature. During sonication, the solution went from a creamy-white opalescent solution to a clear dark yellow solution.

The excess methyl iodide was removed from the solution by rotary evaporation at room temperature. After three-fold dilution in water, the methylated polysaccharide was dialyzed free of excess reagents against running water (24 hours) in a dialysis tube that had been previously

boiled in distilled water (3 X 30 minutes). After further dialysis against distilled water for 16 h, the methylated polysaccharide was recovered by freeze-drying and stored desiccated at room temperature.

The dry methylated polysaccharide was dissolved in chloroform and quantitatively transferred to a clean, dry 25 ml Quickfit pear-shaped flask, and the chloroform removed under a stream of nitrogen. Formic acid (90% (v/v), 2 ml) was added, and the flask heated on a boiling water bath (98°C) for one hour, after which the formic acid was removed by rotary evaporation at 40°C. The last traces of formic acid were removed by co-distillation with methanol (3 X 3 ml). After adding sulphuric acid (0,13 M, 3 ml) the contents of the flask were transferred to a 10 ml ampoule which was sealed, and heated at 98°C for 16 hours to complete hydrolysis.

The ampoule was opened, and the solution neutralized (final pH about 6,5, pH paper) by the addition of solid barium carbonate. Nitrogen gas was bubbled through the solution to continuously strip the solution of carbon dioxide. Care was taken not to add too great an excess of barium carbonate to the solution, as it made subsequent filtration difficult. The sample was passed through a Millex filter (0,45 μm, Millipore), and the filtrate collected in a dry, clean Quickfit pear shaped-flask. The solid residue was washed with distilled water (3 X 2 ml), and the washings passed through the same Millex filter. The pooled filtrate was reduced in volume to about 2 ml on the rotary evaporator at 40°C, and NaBH$_4$ (25 mg) added. After reduction of the methylated sugars for two hours at room

temperature, the excess borohydride was destroyed by the addition of Dowex-50 X8 (H+)(previously washed with methanol and distilled water) until the pH had dropped to about 3,5 (pH paper). The ion-exchanger was removed from solution by filtration through a glass-wool plugged Pasteur pipette, and the resin washed with water (3 X 1 ml). The pooled filtrate was concentrated to dryness by rotary evaporation, and the borate removed, as the volatile methyl borate, by co-distillation with methanol (3 X 5 ml).

The methylated alditols were converted to the acetates by acetylation with a mixture (1:1) of dry pyridine and acetic anhydride (2 ml) for one hour at 90-100°C and worked up as described previously (Section 4.1.16). The sample was dissolved in methylene chloride for analysis by GLC or GC-MS.

4.1.27. Periodate Oxidation.

<u>Procedure.</u>

Polysaccharide (5-10 mg ml^{-1}), clarified by centrifugation (27 000 X g, 20 minutes, 20°C), was made up to about 0,033 M in sodium periodate by the addition of an appropriate volume of ice-cold 0,1 M sodium periodate. A blank containing water in place of the polysaccharide was prepared in parallel. Aliquots were immediately removed for the determination of the periodate concentration by either the TPTZ or A_{260} method. Oxidation was

carried out in the dark at 4ºC, aliquots being removed at different times for the measurement of periodate consumption.

When periodate consumption ceased, the excess periodate was destroyed by the addition of a 10-fold excess of ethylene glycol, and the samples kept at room temperature for 30 minutes. The oxidized polysaccharide was dialyzed for 24 hours against 3 changes of distilled water, and recovered by lyophilization.

4.1.28. Smith Degradation.

Procedure.

The periodate oxidized polysaccharide was dissolved in water (2-5 mg ml^{-1}), and a calculated ten-fold excess of solid NaBH$_4$ added. The periodate oxidized polysaccharide was difficult to dissolve in water, but dissolved readily after the addition of the borohydride.

Reduction was allowed to proceed overnight (16 hours), and the excess borohydride was destroyed by the careful addition of Dowex-50 X8 (H$^+$). The pH of the solution was monitored continuously with a pH meter and not allowed to drop below 3,5 as it was found that the polyalcohol was exceptionally acid labile.

The ion-exchange resin was removed by filtration through a funnel plugged with glass-wool, and the polyalcohol recovered by lyophilization. The

resulting material contained borate which was removed by co-distillation with methanol (3 X 5 ml) by rotary evaporation. After dissolution of the residue in water, the polyalcohol was recovered by freeze-drying, and stored in a desiccator. This procedure produced a material that was easy to work with, compared to the gummy material resulting from the removal of the borate with methanol.

A sample for methylation and monosaccharide analysis was removed, and the residue dissolved in water (2-5 mg ml^{-1}), made to 0,5 M with respect to TFA by the addition of an equal volume of 1 M TFA, and kept at room temperature overnight (16-18 hours). After removal of the TFA by freeze-drying, the resulting material was dissolved in water (4 mg ml^{-1}), and fractionated on a Bio-Gel A-1,5m column (1,5 X 82 cm). The effluent was continuously monitored by a RI detector, and 1,4 ml fractions were collected. Aliquots from the RI positive fractions were assayed for neutral sugars by the phenol-sulphuric method.

In some experiments, the lower molecular mass fragments were re-chromatographed on a Bio-Gel P-2 column (1,6 X 62 cm).

Carbohydrate positive peaks were pooled, recovered by lyophilization and stored desiccated while awaiting monosaccharide and methylation analysis.

4.2.   RESULTS AND DISCUSSION.

In this chapter, a departure is made from the previous format of the thesis in that the results and discussion are presented together. This is necessary in order to show clearly how the structure of the EPS is deduced step by step from the data.

4.2.1.   Correction of the Carbazole Assay for Interference by Neutral Sugars.

The glucose and mannose standard curves were virtually superimposable (Fig. 28). Both the rhamnose assay and the glucuronic acid assay were less sensitive than the glucose assay (15% less and 67% less respectively).

Neutral sugars, particularly glucose, contribute substantially to the absorbance at 530 nm in the carbazole assay. If the uronic acid content of a polysaccharide is to be estimated accurately, correction must be made for the contribution of the neutral sugars to the absorbance at 530 nm. Correction is relatively simple in the case of polymers containing only a single monosaccharide in addition to the uronic acid. It becomes more difficult, however, when there is more than one monosaccharide in the polymer, particularly if each contributes differently to the absorbance at 530 nm.
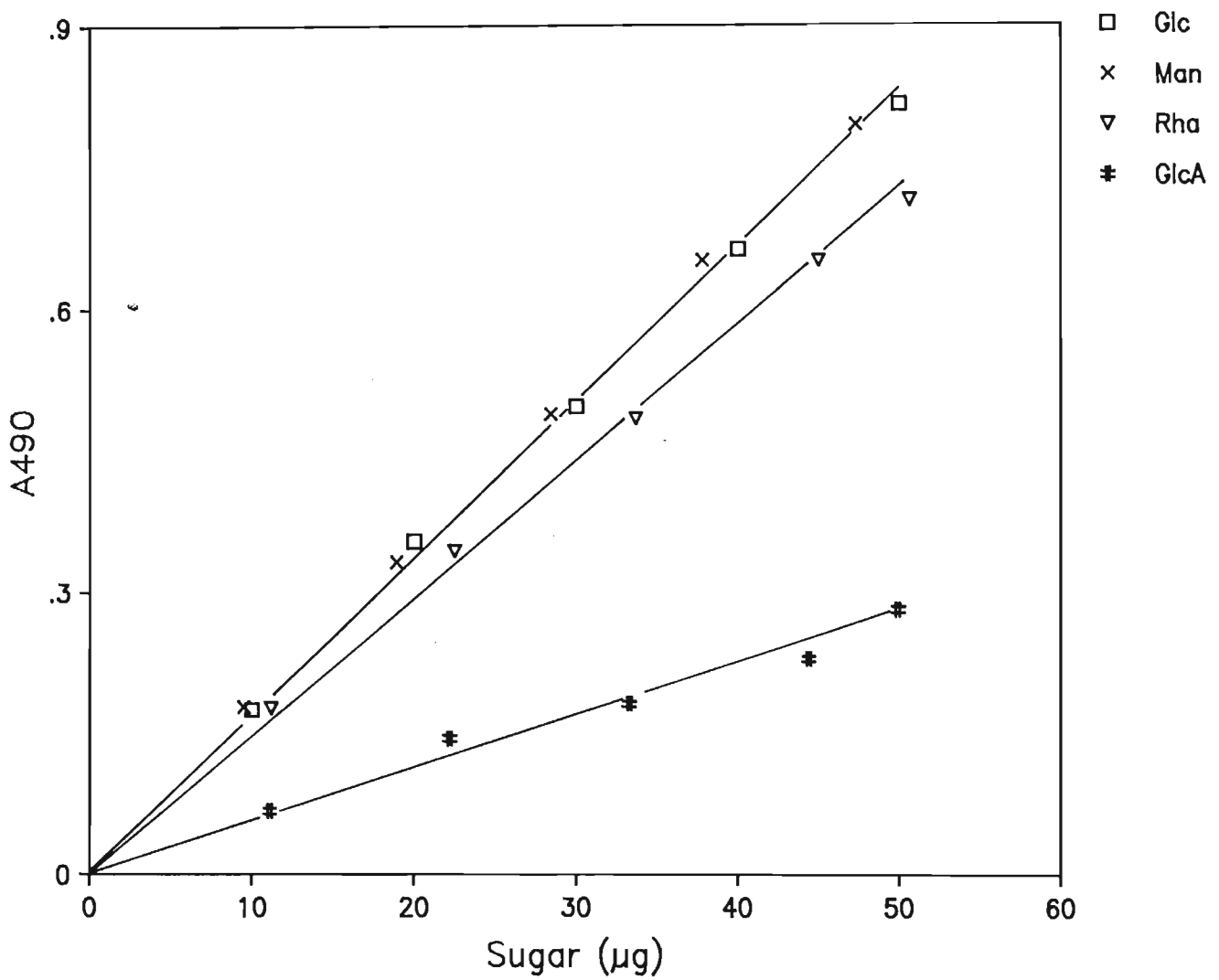
Fig. 28. Comparison of phenol-sulphuric acid standard curves for glucose, mannose, rhamnose, and glucuronic acid.

However, it is possible to correct for the contribution of the neutral sugars to the absorbance at 530 nm provided the approximate monosaccharide composition polysaccharide is known. Since the monosaccharide composition of the Erwinia EPS was known, it was possible to apply the correction in calculating both the uronic acid content of the EPS, and the uronic acid/neutral sugar ratios of the EPS in column effluents.

The principles involved in making the correction are illustrated by the following example.

Let A_{530}(Rha) be the absorbance at 530 nm of W_{Rha} μg of rhamnose.

Let A_{530}(Man) be the absorbance at 530 nm of W_{Man} μg of mannose.

Let A_{530}(Glc) be the absorbance at 530 nm of W_{Glc} μg of glucose.

If the ratio of each sugar is known in the polysaccharide, a value can be determined for the contribution of each sugar to the absorbance at 530 nm for any given weight of polysaccharide. As the amount of sugar can be readily determined from the phenol-sulphuric acid procedure, this assay, in conjunction with the carbazole assay, can be used to determine the approximate uronic acid content of the polysaccharide.

Contribution of glucose to the carbazole assay =

$$A_{530}(Glc)/W_{Glc} \text{ X (amount of glucose in polysaccharide)}$$

$$= x$$

Contribution of mannose to the carbazole assay =

$$A_{530}(Glc)/W_{Glc} \text{ X (amount of mannose in polysaccharide)}$$

$$= y$$

Contribution of rhamnose to the carbazole assay =

$$A_{530}(Rha)/W_{Rha} \text{ X (amount of rhamnose in polysaccharide)}$$

$$= z$$

Total contribution to the absorbance at 530 nm = x+y+z

This value is subtracted from the absorbance at 530 nm.

If the weight of the polysaccharide is known, the correction is easy to apply, but becomes more difficult for column effluents where the exact weight of the polysaccharide is not known. A problem exists with using the phenol-sulphuric acid to quantify the weight of each monosaccharide, as rhamnose and glucose/mannose contribute differently to the absorbance at 490 nm, but an approximate value for each sugar can be obtained.

The A_{490} of a polysaccharide following reaction with the phenol-sulphuric acid carbohydrate assay reagents is made up of the weighted contribution of the A_{490} of each monosaccharide present in the polysaccharide. From the standard curves, the absorbances of equal weights of glucose and mannose are about equal, whereas that of rhamnose is about 15% lower. A

preliminary GLC analysis of the <u>Erwinia</u> EPS gave the following approximate ratio.

$$3 \text{ Rha} : 1 \text{ Glc} : 1 \text{ Man}$$

The contribution of each sugar to the absorbance at 490 nm is, therefore,

$$3 \text{ X } 0,85x + x + x = 4,55x.$$

It follows that rhamnose contributes 2,55/4,55 of the total A_{490}, while glucose and mannose each contribute 1,0/4,55 of the total A_{490}.

Thus, for a total A_{490} of T,

$$A_{490} \text{ (Rha)} = 2,55/4,55 \text{ X T},$$
$$A_{490} \text{ (Glc)} = 1,0/4,55 \text{ X T}$$
$$A_{490} \text{ (Man)} = 1,0/4,55 \text{ X T}$$

Once the individual absorbances are known, the relevant standard curves can be used to determine the weights of each monosaccharide present, and their contribution to the absorbances at 530 nm calculated as explained above.

The contribution of the uronic acid to the absorbance at 490 nm is calculated as follows. In the <u>Erwinia</u> EPS, about 1 residue in 6 residues is glucuronic acid i.e. the maximum total contribution to A_{490} is 0,33 X 1/6 = 5,5%.

It may be noted that an error in the estimation of the rhamnose content of the EPS introduces only a small error in the subsequent correction factor. The same cannot be said for glucose. For example, suppose the ratio of neutral sugars was 4 Rha:1 Glc:1 Man instead of 3:1:1. The rhamnose contribution to the absorbance at 490 nm would go up by 0,85 in 5,4 (=4,55 + 0,85) i.e. 15%. However, the absorbance at 530 nm of a particular weight of rhamnose is about 10% of that of an equivalent weight of glucose i.e. a final error of about 1,5% is introduced. A larger error (3,4%) is introduced by overestimating the amount of glucose (1 in 4,55 as opposed to 1 in 5,4).

Although the method could probably be further refined, it did provide a working solution to the carbazole assays reported in this chapter.

4.2.2. Hydrolysis Conditions.

Trifluoroacetic acid was chosen as the acid for hydrolysis as Albersheim et al.(1967) have found that it was as effective as other mineral acids used for the hydrolysis of polysaccharides with the added advantage that it was readily removed by evaporation.

Hydrolysis of the EPS in 2 M TFA offered no advantage over hydrolysis in 1 M TFA other than a slightly more rapid release of the monosaccharides (Fig. 29). There was however, a more rapid degradation of some monosaccharides during hydrolysis in 2 M TFA (Fig. 29).
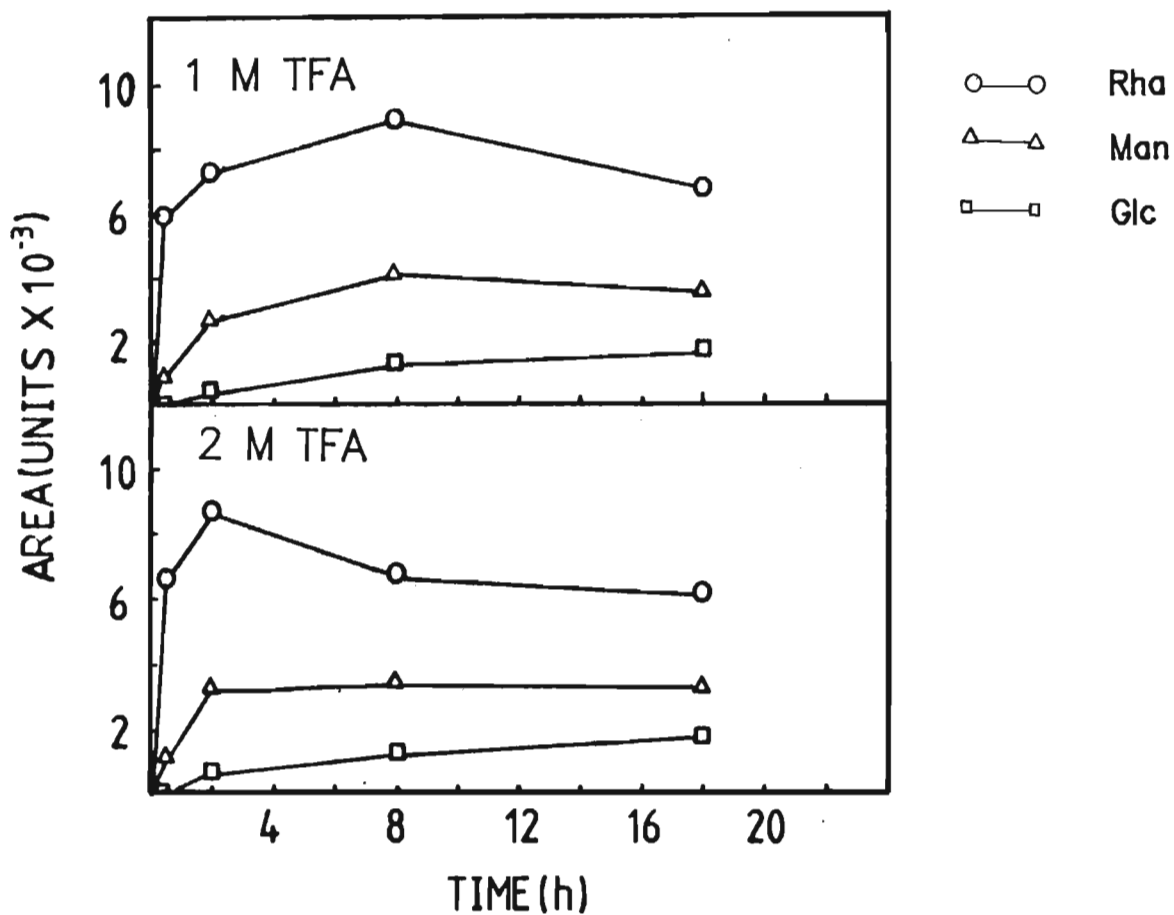
Fig. 29. Comparison of EPS hydrolysis by 1 and 2 M TFA. EPS (3X ethanol precipitated, 4,45 mg) was dissolved in water (5,0 ml) and 0,5 ml aliquots were hydrolysed by either 1 or 2 M TFA for the stated periods. The samples were worked up as described in Materials and Methods, and analyzed on a Hewlett-Packard 5710A gas chromatograph and a SE-54 capillary column operated under the following conditions:

Column temperature: 190°C isothermal.
Carrier: Helium, 30 cm sec^{-1}.
Injector temperature: 220°C.
Detector temperature: 250°C.

The optimum monosaccharide ratios obtained from both TFA concentrations were similar (Table 19), and provided a time course of hydrolysis is done, the choice of concentration of acid would appear to make little difference. However, when only a single hydrolysis time is used (18 h), it is better to use 1 M TFA. Therefore, 1 M TFA was used in all further experiments reported in this study unless otherwise stated.

4.2.4   Purity of the EPS.

It is very important that structural studies be performed on a pure polysaccharide preparation, and therefore considerable effort was expended in demonstrating the purity of the Erwinia EPS used in this study.

Chromatography of the crude polysaccharide on Bio-Gel A-1,5m (Fig. 30) demonstrated contamination by a substantial amount of low molecular mass material. Two major carbohydrate peaks eluted from the column, one near the void volume of the column, and the other at the total volume of the column (Fig. 30). The uronic acid eluted with the high molecular mass peak. Analyses of the monosaccharide composition (Table 20) of selected fractions from the column suggested that the high molecular mass material was of a single molecular species, although polydisperse in size. This conclusion was substantiated by the uronic acid/neutral sugar ratios of

Table 19. Sugar ratios obtained after hydrolysis of native EPS by 1 M or 2 M TFA. Experimental conditions are given in the legend to Fig. 29.

[TFA] M	Sugar Ratio		
	Rha	Glc	Man
1	4,10	2,38	1,00
2	4,28	2,28	1,00
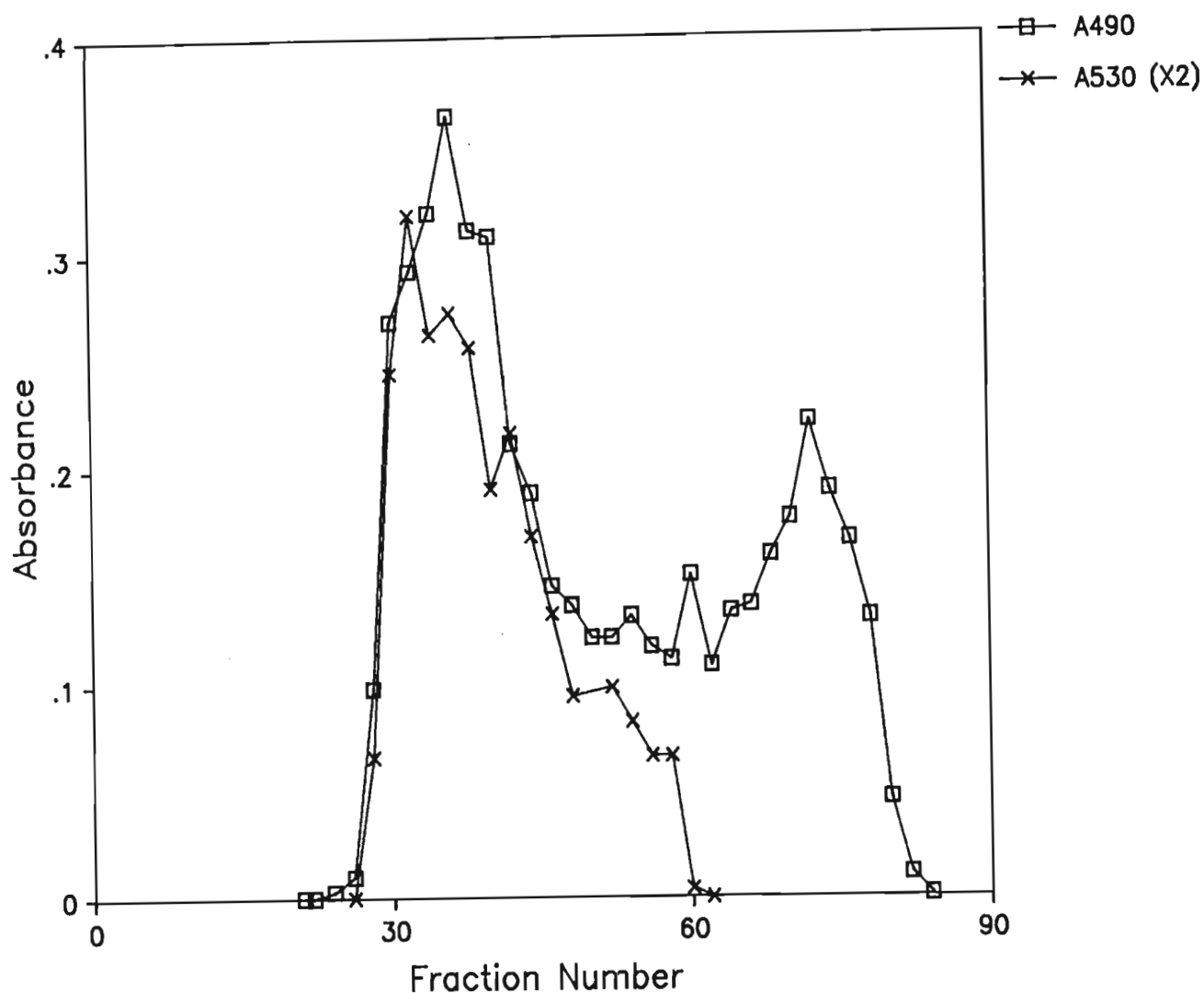
Fig. 30. Chromatography of crude EPS on Bio-Gel A-1,5m. Crude EPS (18 mg) in 50 mM ammonium formate (6 ml) was clarified by centrifugation (27 000 X g, 30 min, room temperature), and 5 ml applied to a Bio-Gel column (1,6 X 82 cm) pre-equilibrated with the same buffer. The column was developed at a flow rate of 46 ml/h, and 2 ml fractions collected. Aliquots were assayed for total neutral sugar (0,1 ml) and uronic acid (0,2 ml) by the phenol-sulphuric acid and carbazole methods respectively.

Table 20. Monosaccharide composition and uronic acid/neutral sugar ratios
of fractions from Bio-Gel A-1,5m chromatography of crude EPS.
Aliquots (0,4 ml) of fractions 29, 31, 34, 38, 60, and 72 were
hydrolysed in 1 M TFA for 18 h and the released monosaccharides
were converted to the alditol acetates as described in Mate-
rials and Methods. The alditol acetates were analysed by GLC as
described in the legend to Fig. 29. Uronic acid and neutral
sugar were assayed as described in Materials and Methods.

Fraction		Sugar Ratio			A_{530}/A_{490}
Number	Rha	Glc	Man	Gal	
29	4,48	2,73	1,00	–	0,218
31	4,78	2,39	1,00	–	0,199
34	4,78	2,31	1,00	–	0,206
38	4,76	2,37	1,00	–	0,206
60	–	1,31	–	1,00	–
72	–	1,00	–	1,03	–

selected fractions across the chromatogram (Table 20). The carbohydrate in the low molecular mass material contained equal amounts of glucose and galactose, and appeared to consist of unmetabolized lactose.

A single precipitation of the polysaccharide by ethanol was not sufficient to remove the low molecular mass material, as analyzed by chromatography on Bio-Gel A-1,5m. However, three precipitations with ethanol gave a product eluting as a single peak near the void volume of the Bio-Gel A-1,5m column (Fig. 31). The width of the peak indicated some molecular mass dispersity, but the monosaccharide composition and uronic acid/neutral sugar ratios (Table 21) suggested that the peak contained a single molecular species.

Preliminary experiments performed to determine the behaviour of the EPS on DEAE-cellulose revealed that EPS was completely adsorbed at 10 mM KCl but not at 250 mM KCl. The DEAE-cellulose column was therefore loaded with EPS dissolved in 10 mM KCl, washed with two bed volumes of 10 mM KCl, and eluted with a 10 to 500 mM linear KCl gradient. Chromatography of the purified EPS on DEAE-cellulose gave a single peak eluting at a KCl concentration of about 150 mM (Fig. 32). The monosaccharide composition and neutral sugar/uronic acid ratios of selected fractions across the peak were similar, again suggesting that the material was a single molecular species (Table 22). No neutral polysaccharide was found in the EPS preparation.
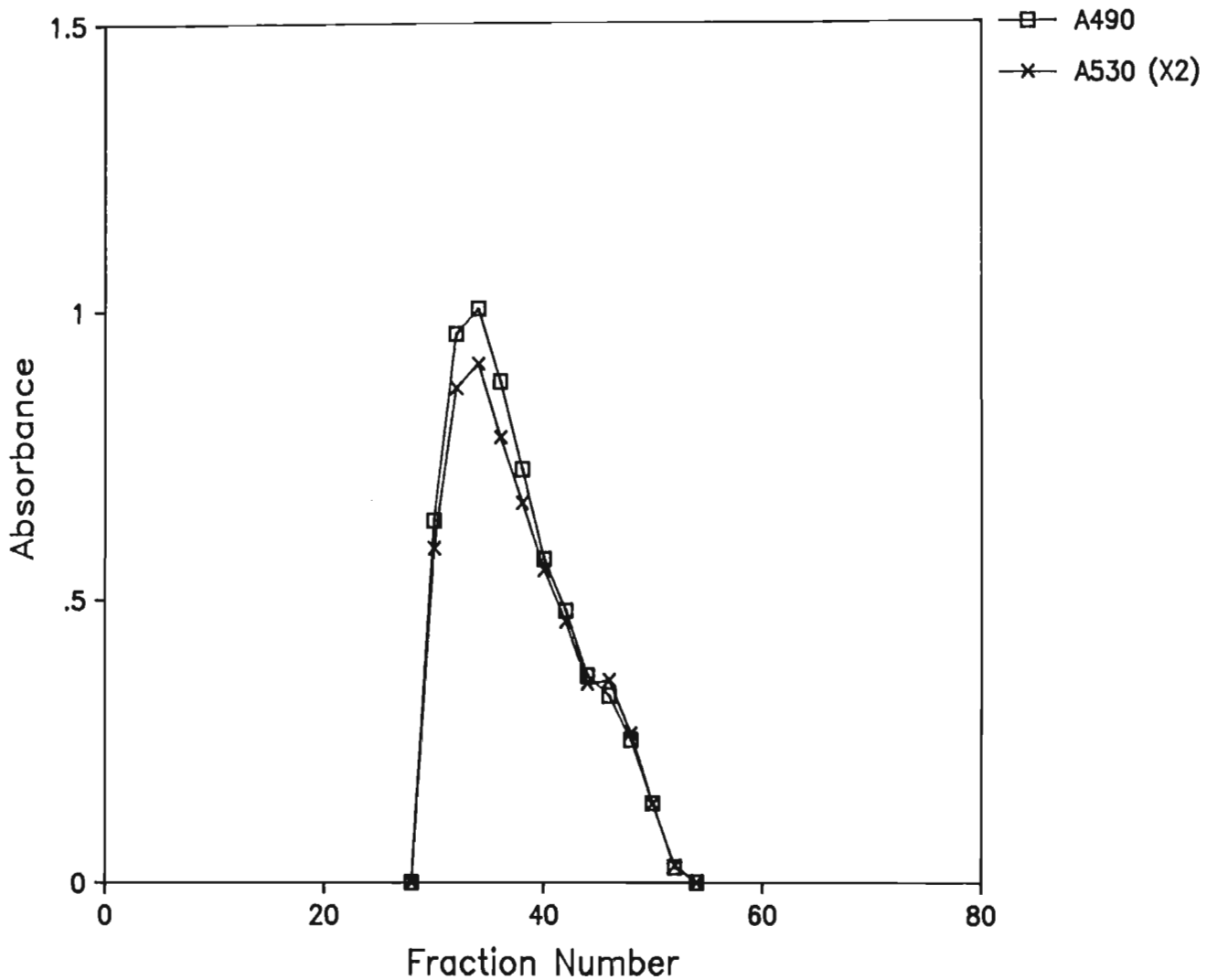
Fig. 31. Chromatography of purified EPS (3 X ethanol precipitated) on Bio-Gel A-1,5m. EPS (20,83 mg) in 50 mM NaCl (5,0 ml) was clarified by filtration (0,45 μm HAWP Millex filter, Millipore), and applied to a Bio-Gel A-1,5m column (1,6 X 82 cm) pre-equilibrated in the same eluant. The column was eluted at a flow rate of 46 ml/h, and 2 ml fractions collected. Aliquots were assayed for neutral sugar (0,100), and uronic acid (0,200 ml) by the phenol-sulphuric acid and carbazole methods respectively.

Table 21. Monosaccharide composition and uronic acid/neutral sugar ratios of selected fractions from Bio-Gel A-1,5m chromatography of purified EPS. Aliquots of selected fractions were analysed for uronic acid and neutral sugar, and for monosaccharide composition as described in Table 20.

Fraction	Sugar Ratio			A_{530}/A_{490}
Number	Rha	Glc	Man	
32	4,33	2,47	1,00	0,231
36	4,16	2,89	1,00	0,226
39	4,24	1,95	1,00	0,226
41	4,81	2,43	1,00	0,230
46	4,55	2,26	1,00	0,239
52	4,87	2,18	1,00	–
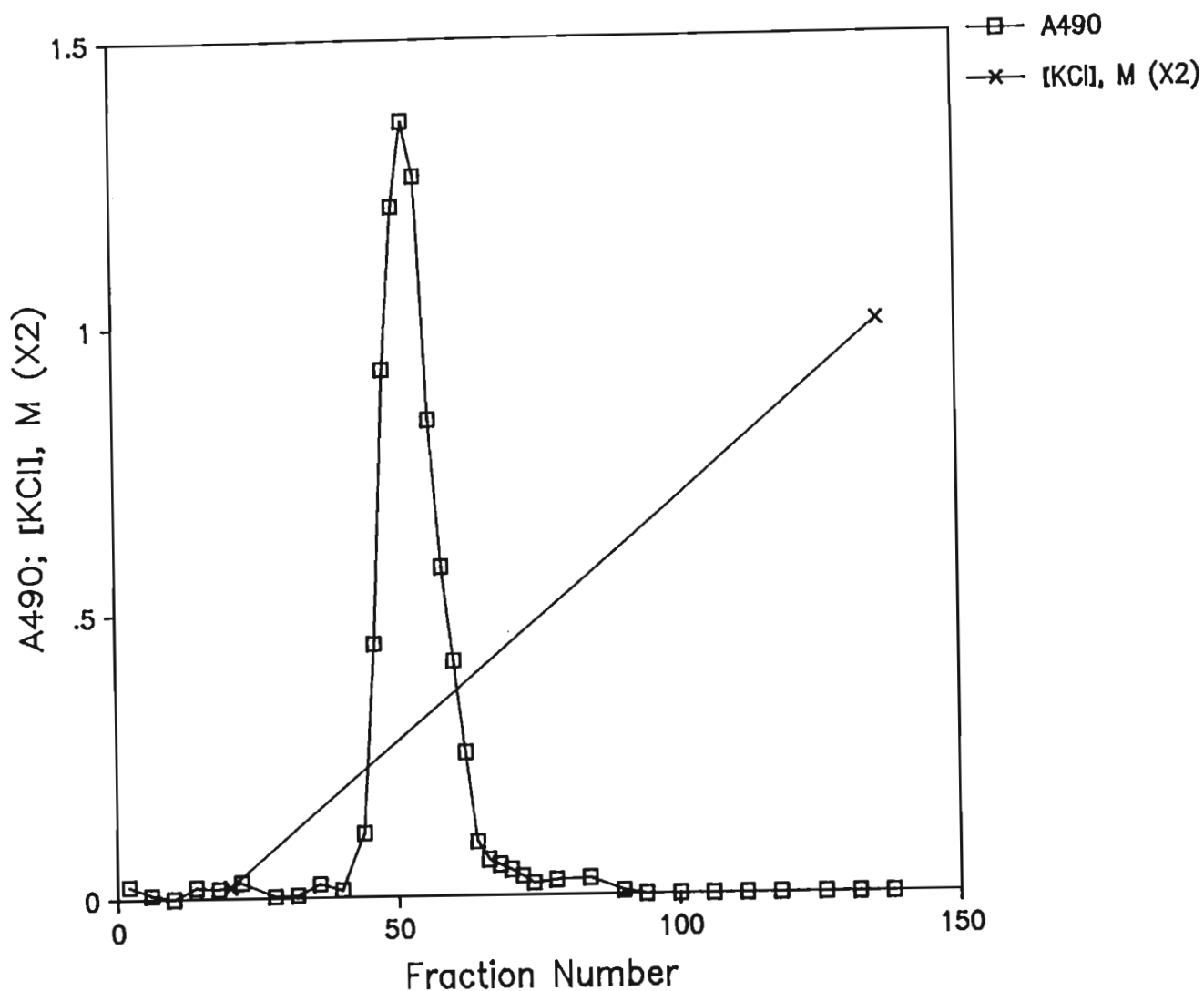
Fig. 32. Chromatography of purified EPS on DEAE-cellulose. EPS (19,22 mg) in 10 mM KCl (30 ml) was clarified by centrifugation (27 000 X g, 30 min, room temperature), and applied to a column of DE-52 DEAE-cellulose (2,5 X 33 cm) pre-equilibrated in the same eluant. The column was washed with about 2 bed volumes of 10 mM KCl, developed with a linear KCl gradient (10 to 500 mM), and fractions (8,4 ml) collected. Aliquots (0,200 ml) were analysed for total neutral sugar by the phenol-sulphuric acid method.

Table 22. Monosaccharide composition and uronic acid/neutral sugar ratios in selected fractions from DEAE-chromatography of purified EPS. Aliquots of fractions from the DE-52 column in Fig. 32 were analysed for uronic acid and neutral sugar as described in Materials and Methods. Fractions 48, 52, and 58 were dialysed for 24 h against distilled water, and duplicate aliquots (1 ml) hydrolysed by 1 M TFA, and the released monosaccharides analysed by GLC as described in Table 20.

Fraction Number	Rha	Glc	Man	A_{530}/A_{490}
44	–	–	–	0,250
48	3,90	1,62	1,00	0,240
50	–	–	–	0,235
52	3,94	1,59	1,00	0,257
54	–	–	–	0,249
56	–	–	–	0,267
58	3,73	1,59	1,00	0,268
60	–	–	–	0,251

Further proof of the homogeneity of the thrice-precipitated ethanol EPS arose from HPLC and ultracentrifugal analysis of the EPS. HPLC of the EPS on a TSK PW 5 000 column revealed a single peak eluting near the void volume of the column, and analysis of the EPS by ultracentrifugation on a Beckman Model E ultracentrifuge revealed a single peak with a sedimentation velocity of 10,8 S.

The conclusion from all these studies was that the thrice ethanol-precipitated EPS used in this study consisted of a single molecular species which was polydisperse with respect to molecular mass.

4.2.3.   Carboxyl Reduction of the EPS.

About 90% of the uronic acid was converted to neutral sugar by a single reduction step. Chromatography of the product on DEAE-cellulose gave a single peak which eluted at about 75 mM KCl. A second reduction of the same material gave a peak that eluted from the DEAE-cellulose column with the 10 mM KCl starting solution, and contained no uronic acid when assayed by the carbazole procedure.

Recovery of the EPS was greater than 90% after the first reduction, and about 60% after the second reduction.

4.2.4.   Protein and Amino Sugars.

The protein content of the EPS was determined to be less than 0,7% amino acid analysis. Neither the Lowry nor the Coomassie Blue methods revealed the presence of any protein. Furthermore, no amino sugars were detected by amino acid analysis.

4.2.5.   Monosaccharide Composition of the EPS.

The only neutral monosaccharides detected in the acid hydrolyzates (1 M TFA, 18h) of both the native and carboxyl-reduced EPS by TLC and paper chromatography were glucose, mannose, and rhamnose. In addition, TLC analysis revealed two extra spots, corresponding to glucuronic acid and its lactone, in the acid hydrolyzates of the native EPS.

No KDO was found in the EPS, demonstrating the absence of any lipopoly-saccharide contamination.

4.2.6.   Quantitative analysis of the native and carboxyl-reduced EPS.

The neutral sugar composition of the electrodialyzed EPS was found to be 6,16 Rha : 2,09 Glc : 1,00 Man by GLC analysis of the alditol acetates (Table 23). The alditol acetate of the uronic acid is not volatile, and therefore could not be analyzed by GLC. Total recovery of the sugars, as determined by the GLC analysis, was 69,9% (Table 23). The uronic acid

content of the EPS was found to be about 12,7% (w/w) by the carbazole assay.

The equivalent weight of the uronic acid in the electrodialyzed EPS was 1269 $\pm$ 14,1 (mean and standard error of the mean of 7 determinations), suggesting a repeat unit of between 7 and 8 monosaccharide residues. The equivalent weight of the uronic acid is a little higher than predicted from the analysis of the monosaccharides. As no precautions, however, were taken to rigorously exclude carbon dioxide from the solutions during the titrations, the equivalent weight of the uronic acid may have been overestimated.

The monosaccharide composition of the reduced EPS was found to be 3,70 Rha : 2,01 Glc : 1,01 Man (Table 24). These results further support the conclusion that the uronic acid is glucuronic acid no since no new sugar was obtained during reduction of the native EPS. Still further support for this identification was obtained from methylation analysis of the native and carboxyl-reduced EPS (see Section 4.2.9).

Total recovery of the sugars from the carboxyl-reduced EPS, as determined by GLC, was 94,8% (Table 24). This figure should be contrasted with the 68% recovery of monosaccharides following hydrolysis of the native EPS. The discrepancy arises from two causes. Firstly, glycosiduronic acid bonds are very resistant to acid hydrolysis (Percival, 1963). Secondly,

Table 23. Neutral sugar composition of the native EPS. Electrodialyzed
EPS (0,837 mg per tube) was hydrolysed with 1 M TFA at 100°C
for different periods of time, and converted to the alditol
acetates as described in Materials and Methods. 2-Deoxy-D-
galactose (1,363 μmole per tube) was used as internal standard.

Time of hydrolysis (h)	Sugar (μmole)		
	Rha	Glc	Man
0,5	1,962	0,209	–
2,0	2,515	0,674	0,110
8,0	2,449	0,935	0,278
12,0	2,544	0,863	0,413
18,0	1,675	0,570	0,228

Maximum values: Rha : Glc : Man

μmole: 2,544 : 0,935 : 0,413

ratio: 6,16 : 2,09 : 1,00

Recovery: Anhydrorhamnose   2,544 μmole = 0,367 mg
            Anhydroglucose   0,935 μmole = 0,151 mg
            Anhydromannose   0,413 μmole = 0,067 mg

            Total   0,585 mg

Weight EPS hydrolysed = 0,837 mg
Weight EPS recovered = 0,585 mg

        % Recovery = 69,9

Table 24. Monosaccharide composition of carboxyl reduced EPS. EPS (2 X reduced, 0,481 mg per tube) was hydrolysed with 1 M TFA at 100°C for different times, and converted to the alditol acetates as described in Materials and Methods. 2–Deoxy–D–galactose (1,363 µmole per tube) was used as internal standard.

Time of	Sugar (µmole)		
hydrolysis (h)	Rha	Glc	Man
0,5	1,263	0,241	–
2,0	1,514	0,827	0,347
8,0	1,660	0,842	0,424
12,0	1,542	0,901	0,449
18,0	1,460	0,851	0,427

Maximum values: Rha   :   Glc   :   Man

µmole sugar: 1,660   :   0,901   :   0,440

ratio: 3,77   :   2,05   :   1,00

Recovery: Anhydrorhamnose   1,660 µmole = 0,239 mg
           Anhydroglucose   0,901 µmole = 0,146 mg
           Anhydromannose   0,440 µmole = 0,071 mg

               Total   0,456 mg

Weight of EPS hydrolysed = 0,481 mg
Weight of EPS recovered = 0,456 mg

       % Recovery = 94,8

monosaccharides are destroyed to some extent during hydrolysis. Taking into account that the contribution of the uronic acid to weight of the native EPS is about 13%, this still accounts for only about 81% of the total weight of the polysaccharide (i.e. 68% + 13%). The unaccounted portion of the EPS consists of the monosaccharide still attached to the uronic acid (i.e. the aldobiouronic acid) and the amount of monosaccharide destroyed during the hydrolysis of the EPS. In the carboxyl-reduced EPS, about 95% of the weight of the EPS is accounted for by hydrolysis and subsequent GLC analysis. The unaccounted 5% is presumably due to the destruction of the monosaccharides during hydrolysis of the EPS. If the same proportion of monosaccharide is destroyed during hydrolysis of the native EPS, then very little of the aldobiouronic acid could have been hydrolysed, and the entire starting weight of the EPS can be accounted for: 70% in neutral sugars, about 25% from the aldobiouronic acid, and 5% destruction of neutral sugars.

4.2.7.   Methylation analysis of the native and carboxyl-reduced EPS.

The native EPS gave rise to four major partially methylated alditol acetates after methylation analysis (Fig. 33). These were identified as indicated in Table 25 column A, by GC-MS (see Section 4.2.11 for further details regarding the GC-MS analyses and a discussion of how the identity of the peaks was determined). The identity of the 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl glucitol was unambiguously confirmed by standards prepared from the methylation of dextran. The following linkages are
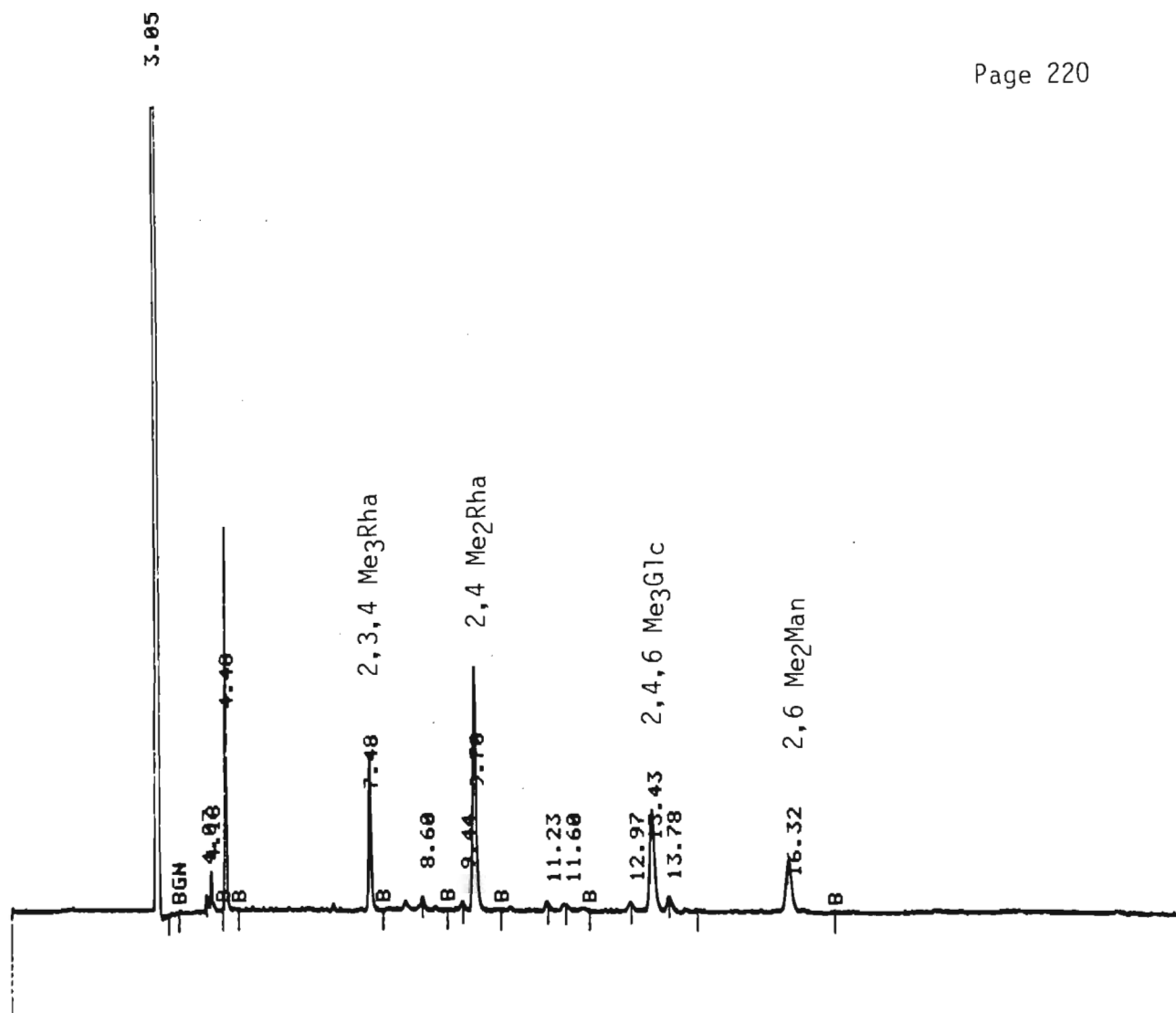
Fig. 33. Gas liquid chromatogram of the partially methylated alditol acetates derived from native EPS after methylation analysis. The partially methylated alditol acetates were analyzed on a Sigma 1 gas chromatograph and a DB5 column operated under the following conditions.

Column temperature: 220 °C isothermal.
Carrier: N_2, 14 cm sec^{-1}.
Injector temperature: 240°C.
Detector temperature: 250°C.

Consult Table 28 for a list of relative retention times for the different partially methylated alditol acetates.

Table 25.   Methylation analysis of native and carboxyl-reduced EPS from E. chrysanthemi.

Me Sugar[1]	Mole %	
	A[2]	B[3]
2,3,4 Me$_3$Rha	16,8	15,3
2,4 Me$_2$Rha	41,2	34,2
2,4,6 Me$_3$Glc	24,2	17,8
2,3,6 Me$_3$Glc	–	17,8
2,3,6 Me$_3$Man	–	–
2,6 Me$_2$Man	17,9	15,0

[1]2,3,4 Me$_3$Rha = 1,5-di-O-acetyl-2,3,4-tri-O-methyl rhamnitol etc.

[2]Methylation analysis of native EPS.

[3]Methylation analysis of carboxyl reduced EPS.

therefore present in the native EPS.

$$\text{Rha}^{1}\text{---} \qquad\qquad \overset{3}{\text{---}}\text{Rha}^{1}\text{---}$$

$$\overset{3}{\text{---}}\text{Glc}^{1}\text{---} \qquad\qquad \overset{4}{\text{---}}\underset{3\,|}{\text{Man}}^{1}\text{---}$$

Five major partially methylated alditol acetates were detected after methylation analysis of the carboxyl reduced EPS (Fig. 34). In addition to the four partially methylated acetates found in the native EPS, 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl glucitol, derived from the carboxyl-reduction of the glucuronic acid, was also found (Table 25 column B). The pattern of methylation of the partially methylated alditol acetates was confirmed by GC-MS (see Section 4.2.11), and the identity of 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl glucitol unambiguously confirmed by standards derived from the methylation of glycogen.

The additional sugar and linkage present in the carboxyl-reduced EPS is therefore $\overset{4}{\text{---}}\text{Glc}^{1}\text{---}$ and $\overset{4}{\text{---}}\text{GlcA}^{1}\text{---}$ in the native EPS.

4.2.8. Periodate oxidation and Smith degradation.

Periodate oxidation is used extensively in carbohydrate chemistry to obtain analytical and structural information about these compounds
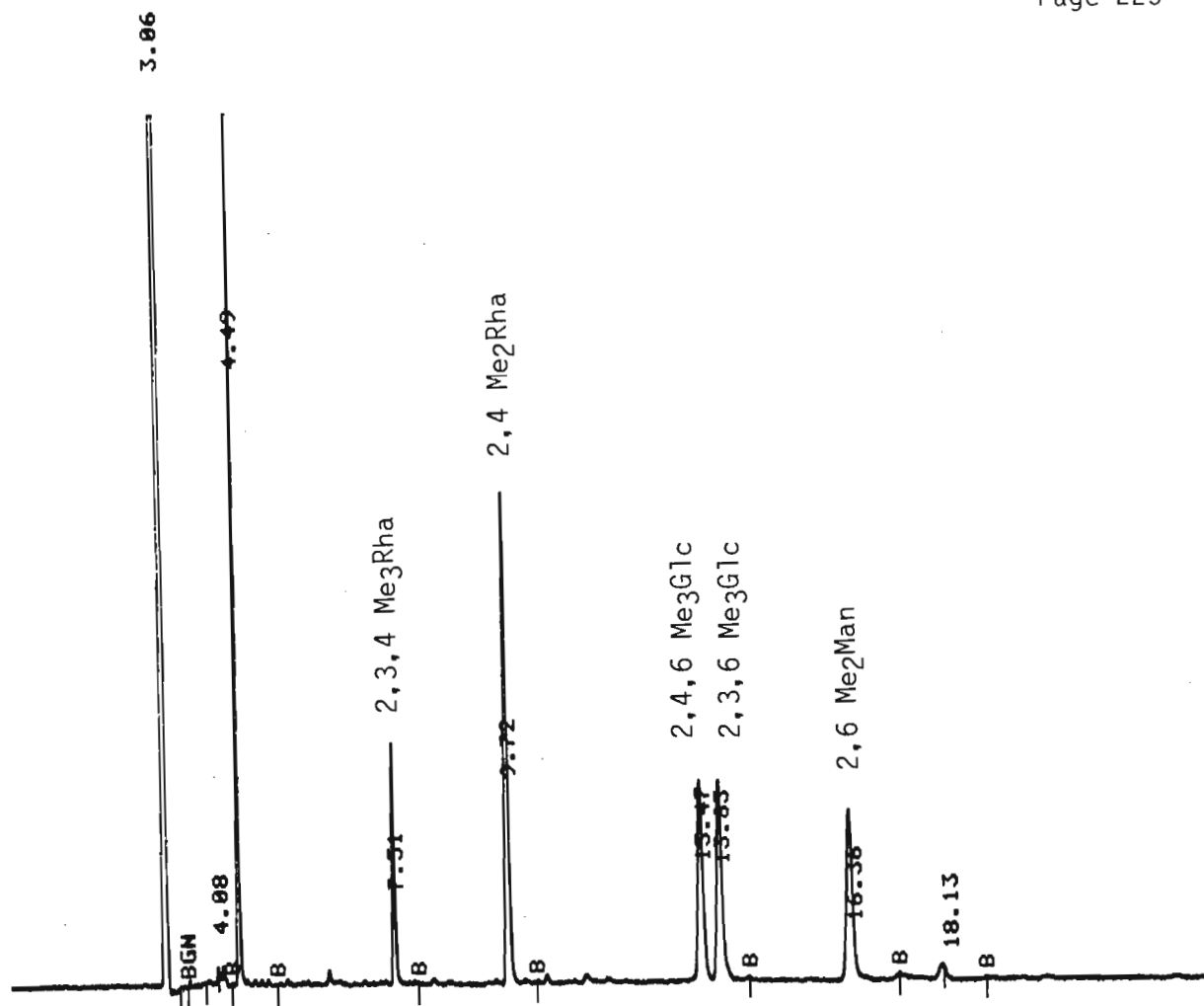
Fig. 34. Gas liquid chromatogram of the partially methylated alditol acetates derived from carboxyl-reduced EPS after methylation analysis. The partially methylated alditol acetates were analyzed on a Sigma 1 gas chromatograph and a DB5 column operated under the following conditions.

Column temperature: 220 °C isothermal.
Carrier: N_2, 14 cm sec^{-1}.
Injector temperature: 240°C.
Detector temperature: 250°C.

Consult Table 28 for a list of relative retention times for the different partially methylated alditol acetates.

(Bobbitt, 1956; Dyer, 1956, Guthrie, 1961; Hay et al., 1965; Hough, 1965; Perlin, 1980). The specific cleavage of 1,2-diols provides a simple means of obtaining information about the nature of the linkages in polysaccharides. In combination with other techniques such as reduction of the polyaldehyde formed during the periodate oxidation and mild acid hydrolysis of the resulting polyalcohol (Smith degradation), smaller oligosaccharide fragments are produced which, after isolation and identification, provide much information about the structure of the polysaccharide (Goldstein, 1965; Lindberg, 1975; Perlin, 1980; Rauvala et al., 1981). This mild hydrolysis is possible, as the polyalcohol produced after reduction of the polyaldehyde contains acyclic acetal groupings which are hydrolyzed much faster than the glycosidic linkages present in the polymer.

Periodate oxidation of the carboxyl reduced EPS resulted in the consumption of about 0,31 mole periodate per mole of anhydroglucose unit (AGU, Fig. 35). This value was obtained by extrapolating the linear part of the oxidation curve to zero time, and is consistent with the methylation analysis reported above i.e. 2 residues in about 7 would be expected to be oxidized.

Smith degradation (i.e. reduction of the polyaldehyde by $NaBH_4$ followed by mild acid hydrolysis) resulted in the production of a high and a low molecular mass fragment which were separated by chromatography on a
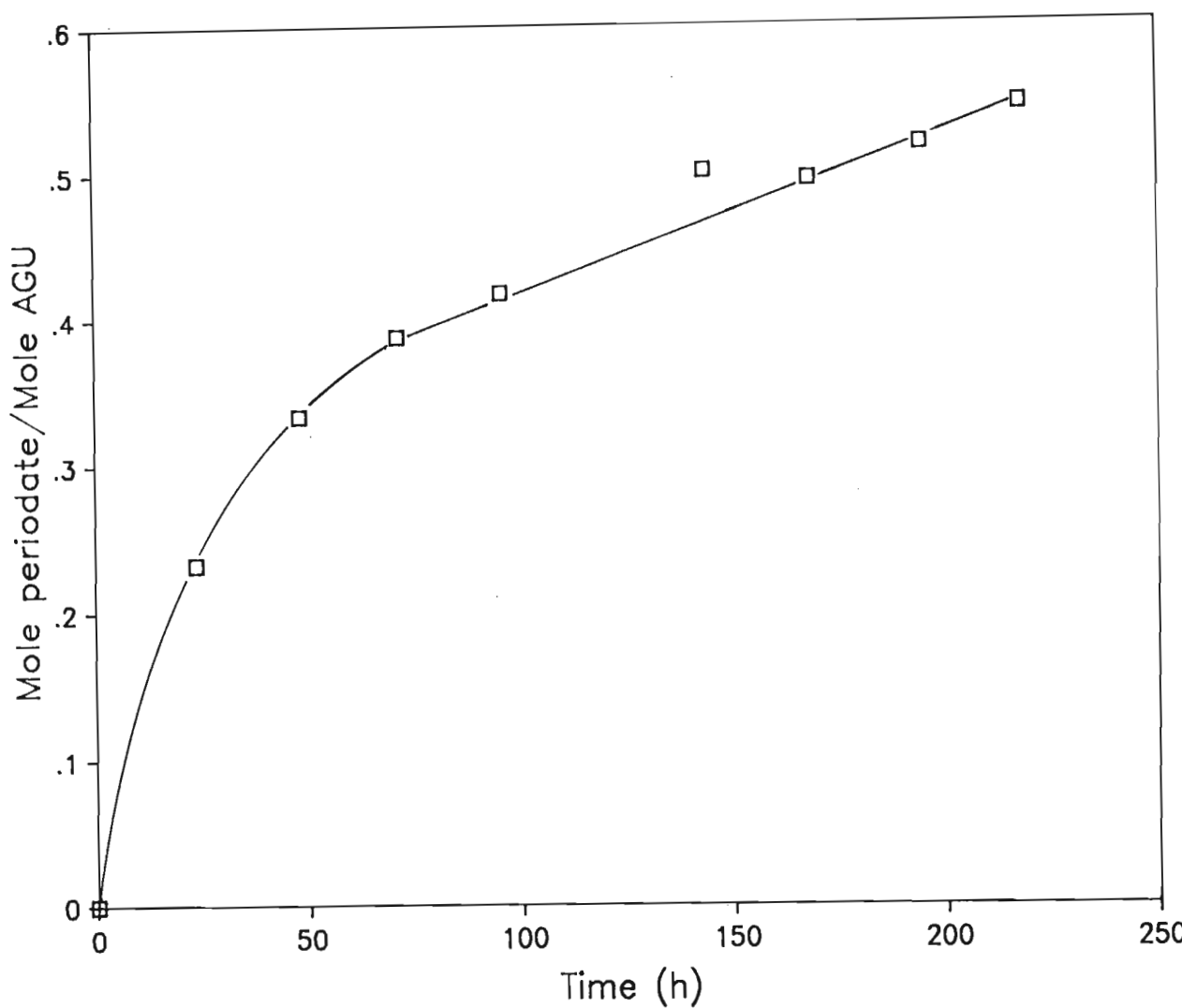
Fig. 35. Periodate oxidation of carboxyl-reduced EPS. Carboxyl-reduced EPS (98,2 mg) in 0,033 M periodate solution (30 ml) was kept in the dark at 4°C, and samples removed at intervals for the determination of residual periodate. A solution in which H_2O replaced the EPS was used as a control.

Bio-Gel A-1,5m agarose column (Fig. 36). The low molecular mass fragment was found to contain only rhamnose by GLC analysis. The high molecular mass fragment was found to contain equimolar amounts of glucose, mannose, and rhamnose.

The terminal rhamnose and the glucose, derived from the carboxyl-reduction of the glucuronic acid in the EPS, were not detected during methylation analysis of the polyalcohol before mild acid hydrolysis (Table 26 column A). However, methylation analysis of the large fragment after mild acid hydrolysis revealed a new partially methylated alditol acetate which was identified as 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl hexitol by GC-MS (Table 26 column B). The appearance of this peak coincided with the disappearance of the 1,3,4,5-tetra-O-acetyl-2,6-di-O-methyl mannitol present in the polyalcohol before mild acid hydrolysis (and in the native and carboxyl-reduced EPS), and was consequently identified as 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl mannitol. Therefore, it was concluded that the side-chain was attached to the EPS backbone via position 3 of the mannose residue.

Methylation analysis of the EPS backbone also gave rise to equimolar amounts of 1,3,5-tri-O-acetyl-2,4-di-O-methyl rhamnitol, 1,3,5-di-O-acetyl-2,4,6-tri-O-methyl glucitol, and 1,4,5-tri-O-acetyl-2,3,6-tri-O-
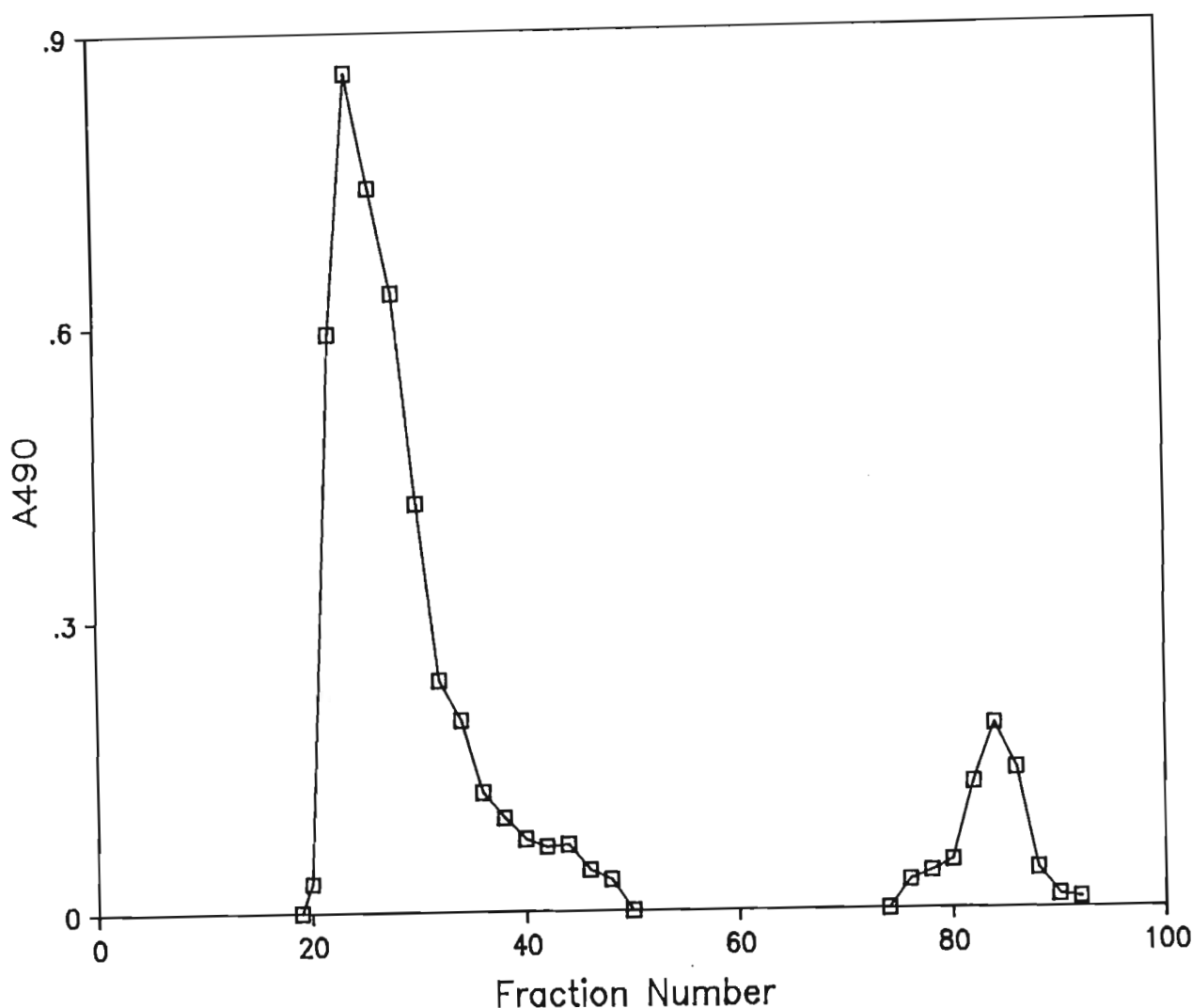
Fig. 36. Chromatography of the Smith degradation products derived from carboxyl-reduced EPS on Bio-Gel A-1,5m. Polyalcohol (53 mg) was dissolved in 0,5 M TFA (20 ml), and kept at room temperature for 16 h. After removal of the TFA by freeze-drying, the residue was dissolved in H_2O (5 ml), clarified by centrifugation (50 000 g, 22°C, 10 min), and chromatographed on a Bio-Gel A-1,5m column (1,5 X 82 cm) with water as eluent, at a flow rate of 10 ml h^{-1}. Fractions (1,54 ml) were collected, and assayed for total carbohydrate by the phenol-sulphuric acid method.

Table 26.   Methylation analysis of the polyalcohol derived during the Smith degradation of native and carboxyl-reduced EPS E. chrysanthemi.

Me Sugar[1]	Mole %		
	A[2]	B[3]	C[4]
2,3,4 Me$_3$Rha	<1,0	3,0	27,0
2,4 Me$_2$Rha	48,8	30,4	36,2
2,4,6 Me$_3$Glc	27,5	33,5	39,6
2,3,6 Me$_3$Glc	<1,5	-	-
2,3,6 Me$_3$Man	-	30,7	12,7
2,6 Me$_2$Man	23,7	2,4	22,2

[1]2,3,4 Me$_3$Rha = 1,5-di-O-acetyl-2,3,4-tri-O-methyl rhamnitol etc.

[2]Methylation of polyalcohol obtained from carboxyl-reduced EPS before mild acid hydrolysis. Oxidation by periodate performed for 200 h.

[3]Methylation of polyalcohol obtained from carboxyl-reduced EPS after mild acid hydrolysis. Oxidation by periodate performed for 200 h.

[4]Methylation of polyalcohol obtained from native EPS after mild acid hydrolysis. Periodate oxidation was performed for 200 h.

methyl mannitol Table 26 column B). The following sugars and linkages are therefore present in the EPS backbone.

$$\underset{}{\overset{3}{\underline{}}}\text{Rha}^{\underline{1}}\underline{} \qquad\qquad \overset{3}{\underline{}}\text{Glc}^{\underline{1}}\underline{}$$

$$\overset{4}{\underline{}}\text{Man}^{\underline{1}}\underline{}$$

Further, it is deduced that the EPS backbone possesses one of the following two repeating units:

$$[\quad \overset{3}{\underline{}}\text{Glc}^{1}\underline{}\overset{4}{}\text{Man}^{1}\underline{}\overset{3}{}\text{Rha}^{1}\underline{} \quad]_n$$

or

$$[\quad \overset{3}{\underline{}}\text{Rha}^{1}\underline{}\overset{4}{}\text{Man}^{1}\underline{}\overset{3}{}\text{Glc}^{\underline{1}}\underline{} \quad]_n$$
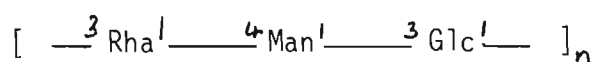
Periodate oxidation of the native EPS was attended by several difficulties. Oxidation never appeared to be complete, and the Smith degradation of the resulting polyaldehyde showed that about 65% of the sidechains were still attached to position 3 of the mannose residue in the backbone of the EPS. This was shown by the presence of 1,3,5,6-tetra-O-acetyl-2,4-di-O-methyl mannitol in the polyalcohol after 1 cycle of Smith degradation (Table 26 column C). A similar problem with a number of other polysaccharides has been reported in the literature, and is thought to arise from the formation of inter- and intra-residue hemiacetals, which

lead to incomplete oxidation of the polysaccharides (Guthrie, 1961; Aspinall and Stephen, 1973).

A possible solution to this might be to restrict work to carboxyl-reduced EPS, or to perform a second periodate oxidation of the poly-alcohol before mild acid hydrolysis is carried out. Neither possibility however, was investigated by the author.

4.2.9. GC-MS analysis and identification of partially methylated alditol acetates.

The mass spectrum of a partially methylated alditol acetate can be used to determine the number and position of the methoxyl groups in the molecule, but cannot be used for the unambiguous identification of the parent hexose, as similar fragmentation patterns are obtained for similarly substituted methylated alditol acetates (Bjorndal et al., 1967; Bjorndal et al., 1970; Jansson et al., 1976). However, with the added dimension of retention time in GC-MS, this technique becomes an extremely sensitive and powerful method for the identification and quantification of the partially methylated alditol acetates.

The identification of peaks by GC-MS obtained during methylation analysis of homopolysaccharides is relatively easy as the substitution pattern, with a few exceptions, can be unambiguously determined. The identification of the methylated alditol acetates obtained from heteropolysaccha-

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rides, however, present a problem, and standards must often be used for the unambiguous identification of the methylated products.

As standards are not available commercially, they must be obtained from other sources, such as from the methylation of polysaccharides of known structure. In this study, dextran and glycogen were used as standards for the identification of the methylated alditol acetates arising from the glucose in the EPS. The identity of the methylated alditol acetates derived from the mannose in the EPS was deduced as described below.

The mass spectra obtained for the various partially methylated alditol acetates are presented in Fig. 37, and the primary fragments expected from each partially methylated alditol acetate in Table 27. The relative retention times on the columns used in this study for a number of partially methylated alditol acetates are listed in Table 28.

In the chromatograms shown in Figs. 33 and 34, the first two peaks were identified as 1,5-di-O-acetyl-6-deoxy-2,3,4-tri-O-methyl hexitol and 1,3,5-tri-O-acetyl-6-deoxy-2,4-di-O-methyl hexitol respectively. As rhamnose is the only 6-deoxy sugar present in the EPS, these were identified as 1,5-di-O-acetyl-6-deoxy-2,3,4-tri-O-methyl rhamnitol and 1,3,5-tri-O-acetyl-6-deoxy-2,4-di-O-methyl rhamnitol respectively. The identities of 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl glucitol and 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl glucitol were unambiguously identified by
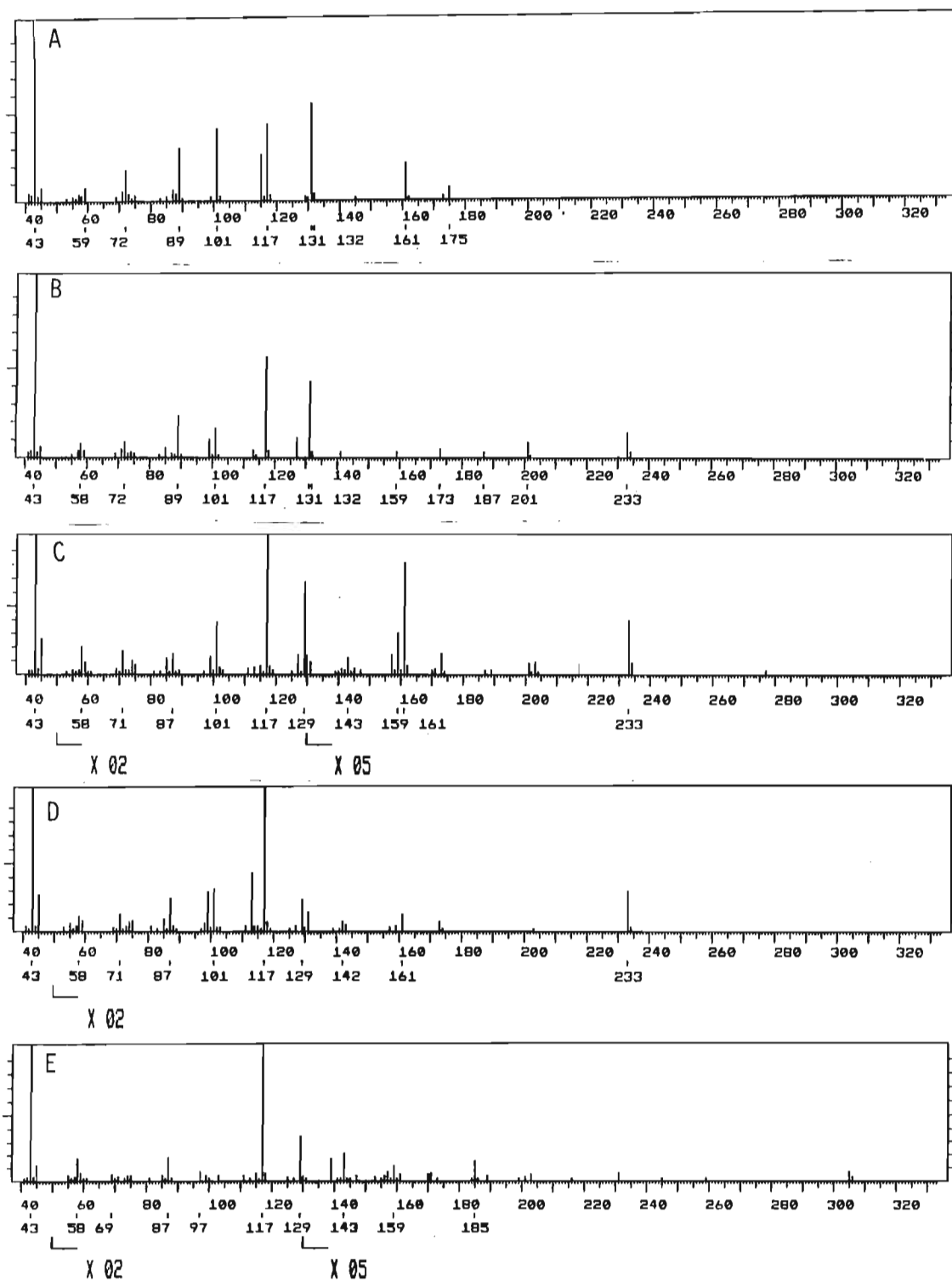
Fig. 37. Mass spectra of the partially methylated alditol acetates derived during methylation analyses of the native and carboxyl-reduced EPS. Instrument operating conditions are described in Section 4.1.22.

A = 2,3,4 Me$_3$Rha, B = 2,4 Me$_2$Rha, C = 2,4,6 Me$_3$Glc, D = 2,3,6 Me$_3$Glc/Man, E = 2,6 Me$_2$Man.

Table 27. Primary fission fragments expected in the mass spectra of the partially methylated alditol acetates obtained from Erwinia EPS and its derivatives after methylation analysis.

Sugar	Position of Me group.	Primary fragments (m/e)
Rha	2,3,4	117,131,161,175
Rha	2,4	117,131,233
Glc	2,4,6	45,117,161,233
Glc/Man	2,3,6	45,117,233
Man	2,6	45,117

Table 28. Relative retention times of the partially methylated alditol acetates derived during methylation analysis of _Erwinia_ EPS and its derivatives for a number of different columns. Conditions of GLC analysis are described in the legend. All columns except for the OV225 column are capillary columns.

Sugar	Position of Me group.	Column			
		SE-54[a]	BP5[b]	BP5[c]	OV225[d]
		Relative Retention Time			
Rha	2,3,4	1,00[e]	1,00[f]	1,00[g]	1,00[h]
Rha	2,4	1,50	1,51	1,29	1,97
Glc	2,3,4,6	1,68	1,63	ND[i]	2,25
Glc	2,4,6	2,48	2,04	1,79	3,96
Glc	2,3,6	2,60	2,10	1,84	5,15
Glc	2,3,4	2,78	2,19	ND	4,88
Glc	2,4	4,24	2,61	ND	9,44
Man	2,3,4,6	1,71	1,64	ND	ND
Man	2,3,6	2,54	2,07	ND	4,42
Man	2,6	3,36	2,36	2,18	6,79

a. Oven 160°C Isothermal. Gas, He 25 cm sec^{-1}

b. Oven 190°C, 15 min, increase to 240°C at 2°C min^{-1}, hold 5 min. Gas, N$_2$ 14 cm sec^{-1}.

c. Oven 220°C Isothermal. Gas, N$_2$ 14 cm sec^{-1}.

d. Oven 160°C Isothermal. Gas, He 30 ml min^{-1}.

e. Retention time = 5,17 min.

f. Retention time = 13,15 min.

g. Retention time = 7,49 min.

h. Retention time = 3,02 min.

i. Not determined.

GC-MS, and by comparison with standards derived from the methylation of dextran (a homopolymer containing mainly 1,6-linked glucose units, with 1,3,6- and 1,4,6-linked branch points) and glycogen respectively. The remaining peak was identified as 1,3,4,5-tetra-O-acetyl-2,6-di-O-methyl hexitol by GC-MS. As mannose was the only residue not accounted for, this peak was assumed to be 1,3,4,5-tetra-O-acetyl-2,6-di-O-methyl mannitol.

After Smith degradation of the EPS, and subsequent methylation of the resulting polyalcohol, a new peak appeared which was identified by GC-MS as 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl hexitol. This peak did not elute with the same retention time as the 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl glucitol derived from glycogen. Its appearance also conincided with the disappearance of 1,3,4,5-tetra-O-acetyl-2,6-di-O-methyl mannitol from the methylation products of the native and carboxyl-reduced EPS. The peak was therefore assumed to be 1,4,5-tetra-O-acetyl-2,3,6-tri-O-methyl mannitol. This result also supported the assumption that the 1,3,4,5-tetra-O-acetyl-2,6-di-O-methyl hexitol observed in the methylation products of the native and carboxyl-reduced EPS was 1,3,4,5-tetra-O-acetyl-2,6-di-O-methyl mannitol.

4.2.10.   Nuclear Magnetic Resonance (NMR) studies of autohydrolyzed EPS and the EPS backbone obtained after Smith degradation.

NMR spectroscopy has become an increasingly powerful and sophisticated method for use in carbohydrate chemistry (Rowe et al., 1970; Coxon, 1972; Bundle and Lemieux, 1976; Jennings and Smith, 1978, 1980; Barker and

Walker, 1980; Hall, 1980; Barker et al., 1982; Dabrowski et al. 1982;
Vliegenthart, et al., 1981, 1983). Proton NMR (^{1}H-NMR) is used extensi-
vely for the determination of the conformation of the anomeric linkages
present in oligo- and polysaccharides and has also been used to identify
and quantify the non-carbohydrate components in oligo- and polysaccha-
rides.

The availability of high-field NMR spectrometers (360-500 MHz) has
allowed the assignment of the resonance signals for all the protons in a
variety of mono- and oligosaccharides. Of particular note is the work
reviewed by Vliegenthart et al. (1981, 1983) in which high-field NMR
spectrometry, in conjunction with methylation analysis, is used to
identify the composition and linkages of complex carbohydrates. The
advent of Fourier-transform (FT) NMR spectrometers has also increased the
power of the technique, making it possible to obtain the [1]H-spectra of
smaller amounts of sample, and in addition to observe nuclei such as ^{13}C,
which are present normally in small amounts.

In ^{1}H-NMR, the anomeric protons resonate further downfield than either
the methine or methylene protons. The anomeric protons usually resonate
in the region δ 4,3-5,5 ppm, the methine protons (i.e. ring protons) in
the range δ 2,5-4,0 ppm, and the methylene protons in the δ 1,0-2,0 ppm
range. The chemical shifts of equatorial ring protons also resonate at
lower field than their axial counterparts. In monosaccharides, the α -
anomeric proton is usually equatorial whereas the β-anomeric proton is
axial. The difference between the chemical shifts of the α- and β-anome-

ric protons are clearly observed in the ^{1}H-NMR spectrum of glucose presented in Fig. 38. The α-anomeric proton resonates at δ 5.2 ppm and the β-anomeric proton at δ 4.9 ppm.

The spectrum presented in Fig. 38 was run at room temperature, and the β-anomeric proton signals are on either side of the HDO peak. These signals are frequently obscured by the large HDO peak, but the problem can be overcome by running the spectrum at 70-90°C, which shifts the HDO peak upfield. Determining the spectra at this temperature has another advantage in that the signals are sharpened. Unfortunately, the NMR spectrometer used in this study did not have a high temperature accessory, and all spectra were therefore determined at room temperature. This was not ideal since some of the anomeric signals may be hidden in the large solvent peak (HDO).

The spin-spin coupling constants for the anomeric proton and the adjacent proton on C-2 also provide information about the nature of the sugar involved in the glycosidic linkage. It has been observed that two protons axial to one another give rise to a coupling constant of about 7-10 Hz, two equatorial protons produce a coupling constant of about 1.5 Hz, and an equatorial and an axial proton possess a coupling constant of about 4 Hz. Exceptions to these values do exist, as will seen below for the coupling constant between the equatorial and axial protons in α-D-glucose and α-D-mannose. However, a combination of the observed chemical shifts
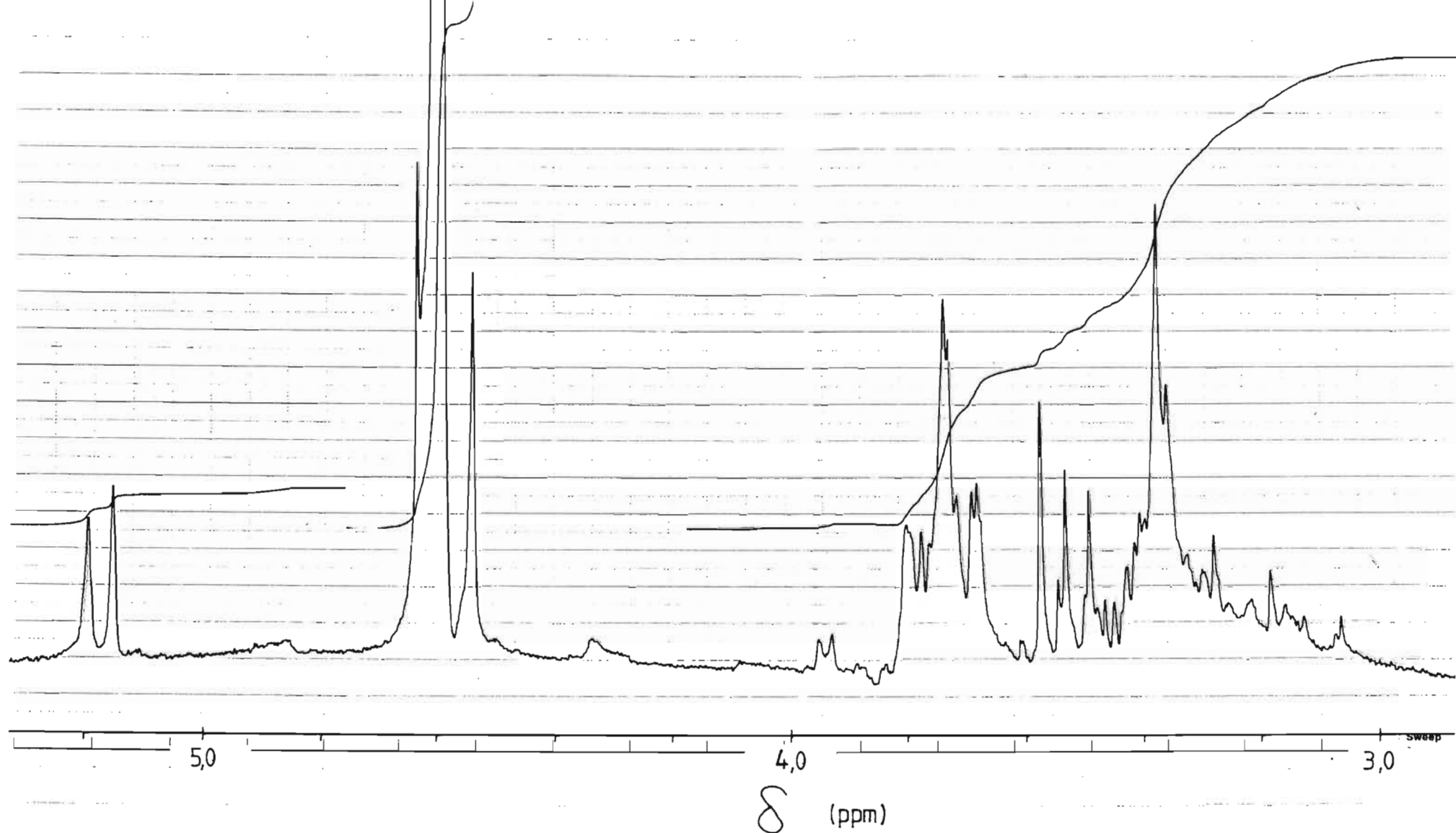
Fig. 38. ¹H-NMR spectrum of D-glucose run at 80 Mz. Glucose (16 mg) was dissolved in D_2O (1 ml), and recovered by freeze-drying. The residue was dissolved in D_2O (1 ml), filtered (0,45 μm Millex, Millipore) into a 5 mm NMR tube, and the spectrum run at room temperature on a Bruker WP 80 SY NMR spectrometer. The chemical shifts were obtained relative to external TMS. Operating conditions: number of accumulated scans, 256; number of points accumulated per scan, 8192 i.e. 8K; pulse width, 1 μsec; sweep width, 200 Hz.

and the coupling constants provide a great deal of information about the configuration of the glycosidic linkage and the nature of the sugar residue involved.

The equilibria between the α- and β-forms of D-glucose and D-mannose are presented in Fig. 39. The protons on C1 and C2 in β-D-glucose are both axial, whereas they are equatorial-axial in α-D-glucose, axial-equatorial in β-D-mannose, and equatorial-equatorial in α-D-mannose. The anomeric protons resonate at δ 5,2 ppm (α-D-glucose), 4,6 ppm (β-D-glucose), δ 5,12 ppm (α-D-mannose), and δ 4,8 ppm (β-D-mannose) (Figs. 38, 40 and Table 29). The coupling constants for the anomeric proton in β-D-glucose (i.e. J_{aa}) was found to be 7,6 Hz, and for the α-anomeric proton (i.e. J_{ea}), 3,3 Hz (Fig. 38 and Table 29). The equivalent coupling constants for α-D-mannose (i.e. J_{ee}) was 1,5 Hz, and for β-D-mannose (i.e. J_{ae}), 1,1 Hz (Fig. 40 and Table 29). The low coupling constants for D-mannose (which are similar to those found in L-Rhamnose) are typical of the D-manno- or L-rhamno- configurations.

The area under each peak is proportional to the number of protons. Thus the number of residues in the repeating unit of an oligo- or polysaccharide can often be obtained by integrating the peak area of the anomeric proton signals.
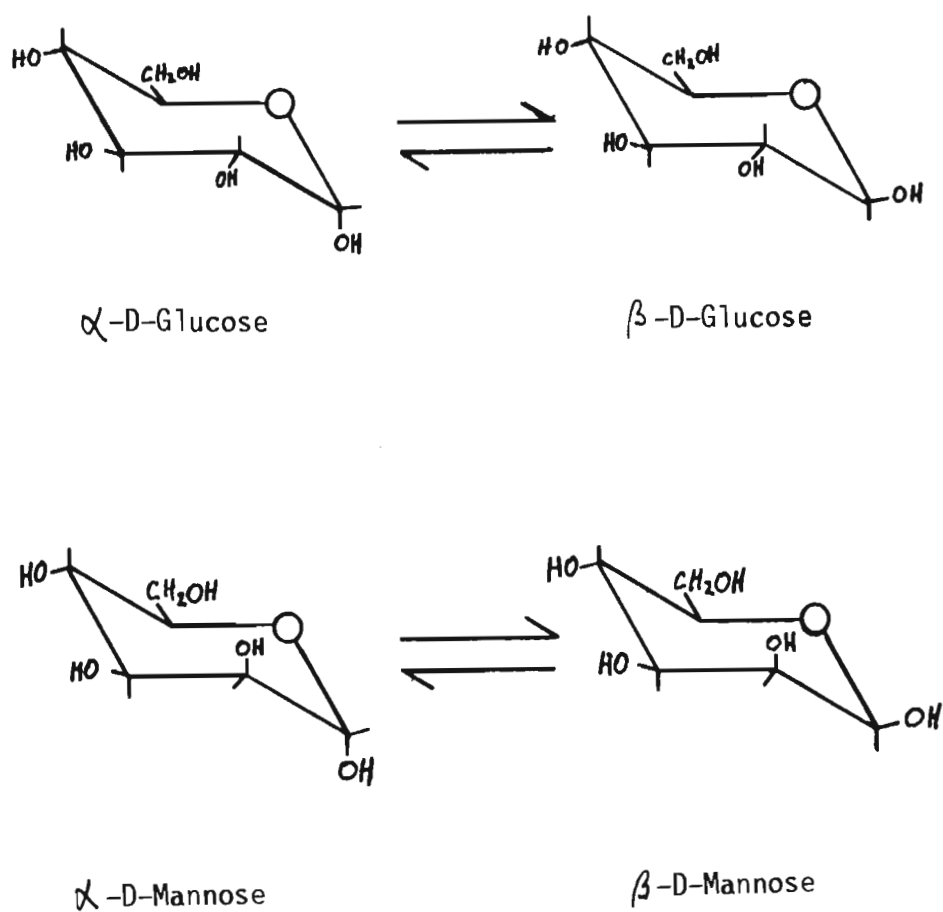
Fig. 39. Diagrammatic representation of the equilibrium between the α- and β-configurations of D-glucose and D-mannose.

Table 29. Chemical shifts and coupling constants for the anomeric protons of α- and β-D-glucose and mannose. Data obtained from Figs. 38 and 40.

| Sugar	Chemical shift of anomeric proton (ppm)	Coupling constant (Hz)
α-D-Glucose	5,20	$J_{ea} = 3,3$
β-D-Glucose	4,60	$J_{aa} = 7,6$
α-D-Mannose	5,12	$J_{ee} = 1,5$
β-D-Mannose	4,80	$J_{ae} = 1,1$
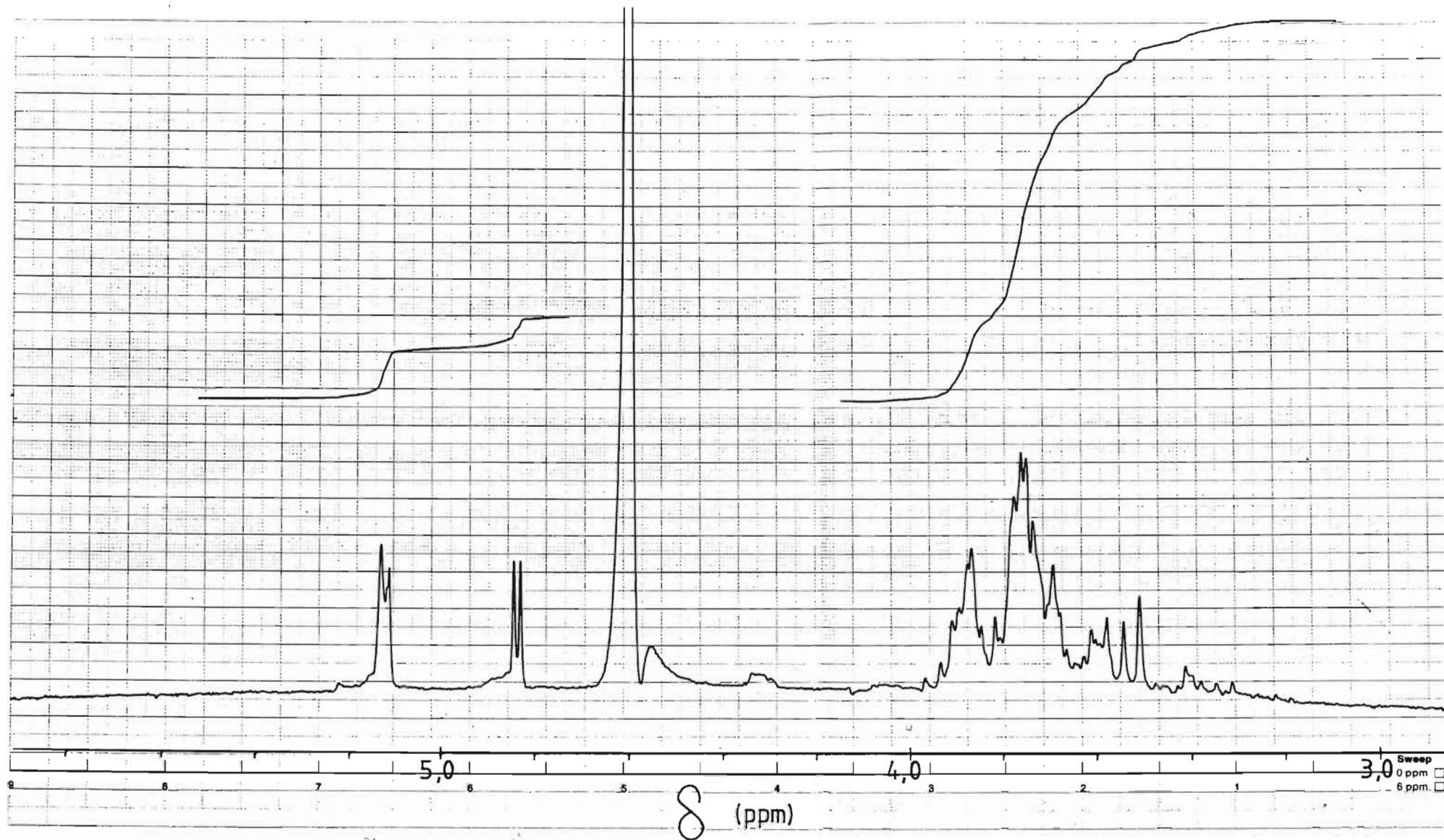
Fig. 40. ^{1}H-NMR spectrum of D-mannose run at 80 Mz. Mannose (12 mg) was dissolved in D_2O (1 ml), and recovered by freeze-drying. The residue was dissolved in D_2O (1 ml), filtered (0,45 μm Millex, Millipore) into a 5 mm NMR tube, and the spectrum run at room temperature on a Bruker WP 80 SY NMR spectrometer. The chemical shifts were obtained relative to external TMS. Operating conditions: sweep width, 250 Hz. All other operating conditions as described in Fig. 38.

In practice, the quality of the ^{1}H-NMR spectrum will depend on a number of factors, including the amount of material available, the solubility of the material, and the presence of particles in the sample, the solvent, and the viscosity of the sample. The temperature at which the spectrum is accumulated is also important. Higher temperatures lead to a shift upfield of the HDO peak, and to sharper resonance lines.

The ^{1}H-NMR spectrum of the electrodialyzed EPS was not very good due to the high viscosity of the solution, and gave broad, poorly resolved signals in the anomeric region. In order to reduce the viscosity of the solution, the electrodialyzed EPS (15 mg ml^{-1}) in D_2O was autoclaved (121°C) for 20 minutes, and then lyophilized. The process of dissolution in D_2O and lyophilization was repeated, and the sample finally dissolved in 1 ml D_2O, filtered (0,45 μm Millex, Millipore) into a 5 mm NMR tube. An improved spectrum was obtained at 80 MHz and this is presented in Fig. 41.

Despite the relatively broad resonance signals, the following information is obtainable from the spectrum. The doublet at δ 1,28 ppm (J=5,8 Hz) is due to the 6-methyl group of rhamnose. The signals in the δ 1,8 to δ 5,3 ppm region are due to the anomeric protons. The doublet at δ 4,88 ppm has a coupling constant of 1,4 Hz, typical of a D-manno or L-rhamno config-uration. The ratio of the integral in the δ 4,88 ppm peak to that of the δ 1,28 ppm peak is 3,9, suggesting that this signal may have arisen from
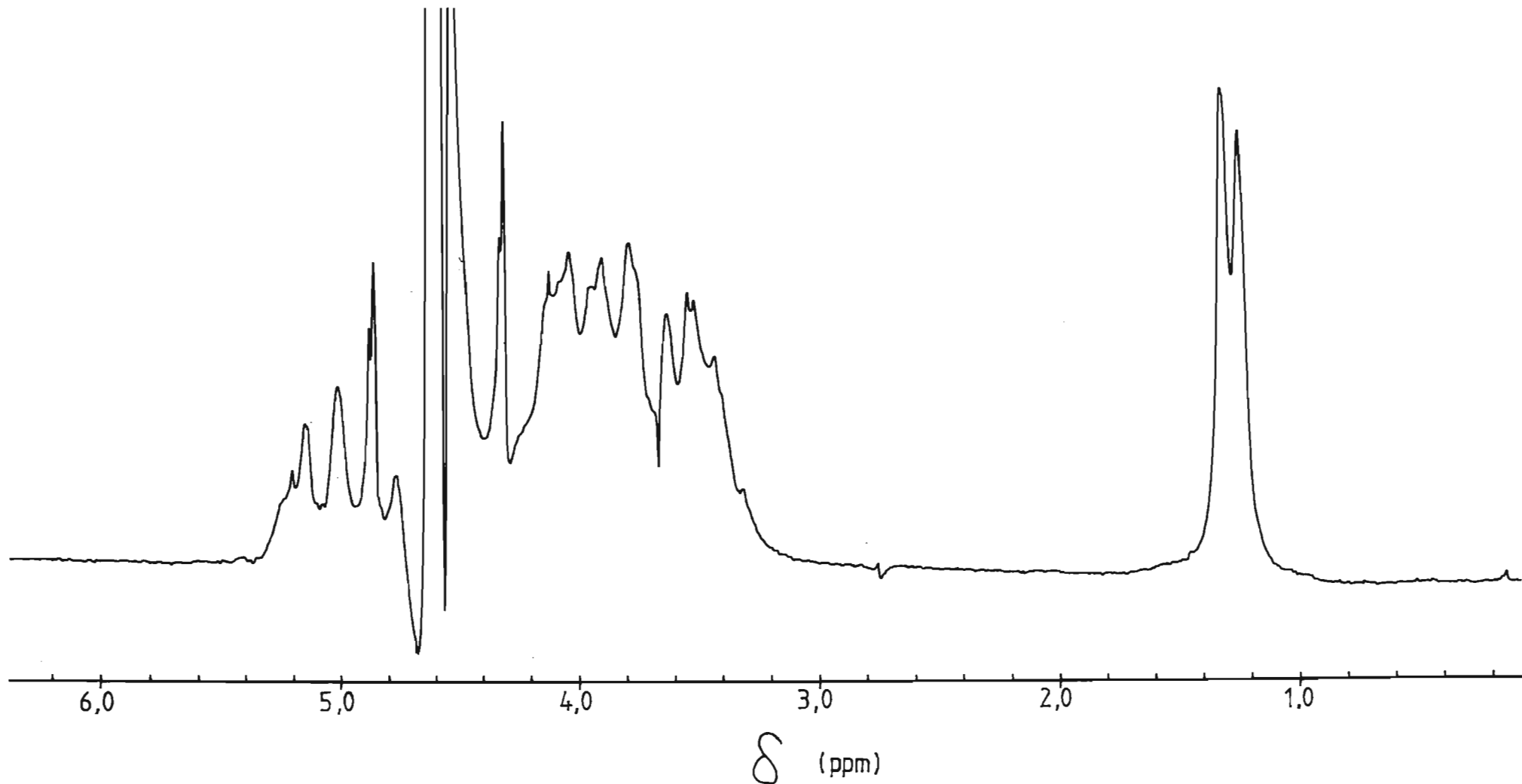
Fig. 41. ^{1}H-NMR spectrum of autoclaved electrodialyzed EPS run at 80 MHz. Electrodialyzed EPS (64 mg) was dissolved in D_2O, and autoclaved (121°C, 20 min) in a test tube fitted with a teflon lined cap. The EPS was recovered by freeze-drying, and the residue dissolved in D_2O (1 ml). After filtering (0.45 μm Millex, Millipore) into a 5 mm NMR tube, and the spectrum run at room temperature on a Bruker WP 80 SY NMR spectrometer. The chemical shifts were obtained relative to external TMS. Operating conditions: number of accumulated scans, 256; number of points accumulated per scan, 8192 i.e. 8K; pulse width, 1 μsec; sweep width, 200 Hz.

the anomeric protons of rhamnose. If this is the case, then the rhamnose residues in the EPS are all β-linked. The other signals in this region are not well resolved. The furthest upfield signal (δ 5.21 ppm) appears to consist of a doublet, with a coupling constant of 4.14 Hz, typical of an α-linked D-gluco- or D-galacto- residue. This signal, which integrates to two protons therefore appeared to be due to the glucose and glucuronic acid in the EPS. Two other signals remain, one at δ 5.02 ppm and the other at δ 4.77 ppm (which, being close to the HDO peak may be an artifact). The singlet at δ 5.02 ppm integrates to 3 protons, and may arise in part from the anomeric proton of mannose, but cannot be definitely assigned. If it is due to the mannose in the EPS, then this residue appears to be α-linked in the EPS.

No resonance other than the doublet arising from the 6-methyl group of rhamnose was observed in the δ 1 to 2 ppm region, indicating the absence of non-carbohydrate components in the electrodialyzed EPS.

The ^{1}H-NMR spectrum of the EPS backbone (obtained after 1 cycle of Smith degradation) is presented in Fig. 42. The poor resolution of the spectrum is due mainly to the viscosity of the solution, but the following information can be obtained from it. Firstly, the broad singlet at δ 1.31 ppm arises from the 6-methyl group of rhamnose, and integrates to about three protons. Secondly, the broad signals between δ 5.09 and δ 5.24 ppm arise from the anomeric protons of glucose, mannose, and rhamnose, and
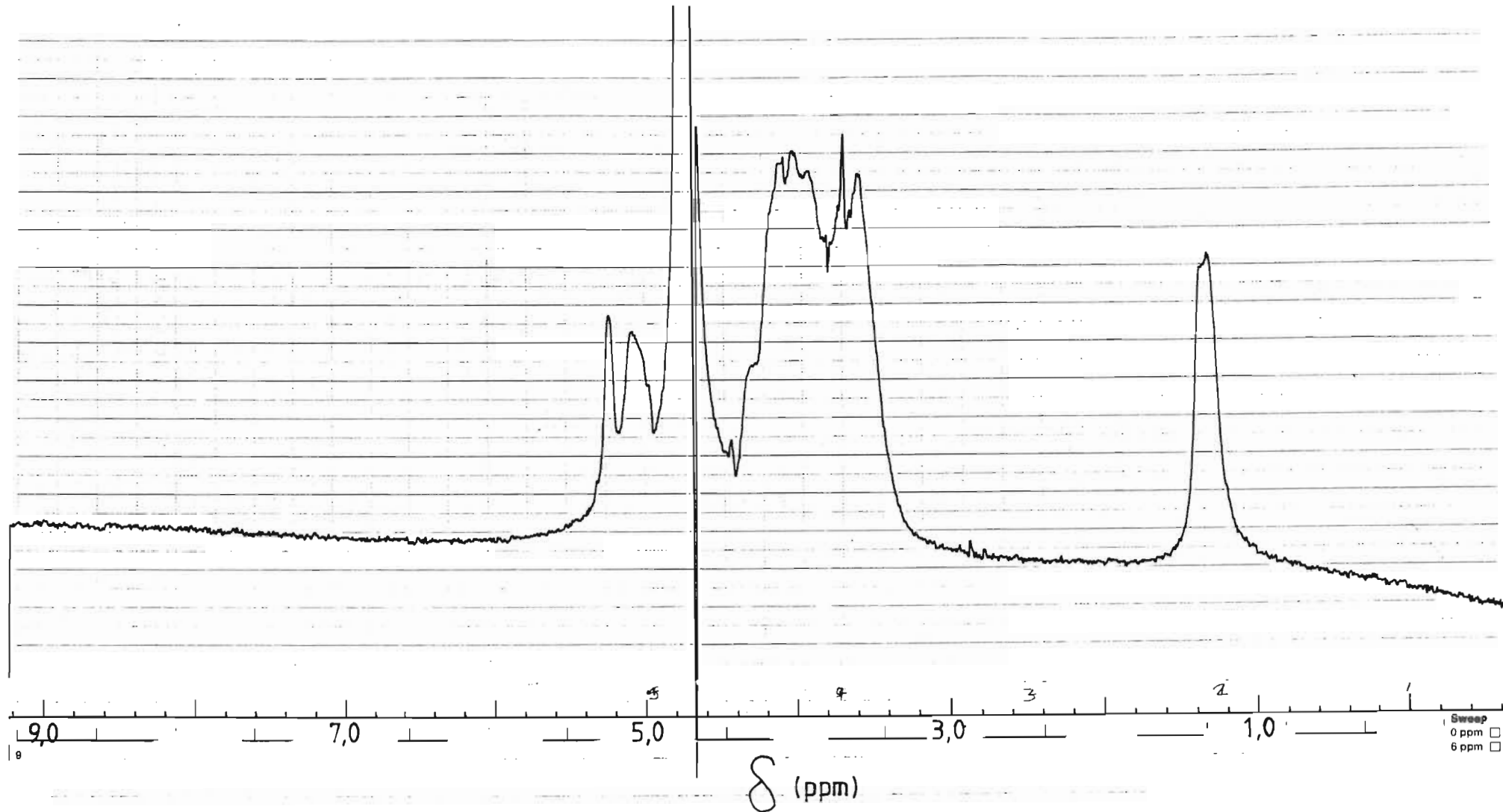
Fig. 42. ^{1}H–NMR spectrum of the EPS backbone run at 80 MHz. The high molecular mass polymer obtained during chromatography of the Smith degraded carboxyl-reduced EPS (5 mg) on Bio-Gel a-1,5m was dissolved in D_2O (1 ml), and recovered by freeze-drying. The residue was dissolved in D_2O (1 ml), and after filtering (0,45 m Millex, Millipore) into a 5 mm NMR tube, the spectrum run at room temperature on a Bruker WP 80 SY NMR spectrometer. The chemical shifts were obtained relative to external EPS. Operating conditions: number of scans, 2072; sweep width, 800 Hz. Other conditions are as described in the legend to Fig. 38.

also integrate to about three protons. The singlet at δ 5,24 ppm appears to be the counterpart of the δ 5,21 ppm signal in the electrodialyzed EPS, and integrates to about one proton. It may therefore be due to an α-anomeric proton of glucose. The broad signal at δ 5,09 ppm integrates to about two protons, and therefore possibly arises from the α- and the β-anomeric protons of mannose and rhamnose respectively.

^{13}C-NMR spectroscopy is increasingly used in carbohydrate chemistry, despite the relative insensitivity of the technique. The use of FT-NMR spectrometers allows the spectrum of less than 10 mg of material to be obtained under suitable conditions. Most spectra are obtained with broad band decoupling in which the contributions by the protons in the sample to the spectrum are suppressed. This results in spectra consisting of a number of sharp lines in contrast to the multiple signals of ^{1}H-NMR spectra. This point is illustrated by comparing the ^{1}H- and ^{13}C-spectra of D-glucose presented in Figs. 38 and 43. As in ^{1}H-NMR, the anomeric carbons resonate at lower field than the other ring carbons. In contrast to ^{1}H-NMR spectroscopy, the carbons bearing the anomeric substituent (i.e. the aglycone) in the equatorial position (normally the β-anomer) resonate downfield from the carbon atom bearing the aglycone in the axial position (usually the α-anomer). In Fig. 43, the anomeric carbon bearing the aglycone in the equatorial position (i.e. the β-configuration) resonates at δ 96,62 ppm, and that bearing the aglycone in the axial position (i.e. the α-configuration) resonates at δ 92,80 ppm.
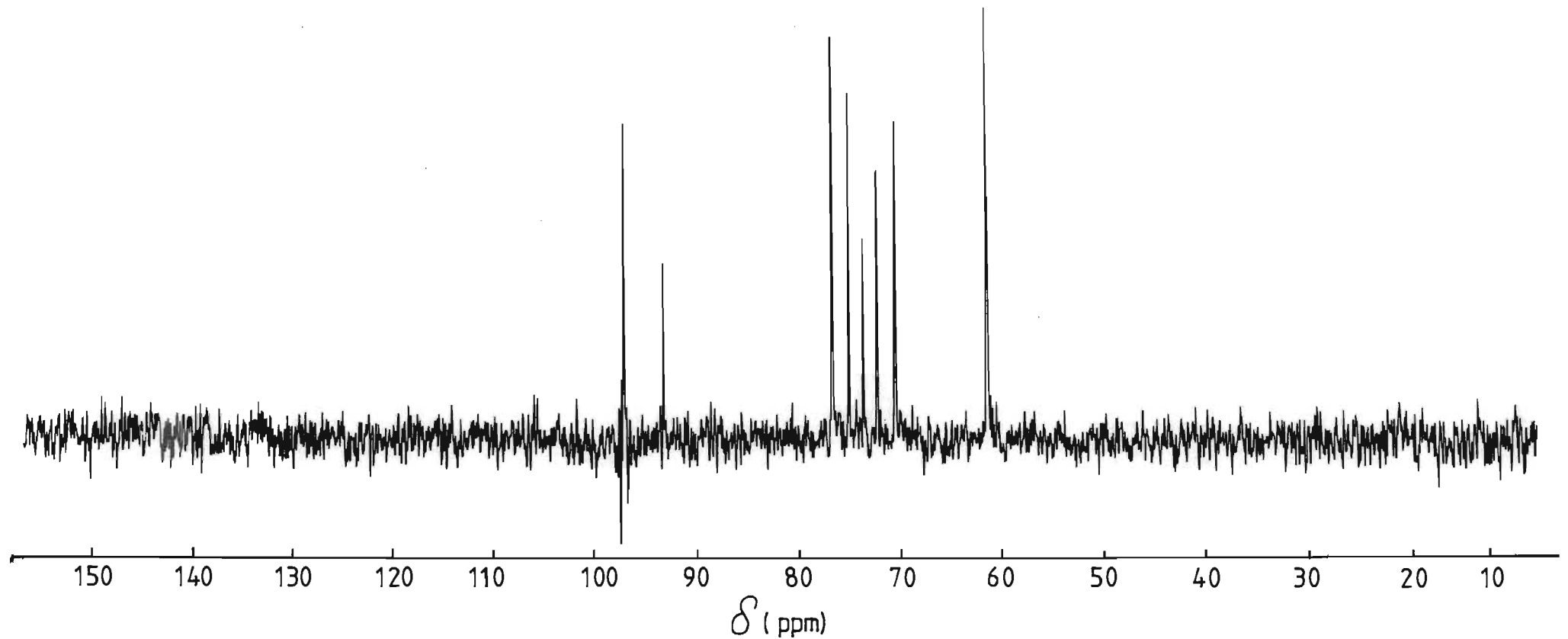
Fig. 43. The proton-decoupled ^{13}C-NMR spectrum of D-glucose run at 20,15 MHz. Glucose (270 mg) was dissolved in D_2O (1 ml), and filtered (0,45 m Millex, Millipore) into a 5 mm NMR tube, and the ^{13}C-NMR spectrum run at room temperature on a Bruker WP 80 SY NMR spectrometer. All chemical shifts were measured relative to C-6 of glucose taken as 61,6 ppm (Johnson and Jankowski, 1972). Operating conditions: number of accumulated scans, 232; number of points accumulated per scan, 8192 i.e. 8K; pulse width, 2,5 sec, equivalent to a pulse angle of 63°C; sweep width, 3000 Hz; broad band decoupling power, 4H; O1, 650 Hz.

In ^{13}C-NMR spectra, the chemical shifts observed for the carbon atoms in oligo- and polysaccharides are generally similar to those observed in the constituent monosaccharides except for usually minor substituent effects. An exception is found in the chemical shifts of directly substituted carbon atoms which show a large increase of 3-7 ppm (i.e. are shifted downfield), while the carbon atoms β to the substituted carbon atoms generally show a change in chemical shift (usually upfield). The determination of the substituted carbon atom as well as the configuration of the glycosidic linkage is thus also made possible by ^{13}C-NMR spectroscopy.

The ^{13}C-NMR spectrum of the autohydrolyzed EPS is presented in Fig. 44. The signal at δ 16,35 ppm is due to the methyl group of rhamnose. Only two signals are present in the anomeric region, one at δ 100,68 ppm and the other at δ 101,98 ppm. Their origin is difficult to determine, but the signal at δ 101,98 ppm may be due to the β-rhamnose residues, and that at δ 100,68 ppm due to the other C1-atoms of glucose, mannose, and glucuronic acid. The only other identifiable signal was at δ 172,54 ppm, which arises from the carboxyl group of glucuronic acid.

No ^{13}C-NMR spectrum could be obtained for the EPS backbone, even after accumulating 250 000 scans.
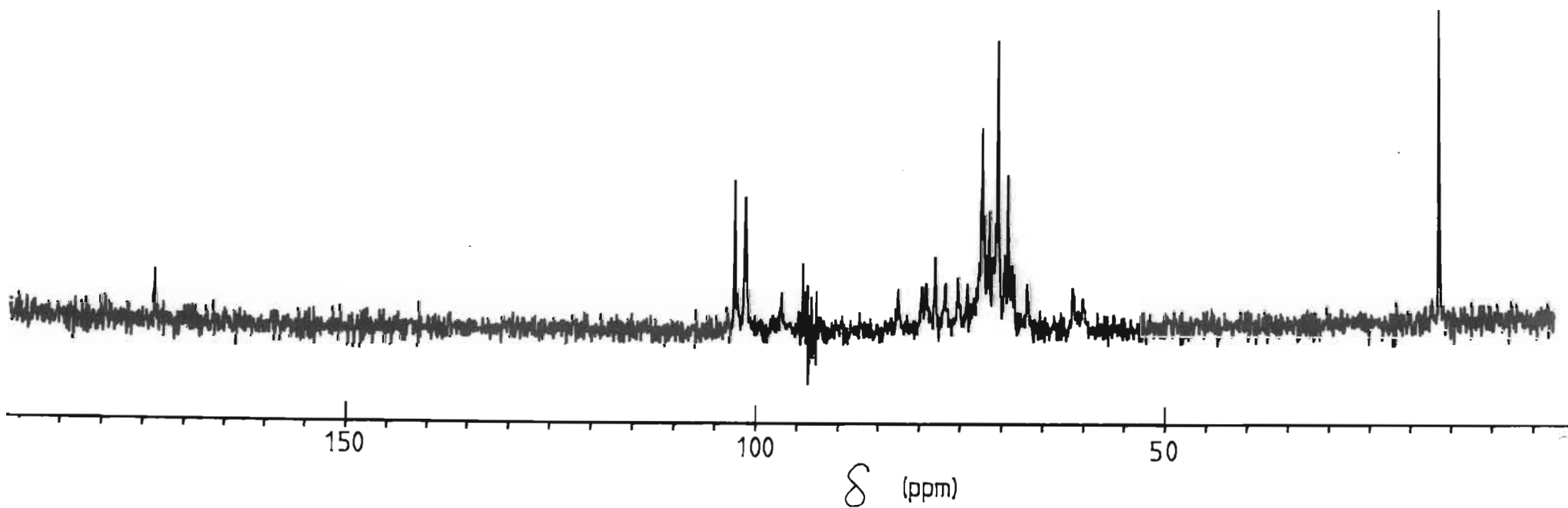
Fig. 44. Proton-decoupled ^{13}C-NMR spectrum of autoclaved EPS (electrodialyzed) run at 20,15 MHz. The ^{13}C-spectrum of the sample described in the legend to Fig. 41 was run at room temperature on a Bruker WP 80 SY NMR-spectrometer. The chemical shifts were obtained relative to C-6 of glucose as described in the legend to Fig. 43. Operating conditions: Number of scans, 74 000; sweep width, 5000 Hz; broad band decoupling power, 6H; 01, 910 Hz. Other conditions are as described in the legend to Fig. 43.

In conclusion, it appears as if the rhamnose residues in the EPS may all be β-linked, whereas all the other sugars may be α-linked. Confirmation will, however, have to come from NMR studies on smaller oligosaccharides produced by an additional series of Smith degradations, or by chemical means, such as chromium trioxide oxidation (Hoffman and Lindberg, 1980).

4.2.11. Isolation and characterization of the aldobiouronic acid.

An aldobiouronic acid was isolated after acid hydrolysis of the native EPS by chromatography on Bio-Rad AG1-X8, and migrated as a single spot during TLC on cellulose plates using solvent II. Further hydrolysis and analysis of the alditol acetates by GLC showed mannose as the major together with a small amount of glucose. The glucose may have arisen from the glucuronic acid in the aldobiouronic acid, which after hydrolysis and drying from acid solution may have formed the lactone (Blake and Richards, 1968). The lactone, on subsequent reduction, would then give rise to glucitol.

The ^{1}H-NMR spectrum of the aldobiouronic acid run at 80 Mhz clearly showed two doublets in the anomeric region. The first was at δ 5,02 ppm (J = 1,9 Hz) and the other at δ 5,16 ppm (J = 3,89 Hz). Other signals were presumably lost in a very broad HDO signal. The signal at δ 5,02 ppm probably arises from the mannose residue in the aldobiouronic acid, whereas that at δ 5,16 ppm is probably due to the glucuronic acid residue. The glycosiduronic acid linkage appears to be an α-linkage, as the coupling constant (3,89 Hz) is characteristic of sugar residues in

the α-D-gluco- configuration. The structure of the aldobiouronic acid therefore appears to be GlcA$\xrightarrow{\text{1} \quad \alpha \quad \text{3}}$Man. This will, however, have to be confirmed by further work.

4.2.12. Conclusion.

Given the available data it appears that the backbone of the EPS is a glucomannorhamnan to which the side chains are attached via α-glucosyl-uronic linkages through the 3 position of mannose. The structure of the side-chain is not known, but is likely to be

$$\text{Rha}\xrightarrow[\text{1}]{\beta \quad 3}\text{Rha}\xrightarrow[\text{1}]{\beta \quad 3}\text{Rha}\xrightarrow[\text{1}]{\beta \quad 4}\text{GlcA}$$
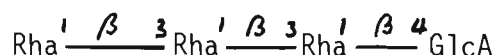
The following two structures for the EPS are therefore consistent with the available evidence.

$$[\xrightarrow{3}\text{Glc}\xrightarrow[\text{1}]{\alpha \quad 4}\text{Man}\xrightarrow[\text{1}]{\alpha \quad 3}\text{Rha}\xrightarrow[\text{1}]{\beta}]$$
$$\begin{array}{c} | 3 \\ \alpha \\ |1 \end{array}$$
$$\text{Rha}\xrightarrow[\text{1}]{\beta \quad 3}\text{Rha}\xrightarrow[\text{1}]{\beta \quad 3}\text{Rha}\xrightarrow[\text{1}]{\beta \quad 4}\text{GlcA}$$

or

$$[\xrightarrow{3}\text{Rha}\xrightarrow[\text{1}]{\beta \quad 4}\text{Man}\xrightarrow[\text{1}]{\alpha \quad 3}\text{Glc}\xrightarrow[\text{1}]{\alpha}]$$
$$\begin{array}{c} | 3 \\ \alpha \\ |1 \end{array}$$
$$\text{Rha}\xrightarrow[\text{1}]{\beta \quad 3}\text{Rha}\xrightarrow[\text{1}]{\beta \quad 3}\text{Rha}\xrightarrow[\text{1}]{\beta \quad 4}\text{GlcA}$$
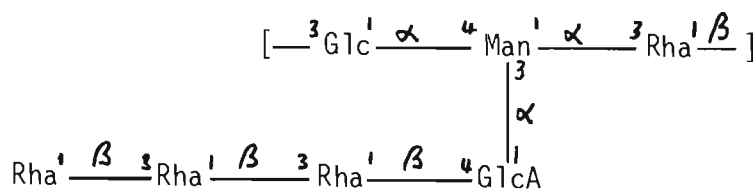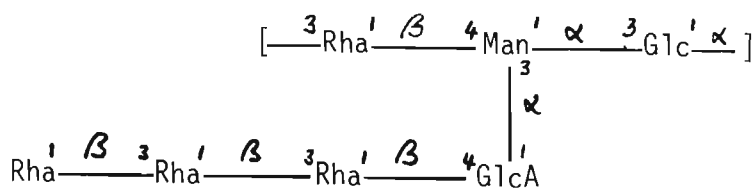
The present investigation has not yielded an unambiguous structure for the E. chrysanthemi (FH1) EPS. It has, however, narrowed down the field to two strong contenders and the results provide a solid foundation for further studies.

CHAPTER 5

GENERAL DISCUSSION

In this thesis a contribution has been made to the further understanding of the biochemistry of <u>Erwinia</u> <u>chrysanthemi</u>. Although the research results have been discussed in previous chapters, it is worthwhile reemphasizing some key features. Wherever possible, reference will be made to pathogenicity, for this aspect is of obvious social and economic importance.

The threat posed by <u>E</u>. <u>chrysanthemi</u> pv. <u>zeae</u> cannot be overestimated. In the first place, the pathogen causes soft rot in maize, the staple food of much of Africa's peoples. At present, much of the maize is produced under dry-land conditions (the commonest form of subsistence farming), and soft rot is not a problem. However, the rapidly increasing population is placing enormous pressure on agriculture to produce food more efficiently, and it is therefore possible that more maize will be grown under irrigation. This in turn will increase the incidence of diseases such as soft rots which thrive under conditions of high humidity.

One way in which to prevent infection by phytopathogens during irrigation is to include chemicals such as chlorine in the water. Alternatively, new plant varieties with increased resistance to disease must be bred. Plant breeding programs are time consuming, often due to the lack of knowledge

of the precise factors involved in the trait being selected for. The development of rapid analytical procedures for the screening of a particular trait, particularly in conjunction with new propagation techniques such as tissue culture, would significantly decrease the time required for the production of a new plant variety. The development of these assays requires a knowledge of the biochemistry of both the host and the pathogen. However, it has been stressed that the host-pathogen interaction is a complex one and biochemical investigations, such as the present one, can do no more than provide clues about important aspects of the process.

In Chapter 2, it was shown that pectate lyase (PL) production was largely unaffected by the presence of a variety of sugars in the growth medium. This observation suggests one reason for the virulence of E. chrysanthemi. The pectate lyase (PL) enzymes of E. chrysanthemi have been shown to be essential virulence factors in the organism and strains of E. chrysanthemi lacking these enzymes are avirulent (Chatterjee and Starr, 1977, 1978). It is important, however, that the organism not only produce PL, but that it continues to do so during growth in the host. Failing this, the further spread of bacteria through the host tissues would be prevented. During infestation, the pathogen is bound to encounter a number of sugars of host origin, particularly those arising from the degradation products of pectic substances, cellulose, and hemicellulose. If these degradation products readily repressed PL production, the spread of E. chrysanthemi would certainly be temporarily halted, perhaps long enough for the host defenses to deal with the invader. Some of the

most likely products of cell wall degradation will be sugars such as cellobiose, L-arabinose, D-mannose, L-Rhamnose, D-xylose, and D-galacturonic acid. In Chapter 2, it was shown that none of these sugars represses PL synthesis by E. chrysanthemi completely. This insensitivity of PL production to repression by the sugars commonly present in cell wall digests underlines the importance of PL to the virulence of the organism.

A preference of E. chrysanthemi for sodium polypectate as a carbon source was demonstrated for the Lac+ mutant which utilizes this polymer in preference to lactose. Polypectate was the preferred carbon source even though the transport of lactose into the cell was constitutive and the β-galactosidase already fully induced before addition of the sodium polypectate to the culture. Thus, E. chrysanthemi (EC-S) induced a completely new set of genes in order to metabolize sodium polypectate rather than continuing to metabolize a substrate which was already being used. Although lactose is not a normal plant constituent, these results still suggest that the pathogen may have a significant degree of control over the sequence of nutrients that it will use when challenged with a variety of different carbohydrates.

The mechanism involved in this pecking order is not clear from the work reported in this thesis, and bears additional investigation. In this regard, simultaneous exposure of E. chrysanthemi to sugars more likely to be encountered in the plant, such as melibiose and raffinose, may be profitable. This latter aspect is of great interest, particularly as it

is now known that lactose transport occurs via either the melibiose or the raffinose permeases.

The discovery of a Lac[+] mutant of E. chrysanthemi (EC-S) is itself of interest. It not only offers an explanation of why lactose metabolism is classed as variable in E. chrysanthemi, but also emphasizes the ability of microorganisms to acquire new metabolic activities. E. chrysanthemi therefore joins the ranks of the ever increasing number of Enterobacteri- aceae found to acquire the ability to metabolize novel compounds (see reviews by Hall, 1982, 1983; Mortlock, 1982).

The finding that E. chrysanthemi did not possess a lac operon analogous to that found in E. coli was unexpected, and illustrates the danger of assuming too much similarity between organisms within a family. In retrospect, it is not surprising that E. chrysanthemi should metabolize lactose differently from E. coli. Lactose is a sugar commonly encountered by E. coli in its environment, whereas E. chrysanthemi is unlikely to come across this sugar in nature. The absence of the lac operon in E. chrysanthemi is therefore an example of adaptational economy in that genes not required for survival become redundant.

Lactose induces a lactose permease in E. coli, but not in E. chrysanthemi where the Lac[+] phenotype of strain EC-S results from a mutation allowing the constitutive expression of a lactose transport system. This is because the genes for lactose transport and hydrolysis are on different operons in E. chrysanthemi, and in this respect, E. chrysanthemi differs

fundamentally from $\underline{E}$. $\underline{coli}$, where the genes for lactose transport and hydrolysis are situated in the same operon, and are co-ordinately induced (Zabin and Fowler, 1980).

Two permeases capable of lactose transport appear to be present in $\underline{E}$. $\underline{chrysanthemi}$, neither of which are induced by lactose. One of these appears to be associated with the $\underline{mel}$ operon, and the other with the $\underline{raf}$ operon. Evidence strongly indicates that it is the constitutive expression of the $\underline{mel}$ operon that is responsible for the Lac$^+$ phenotype of $\underline{E}$. $\underline{chrysanthemi}$ strain EC-S. Definitive proof can, however, only be obtained by the isolation of the relevant mutants in which any interfering operons and genes have been eliminated.

Further differences between $\underline{E}$. $\underline{coli}$ and $\underline{E}$. $\underline{chrysanthemi}$ become apparent when the properties of the $\underline{E}$. $\underline{coli}$ $\underline{mel}$ permease are contrasted with the data of Chapter 3 relating to the specificity of the $\underline{E}$. $\underline{chrysanthemi}$ melibiose induced permease. Firstly, the $\underline{mel}$ permease in $\underline{E}$. $\underline{coli}$ is unable to transport lactose, whereas the $\underline{E}$. $\underline{chrysanthemi}$ melibiose induced permease can. Secondly, the $\underline{E}$. $\underline{chrysanthemi}$ melibiose induced permease is unable to transport TMG, whereas the $\underline{mel}$ permease of $\underline{E}$. $\underline{coli}$ can (Rotman $\underline{et}$ $\underline{al}$., 1968). Additionally, the α-galactosidases induced in both systems are different; the $\underline{E}$. $\underline{coli}$ enzyme is inactivated by toluene, requires a cofactor for activity, and is stabilized by Mn^{2+} ions and dithiothreitol (DTT), whereas the $\underline{E}$. $\underline{chrysanthemi}$ does not require a cofactor, is not inactivated by toluene, nor activated by Mn^{2+} or DTT. In fact, the $\underline{E}$. $\underline{chrysanthemi}$ melibiose induced permease resembles more

closely that of <u>Klebsiella</u> <u>aerogenes</u> (Reeve and Braithwaite, 1973a) in that it can transport lactose, although the insensitivity of the α-galactosidase to inactivation by toluene resembles the raffinose induced α-galactosidase in <u>E</u>. <u>coli</u> (Schmid and Schmitt, 1976).

Bacterial polysaccharides are widely distributed in nature and constitute a heterogeneous group of polymers. Many phytopathogenic bacteria appear to produce extracellular polysaccharides (EPS's) of widely differing structures. Most EPS's are heteropolymers consisting of repeat units of two to twelve monosaccharide residues or more (Sutherland, 1977). Non-carbohydrate components (such as pyruvate, succinate, acetate, and sulphate) are often covalently associated with these polysaccharides, and confer special solubility and gelling properties on these polymers. In addition, during growth there is the possibility of non-covalent contaminants such as other carbohydrates, nucleic acids and proteins becoming associated with the EPS. It is therefore important that the polysaccharide be purified of these contaminants before attempting to elucidate its structure.

Because of wide differences in the structures of bacterial EPS's, it is extremely difficult to draw meaningful comparisons between the structure of the <u>E</u>. <u>chrysanthemi</u> EPS reported in Chapter 4 and other bacterial EPS's. Comparisons are more difficult because it appears that no other study, structural or otherwise, apart from the present one, has been made of the EPS's from <u>E</u>. <u>chrysanthemi</u>. It is therefore particularly diffi-

cult to add anything to what has already been said about the structure of the E. chrysanthemi polysaccharide in Chapter 4.

The connection between virulence and polysaccharide production in E. chrysanthemi is not clear at present, however in the case of Pseudomonas solanacearum, a definite correlation has been established (see Sequeira 1982, 1983, 1984a,b for reviews). The EPS⁺ form of this organism is virulent, whereas the EPS⁻ mutant is avirulent. When virulent cells are washed free of EPS, they become avirulent (Sequeira and Graham, 1977). Conversely, when the washed cells are mixed with EPS prior to infection, they regain their virulence. Therefore, it is unlikely that some other factor, which coincidentally accompanies EPS expression, might be actually responsible for the observed virulence. Available evidence suggests that in this case EPS masks the core region of the lipopolysaccharide (LPS) of the bacterium, which is, consequently, not recognized by the host defense system.

A further example of a connection between virulence and EPS production is provided by E. amylovora. This organism produces an EPS, amylovorin, that has been shown to occlude the xylem vessels of the host, resulting in wilting (Suhayda and Goodman 1981). Little is known about the E. chrysanthemi EPS, and its role in pathogenicity. However, one of the early symptoms during infection of maize by E. chrysanthemi is a wilting of the plant prior to the onset of the soft rot. As E. chrysanthemi produces EPS on a number of sugars other than lactose, it is possible that this organism produces EPS after exposure to the carbohydrate

breakdown products of host origin during infection. This may explain the wilting observed as one of the early symptoms of E. chrysanthemi infection. It is not known if the EPS has a similar role in E. chrysanthemi to that in P. solanacearum mentioned above.

It may be mentioned in passing that many microbial polysaccharides are finding uses in industry, as for example in oil-drilling and recovery. As first it was thought that the E. chrysanthemi might be useful in this respect. The lability of the rhamnose residues in the polysaccharide would, however, appear to limit the usefulness of the E. chrysanthemi polysaccharide in this respect.

Comparatively little is known about either the general biochemistry of E. chrysanthemi or the more specific relationship between biochemistry and pathogenicity. The findings of this thesis have a bearing on both topics, and it is hoped that the results presented provide some incentive for others working in these, or related, fields.

SUMMARY

1.   Pectate lyase (PL) production by <u>Erwinia</u> <u>chrysanthemi</u> (FH1) was studied during growth on a number of media. Best enzyme production was found on media supplemented with yeast extract and high levels of either phosphate (0,1 M) or calcium ions (7,55 mM). The presence of sodium polypectate (NaPP) in the medium was essential for high levels of PL expression by <u>E</u>. <u>chrysanthemi</u>.

2.   Two extracellular PL isoenzymes were detected in the spent medium by isoelectrofocusing, one with a pI of about 5,0, and the other with a pI of about 9,2. The PL isozymes exhibited a pH optimum of 8,5 when assayed on NaPP, and 9,0 when assayed on pectin, and both exhibited an absolute requirement for calcium ions for activity, being completely inactivated by the addition of EDTA to the assay mixture. Activity was restored by the adding calcium ions back to the assay mixture.

3.   PL was found to be an inducible enzyme that was also subject to catabolite repression. A number of carbohydrates were tested for their ability to repress PL synthesis, and were found to be divisible into three groups. The first group repressed enzyme synthesis severely (> 90%), and included D-glucose, D-fructose, sucrose, and D-mannitol. The second group, which included D-galacturonic acid, D-xylose, D-galactose, L-arabinose, and cellobiose, all repressed enzyme activity to an intermediate degree (30-80%), whereas the

final group, including glycerol, D-arabinose, L-rhamnose, lactose, D-mannose, and xylan, had little or no effect on PL synthesis (< 30%).

4. The wild type strain of E. chrysanthemi (FH1) was found to be Lac⁻ (i.e. unable to grow on lactose). It was found that Lac⁺ strains could be isolated from the Lac⁻ parent after extended incubation on a poor medium containing lactose as the major carbon source. The Lac⁺ strains were readily distinguished by their appearance on a variety of differential media, and by their slimy appearance on lactose containing media. Because of its slimy appearance, the Lac⁺ strain was named EC-S (i.e. E. chrysanthemi-slimy), while the parent strain was called EC-C (i.e. E. chrysanthemi-clear). The production of slime by EC-S from lactose is not due to the acquisition of a new polysaccharide biosynthetic pathway, as both EC-C and EC-S produce copious amounts of slime during growth on D-glucose, D-galactose, and sucrose.

4. The effect of lactose on PL synthesis in strain EC-S was investigated. It was found that NaPP was always the preferred carbon source, even by cells pre-grown on lactose, and whose β-galactosidase was induced. NaPP and its PL-digestion products were found to repress β-galactosidase synthesis in strain EC-S growing in the presence of either lactose or glycerol+isopropyl-β-D-thiogalacto-pyranoside (IPTG). The synthesis of PL increased shortly after addition of NaPP or its degradation products to the cells growing on

lactose, and was accompanied by a decrease in the rate of β-galacto-sidase synthesis. NaPP, or its digestion products, appeared to be directly involved in the repression of β-galactosidase, as the rate of β-galactosidase synthesis in cells of EC-S growing on a mixture of NaPP+lactose increased to a rate characteristic of growth on plain lactose once all the NaPP in the medium was depleted. The mechanism of repression did not appear to be similar to that exerted by glucose, as this monosaccharide repressed both β-galactosidase and PL synthesis.

5. The β-galactosidase from both EC-S (Lac$^+$) and EC-C (Lac$^-$) were compared and found to be similar. Both enzymes had similar Km values, and were similarly activated by Na$^+$, Mg^{2+}, Mn^{2+}, and 2-mercaptoethanol. They were also similarly sensitive to inhibition by sodium dodecyl sulphate (SDS) and insensitive to sodium deoxycholate (DOC) inhibition. Finally, both enzymes hydrolyzed lactose equally well. It was concluded that the Lac$^-$ phenotype was not due to the inability of the β-galactosidase to hydrolyze lactose.

6. The transport of lactose by strains EC-C and EC-S grown on a number of different carbon sources was investigated. Lactose transport by strain EC-S was found to be constitutive, and induced by melibiose, raffinose, and to a lesser extent, galactose in strain EC-C. Neither IPTG, nor thiomethyl-β-D-galactopyranoside (TMG), both of which induced high levels of β-galactosidase in strain EC-C, induced a lactose transport system in this strain. Lactose, which is not an

inducer of β-galactosidase in strain EC-C, also failed to induce a lactose transport system in strain EC-C. As EC-C grown in the presence of both IPTG (to induce β-galactosidase) and lactose was still Lac⁻, it was concluded that the absence of a lactose permease, inducible by lactose, was responsible for the Lac⁻ phenotype of strain EC-C. It was further concluded that the Lac⁺ phenotype of strain EC-S was due to a mutation allowing constitutive expression of a lactose permease. Neither EC-C nor EC-S were able to transport IPTG or TMG into the cell.

7. The presence of two lactose transport systems was detected in E. chrysanthemi (FH1) by lactose transport competition experiments. One lactose transport system appeared to be associated with the raf operon, and was induced by raffinose in strain EC-C, and the other with the mel operon. Lactose transport by both permeases was equally inhibited by melibiose, whereas lactose uptake by the raffinose induced permease was more severely inhibited by raffinose than was lactose transport by the melibiose induced permease. As the constitutively expressed lactose transport system in strain EC-S was only slightly inhibited by raffinose, it was concluded that a mutation allowing constitutive expression of the mel operon was responsible for the Lac⁺ phenotype of strain EC-S.

In support of this conclusion, a constitutively expressed α-galactosidase was detected in strain EC-S, which was co-ordinately induced together with the lactose transport system by melibiose in strain

EC-C. This α-galactosidase was found to be resistant to toluene inactivation, was not activated by either dithiothreitol or Mn^{2+}, and did not require a cofactor for activity. These factors all distinguished this enzyme from the E. coli mel α-galactosidase.

8. The extracellular polysaccharide (EPS) produced by strain EC-S during growth on lactose was purified by ethanol precipitation. The purity of the EPS was established by a combination of molecular exclusion chromatography, ion exchange chromatography, HPLC, and analytical ultracentrifugation.

9. The structure of the EPS was investigated using a combination of techniques including acid hydrolysis, periodate oxidation and Smith degradation, methylation analysis, gas chromatography and gas chromatography-mass spectrometry, carboxyl-reduction, and NMR spectroscopy.

10. The polysaccharide was found to have a heptasaccharide repeat unit composed of 4 Rha, 1 Glc, 1 Man and 1 GlcA residues. Methylation analysis of the native and carboxyl reduced EPS revealed that the Rha residues were either terminal, or were 1,3-linked, that the Glc residue was 1,3-linked, that the GlcA residue was 1,4-linked, and that the Man residue was 1,3,4-linked.

11. The backbone of the EPS, obtained after 1 cycle of Smith degra-
dation, was found to have a trisaccharide repeat unit composed of
equimolar amounts of Rha, Glc, and Man. Methylation analysis
revealed that the Rha and Glc were both 1,3-linked, and that the Man
was 1,4-linked. These results indicted that the side-chain was
attached directly to the glucomannorhamnan backbone via the glucuro-
nic acid through position 3 of the mannose residue.

12. Isolation of the aldobiouronic acid revealed that the main neutral
monosaccharide present was Man. NMR analysis of the disaccharide
revealed that the glucosyluronic acid linkage was in the α-config-
uration.

13. NMR analysis of the electrodialyzed EPS which had been autohydro-
lyzed to reduce its viscosity (121°C, 20 min), suggested that the
Rha residues were all β-linked, and that the other residues were
possibly α-linked. This conclusion was partially confirmed by the
NMR analysis of the EPS backbone. However, due to the viscosity of
the backbone, the NMR signals were broad and hard to analyze. No
non-carbohydrate components were found to be present in the EPS by
NMR analysis.

14. The available data allowed the proposal of the following two possible structures for the EPS.

$$[\overset{3}{\longrightarrow} Glc\overset{l}{\underset{}{}}\overset{\alpha}{\longrightarrow}\overset{4}{} Man\overset{l}{\underset{}{}}\overset{\alpha}{\longrightarrow}\overset{3}{} Rha\overset{l}{\underset{}{}}\overset{\beta}{\longrightarrow}]_n$$

$$\overset{}{\underset{3}{\big|}}$$
$$\alpha$$
$$\overset{l}{\underset{}{}}$$

$$Rha\overset{l}{\underset{}{}}\overset{\beta}{\longrightarrow}\overset{3}{} Rha\overset{l}{\underset{}{}}\overset{\beta}{\longrightarrow}\overset{3}{} Rha\overset{l}{\underset{}{}}\overset{\beta}{\longrightarrow}\overset{4}{} GlcA$$

or

$$[\overset{3}{\longrightarrow} Rha\overset{l}{\underset{}{}}\overset{\beta}{\longrightarrow}\overset{4}{} Man\overset{l}{\underset{}{}}\overset{\alpha}{\longrightarrow}\overset{3}{} Glc\overset{l}{\underset{}{}}\overset{\alpha}{\longrightarrow}]$$

$$\overset{}{\underset{3}{\big|}}$$
$$\alpha$$
$$\overset{l}{\underset{}{}}$$

$$Rha\overset{l}{\underset{}{}}\overset{\beta}{\longrightarrow}\overset{3}{} Rha\overset{l}{\underset{}{}}\overset{\beta}{\longrightarrow}\overset{3}{} Rha\overset{l}{\underset{}{}}\overset{\beta}{\longrightarrow}\overset{4}{} GlcA$$
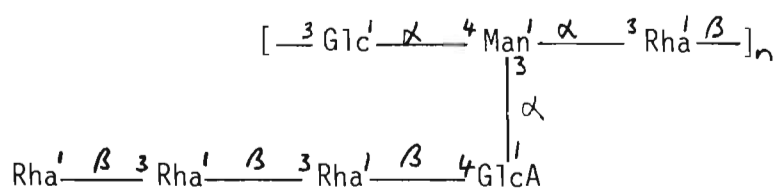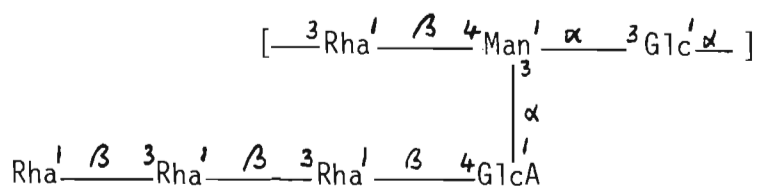
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