THE BIOSYNTHESIS OF BACTERIAL ALGINATE

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Submitted for the Degree of Ph.D

University of Edinburgh

1978

ACKNOWLEDGEMENTS

I would like to thank Dr. I. W. Sutherland for his advice and encouragement throughout this work, Dr. T. R. Jarman (Tate and Lyle, Limited) for his useful advice, Professor J. F. Wilkinson for allowing me to work in the Microbiology Department of the University of Edinburgh and the Science Research Council for a Research Studentship and Tate and Lyle for a C.A.S.E. award.

I am also indebted to my friends and colleagues at Tate and Lyle Research for many useful discussions and to Dr. J.R.W. Govan and Ms. J.A.M. Fyfe for supplying the PAO strains. I would also like to thank Dr. P. Jefferies for the electron micrograph.

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SUMMARY

The cultural characteristics of various mucoid strains of Ps.aeruginosa were examined; a wide variation in the time when the polymer was produced, the levels synthesised and the composition of the polymer occured. The only similarity between these strains is that they produced an acetylated polyuronide.

Mucoid strains were found to be highly unstable in ammonia limited chemostat cultures. Small colonies appeared at a high rate which led to a rapid displacement of the parental type indicating that some of these variants possessed a competitive advantage over the wild type. Ultimately selection for a non mucoid strain with a higher yield of biomass on the growth-limiting substrate occurred.

The pathway for the biosynthesis of alginate appears to differ very little from the reaction sequences proposed in both Az.vinelandii and Fucus gardneri. In the study of enzymes involved in the biosynthesis by PAO strains similarities occurred to the biosynthetic pathway of colanic acid found in E.coli K12. The wild type contains all the enzymes necessary to produce alginate but it is not expressed. Synthesis of alginate leads to an increase in the levels of enzymes involved in the formation of the precursor GDP-Mannuronic acid.

In non mucoid strains both sup^+ and sup^-, GDP-Mannose dehydrogenase was absent. Also in these strains either GDP-Mannose pyrophosphorylase or phosphomannose isomerase was absent. In some sup^- strains elevated levels of certain carbohydrate enzymes were found. The regulation/repression of the enzymes is complex but the pathway lacks the fine control mechanisms present in other biosynthetic pathways.

Synthesis of alginate led to an increase in resistance to carbenicillin and a decrease in resistance to tetracycline. In salts media both mucoid and non mucoid sup^+ and sup^- strains showed a slight

ABBREVIATIONS

$AcGlcNH_2$	N-acetylglucosamine
AMP	Aldenosine 5' monophosphate
ATP	Adenosine 5' triphosphate
DCPIP	Dichlorophenolindophenol
dTDP	deoxy-Thymidine 5' diphosphate
EtN	Ethanolamine
FPA	p-Fluorophenylalanine
EDTA	Ethylenediaminetetra acetic acid
Gal	Galactose
$GalNH_2$	Galactosamine
GDP	Guanosine 5' diphosphate
Glc	Glucose
$GlcNH_2$	Glucosamine
Hep	Heptose (L-glycero-D-mannoheptose)
KDO	Ketodeoxyoctonate
LPS	Lipopolysaccharide
POPOP	1,4-di-(5-phenyloxazolyl)-benzene
PPO	2,5-diphenyloxazole
Rib	Ribose
Rha	Rhamnose
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
TTP	Thymidine 5' triphosphate
UDP	Uridine 5' diphosphate
UTP	Uridine 5' triphosphate

MORPHOLOGY OF BACTERIAL CELL ENVELOPE

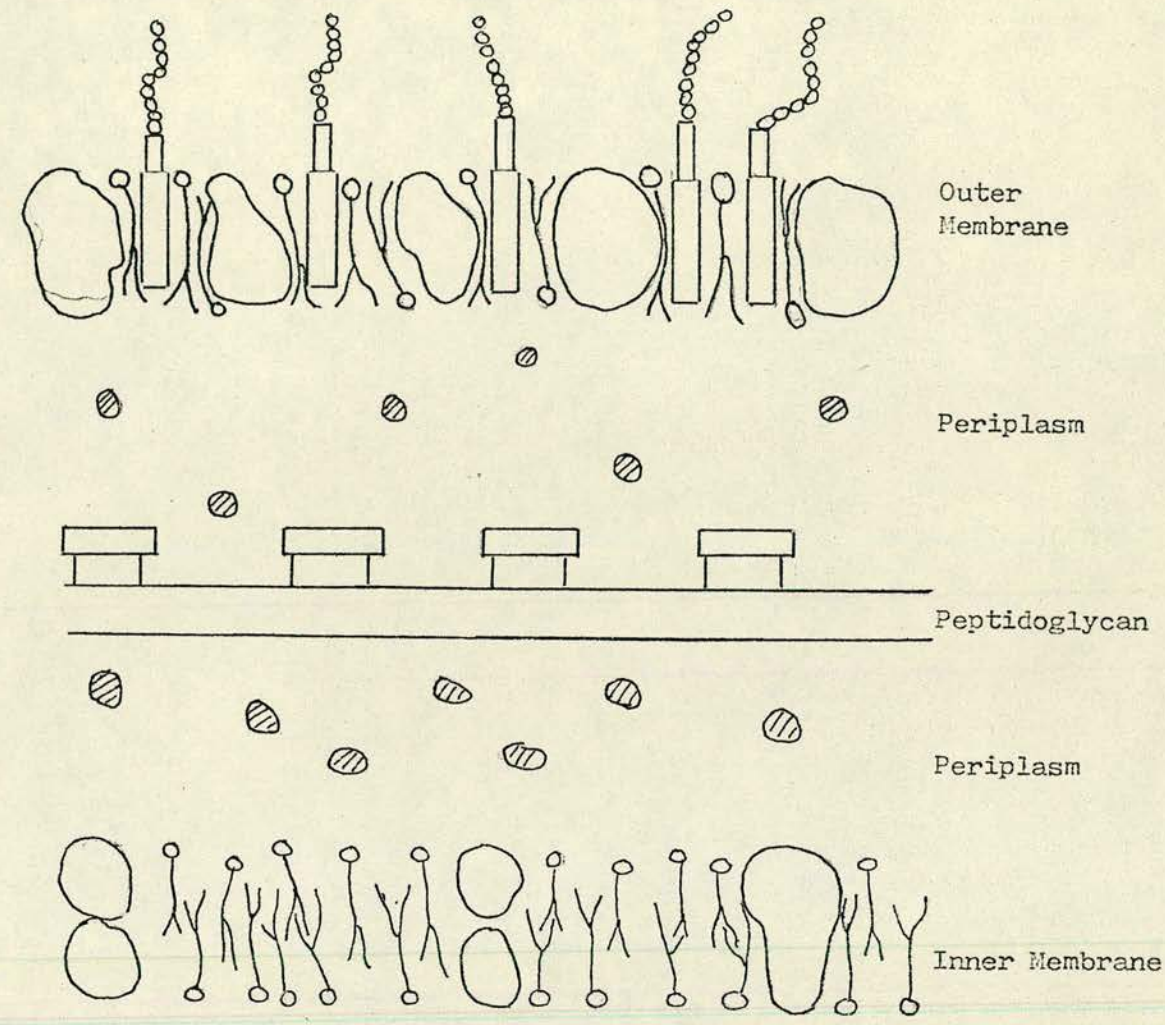
The prokaryotic cell is surrounded by two or three surface layers which differ in chemical composition, fine structure and function. These surface layers are the cell membrane and cell wall which are always present and, external to these, many but not all bacteria have a capsule or slime layer. The exact morphology of the wall depends upon the bacterium studied. Bacteria may be classified into two broad classes based on the Gram stain. This involves the ability of bacteria to retain crystal violet dye after washing with alcohol. The bacteria which retain the dye are classified as Gram+ve whilst those which are decolourised are designated Gram -ve.

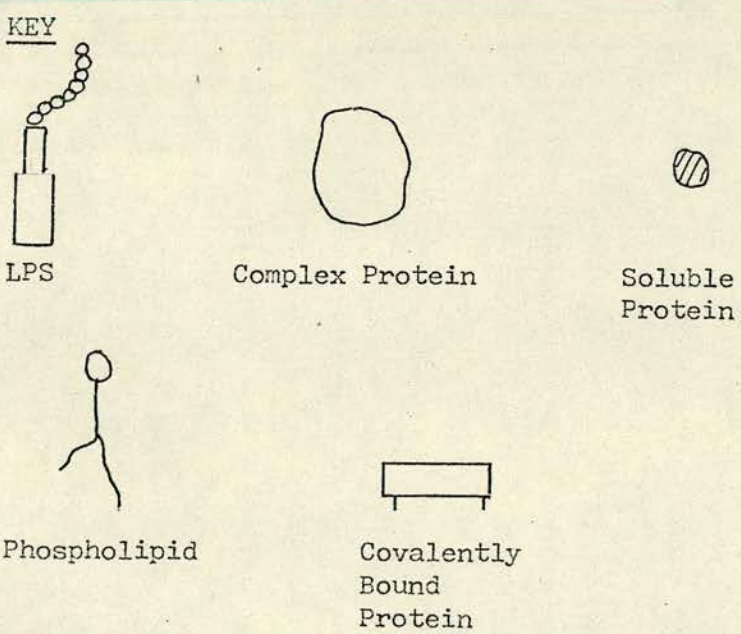
The fine structure of the cell wall and membrane may be visualised using the electron microscope when thin sections are stained with heavy metals.

The cell wall of Gram -ve bacteria is multi-layered, consisting of an inner layer 2-3 nm across, covered by one or more layers 10-30 nm thick. Whilst that of a Gram +ve bacterium is usually an electron dense layer some 20-80 nm thick. A stylised diagram of the cell wall of a gram -ve bacterium is shown in Fig. 1. For a review see Costerton, Ingram and Cheng, (1974).

Common to both classes of bacteria are the cytoplasmic membrane and peptidoglycan layer. The former consisting of 45-70% protein and 10-35% lipid. The major type of lipid present is in the form of phospholipid (usually phosphatidyl glycerol or diphosphatidyl glycerol) and a smaller component of glycolipid. The cytoplasmic membrane is multifunctional being the site of the electron transport system and site of attachment of ribosomes. It also acts as a selective barrier for nutrient uptake as well as being the site of many enzymic activities,

FIG. 1 STYLISED DIAGRAM OF A GRAM NEGATIVE BACTERIAL CELL WALL (redrawn after Meadow, 1975).





including those involved in synthesising the outermost cell structures.

Situated externally to the cytoplasmic membrane is the peptidoglycan, which is a co-polymer of alternating N-acetyl-D-glucosamine and N-acetyl-D-muramic acid residues linked by β 1, 4 linkages. The glycan chains are cross-linked by peptide chains. The main function of this polymer is to confer both shape and rigidity on the cell, as its loss causes osmotic instability and protoplast or spheroplast formation. In Gram +ve bacteria this is the major cell wall component which often accounts for more than 50% of the dry weight (Ghuysen et al 1968); for Gram -ve bacteria this figure is much lower, the value for E.coli being 2% (White, Dworkin and Tipper, 1968) and 1.2% for a marine pseudomanad (Forberg et al, 1972). For comprehensive reviews see Ghuysen (1968) and Ghuysen and Schockmann (1973).

Next to this layer lies the periplasmic space, which contains some proteins which are released by osmotic shock. Chatterjee (1976) showed that the non-cytoplasmic enzymes acid phosphatase, ribonuclease and cyclic phosphodiesterase were released from LPS deficient Salmonella mutants. By washing cells of Ps.aeruginosa with 100-200 mM Mg^{2+} Ingram et al (1973) caused the release of alkaline phosphatase which was shown to be periplasmically located by the Gormi reaction in thin section.

External to this, in Gram -ve bacteria is the outer membrane which was shown to consist of 26% lipid and 60% lipopolysaccharide (LPS) to form a lipid bilayer. Also some proteins (11%) which may be in the form of lipoproteins or glycoproteins are present. The LPS are polymers of polysaccharide and associated lipid, the structure of which was initially proposed by Lüderitz and Westphal (1965) for the Enterobacteriaceae. Such polymers consist of a serotype specific side chain (oligosaccharide) attached to a core polysaccharide which is in turn attached to a lipid molecule designated lipid A.

Gram positive bacteria do not possess an outer membrane, instead the major cell wall component is the peptidoglycan. Other cell wall components are found in association with the peptidoglycan either covalently bound or attached by weaker bondings (Ghuysen et al, 1968). Both teichoic acids and teichuronic acids come into this category, as in a few species do lipomannans.

Different structural types of teichoic acids exist which are divided into wall and membrane teichoic acids. Wall teichoic acids can account for up to 50% of the dry weight of the wall. Polymers of glycerol phosphate, ribitol, phosphate substituted with D-alanine and various glycosyl residues as well as sugar-1-phosphates are known to occur. One end of the polymer is attached to the peptidoglycan whilst the other end is exposed on the surface. Thus these molecules may be compared to the O-antigen of Gram -ve bacteria. Both are long flexible molecules extending from the surface, which are therefore accessible to phage (Archibald and Coapes, 1971), lectins (Lotan et al, 1975) and antibodies.

Membrane teichoic acids or lipoteichoic acids are polymers of glycerol phosphate and are attached to lipid residues. Both membrane and wall teichoic acids have a function in binding Mg^{2+} ions. Heptinstall et al (1970) found that Mg^{2+} binding to wall was dependent upon the presence of teichoic acids. The amount bound is related non quantitatively to the alanine content of the polymer. Hughes et al, (1973) showed that biosynthesis of B.licheniformis wall polymers is dependent upon the Mg^{2+} conc, these ions could be supplied by both types of polymers. These polymers were regarded as being uniquely found in Gram +ve bacteria but recently they have been isolated from a Gram -ve bacterium Butyrivibrio fibriosolvens N.37 (Hewett et al, 1976). For comprehensive reviews see Baddiley (1970) and Duckworth (1977).

External to these layers is the capsule or slime layer however not all bacteria possess one. The capsule is tightly bound to the cell surface whereas, slime is apparently unattached to the bacterial cell surface. There is no apparent difference in the chemical composition between the capsule and slime produced by the same bacterium. Visualisation of the capsule is made possible by use of the India ink method (Duguid, 1951). However a more exact method of visualisation of the relationship between the cell wall and exopolysaccharides uses ruthenium red stains and examination using the electron microscope. The exact function of these exopolysaccharides is unknown; however suggested functions include the binding of ions, prevention of dessication, protection against deleterious agents e.g. phage, chelating agents and lysozyme. A comprehensive review of polysaccharides has been recently published by Sutherland (1977).

The first step in the preparation of cell wall components usually involves breakage of the cell by mechanical methods e.g. pressure cell, glass beads or sonication followed by cycles of differential centrifugation to yield bacterial cell walls; other cell wall constituants may be extracted from whole cells by chemical means. Lipopolysaccharide (LPS) may be removed from either freeze dried whole cells or walls of Gram -ve sp. by aqueous phenol (Westphal and Jann, 1965). Teichoic acids can be extracted by treatment with hot formamide or treatment with cold TCA solution followed by precipitation with alcohol or acetone, (Archibald, 1972). Peptidoglycan may be extracted with hot phenol or with detergents followed by proteases, (Ghuysen, 1968). The inner and outer membrane may be isolated from spheroplasts by centrifugation on a sucrose gradient (Osborn et al, 1972). A major problem is the isolation of pure cell wall components which are not contaminated with

cytoplasmic components such as RNA, DNA or protein. These may be removed by treatment with RNA 'se DNA'se and proteases followed by dialysis to remove the low molecular weight products.

STRUCTURE AND FUNCTION OF CARBOHYDRATE CONTAINING CELL WALL POLYMERS

The discussion is restricted to the polymers which were studied, also only Gram -ve bacteria are discussed. (Detailed reviews of LPS structure and properties can be found elsewhere e.g. Wilkinson, 1977).

1. LIPOPOLYSACCHARIDE

The LPS is found in association with the outer cell membrane of Gram -ve bacteria. Gel filtration and sedimentation studies give a molecular weight of several million; this complex is readily dissociable by detergents and EDTA. A general property of most lipopolysaccharides is that of binding cations, this being of vital importance to Ps.aeruginosa (Wilkinson, 1975). Most of the studies have been performed on the Enterobacteriaceae notably E.coli and Salmonella sp.

The architecture of the molecule is that of a chain of oligosaccharide units (O-specific chain) linked to an oligosaccharide (core) which is attached to an acetylated glucosamine disaccharide (lipid A). Cleavage of the lipid A from the polysaccharide is achieved by mild acid hydrolysis, usually with 1% acetic acid for 1-2 h. The polysaccharide may then be freeze dried and applied to a Sephadex column which separates high (side chain) and low (core) mol. wt. fragments (Chester, Meadow and Pitt, 1973).

The lipd A moeity in the Enterobacteriaceae has been extensively studied, it consists of β 1, 6 or β 1, 4 linked disaccharide units of glucosamine to which are attached long chain fatty acids. The major fatty acid in most strains of gram negative bacteria which have been studied is β-OH myristic acid which is absent in many of the Ps. aeruginosa strains so far studied (Fenson and Meadow, 1970).

FIG. 2 Partial Structure of R Form Core Polysaccharide from *Salmonella* Mutants

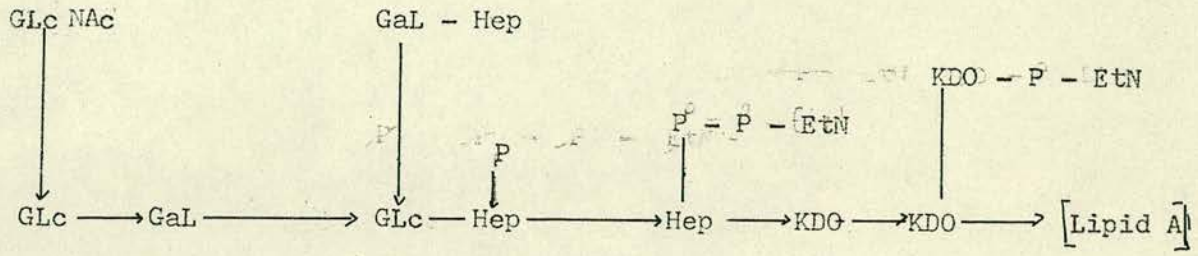
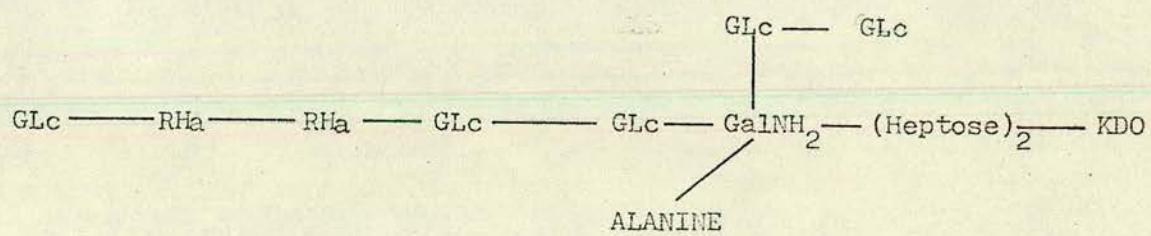


FIG. 3 Partial Composition of Core LPS obtained by work by Koval and Meadow and Drewry *et al* on *Ps. aeruginosa* PacI and *Ps. aeruginosa* 1999 respectively



Chester et al, (1973) found there were three fatty acids present 3-OH 10:0, 2-OH 12:0 (both minor components) and the major acid was 3-OH 12:0.

The most elaborate studies on chemical composition of the LPS have been performed using Salmonella mutants. Lüderitz (1977) using a variety of rough (R) mutants which produce an LPS lacking the O-antigen (Ra) amd mutants which lack parts of the core has proposed the structure of the core polysaccharides (Fig. 2).

Studies by Koval and Meadow (1977)on Ps.aeruginosa strain PACI and Drewy et al (1975) using Ps.aeruginosa 1999 have suggested that there is a common core polysaccharide (Fig. 3). This uniformity in core composition in both Enterobacteriaceae and Pseudomonas is in contrast to the variability which occurs in the O-specific side chains. The LPS of these Pseudomonad strains are rich in phosphorous; the exact location is unknown, but it is likely to be the inner region of the core in which the heptose residues are located. Much of this phosphorous is released as mono, di and tri phosphates or ethanolamine on mild acid hydrolysis.

In the studies by Meadow and her associates the O-specific side chain varied in both amount and composition. The only major neutral sugar commonly present is rhamnose (Fensom and Meadow, 1970; Wilkinson and Galbraith, 1975). This fraction is also rich in amino sugars e.g. glucosamine, galactosamine and fucosamine (Koval and Meadow, 1977). The composition of these high mol. wt. side chains may be characteristic of the serotype.

2. BACTERIAL POLYSACCHARIDES

Bacteria produce a variety of intracellular polysaccharides which may be structural or act as storage compounds. External to the cell wall

TABLE 1 The Variety of Monosaccharides and Substituents found in Bacterial Exopolysaccharides

MONOSACCHARIDE COMPONENT	BACTERIAL SP.	REFERENCE
Man GLc GLcA Ac Pyruvate	Xanthomonas campestris	Sloneker and Jeanes (1962)
Glc Gal Ac pyr	Rhizobium meliloti	Bjorndahl (1971)
Glc, succinic acid pyr	Alcaligenes faecalis var. mycogenes	Harada (1965)
GLc	Acetobacter	Hesterin and Schramm (1955)
GLc, GaL, Man, $GLcNH_2$	Glostridium perfringens	Baine and Cherniak (1971)
Mannuronic Guluronic acids	Az. vinelandii	Carlson and Mathews (1966)
	Ps. aeruginosa	Evans and Linker (1973).

TABLE 2 Cultural Conditions and Monosaccharide Components of Exopolysaccharides produced by Ps. aeruginosa strains. The nature of the monosaccharide(s) present were determined by chromatography.

Authors	Cultural Condition	Major Components	Minor Components	Containants
Brown et al (1969)	5-day static culture 37°C	Glucose	Rhamnose Glucosamine Hyaluronic Acid	Protein (5%) RNA DNA
Eagon (1962)	Static culture 37°C	50% Mannose	-	2-3% Protein 10-12% DNA 4-10% RNA
Warren and Gray (1957)	Glucose agar 72-96h, 37°C	Glucuronic Acid. N-acetyl Glucosamine	-	
Evans and Linker (1973)	High protein/glucose Agar medium	Mannuronic Acid Guluronic Acid	-	5% Nucleic Acid
Bartell et al (1973)	3-4 day static cultures Trypticase soy sugar	RNA acetate $GLcNH_2$ $GaLNH_2$	GLc Man	DNA RNA Protein (18%)

many bacteria secrete a capsule or slime layer which are termed exopolysaccharides. This latter field has been extensively reviewed by Sutherland(1972, 1977). Table 1 shows the diversity of monosaccharides and other constituents present in the polysaccharide produced by a variety of bacteria. Consequently this discussion is restricted to exopolysaccharides produced by Pseudomonas sp. and alginate (both microbial and algal).

Under suitable conditions Pseudomodaceae secrete expoly-saccharides of differing composition. Several investigations have found a variety of monosaccharide components from polymers produced by pathogenic strains of Ps.aeruginosa. These include polymers containing glucose and mannose (Brown et al, 1969) uronic acids (Evans and Linker, 1973; Carlson and Mathews, 1966), mannan (Eagon, 1962) hyaluronic acid (Warren and Gray, 1955), galactosamine and glucosamine (Bartell et al, 1973). The monosaccharide components are summarised in Table 2.

The expopolysaccharides were heavily contaminated with RNA, DNA and protein and probably, with wall material. No attempt was made to purify the polysaccharide and thus some doubt must exist over the presence/absence of the minor components.

Alginate producing strains of Ps.aeruginosa have been isolated from pulmonary infections of children with cystic fibrosis (CF). The infecting strain is non mucoid which characteristically changes to mucoid (Iacocca, et al, 1963). A non mucoid strain always precedes the mucoid strain. This is unusual as mucoid strains are not commonly isolated from patients (Doggett, 1969). A suggested causative factor in the emergence of these mucoid strains had been prolonged antibiotic treatment (Iacocca, et al, 1963). When isolated from the same specimen they both belong to the same pyocine type (Williams and Govan, 1973)

and serotype (Diaz et al, 1970). This suggests that the mucoid strain originates from the non-mucoid strain in vivo. Mucoid strains are persistent in vivo, but in vitro reversion to the non mucoid form usually occurs. The slime produced by Ps.aeruginosa in experimental infections was shown to possess the characteristics of a virulence factor by Dimitracopoulos et al (1974), although no attempt was made to purify or analyse the polysaccharide.

Martin (1973) observed mucoid colonies immediately around areas of phage lysis, these were considered not to have arisen spontaneously, but to depend upon the phage for their initiation and continued existence.

Govan (1975) determined the influence of various compounds on the stability of alginate production in shake flask culture. Both anionic, cationic and neutral surfactants were found to stabilise alginate production. Jones et al, (1977) demonstrated that all mucoid strains tested on batch subculture in nutrient broth were unstable, this effect was enhanced by the addition of deoxycholate. However, other surfactants polysorbate 80, SDS had no effect. In a salts medium both stable and unstable strains occurred and the presence of surfactants decreased stability. The same authors (Jones et al, 1977) using continuous culture of mucoid strains determined the effect of the growth limiting substrate upon stability. Both sulphate and magnesium limited cultures were fully stable whereas iron limited cultures had 10% revertants after 16 generations. With nitrogen, phosphorous and carbon limitation the percentage of non mucoid bacteria present after 16 generations was 55%, 75% and 98% respectively.

ALGINATE

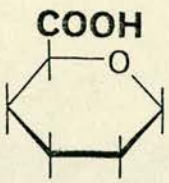
There are two sources of alginate, algal and bacterial. The

former is a major constituant of the cell wall of the brown sea weeds (Phaeophycae) being located in the middle lamella and outer cell wall. Bacterial alginate is an extracellular polymer synthesised by two species of bacteria, Azotobacter vinelandii and Ps. aeruginosa. The only difference between the two types of polymer is that the bacterial alginates contain O-acetyl groups which are absent from the algal alginate.

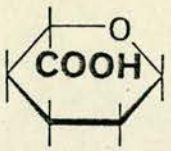
ALGAL ALGINATE

Alginic acid was originally isolated from brown sea weed by Stamford (1883), the material was heavily contaminated with nitrogen that the polymer was considered proteinaceous. Hoagland and Leib (1912) showed that alginate had the empirical formula of polysaccharide. Nelson and Cretcher (1929) by boiling alginate with 12% hydrochloric acid and determining the amount of carbon dioxide evolved (this can be correlated stoichemetrically with the amount of uronic acid present), determined that the polymer was 100% uronic acid. The same authors (1930) demonstrated that alginate was a polymer of mannuronic acid by preparing diamide and diphenylhydrazine derivatives and comparing them with derivatives made from mannuronic acid. For many years alginate was thought to be a homopolymer of mannuronic acid. Hirst et al (1964) studied L.digitata alginate which had been converted to the di-O-proprionyl derivative; this was reduced, desterified and partially hydrolysed with acid, separation of the products by chromatography indicated that L-gulose was a constituent, (Fig. 4). This was characterised as 1, 6 anhydrotri-O-benzonyl-L-gulose, other components isolated were 4-O-D-mannosyl gulose and mannobiose. The same authors demonstrated that separation of guluronic rich and mannuronic rich fractions was achieved by partial precipitation using manganous chloride, followed by calcium chloride and aqueous potassium chloride. Fischer

FIG. 4 The Structure of D-Mannuronic Acid and L-Guluronic Acid

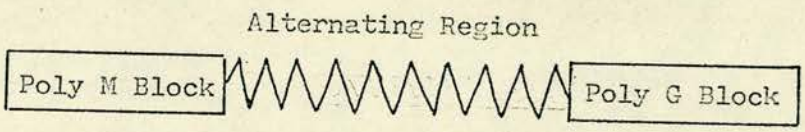


D-Mannuronic



L-Guluronic

FIG. 5 Postulated Structure of the Alginate Molecule



Poly (M-G)

and Dorfel (1955), using alginate obtained from 17 sea weed genera also demonstrated the presence of L-guluronic acid. Hirst et al (1964) were also able to show that the linkage between the sugars were 1, 4. Using 1M oxalic acid, (Haug and Larsen, 1965) only 30% of the polymer hydrolysed under defined conditions. The insoluble residue was found to be a mixture of mannuronic rich and guluronic acid rich fragments. These could be separated by partial precipitation at pH 2.85, the guluronic acid blocks remaining in solution. Rees and Samuel (1967) using nine samples of alginate which were methylated, reduced and partially hydrolysed with acid indicated that the polymers were linear and probably contained 1, 4 linkages. Atkins et al (1970) using x-ray crystallography on samples of poly M and poly G indicated that L-guluronic acid residues were linked in the CI fashion. Using n.m.r. spectroscopy Penman and Sanderson (1972) confirmed the work of Atkins et al. This gave rise to the postulated structure of alginate (Fig. 5).

The relationship of alginate to sea weed cell wall and its function have been considered previously (Preston, 1964; MacDowell and Percival, 1974) and are not considered here.

BACTERIAL ALGINATE

Analysis of the polysaccharide produced by Az. vinelandii by Gorin and Spencer (1966) using periodate oxidation followed by borohydride reduction and separation of the products formed by hydrolysis showed the polymer contained mannuronic acid and guluronic acid. The same authors also demonstrated that the guluronic acid units have the L-configuration and are glycosidally linked in the 1, 4 position. Also the polymer was found to be partly acetylated, containing one acetyl group per 5.2 sugar residues. Using n.m.r. spectroscopy, Penman and

and Sanderson (1972) demonstrated that Azotobacter alginate also contained poly M and poly G blocks although less than that found in the algal samples. The same authors also demonstrated that both mannuronic and guluronic acid residues were in the same conformation as found in the algal samples.

Using Az. vinelandii NCIB 9068 Bucke et al (1975) employing the conditions described by Gorin and Spencer(1966) obtained a 5% yield of polysaccharide. Acetyl groups were present in a low and variable degree and a number average value for the molecular wt of 5×10^5 was obtained using an osmometry technique. Using polyacrylamide gel electrophoresis (Bucke, 1974) was able to show the polydisperse nature of the polysaccharide in comparison with many samples tested. Using light scattering techniques Smidsrod and Haug (1968) demonstrated a weight-average molecular weight of 5×10^5 for algal samples.

Isolation of mucoid strains of Ps. aeruginosa from patients with cystic fibrosis (CF) and analysis of the polysaccharide indicated that the polymer was a polyuronide (Linker and Jones, 1964; Carlson and Mathews, 1966; Evans and Linker, 1973). Comparison with algal alginate by means of I.R. spectra, chromatography of the hydrolysed polysaccharide and action of alginate-degrading enzymes indicated that the polymer was alginic acid. Analysis of a variety of alginates from Ps. aeruginosa strains indicated that the ratio of mannuronic to guluronic acid differed widely as did the number of O-acetyl groups (Evans and Linker, 1973). The same authors determined the molecular weight by a viscometry technique found that it varied from 90,000 - 480,000.

APPLICATION OF ALGINATE

Alginate is a commercially important polysaccharide with gelling and colloidal properties and has widespread application.

These include coatings for printing paper and cloth, dental impression material, papermaking and walings for photographic emulsions. It is also an additive in various foodstuffs including ice cream, jellies and cakes. For additional and fuller discussion of applications of alginate see Percival and McDowell, 1967 and C.I.B.A. review, 1969.

BIOSYNTHESIS OF ALGINATE

1. ALGAL

There has only been one study on the nucleotides found in brown sea weed which was performed by Lin and Hassid (1966a). Working on Fucus gardneri silva they isolated 3 μ moles of GDP-Mannose and a component which was identified as GDP-Gulose from 14 kg of plant material. The major component (80%) was GDP-Mannose which was identified by its spectral properties, the quantitative analysis of its components, chromatographic and electrophoretic mobilities GDP-Gulose was identified by detection of gluconate after borohydride reduction and isolation of the gulurone and glucitol hexacetate derivatives.

In a subsequent study by the same authors (Lin and Hassid, 1966b) they determined the presence of the following enzymes which could account for the conversion of D-mannose to GDP-Mannuronic acid. Hexokinase, phosphomannose isomerase, GDP-Mannose pyrophosphorylase and GDP-Mannose dehydrogenase. Lin and Hassid suggested that the guluronic acid moeity of the alginate was derived from the C5 epimerisation of GDP-Mannuronic acid, although no enzyme capable of such an epimerisation was isolated. Lin and Hassid were also able to demonstrate the incorporation of radioactivity from C^{14} labelled GDP-Mannuronic acid into a polyuronide fraction. Thus the existence of a mannuronic acid transferase was indicated. It was suggested by Lin and Hassid that guluronic acid was incorporated from GDP-Gulose into alginate by a transferase reaction. The authors were unable to demonstrate such a reaction and thus the method of incorporation of gulose into alginate was left uncertain.

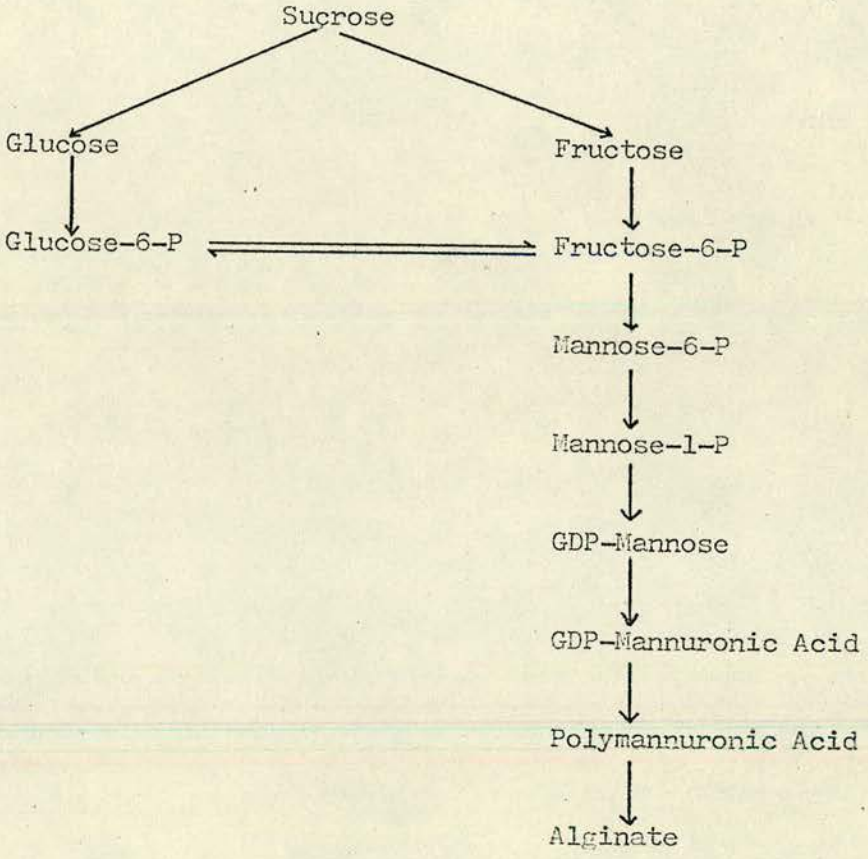
A study by Madgwick et al (1973) provided evidence which demonstrated that the conversion of mannuronic acid to guluronic acid occurred after the polymer was formed. The authors working with Pelvetia canaliculata isolated a soluble enzyme system by ammonium sulphate precipitation which caused the epimerisation of mannuronic acid to guluronic acid. The epimerisation was followed by following changes in carbazole reactivity (Knutson and Jeanes, 1968), and incorporation of tritium into the polyuronide fraction (Larsen and Haug, 1971). This indicated that epimerisation occurred after polymerisation.

Hellebust and Haug (1972) provided additional, although indirect evidence of an epimerisation reaction occurring at the polymer level. Using Laminaria digitata the time course of $NaH^{14}CO_3$ incorporation into alginate was followed. The authors found an increase in incorporation into guluronic acid moiety when little alginate sythesis was occurring.

2. BACTERIAL

All studies so far have been concerned with Az. vinelandii strains which synthesize alginate. Couperwhite and McCallum (1975) were able to demonstrate the presence of GDP-Mannose dehydrogenase in crude extracts. In a much more thorough investigation Pindar and Bucke (1974) studied the enzymes involved in conversion of sucrose to alginate. Crude extracts of bacteria were fed labelled intermediates and the products formed were studied. They were able to show the conversion of sucrose to GDP-Mannuronic acid (Fig. 6). The authors demonstrated the first polymeric compound was polymannuronic acid although they were unable to demonstrate the presence of an epimerase. An attempt to isolate GDP-Gulose was also unsuccessful.

FIG. 6 Pathway of Biosynthesis of Alginate in Az. vinelandii (Pindar and Bucke, 1975).



In an earlier investigation by Larsen and Haug (1970a, 1970b, 1971) an extracellular epimerase was isolated by ammonium sulphate precipitation of cell free supernatant of Az. vinelandii. They found that at low concentrations of calcium a polyuronide rich in mannuronic acid was formed, and by increasing the calcium concentration, the concentration of guluronic acid in the polymer increased. Using an assay based on the difference in colour intensity of mannuronic and guluronic acid in the carbazole reaction the effect of ions, pH and temperature on the enzyme was investigated.

No significant activity was found in the absence of calcium ions, no energy-rich compounds were involved in the reaction. It was postulated that the energy required for this reaction to occur comes from the binding of the calcium ions. The polyuronide was found to exhibit even stronger binding to strontium ions but they were less effective in promoting epimerisation. At high calcium concentrations (3.4mM) there was an increase in the optimum temperature of the reaction compared to low calcium (0.34mM). This would indicate that calcium ions also function in the stability of the enzyme. Addition of sodium or magnesium ions caused a decrease in epimerisation due to competition for binding sites (carboxyl groups). The authors were also able to show incorporation of tritium into the polyuronide from labelled water. Hydrolysis of the product and separation of the uronic acids indicated that 92% of the tritium was associated with mannuronic acid. It was suggested that the first step in epimerisation was the extraction of H-5.

In a similar experiment with P. canaliculata, Madgwick et al (1973) demonstrated this incorporation of tritium into guluronic acid but higher levels were found in association with the mannuronic acid. This led to the suggestion that the algal enzyme showed a higher

degree of reversibility than the Azotobacter enzyme.

Couperwhite and McCallum (1973) were also able to provide indirect evidence for the presence of an epimerase. Growth of Az. vinelandii in the presence of 50 mgl^{-1} of EDTA caused the production of a polyuronide rich in mannuronic acid. When the EDTA was omitted an increase in the amount of guluronic acid present was observed.

[illegible] were conducted using alginate isolated from an al[illegible] were thus absent. It has been suggested by Davidson et al (1977) that O-acetylated mannuronic acids are resistant to epimerisation. This mode of controlling the amount of conversion to guluronic acid, remains uncertain.

The acetyl donor and the point at which acetylation occurs, whether at the nucleotide sugar level or at the polymer level remains uncertain. In a study of acetylation of Mycobacterial LPS Tung and Ballou (1973) demonstrated that the acetyl donor was acetyl coA. For a review on acetylation see Sutherland 1978.

PHYSIOLOGY OF EXOPOLYSACCARIDE PRODUCTION IN PSEUDOMONADS

Studies on the effect of environmental conditions on polysaccharide synthesis in Pseudomonas have been relatively few. Much of the material used in early work especially on composition, the polysaccharide was usually contaminated with large amounts of nucleic acid or protein. Consequently only recent studies are considered.

Goto et al (1972) working with Ps. aeruginosa IFO 3445 investigated the nutritional requirements and environmental conditions. The measure of polysaccharide production used was the determination of relative viscosity after removal of the cells. Using a cellophane plate method a synthetic medium containing glucose, glutamate (as nitrogen source) sodium and potassium phosphates and magnesium sulphate was described. The optimal incubation period was three days at 37°C. The best carbon source was shown to be gluconate; ferrous sulphate, yeast RNA and yeast extract were shown to have a stimulating effect on polymer synthesis.

Evans and Linker (1973) testing alginate synthesising strains of Ps. aeruginosa used culture plates containing different media to estimate optimal conditions for maximal slime production. These were ascertained by visual estimation of the ratio of transparent slime (estimated by the amount of slime surrounding the bacterial cell by the India ink method of Duguid (1951)) to the amount of opaque cellular material. No attempt was made to determine levels of biomass, polysaccharide or pH and thus the results are open to question.

Williams and Wimpenny (1978a, b) in a study of exopolysaccharide synthesis of Pseudomonas NCIB 11264 found polysaccharide synthesis in batch culture started late in exponential phase and continued maximally until growth had ceased. The polymer was found to consist of glucose

and galactose in the ratio 7:1 with acetate and pyruvate also being present. The composition of the polymer was found to be independent of the carbon source. In common with many other exopolysaccharide synthesising strains nitrogen limitation favoured exopolysaccharide production (Wilkinson and Duguid, 1953). Optimal conditions for exopolysaccharide synthesis with respect to ammonium, phosphate, initial glucose or sucrose concentrations were described.

Using continuous culture of NCIB 11264 grown under nitrogen limitation Williams and Wimpenny demonstrated that the rate of polymer synthesisis was independent of the specific growth rate varying by 25% about the mean. The polysaccharide concentration varied proportionally to the residence time of the culture. Steady states were attained for 500h without any form of cultural degeneration. The optimal conditions for synthesis were found to be pH 7.0 and 30°C.

Work by Deavin et al (1976) indicated that alginate synthesis by Az. vinelandii in batch culture was growth associated. Phosphate limitation was also shown to enhance alginate synthesis. Using continuous culture, the rate of polysaccharide production was shown to be independent of growth rate over a dilution rate range of 0.05 - 0.25. Using a fixed dilution rate the effect of varying the limiting substrate was determined. A relatively constant rate of synthesis was observed over a wide range of limitations including carbon limitation. The authors were unable to distinguish if alginate synthesis was finely controlled or relatively uncontrolled.

CONTROL OF EXOPOLYSACCHARIDE BIOSYNTHESIS

There are numerous possible sites for control of exopolysaccharide synthesis, some or all of which may operate in different bacterial species.

These are:-

(1) Uptake mechanisms - this is discussed later as it specifically applies to Ps. aeruginosa. In those micro-organisms which have been adequately studied, complex systems for control of nutrient uptake exist. (For example, see review by Saier, 1977).

(2) Regulation of enzyme levels involved in the production of nucleotide precursors or in those enzymes which are specific to the formation of expopolysaccharide.

(3) Alteration of nucleotide pool levels through hydrolytic enzymes.

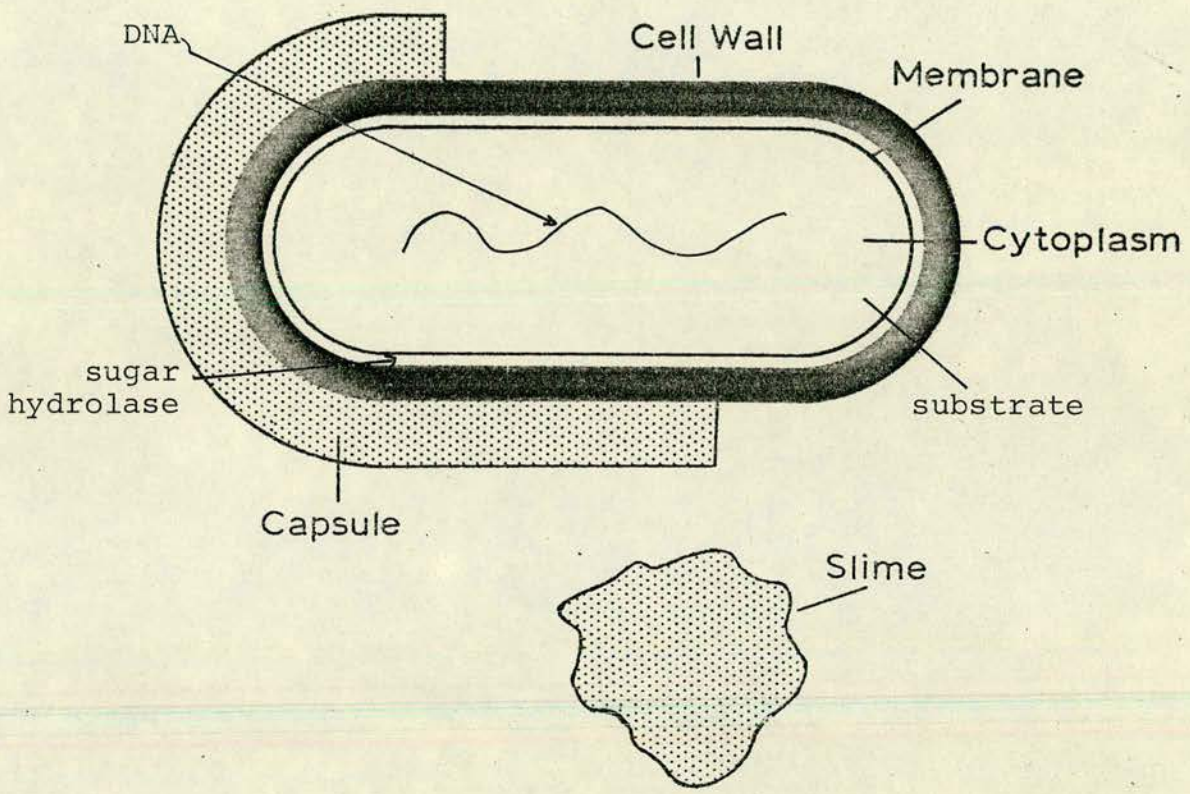
(4) The availability of isoprenoid lipid.

(5) Genetic control - which involves regulation genes, mutations in which lead to enzyme depression and increased polysaccharide synthesis.

Some aspects of these control mechanisms have been extensively reviewed elsewhere and will not be considered here. For reviews including the availability of isoprenoid lipid see Sutherland, 1976, 1977. The genetic control of colonic acid biosynthesis has been reviewed by Markovitz (1977).

Regulation of nuclectide sugar precursors of polysaccharides has been studied mainly in the Enterobacteriaceae notably E. coli and Salmonella sp. The occurrence and properties of nucleotide sugar hydrolases have been studied by Glaser and colleagues (Glaser et al, 1967). An enzyme was isolated from E. coli which hydrolysed UDP-Glucose to uracil,

FIG. 7 : The relationship of sites of control of Polysaccharide Biosynthesis



glucose-1-P and pyrophosphate. This enzyme was shown to be identical with the 5' nucleotidase. An absolute requirement for the addition of a divalent ion at pH 8 was demonstrated.

The hydrolase exhibited little sugar specificity, hydrolysing a variety of nucleotide sugars, but UDP sugars (UDP-Glc, UDP-Gal, UDP-$AcGLcNH_2$, UDP-$AcGalNH_2$) were the preferred substrates. On formation of spheroplasts the activity was released into the medium; however with some Salmonella species hydrolytic activity was firmly bound to the particulate cell fraction obtained after sonication. The same authors were able to isolate a protein inhibitor of the hydrolase from E. coli. This compound was destroyed by mild heating at $55^{o}C$ for ten minutes. An inhibitor was also found in S. weskco, but in S. typhimurium the hydrolase was found to have a high $K_m > 10^{-3}$ M so as not to compete for substrate with biosynthetic enzymes. Ward and Glaser (1969) were able to isolate mutants of E. coli was altered nucleotide hydrolases which had a reduced rate of nucleotide pool turnover. This is the suggested function of the hydrolase but it is difficult to see how they gain access to the nucleotide pool due to this compartmentalisation in the cell.

Beacham et al (1973) using E. coli K12 were able to isolate mutants deficient in UDP sugar hydrolase activity by selecting for resistance to 5' fluorouracil.

In another form of control of the nucleotide pool Melo and Glaser (1965) were able to demonstrate feedback control of TDP - Glucose pyrophosphorylase. This enzyme was able to use a variety of substrates including dTTP, dUTP, UDP-Glc, dUDP-Glc and dTDP-Glc, TDP - Glucose pyrophosphorylase was allosteric as it was inhibited by TDP-Rhamnose which bound to a site other than the active site. Bernstein and Robbins (1965) demonstrated that both TDP-Glucose pyrophosphorylase

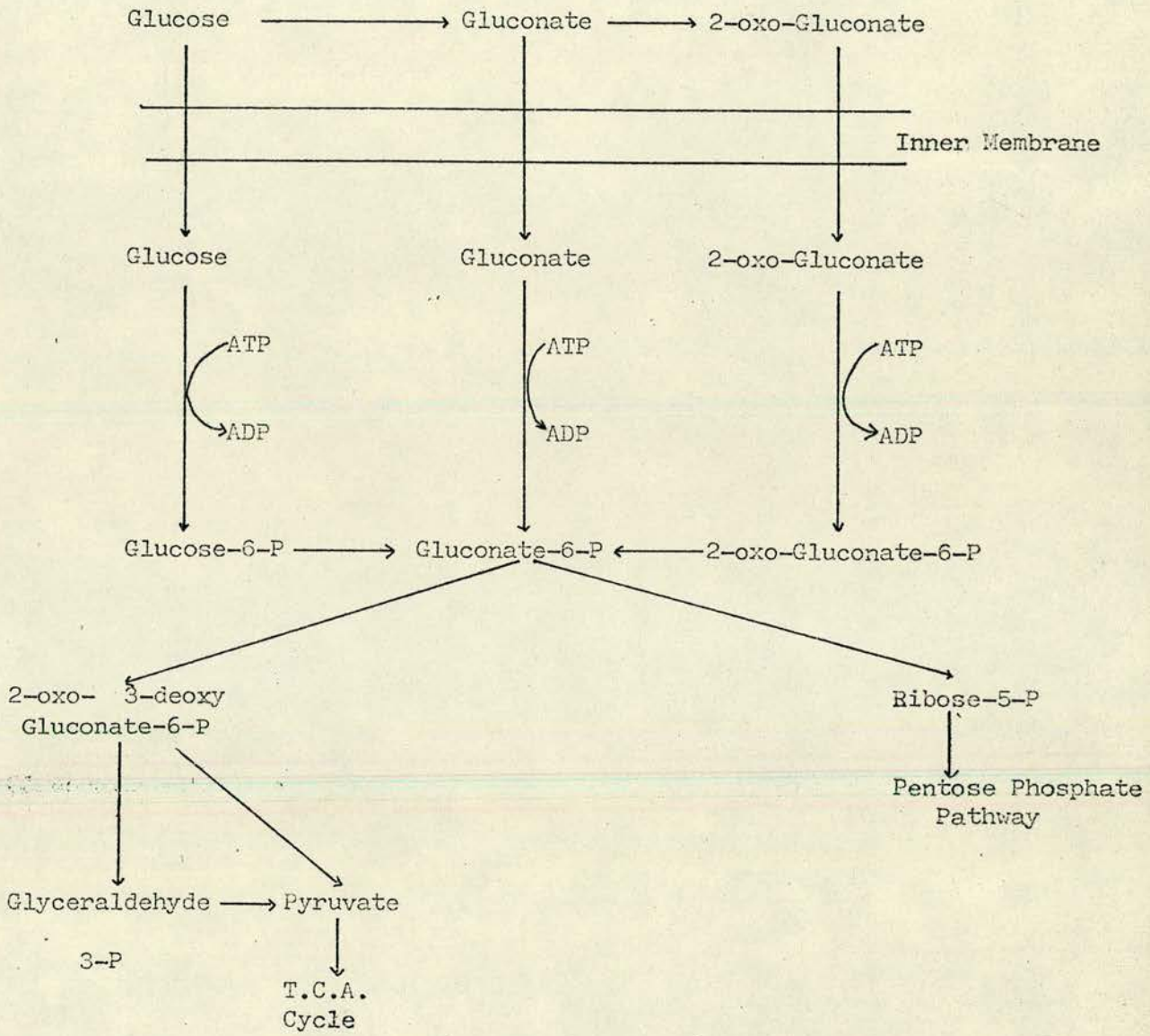
and UDP-Glucose pyrophosphorylase were constitutive in E. coli. The former enzyme was competitively inhibited by TDP-Glucose and TDP-Rhamnose.

Similar system for control of GDP-Mannose and GDP-Fucose synthesis is known to occur (Kornfield and Ginsburg, 1966). In bacteria which contain D-mannose in their polysaccharide, the rate of GDP-Mannose synthesis is regulated by feedback inhibition through GDP-Mannose pyrophosphorylase. In polymers which contain L-fucose the level of GDP-Fucose is controlled through both GDP-Mannose pyrophosphorylase and GDP-Mannose hydrolase. In bacteria which contain both mannose and fucose in their polysaccharides each nucleotide sugar controls its own synthesis.

A comprehensive review of both the genetics and biochemistry of the genus Pseudomonas has been published (Clark and Richmond, 1974). Consequently consideration is restricted here to carbohydrate metabolism. Glucose metabolism has been studied by Dawes and colleagues, (Hamlin, Ng and Dawes, 1967; Dawes, Midgeley and Whiting, 1975).

Glucose was shown to be metabolised by an extracellular (periplasmic) and an intracellular pathway which are linked by transport systems. Using C^{14} labelled glucose these 2 components were identified, one being a high affinity (K_M = 8mM) system. A low affinity system ($K_m \triangleq$ 2mM) was also identified as an active transport system. Glucose transport is via a system of relatively broad specificity taking up a range of hexoses (Midgeley and Dawes, 1973). Uptake can be competitively inhibited by mannose, galactose, xylose, fucose, 6-deoxyglucose, 2-deoxyglucose, methyl-β-glucoside and glucosamine. That is, by both metabolites and non-metabolites. Using a glucose dehydrogenase negative mutant no activity of the high K_M system could be demonstrated. Glucose dehydrogenase was known to be membrane bound and the high K_M system was associated with this. Glucose, at a concentration greater

Fig. 8 : Glucose Metabolism in Ps. aeruginosa



than 15mM caused the repression of glucose uptake, by use of a glucose dehydrogenase mutant gluconate was shown to be the main inhibiting agent (Whiting et al, 1976). As well as gluconate accumulating in the medium 2-oxo-gluconate was shown to be present. Glucose dehydrogenase and gluconate dehydrogenase were shown to be orientated in the membrane so that they oxidised their substrates extracellularly in the periplasmic space. The products formed, gluconate and 2-oxo-gluconate are taken up by transport systems prior to phosphorylation; following phosphorylation, these products were then converted to gluconate-6-P and then further metabolised via the Entner-Doudoroff pathway and to a small extent the pentose phosphate pathway (Fig. 8). As Ps. aeruginosa lacks phosphofructokinase there is no functional glycolytic pathway (Tiwari and Campbell, 1969).

When Ps. aeruginosa was grown under conditions of nitrogen limitation, the levels of enzymes comprising the glucose transport system were increased five fold. The levels of gluconate transport enzymes decreased seven fold and the enzyme for 2-oxo-gluconate transport decreased five fold (WHITING et al, 1976). The fall in the level of transport was correlated with a reduction in the concentration of gluconate and 2-oxo-gluconate. Nitrogen limitation also resulted in a decrease in the levels of gluconate dehydrogenase, glucose dehydrogenase, gluconate kinase and 2-oxo-gluconate-metabolising enzymes. Associated with this was an increase in the levels of hexokinase and glucose-6-P dehydrogenase.

When grown under nitrogen limitation the pathway chosen is extracellular, leading to the accumulation of gluconate and 2-oxo-gluconate in the medium. This acculates in the medium as the rate of production exceeds the rate of metabolism. However under glucose metabolism the extracellular pathway is repressed and glucose is taken directly into the cell.

MATERIALS AND METHODS

Bacterial Strains

Pseudomonas aeruginosa strain B. (obtained from I. W. Sutherland) and mutants derived from this wild type (see Results section).

PAO strains of Ps. aeruginosa were obtained from J.R.W. Gowan. The relationship of the strains to each other is shown in Figure 9.

All mucoid strains were from a freeze dried stock and for each experiment a new culture from the stock was used. Non mucoid colonies were maintained on nutrient agar slopes in screw capped vials or plates at 4°C. A list of all strains used is shown in Table 3.

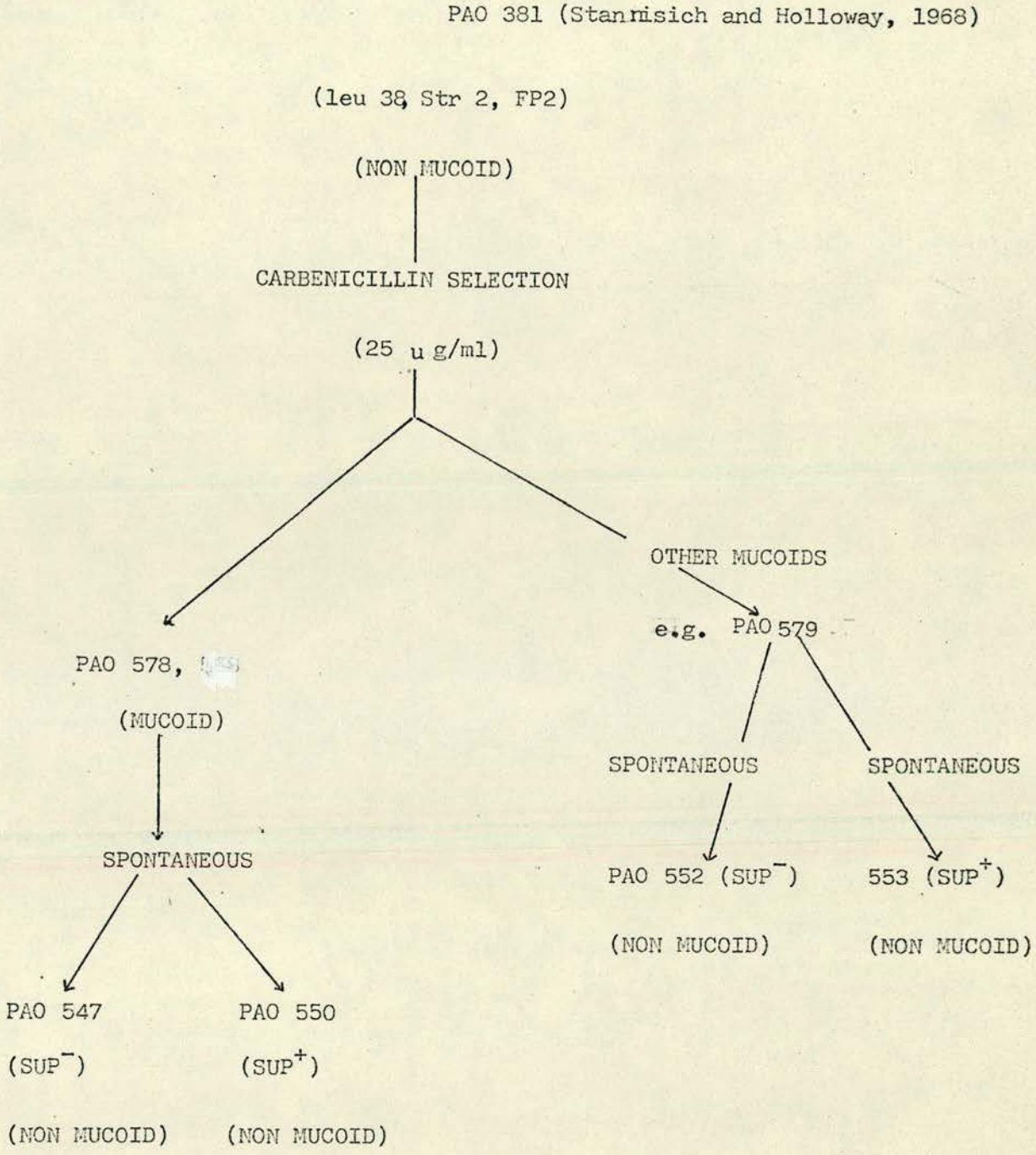
Batch growth of bacteria

Liquid cultures were routinely grown in 1 litre volumes in 2 litre Erlenmeyer flasks incubated at 30°C or 37°C on an orbital shaker. Smaller volumes of 100 ml were grown in 250 ml Erlenmeyer flasks. Sterilisation was carried out by autoclaving at 15 p.s.i, 121°C for 15 minutes. Heat labile compounds were filter sterilised using a Millipore filter (Millipore U.K. Ltd., Wembley, Middlesex) of pore size 0.45u or 0.22u and a Swinney hypodermic syringe (Millipore Ltd.) or a Seitz filter.

A variety of different media were used:-

(1) Yeast extract (gl^{-1}) yeast extract 1.0, casamino acids 1.0, K_2SO_4 1.0, NaCl 1.0, Na_2HPO_4 3.0, $MgSO_4$ 0.2, $CaCl_2$ 0.02, $FeSO_4$ 0.025 and 1 ml stock trace elements solution (mgl^{-1}, $MnSO_4$ 0.09, H_3BO_4 2.9, $CoCl_2$ 1.2, $CuSO_4$ 0.1, $ZnSO_4$ 1.2), and water to 1l., pH 7 (Sutherland and Wilkinson, 1965). For growth of the PAO strains the casamino acids were omitted and replaced by 1g of leucine. The carbon source was 2% glucose or gluconate.

FIG. 9 RELATIONSHIP OF PAO STRAINS OBTAINED FROM J.R.W. GOVAN



(2) Glutamate/gluconate (GG) (gl^{-1}) glutamate 5.0, gluconate 20.0, Na_2HPO_4 1.5, KH_2PO_4 1.5, NaCl 1.0 , $MgSO_4$ 0.2, $CaCl_2$ 0.02, $FeSO_4$ 0.005, 1 ml stock trace elements and water to 1l.pH 7.

(3) Nutrient broth (gl^{-1}) yeast extract 2.0, peptone 5.0, NaCl 5.0, lab lemco powder 1.0, water to 1l pH 7.4.

(4) Nutrient broth salts (gl^{-1}) nutrient broth (as previously described) plus K_2HPO_4 2.2, NaH_2PO_4 0.8, $MgSO_4$ 0.3, $CaNO_3$ 0.02, $FeSO_4$ 0.005, 1 ml stock trace elements and water to 1l pH 7.4.

(5) MacConkey (gl^{-1}) peptone 20.0, lactose 10.0, bile salts 5.0, NaCl 5.0, neutral red 0.075, water to 1l pH 7.4.

(6) Basal medium (gl^{-1}) K_2HPO_4 2.2, KH_2PO_4 0.8, $(NH_4)_2SO_4$ 0.6, NaCl 0.2, $MgSO_4$ 0.3, $CaCl_2$ 0.05, $FeSO_4$ 0.005, 1 ml stock trace elements, water to 1l pH 7.0. The carbon source was gluconate 2%.

(7) Minimal FPA medium (gl^{-1}) Basal medium (as described previously) was supplemented with D,L-p-fluorophenylalanine (FPA). The FPA was sterilised by membrane filtration and added to a final concentration of 5.45×10^{-5} M in liquid medium and 10^{-6} M in solid medium.

(8) Burkes medium (gl^{-1}) Na_2HPO_4 0.19, K_2HPO_4 0.01, $MgSO_4$ 0.20, $FeSO_4$ 0.006, NaCl 0.01, $CaCl_2$ 0.02, 1 ml stock trace elements, sucrose 2% water to 1l pH 7.4.

(9) Continuous culture medium (gl^{-1}) The basal medium (as described previously) was used for fermentation studies, this was supplemented with 50 u g ml^{-1} when the PAO strains were studied. All the salts except $FeSO_4$ were dissolved in 17.3l water and autoclaved for 45 minutes in a 20l aspirator. The $FeSO_4$ was filter sterilised in a Seitz filter. Glucose (400g) was dissolved in 1.5l water in a 3l flask and auto-

claved for 30 minutes at 121°C and all components added aseptically to the 20l aspirator.

TABLE 3 - NON MUCOID PAO STRAINS

STRAIN NO.	GENETIC TYPE	PARENT MUCOID
381	Wild type	-
555 553	Sup $^{+}$	579
552 554	Sup $^{-}$	579
557 558	Sup $^{+}$	568
556 575	Sup $^{-}$	568
547 549	Sup $^{+}$	578
551 550	Sup $^{-}$	578

Sup $^{+}$ - these strains are demonstrably supressed, giving rise to mucoid recombinants when used as donors in plate matings with FP 2 (Hayes, p213).

Sup $^{-}$ - no mucoid recombinants are observed from several hundred colonies when used as donors in plate matings with FP 2 (however these strains could still be supressed)

The following mucoid strains were also used:-

566 568 578 579 581 585 586 591

Azotobacter vinelandii NCIB 9068. Provided by T. R. Jarman.

CONTINUOUS FERMENTATION

Continuous culture vessel was held in a LHE 1/1000 fermentation unit (L. H. Engineering, Bells Hill, Stoke Poges, Bucks) Continuous fermentations were performed in a 4l bottom stirred tank fermenter of 2l working volume, with two side arms for electrode entry. (Figs. 10a, b and 11 show the design features. The vessel was glass and the top and bottom plates and internal structure were stainless steel.

Controls:

(1) Aeration-air was metered via a Planton Flostat pressure regulator and gap meter flow controller. It then passed through an air filter and was released into the fermenter directly below the impeller.

(2) Temperature - the temperature was controlled by the use of hot and cold fingers and a Nobel proportional controller.

(3) pH - This was monitored using a EIL 91B pH controllers with steam sterilisable glass electrodes and remote reference electrodes. Acid and alkali (1M HCl and 1M NaOH) were added via delta pumps with adjustable process timers giving variable de-rating of the pumps.

(4) Dissolved oxygen - This was monitored using a steam sterilisable, Ag/Pb galvanic oxygen electrode with a teflon membrane (L.H.E.) connected to an oxygen meter (L.H.E).

(5) Antifoam - This was added via a delta pump using two timers which caused antifoam (Polypropylene glycol 2025) to be pumped in at pre-determined intervals. Both oxygen and pH electrodes were inserted into the fermenter via the two side arms with compression bung fittings. Acid, alkali

and antifoam were sterilised by autoclaving at 121°C and 15 p.s.i. for 30 minutes and added through ports in the top plate.

(6) Oxygen and carbon dioxide measurements - the oxygen of the effluent gas was measured using a paramagnetic oxygen analyser and carbon dioxide by a Grubb-Parsons IR analyser. The effluent gas was dried using a cold water condensor and then passed through a sterile air filter.

(7) Sample port-samples were taken aseptically via a stainless steel hood with a universal thread fitting connected by a short length of silicone tubing to a 5mm. diameter stainless steel tube through the fermenter base plate.

(8) Inoculum port - a short piece of silicone tubing which was connected to an inoculating device - a piece of glass tubing which passed through a rubber bung which could be fitted to a 250 ml flask.

Continuous flow

The layout is shown in Fig. 11. Medium was pumped from the reservoir with L.K.B. multi perspex pumps through a glass growback tube into the fermenter. The culture level was maintained at 2l by the use of a stainless steel overflow wier. The fermenter vessel was calibrated to 2l in 100 ml intervals with all probes in position. The effluent was passed into a sterile 20l vessel. Silicone tubing with walls at least 1.5 mm thick were used and all joints were secured with Shuco tie straps. The flow rate of medium was checked using an in-line pipette.

Fermenters were autoclaved for 30 minutes at 121°C and 15 p.s.i. with all probes in position, immediately returned to the frame and a positive air pressure applied. The medium was autoclaved separately

KEY FOR FIG. 10A, B

A = Air inlet

B = Baffle

C = Cooling Loop

D = Draught Tube

F = Cold Finger

I = Impeller Shaft

H = heater

M = Medium inlet

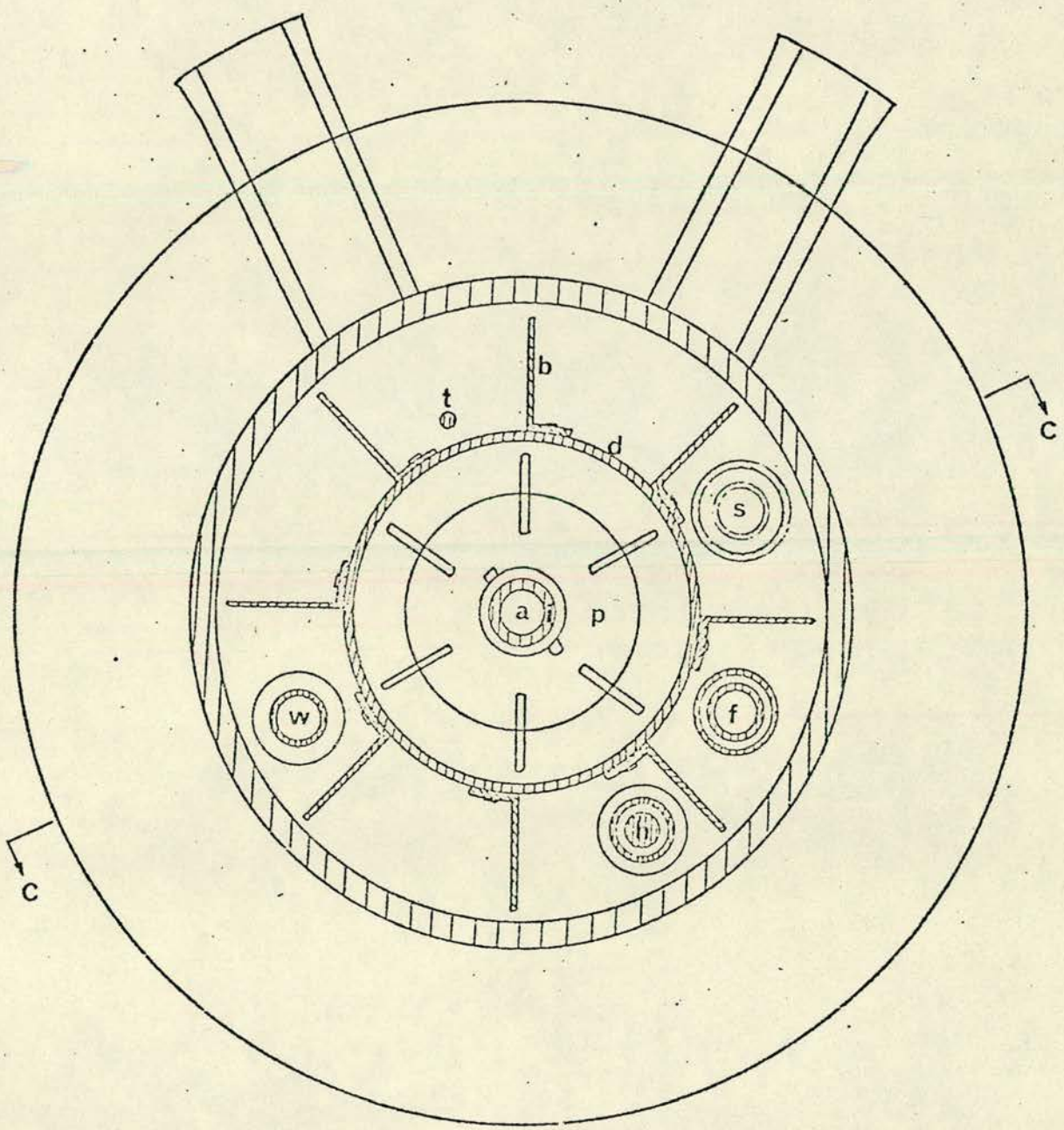
P = Impeller

S = Sample Port

T = Temperature Sensor

W = Weir

FIG. 10B Transverse section through the fermenter pot



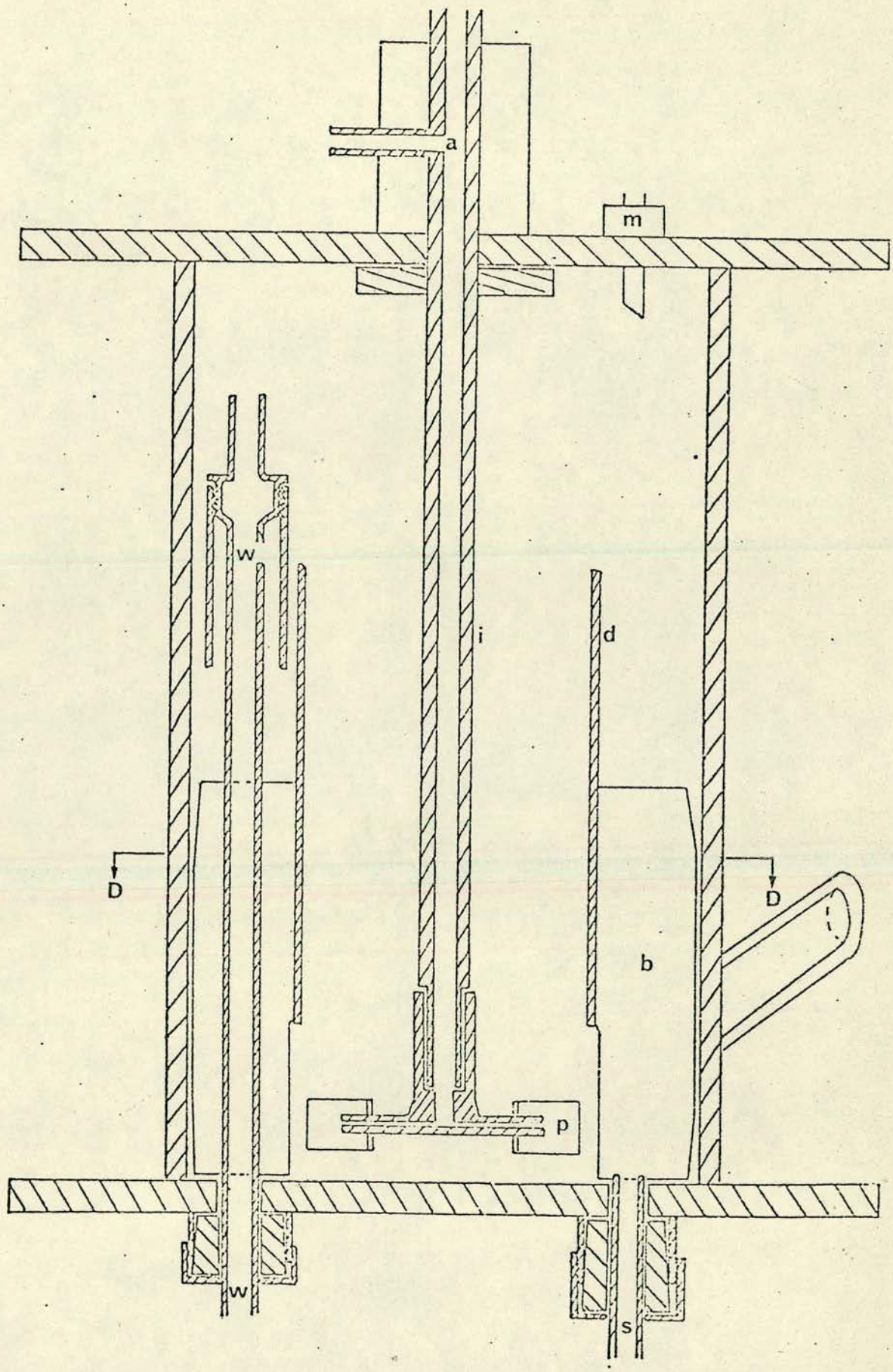
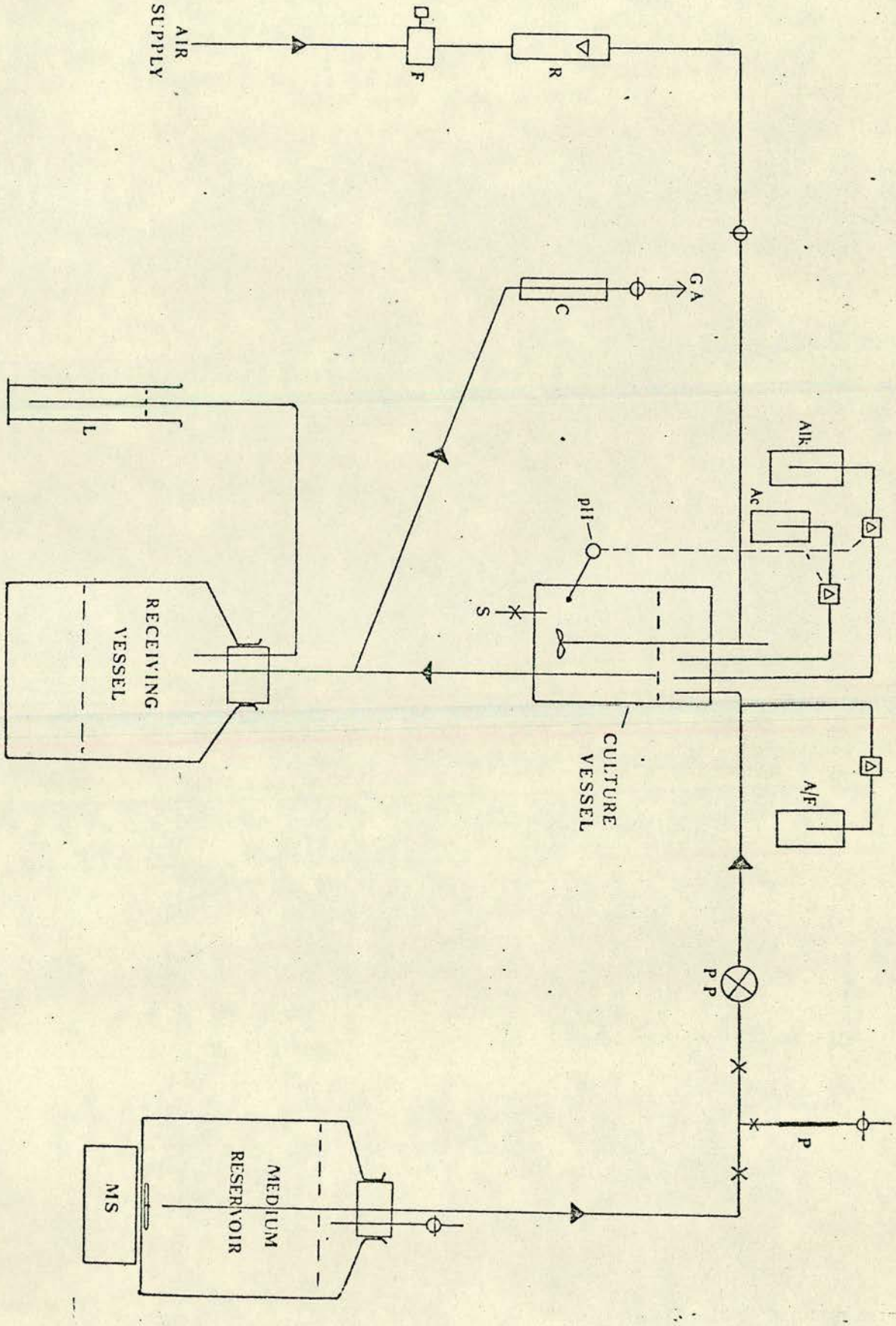


FIG. 10A Longitudinal section through the fermenter pot

FIG. 11 Plan of the fermenter layout



in an aspirator which was calibrated to 20l (in 500 ml increments) and connected aseptically to the fermenter to add the medium. The temperature was allowed to stabilise, samples taken to check the pH on an external meter and the fermenter set accordingly, the pH control point was set and the oxygen electrode was calibrated using oxygen free nitrogen and air.

A magnetic stirring bar was placed in the medium bottle which was stirred vigorously during the experiment. The medium exit line from the reservoir was connected to the fermenter feed line by a plastic connecting tube.

The fermenter was inoculated with a 3% ($^{v}/v$) of an overnight culture which had been stood at $4^{o}C$ for 24h (to allow the culture to be checked for the presence of small colonies and contaminants by plating out the culture) and allowed to grow in batch for at least 18h and fresh medium was added to give a dilution rate of $D = 0.05.h^{-1}$. The medium flow was checked by sucking medium from the resevoir into the in-line pipette, clipping off the medium resevoir and pumping medium from the pipette into the fermenter. The time for 10 ml to flow from the pipette was determined, the medium flow was also checked by noting the medium level in the reservoir at 24h intervals and calculating the volume used per hour.

The fermentation was monitored in samples taken directly from the fermenter using extinction at 520nm, dry weight, polysaccharide concentration, viscosity, viable counts, dissolved oxygen uptake and carbon dioxide output. A steady state was assumed to have been obtained after three residence times. When this was achieved a 1000 ml sample was taken for complete analysis.

To check the limiting nutrient the culture was allowed to return to its steady state (if this was possible as culture degeneration

occurred very rapidly) and the concentration of the growth limiting compound (ammonium sulphate) doubled. The cell density was monitored for 4-5h and if it increased significantly the suspected nitrogen limitation was confirmed.

Twice daily checks by plating the culture onto nutrient agar to determine colony size and the presence of non mucoid colonies were performed.

BIOCHEMICALS, CHEMICALS, ENZYMES

Both biochemicals and chemicals were obtained from either the Sigma Chemical Company (London) Ltd., B.D.H. Chemicals Ltd., Poole, England or Koch-Light Laboratories Ltd., Colnbrook, Bucks., England, and were of the highest grade available.

All the enzymes used were obtained from the Boehringer Co. (London) Ltd.

Radioisotopes were obtained from the Radiochemical Centre, Amersham, Bucks .

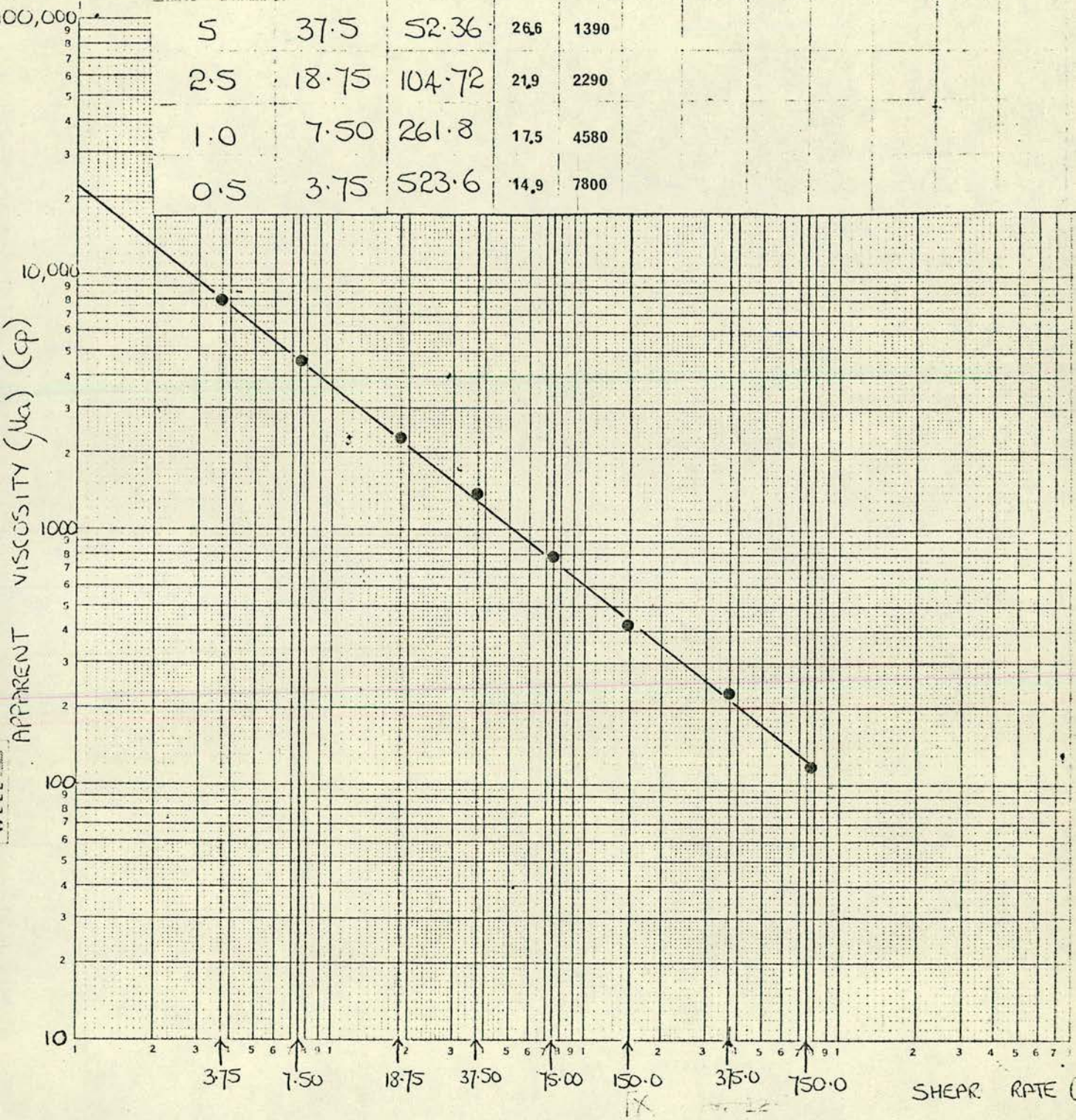
PREPARATION OF POLYSACCHARIDE

The mucoid bacteria were grown in liquid batch culture for 48h, and the polysaccharide was harvested by precipitation with 2 volumes isopropanol (IPA) or acetone. The culture was shaken vigorously and left to stand for at least 20 minutes. The polysaccharide was collected by filtering through muslin and redissolved in distilled water, to this was added 5M NaCl. The solution was then centrifuged at 18,000 r.p.m. for 60 minutes, (6 x 100 ml head) and the polysaccharide was reprecipitated. This procedure was repeated prior to analysis on the now purified polymer.

CELL DRY WEIGHT AND POLYSACCHARIDE DRY WEIGHT

To 30 ml of culture broth 1 ml 5M NaCl was added and this was mixed well diluted 4 x if viscous and centrifuged at 18,000 r.p.m. This was divided into sediment (cells) and supernatant. The cells were transferred to a predried and pre-weighed dish, dried at 105^{o}C overnight, cooled in a dessicator and the cell dry weight was calculated.

SPEED (rpm)	RATE (sec^{-1})	FACTOR	APP. READING	APP. VISCOSITY (cP)	APP. READING	APP. VISCOSITY (cP)	APP. READING	APP. VISCOSITY (cP)	APP. READING	APP. VISCOSITY (cP)	APP. READING
100	750	2·618	46.6	120							
50	375	5·236	33.2	230							
20	150	13·09	31.8	420							
10	75	26·18	30.3	790							
5	37·5	52·36	26.6	1390							
2·5	18·75	104·72	21.9	2290							
1·0	7·50	261·8	17.5	4580							
0·5	3·75	523·6	14.9	7800							



BROOKFIELD

HBT

(0·8° CONE)

FIG. 12

FERMENTER RUN NO.:

SAMPLE 1 : 1% (w/v) Ps B Alginat

SAMPLE 2 :

SAMPLE 3 :

SAMPLE 4 :

SAMPLE 5 :

To the supernatant 2 volumes acetone or IPA was added and the mixture vigorously shaken for 30 seconds. The polysaccharide was filtered onto a prepried and preweighed glass fibre filter (Whatman GF/A) and dried in a vacuum oven at 45°C or in an oven at 80°C for at least 24 hours. It was cooled in a dessicator, reweighed and polysaccharide dry weight calculated.

The following analyses were performed on the purified polysaccharide; the viscosity of a 1% (w/v) soln and a 0.50 (w/v) soln in 50mM EDTA, acetate content (McComb and McReady, 1949), percent mannuronic acid, (Fillipov and Kohn, 1974) polyacrylamide gel electrophesis (Bucke, 1974) and block structure (Penman and Sanderson, 1974).

VISCOSITY MEASUREMENTS ON PURIFIED ALGINATE SAMPLES

The polysaccharide was dried for at least 16h in an evacuated dessicator over P_2O_5. The dessicator was opened in a dry atmosphere (a glove box which had been flushed through with nitrogen) restoppered and reweighed. The polysaccharide was dissolved in 99 x volume of distilled water to give a 1% (w/v) solution. The apparent viscosity was determined by measuring the viscosity at various shear rates and plotted onto log/log paper (Fig. 12). A Brookfield LVT (for low viscosity samples) or a Brookfield HBT (for high viscosity samples) viscometer at 30°C.

A suitable volume of a 1% (w/v) solution was added to an equal volume of 100mM EDTA pH 7.3 to give a 0.50% (w/v) solution of alginate at 50mM EDTA [(a 1% soln of Azotobacter alginate has been shown to have a density of about 1 (T. R. Jarman, personal communication)].

The effect of salts on viscosity-to an equal volume of 1% (w/v) solution of alginate an equal volume of salt soln was added to give a 0.5% (w/v) alginate solution in the required salt concentration.

MANNURONIC TO GULURONIC ACID RATIO (M/G) BY INFRA-RED

To 2 ml of a deacetylated 0.2% solution of alginate 2 ml of 40% soln of KBr was added (giving 200:1, KBr: alginate), this was freeze dried and a KBr disc prepared. The sample (150 mg) was milled in a ball mill for 15 minutes and this transferred with a paper ring into a die, the surface levelled and the die assembled. This was placed in a press and evacuated for 5 minutes and 10 tons of pressure/sq.in. applied for 5 minutes. The vacuum was released and the disc removed and placed in a holder in a Hitachi-Perkin Elmer 457 spectrophotometer. The spectrum from 1800-800cm^{-1} was measured at medium speed. The chart was removed and a baseline drawn between adsorption minima at 850 and 1500 cm^{-1}. The peaks at 1125 and 1030 cm^{-1} were measured and the ratio 1125/1030 calculated and percent mannuronic acid read from a standard curve.

DEACETYLATION OF ALGINATE

To 40 ml of a 0.5% (w/v) solution of alginate, 20 ml of 0.3M NaoH was added and stirred at room temperature for 60 minutes. To this 2 volumes of IPA was added and the precipitate was freeze-dried.

GRAVIMETRIC DETERMINATION OF BLOCK STRUCTURE

Pre-dried (Gravimetric) deacetylated alginate (200 mg) was dissolved in the minimum volume of water and hydrochloric acid (12M) was added to give a final concentration of 0.3M. The solution was heated in a pre-dried and pre-weighed screw-topped test tube on a boiling water bath for 5h, and the solution (Poly M-G) was removed from the precipitate by centrifugation (5 min x 5000 r.p.m.) The precipitate was washed with 0.3M HCl collected by centrifugation and redissolved in water and NaCl added to a final concentration of 100mM and 1M NaoH was added to neutrality. The blocks were precipitated by the addition of 2 volumes

of ethanol, collected by centrifugation and dried over P_2O_5 and the weight of the blocks and test-tube determined. From this the weight of blocks and alternating region was calculated.

From the homopolym eric blocks, 50mg were deuteriated by evaporation 3 times with D_2O (1.5ml). This was dissolved in 0.5ml D_2O, filtered through a 0.45 u millipore filter and examined on a Varian HA-100 spectrophotometer using tertiary butyl alcohol as an internal standard. The spectra were recorded at 80°C with a sweep width of 1000Hz. From the area under the two peaks of poly M and Poly G (if present), the ratio between them was calculated.

POLYACRYLAMIDE GEL ELECTROPHESIS (P.A.G.E.P OF ALGINATE

This was performed by the method of Bucke (1974). The polyacrylamide gel for the study of alginate samples consisted of 6 (w/v) of total monomer with bis-acrylamide constituting 2% (w/v) of the total monomer. Gels were prepared in glass tubes 5 m.m. internal diameter. Monomers were dissolved in 250mM Tris-HCl pH 8.3 containing 0.05% TEMED and ammonium persulphate to a final concentration of 0.08%. Aliquots were transferred to the tubes using a syringe, polymerisation being complete 20 minutes after the addition of the ammonia persulphate. Alginate samples (20-60 ug) in 80 ul water plus 20 ul glycerol and 10 ul of 0.01% bromophenol blue). The running buffer (100mM Glycine-Tris pH 9) was layed on top of the sample gel. Electrophesis was carried out for 90 minutes in a Quickfit (Quickfit & Quartz Ltd., Stone, Staffs.,) P.A.G.E. equipment set at 4mA per gel.

Gels were removed by rimming with a syringe needle lubricated

with 7% acetic acid, and stained overnight in 0.08% Alcian blue in 7% (v/v) acetic acid. Excess stain was removed by standing in 7% acetic acid and the gels scanned with a Joyce Loebl chromoscanner at 620 nm.

PREPARATION OF LIPOPOLYSACCHARIDE

10-30 mg was dissolved in 2 ml 1% acetic acid and hydrolysed on a boiling water bath for 60-120 minutes, depending on the strain. The coagulated Lipid A was removed by centrifugation on a M.S.E. (Measuring and Scientific Equipment Ltd., London) bench centrifuge or by chloroform extraction. The soluble polysaccharide was then freeze-dried.

HYDROLYSIS OF LIPOPOLYSACCHARIDE

5mg of crude LPS was heated with 200-500 µl of 1M H_2SO_4 in a sealed tube at 100°C for 5h. After hydrolysis, neatralisation was carried out using excess Dowex (HCO_3^- form) resin. The hydrolysate was washed free of resin and concentrated to a small volume by rotary evaporation.

CHROMOTOGRAPHY

1. Paper - descending chromotography was carried out using Whatman number 1 paper irrigated with Butanol:Pyridine: Water (6:4:3) for 16-24 h.

2. Colum chromotography - partially hydrolysed LPS samples were chromotographed using gel filtration on a column of Sephadex (Pharmacia, Uppsala, Sweden) G-50 (fine grade). The eluting buffer was pyridinium acetate pH 5.4 (10 ml glacial acetic acid, 4ml pyridine and water to 1l). Details of column dimensions are given in the Results section.

WASHED CELL SUSPENSIONS

They were performed according to the method of Wilkinson and Stark (1956). At intervals cells were harvested from 1l of medium by centrifugation at 12,000 r.p.m. for 15 minutes. The pellet was added to 250 ml flasks containing 25ml phosphate buffer pH 7.2, 5 ml 5% KCl, 10 ml gluconate (100mg ml^{-1}), $MgSO_4$ to a final concentration of 0.20 mg ml^{-1} and 5 u Ci of C^{14} labelled gluconate. Incubation was carried out on a shaking water bath at 30°C. At intervals 5ml samples were removed, formalised, the cells removed by centrifugation and the supernatant dialysed against running water for 40h. Aliquots were then counted in an ambient temperature liquid scintillation counter. From this the rate of incorporation of radioactivity/mg dry weight was calculated.

Diagnostic tests

To differentiate between Pseudomonas species tests were carried out using the methods outlined in Medical Microbiology (Cruikshank, 1965).

β-Galactosidase estimation

This was determined according to the method of Pardee, Jacob and Monod (1959).

Negative Staining

A soln of 2% aq. uranyl acetate was added to the bacteria (Y.E. GROWTH) on a plastic coated carbon stablised 400 mesh copper specimen support grids. These were examined in a Philips EM 300 electon microscope at an operating kV of 60.

ESTIMATION OF ALGINASE ACTIVITY

Cells were harvested by centrifugation at 10,000 r.p.m. for 20 minutes at 4°C and resuspended in cold 10mM MOPS pH 7.5. Small volumes of cell suspensions were broken in a MSE 100 watt ultrasonic disintegrator set at maximum amptitude for 3 x 30 seconds, the suspension being cooled in ice/ethanol mixture. Afterwards cell debris was removed by centrifugation at 10,000 r.p.m. for 20 minutes and the supernatant dialysed against a large volume of cold 10mM MOPS pH 7.5 for 16 h. The production of T.B.A. positive material was assayed using a reaction mixture containing 0.10 ml 1% (w/v) alginate soln, 0.90 ml MOPS buffer pH 7.5 and 200 µl supernatant. After incubation at 30°C on a shaking water bath the enzymic reaction was stopped by boiling at 100°C for 5 minutes, T.B.A. + ve material was estimated using the periodate-thiobarbituric acid assay.

PREPARATION OF SPHAEROPLASTS

Sphaeroplasts were prepared according to Osborn, Gander, Parisi and Carson (1972) 100 ml of an overnight culture was innoculated into 1l of nutrient broth and incubated at 30°C with shaking for 2½h when OD_{600} measurements showed 2 x 10^8 bacteria ml^{-1}.

Bacteria were harvested by centrifugation at 15,000 r.p.m. for 10 minutes at 4°C, the pellet was drained of excess medium and rapidly resuspended in 50ml of 0.75M sucrose in 10mM TRIS HCl pH 7.8. Immediately 2.50ml lysozyme (20 mg ml^{-1}) were added and incubated on ice for 2 minutes. The suspension was slowly diluted with 100ml 1.5mM EDTA (Na^+) pH 7.5 over a period of 8-10 minutes, swirling gently. Sphaeroplasts formation being checked by phase contrast microscopy.

The sphaeroplasts were lysed by sonification discontinuously surrounding the sonicating vessel with ice/ethanol. The unlysed cells were removed by centrifugation at 15,000 r.p.m. for 10 minutes and the sphaeroplast membranes were pelleted at 100,000g for 60 minutes.

QUANTITATIVE ANALYTICAL PROCEDURES

1) Protein was determined by the method of Lowry, Rosebrough, Farr and Randell (1951) using BSA as a standard.

2) Total carbohydrate was determined by the method of Dubois, Gillies, Hamilton, Rebers and Smith (1956).

3) Total phosphorous was determined by the method of Bartlett (1959).

4) Heptose was determined by the method of Osborn (1963).

5) D-Galactose was determined by the "Galactostat"-Galactose oxidase reagent (Worthington) and glucose by the D-glucose oxidase reagent (Boehringer).

6) Gluconate was determined by the use of the gluconate dehydrogenase assay (see section on enzyme assays).

7) Amino sugars were determined by use of the Elson-Morgan method (1933).

8) Unsaturated uronic acids were estimated using the thiobarbituric acid method (Weissback and Hurwitz, 1958).

SENSITIVITY TO ANTIBIOTICS

1ml of an overnight culture of bacteria grown in nutrient broth was diluted 100 fold and 0.05ml added to 2ml of sterile nutrient broth containing varying amounts of antibiotic in one quarter oz. vials. These were shaken for 24h at 30°C on an orbital shaker and the end point taken as the amount of antibiotic (μg) which totally inhibited growth.

SENSITIVITY TO SURFACE ACTIVE AGENTS

An overnight culture of bacteria grown in nutrient broth was centrifuged 15,000 r.p.m. x 15 minutes, resuspended in 50mM TRIS pH 7.3 and repelleted. The pellet was resuspended in 50mM TRIS pH 7.3 to an O.D. 600 ≏ 1.50, to 2 ml of this suspension 1 ml of surface active agent was added. Readings at 600 nm were taken over 90-120 minutes at 10 minute intervals.

SPECTROPHOTOMETRY

All measurements were performed using a Unicam (Unicam Instruments Ltd., Cambridge) SP 500, SP 600 or SP 6-500, spectrophotometer, of a Zeiss (PMQ II) (Carl Zeiss, Oberkochen, W. Germany) spectrophotometer.

RADIOACTIVE COUNTING

Scintilation counting was performed in a Packard Tricarb liquid scintillation spectrophotometer (Packard Instruments Ltd., Caversham, Berks), model 3330. Aqueous samples were counted in a scintillant containing naphthalene 60g, PPO 49q, POPOP 0.29g, methanol - 100 ml, ethylene glycol 20ml and 1,4 dioxane to a final vol. of 1 litre. Samples on paper were counted using a scintillant containing 0.5% PPO in triton.

PREPARATION OF GULURONIC SPECIFIC ENZYME 92191)

This was isolated from a marine pseudomonad (obtained from I. W. Sutherland) for use in an epimerase assay. The bacteria were grown in half strength nutrient broth containing 0.40M NaCl, 0.10M KCl and 0.50M Na alginate. After 48h growth the bacteria were centrifuged (12,000 r.p.m. x 20 minutes) suspended in water and ruptured in a French press. The lysate was centrifuged at 12,000 r.p.m. x 20 minutes to pellet the unbroken cells and the supernatant made 20%

saturated with $(NH_4)_2SO_4$. This was left standing for 2-3h at 4°C centrifuged at 14,000 r.p.m. for 20 minutes and the pellet discarded. The supernatant was made 20% saturated with $(NH_4)_2SO_4$ and centrifuged at 14,000 r.p.m. for 20 minutes and the pellet discarded. The supernatant was made 50% saturated with $(NH_4)_2SO_4$ and centrifuged at 18,000 r.p.m. for 30 minutes and the pellet resuspended in 10mM MOPS buffer pH7 This was dialysed overnight against a large volume of the same buffer (stored frozen at - 20°C) and used as a source of enzyme.

EPIMERASE ACTIVITY IN CELL FREE SUSPENSION

An 18h culture of PsB (grown in GG medium) or PAO 579 (grown in Y.E. medium) was centrifuged at 14,000 r.p.m. for 20 minutes. To 1.8ml of the supernatant 0.2ml of Ca^{2+} ions was added to give a final concentration of 0.10 - 0.72mM, this was incubated for 24h at 30°C in the presence of 1µl toluene to prevent bacterial growth. The reaction was stopped by heating at 100°C for 60 seconds. A control where no calcium was added was also used. The presence of guluronic acid was estimated after the addition of 0.9ml 10mM MOPS pH 7.5 and0.1ml of the 2191 preparation. At intervals samples (0.10ml) were taken, heated to 100°C to stop the reaction and assayed for the presence of unsaturated uronic acid by the T.B.A. method.

ISOLATION OF EPIMERASE ACTIVITY

A 20-50% $(NH_4)_2SO_4$ precipitate was collected in an identical fashion as described above and added to Poly M blocks as prepared by the method of Penman and Sanderson (1974). The reaction mixture contained:- Poly M blocks (1mg ml^{-1}) 1ml Ca^{2+} ions (to a final concentration of 0.72mM) 0.20ml, epimerase preparation 0.20ml and water to 4ml. Guluronic acid was estimated as described above.

Enzyme asays

i) Preparation of cell free extracts - cells were harvested at 12,000 g for 15 minutes and washed with glass distilled water, all subsequent procedures being carried out at 4°C. After this washing 15 ml of 10mM MOPS buffer pH 7.0 was added, the cells resuspended and sonicated in a MSE 100 watt ultrasonic disintegrator set at 70% amplitude for 3 x 30 seconds the cells being cooled in ice/ethanol. After centrifugation at 7,000 r.p.m. for 15 minutes to pellet the unbroken cells, which was repeated, the supernatant was centrifuged at 100,000 g for 45 minutes to pellet the membranes. The supernatant was used to determine the activity of GDP-Mannose pyrophosphorylase and GDP-Mannose dehydrogenase. A Zeiss spectrophotometer was used to determine enzymic activity. Silica cells with a 1 cm path length were used throughout. Activities were measured by observing the changes in absorbance and results calculated from the linear region of the graph; endogenous activity being calculated using an assay system without substrate. Specific activities were calculated as mM product formed min/mg protein using a molar extinction coefficient of 6.22×10^3 for both NAD, NADP and NADH (Horecker & Kornberg 1948). All activities were measured at concentrations of cell free extracts which gave a variation in extinction of 0.01-0.04/min. (Hostalek et al, 1969). Reaction mixtures for the following enzymes consisted of:-

1) PHOSPHOMANNOSE ISOMERASE - assay by modification of the method of Slein (1955) - 10 µl 1.0 M TRIS HCl pH 7.5, 100 µl BICINE-NaOH pH 8.5, 100 µl H_2O 10 µl 1.0M $MgCl_2$, 40 µl of 50mM Mannose

6-P, 20 µl NADP 0.02M, 1 µl glucose - 6-P deH (0.14 units).

ii) GDP-MANNOSE PYROPHOSPHORYLASE - assay by modification of Munch-Petersen (1962) - 100 µl 1.0M TRIS HCl pH 7.5, 100 µl H_2O, 50 µl 0.5M NaF, 10 µl 0.005M ADP, 20 µl 0.05M NaPPi, 20 µl 0.01 M GDP-Mannose, 2 µl hexokinase.

iii) GDP-MANNOSE DEHYDROGENASE - method according to Preiss (1964) - 0.05 ml TRIS HCl pH 8.3, 0.1 ml of 0.1 M NAD, 0.03 ml 0.01M GDP-Mannose 0.01 ml BSA (10 mg/ml) and dehydrogenase to a final volume of 1.00 ml.

iv) HEXOKINASE - 120mM TRIS pH 8.2 (0.33ml); 500mM glucose (0.13ml); 250mM $MgCl_2$ (0.04ml); 36mM ATP (0.27ml); 10mM NADP (0.10ml); G-6-P Deh 15 units/ml (0.07ml) and cell extract (0.03ml).

v) GLUCONOKINASE - 120mM Tris pH 7.6 (0.33ml); 50mM Na gluconate (0.17ml); 250mM $MgCl_2$ (0.04ml); 36mM ATP (0.27ml); 10mM NADP (0.10ml); 6-Phosphogluconate Deh (0.03ml), 2mg/ml; water (0.02ml) and cell extract (0.03ml).

vi) GLUCOSE-6-P DEHYDROGENASE - 120mM Tris pH 7.6 (0.33ml); 20mM Glucose-6-P (0.25 ml); 250mM $MgCl_2$ (0.06ml); 10mM NADP (0.10ml); water (0.33ml) and cell extract (0.03ml).

vii) 6-PHOSPHOGLUCONATE DEHYDROGENASE - 120mM Tris pH 7.6 (0.33ml); 10mM 6-Phosphogluconate (0.15ml); 10mM NADP (0.07ml); 250mM $MGCl_2$ (0.06ml); water (0.36ml) and cell extract (0.03ml).

viii) ISOCITRATE DEHYDROGENASE - 120mM Bicine (pH 8.8) (0.33ml); 10mM Isocitrate (0.20ml); 10mM NADP (0.07ml) 250mM $MgCl_2$ (0.06ml); water (0.34ml) and cell extract (0.03ml).

ix) ACONITASE - 60mM Na_3 citrate - 50mM KH_2PO_4 (0.97ml) and cell extract (0.03ml).

x) 2-OXO-GLUCONATE KINASE AND 2-OXO-GLUCONATE 6-P REDUCTASE - 120mM Tris pH 7.6 (0.33ml); 250mM $MgCl_2$ (0.08ml); 2mM NADPH

(0.10ml); 36mM ATP (0.10ml); 50mM 2-oxo-gluconate (0.03ml); water (0.33ml) and cell extract (0.03ml).

xi) GLUCOSE DEHYDROGENASE - 100mM Sorensons phosphate pH 6.6 (0.33ml); 75mM Glucose (0.11ml); 15mM KCN (0.03ml); DCPIP (0.50mg/ml) (0.04ml); water (0.46ml) and cell extract (0.03ml).

xii) GLUCONATE DEHYDROGENASE - 100mM Na Acetate pH 5.5 (0.20ml); 75mM Na Gluconate (0.08ml) 15mM KCN (0.03ml); DCPIP (0.50mg/ml) (0.02ml); water (0.62ml) and cell extract (0.03ml).

For the determination of enzyme levels utilising NAD(P) increase in absorbance at 340nm was followed for 2-4 minutes for NADPH the decrease in absorbance at 340nm was measured over the same time period. In assays utilising DCPIP the decrease in absorbance at 600nm and 576nm was determined over 3 minutes for glucose dehydrogenase and gluconate dehydrogenase respectively.

RESULTS

CHAPTER 1

BATCH FERMENTATION OF MUCOID STRAINS OF PS. AERUGINOSA

CULTURAL CHARACTERISTICS

Exopolysaccharide production by bacteria leads to the formation of domed glistening colonies on solid media. All mucoid strains of Ps. aeruginosa exhibited this characteristic morphology even on media which did not favour exopolysaccharide production, e.g. nutrient agar. The appearance of such colonies is shown in plate 1. A characteristic of these mucoid colonies was the range of colony diameters. After 48h incubation on nutrient agar plates (24h at 30°C and 24h at room temperature) sizes varied between 1 and 3 mm diameter. This was especially noticeable in strain PAO 579, the significance of which is discussed later.

Growth of mucoid bacteria in liquid culture usually gave rise to a culture of high viscosity which could vary in the range 10-1000 cp. This was dependent upon the strain and cultural conditions used. Microscopic examination of these mucoid bacteria by the method of Duguid (1951) indicated the presence of a small capsule. However most of the polysaccharide was present as an extracellular slime apparently unattached to the bacterial cell surface.

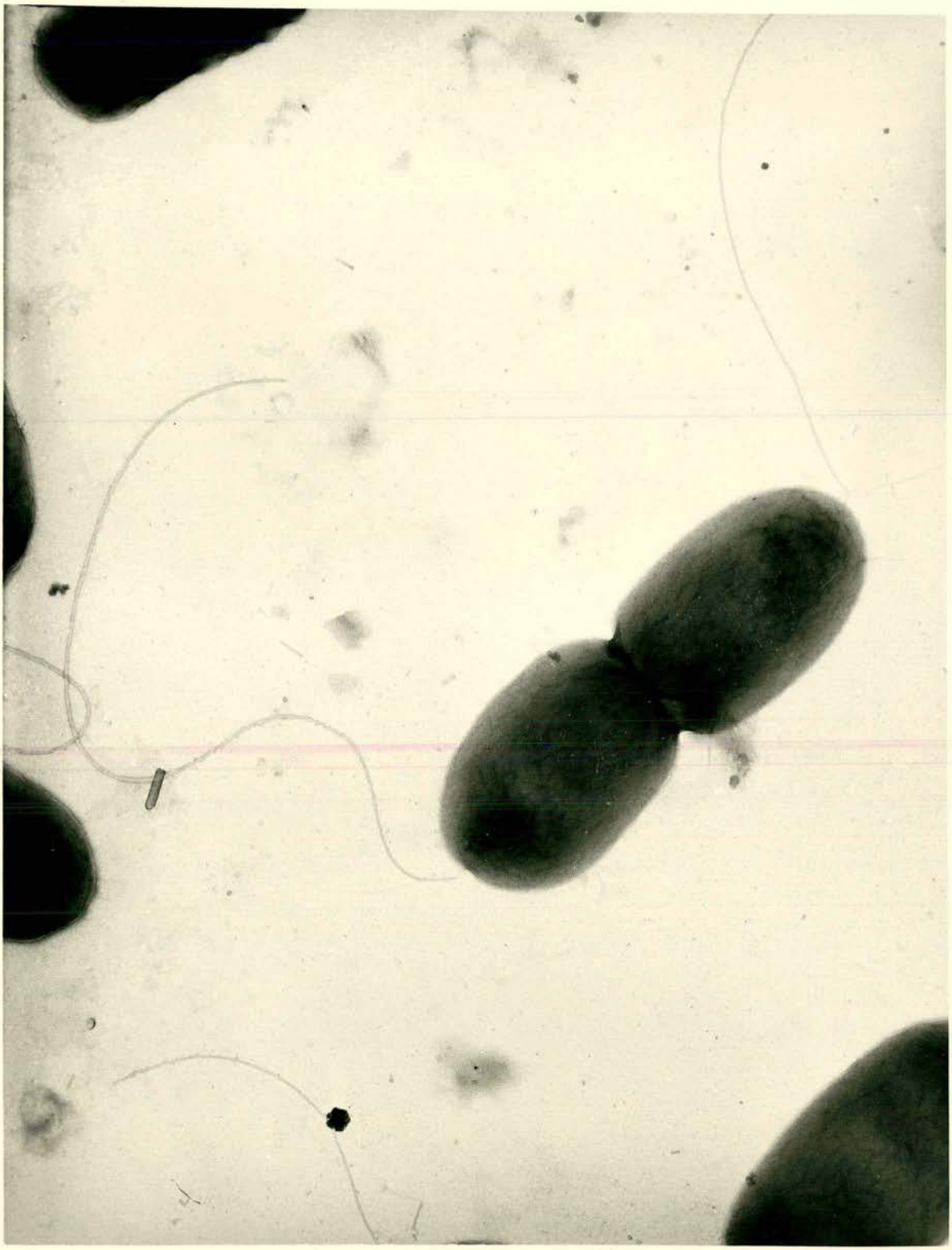
Prolonged growth of mucoid bacteria, especially in a chemostat, gave rise to a wide range of colonial variants when plated onto solid media. In an ammonia limited chemostat culture Ps. aeruginosa strain B (PsB) gave rise both to small mucoid and small non mucoid bacteria. Such a culture is shown on plate 2. However, when PAO 579 was grown in this same medium only small mucoid colonies were present. With the PAO strains reversion of mucoid to non mucoid occurred on solid media as outgrowths from the edge of a mucoid colony (Govan, 1976). This type

PLATE 1 Ps. aeruginosa Strain B grown on nutrient broth plate for 48h at 30°C.

PLATE 2 Ps. aeruginosa Strain B grown in ammonia limited chemostat culture showing mucoid and non mucoid colonies.

PLATE 3 NON MUCOID PAO 381 GROWN 48h AT 30°C ON NUTRIENT BROTH PLATE

PLATE 4 NEGATIVELY STAINED ELECTRON MICROGRAPH OF MUCOID PAO 566.



of behaviour was not observed for the PsB strain. An incubation of bacteria from non mucoid colonies in shake flasks, a culture of uniform turbidity was observed with no increase in viscosity. The cultural characteristics of these non mucoid bacteria were quite different from the parental mucoid type. The non mucoid colonies were smaller than the mucoid colonies and flat when plated on solid media. Colonies of PAO strain 381~~aie~~ shown in Plate 3.

A negatively stained electron micrograph of the mucoid strain PAO 566 is shown in Plate 4.

Analysis of the polysaccharide to determine its nature was initially performed by infra red spectroscopy using a KBr disc method (Filippov and Kohn, 1974). Infra-red spectra of the polysaccharide potassium salt and potassium alginate (both from an algal source and Azotobacter vinelandii) showed no major differences in the position of the peaks (Fig. 13). To confirm that the polymer was alginate a 0.1% (w/v) solution of polysaccharide was treated with an alginate lytic enzyme with specificity towards guluronic acid residues of the molecule (i.e. a polyguluronide lyase) (obtained from I.W. Sutherland) as in Methods. This led to the release of TBA +ve material and the polymer was confirmed as an alginate-like polysaccharide (Fig. 14).

POLYSACCHARIDE PRODUCTION IN BATCH CULTURE

Polysaccharide production by Ps.B was investigated in a series of shake flask experiments. The medium (11) was inoculated with 100 ml of an overnight culture and incubated at 30°C on an orbital shaker. Cell growth was estimated by measurement of turbidity at 600 nm or of cell dry weight. The yield of alginate was estimated following isopropanol precipitation. Upon inoculation a lag of 15-30 minutes occurred followed by expotential growth until the bacteria entered stationary phase after 10h.

FIG. 13 INFRA RED SPECTRA OF ALGAL AND BACTERIAL ALGINATES

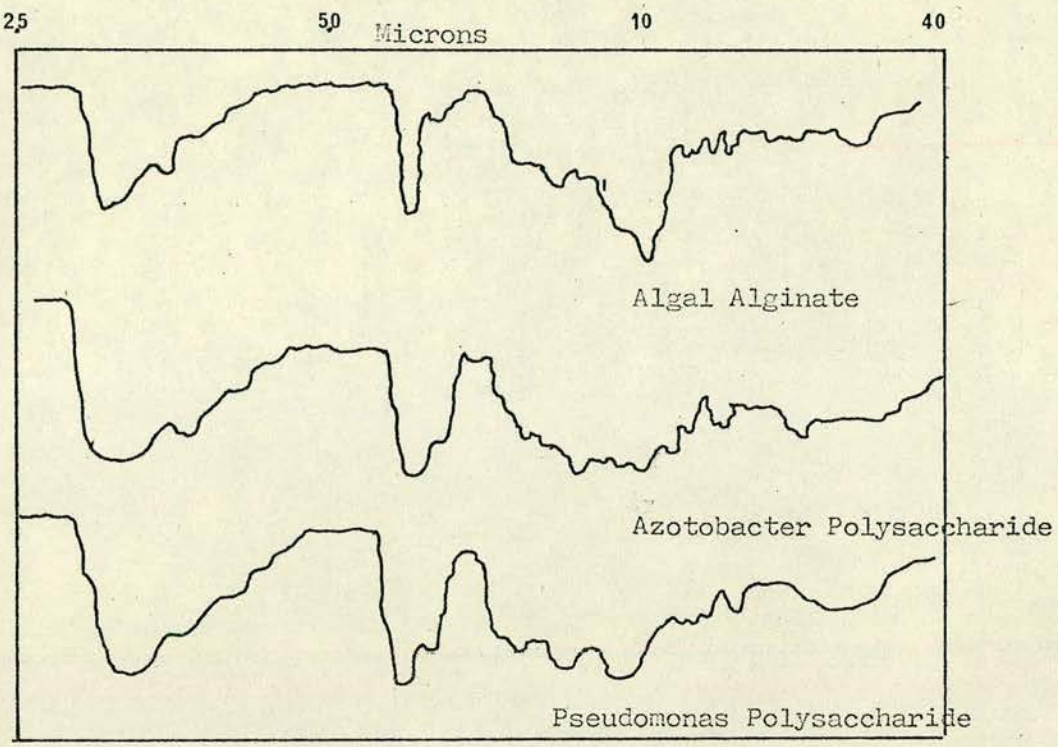


FIG. 14

Action of guluronate lyase on alginate from PsB (□) and PAO strains 587 (△) 591 (▲) enzyme from Beneckia Neptune: 2191.

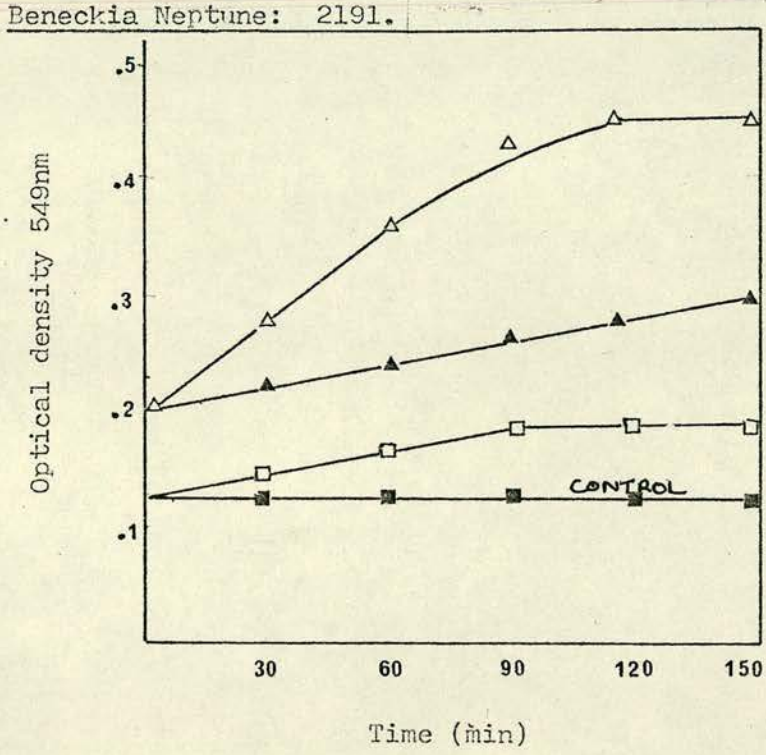
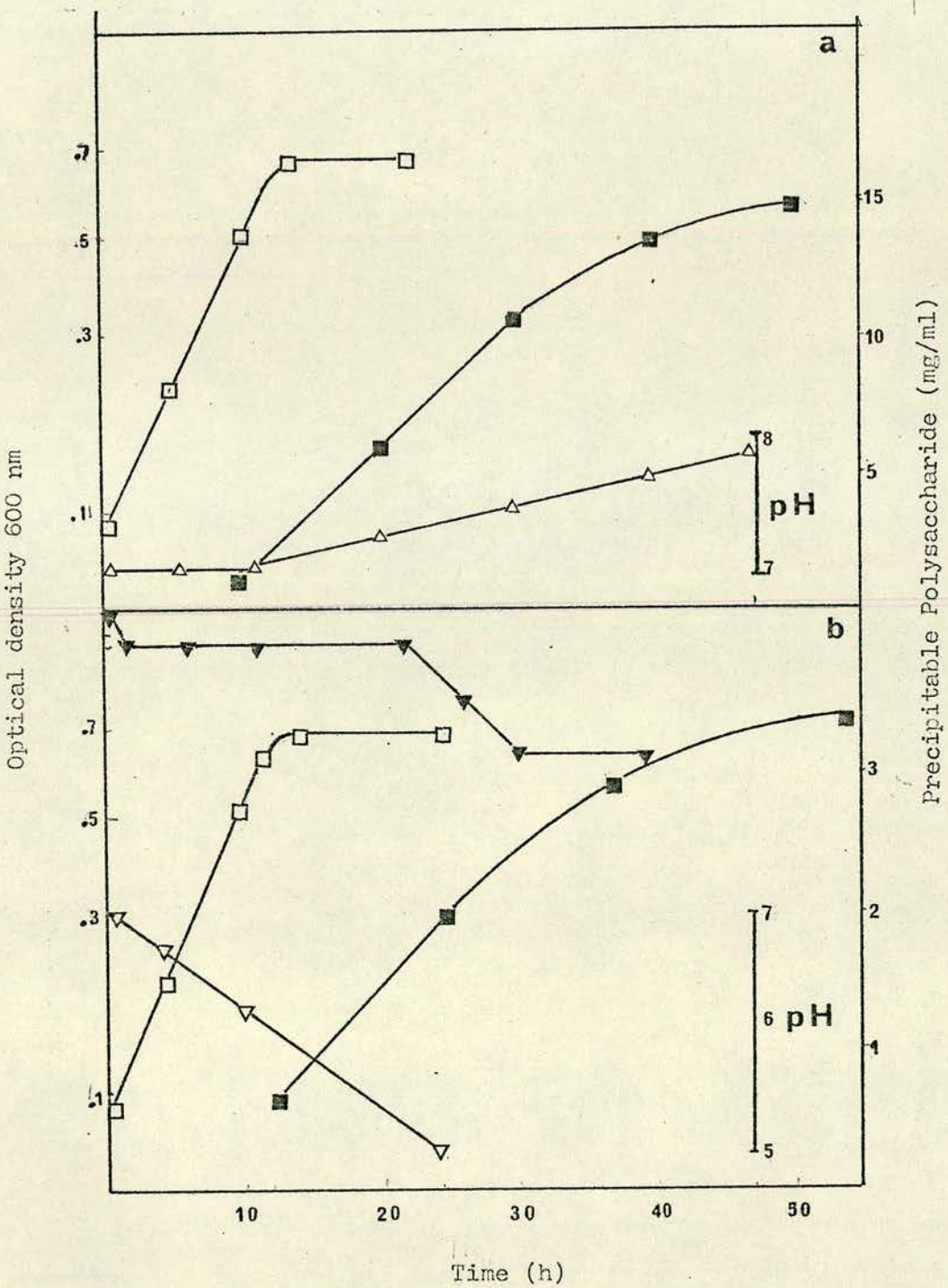


FIG. 15

Time course 11 batch fermentation grown in yeast extract medium pH 7.2, bacterial growth (□) precipitable polysaccharide (■), pH (△) on gluconate 15A and glucose (▼) 15B.



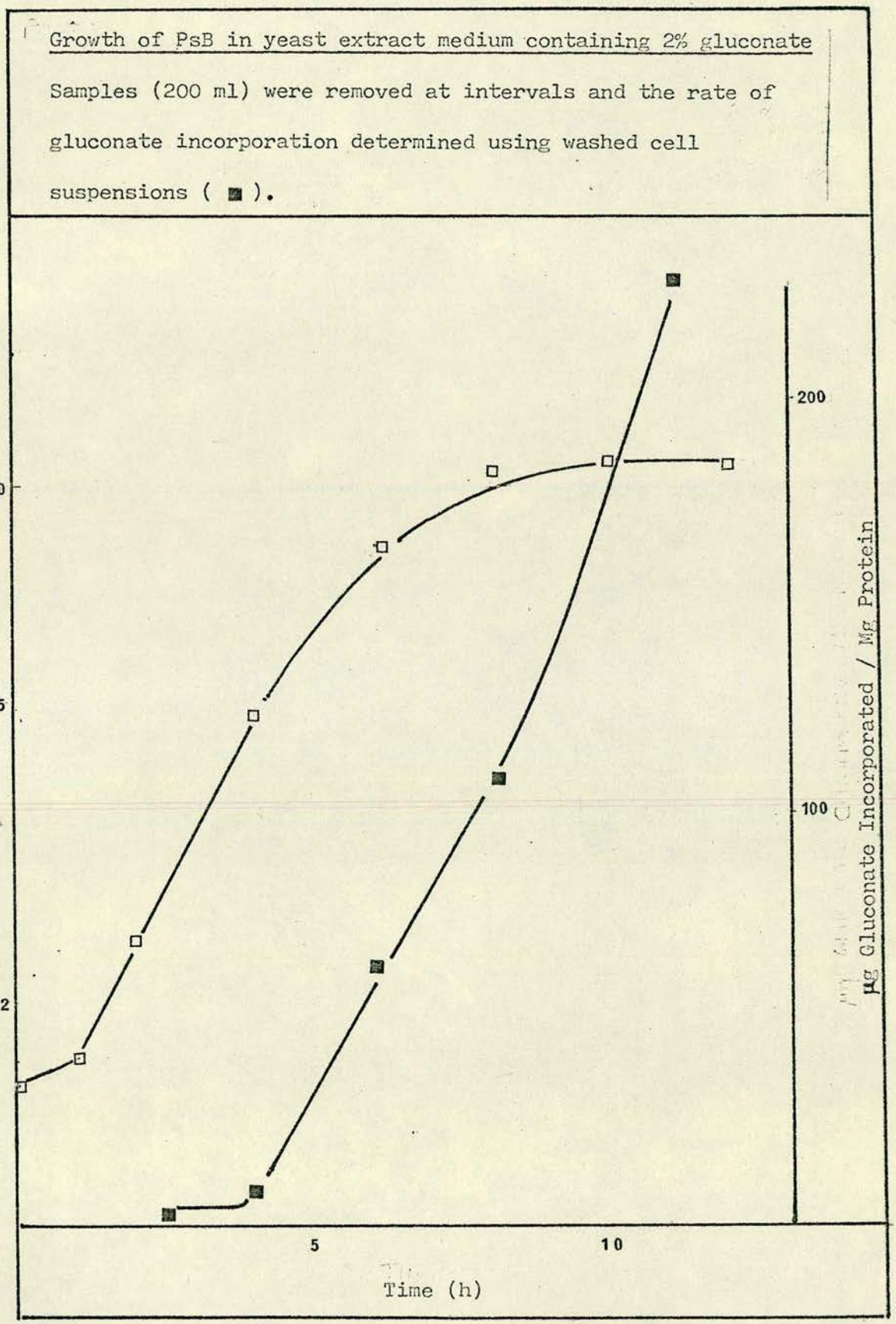
Yeast extract medium containing glucose or gluconate as carbon sources and nutrient broth were used. The cultures exhibited the same general pattern (Fig. 15a,b). Precipitable polysaccharide was only detectable towards the latter stages of exponential growth. A linear rate of polysaccharide synthesis was observed over 10-26h followed by a declining rate of synthesis until its cessation at 36-40h.

The rate of synthesis as expressed in mg alginate OD_{600}^{-1} h^{-1} were calculated from these experiments from the linear portion of the graph (Table 4). Gluconate was found to be the preferred carbon substrate in batch culture and was used in all following work in shake flasks.

The level of alginate was followed over a period of 100h and remained constant throughout. As the level remained constant this was indicative of the absence of an alginate depolyerising enzyme. To confirm this a cell free extract of both log and late stationary phase cells were prepared as in Methods and added to 1% (w/v) solution of alginate. The amount of unsaturated uronic acid material produced by lyase activity was estimated as in the Methods. A positive control of SA1 lyase (Davidson et al, 1976), an alginate depolyerising enzyme obtained from a marine pseudomanad, was used. There was no detectable release of unsaturated uronic acids and the absence of an alginase was confirmed.

In order to determine if there was continuous synthesis of polymer or a "switch on" of polysaccharide synthesis a washed cell experiment using a modified method of Wilkinson and Stark (1956) was used. 1l of yeast extract medium was inoculated with 3% (w/v) of an overnight culture and samples (200ml) were removed at intervals and used to determine the incorporation of radioactivity from ^{14}C gluconate into alginate as described in the Methods. Synthesis of the polysaccharide

FIG. 16


Growth of PsB in yeast extract medium containing 2% gluconate
Samples (200 ml) were removed at intervals and the rate of gluconate incorporation determined using washed cell suspensions (■).
Optical density (600 nm) [□]
1.0
.5
.2
200
100
µg Gluconate Incorporated / Mg Protein
5
10
Time (h)

shown to commence when the cells start to enter stationary phase i.e. a "switch on" point occurs (Fig. 16). Unfortunately there was a 75% reduction in synthesis of alginate when transferred to a washed cell suspension probably due to loss of membrane integrity. Alternatively these conditions are not the optimal ones for polysaccharide synthesis.

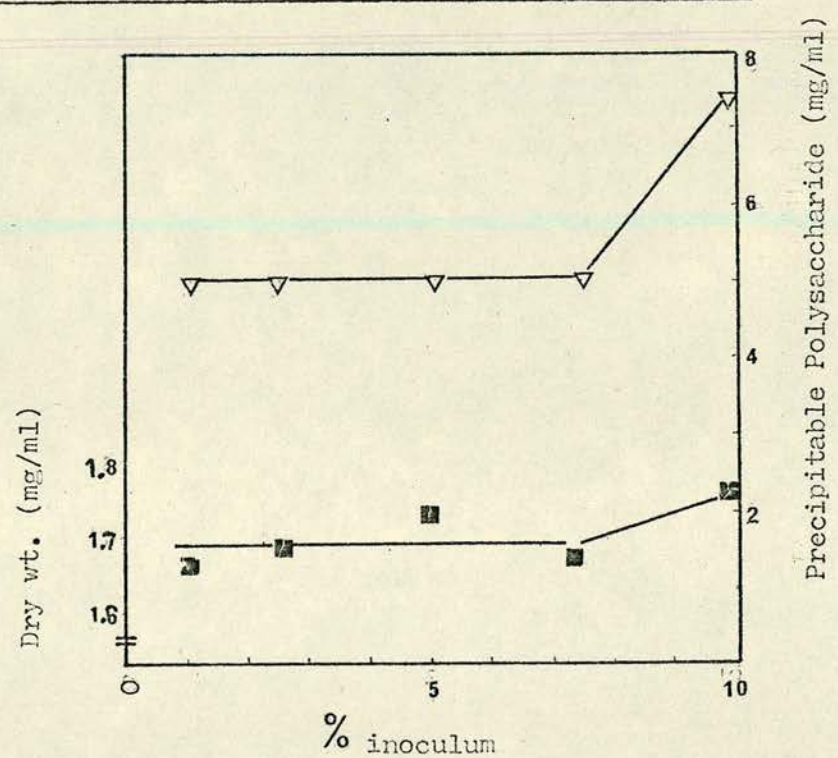
In order to determine the percentage conversion of carbon source to alginate, triplicate flasks of yeast extract medium were set up as previously described. Two carbon sources were used, glucose and gluconate, and the flasks incubated at 30°C for 100h. In shake flasks gluconate resulted in a higher yield of polymer, 65% conversion compared to 13-19% for glucose. One explanation for such a large difference is that the pH drops rapidly to a value of about pH 5 when glucose is metabolised. However with gluconate the pH rises slowly to a value of 7.4-7.8. In these experiments decrease in residual glucose in the medium was variable (Fig. 15b). There was an initial decrease of 5% of the glucose over 2h, followed by a period of no net decrease until 22h after inoculation. Uptake again commenced at a slower rate, ceasing when 25% of the glucose had been removed from the medium. It is known from previous work (Midgley and Dawes, 1973; Whiting, Midgley and Dawes, 1975) that glucose transport involves an extracellular (periplasmic) and intracellular pathway linked by transport systems. At high glucose concentrations a significant proportion is metabolised via the extracellular pathway. This leads to accumulation of both gluconate and 2-oxo-gluconate in the medium since their rate of production exceeds the rate of transport into the cell. The glucose transport system is repressed by gluconate and its activity is inhibited by both this compound and organic acids (Dawes, Midgley and Whiting 1976).

TABLE 4 RATE OF SYNTHESIS OF ALGINATE BY PsB GROWN IN VARIOUS MEDIA

Growth Medium	Rate of Synthesis (mg Alginate h^{-1} OD_{600}^{-1})
Yeast Extract + 2% Gluconate	0.80
Yeast Extract + 2% Glucose	0.13
Nutrient Broth	0.40

FIG. 17

The effect of inoculum size on the levels of alginate (▽) and biomass (■) produced by PsB in GG medium pH 7



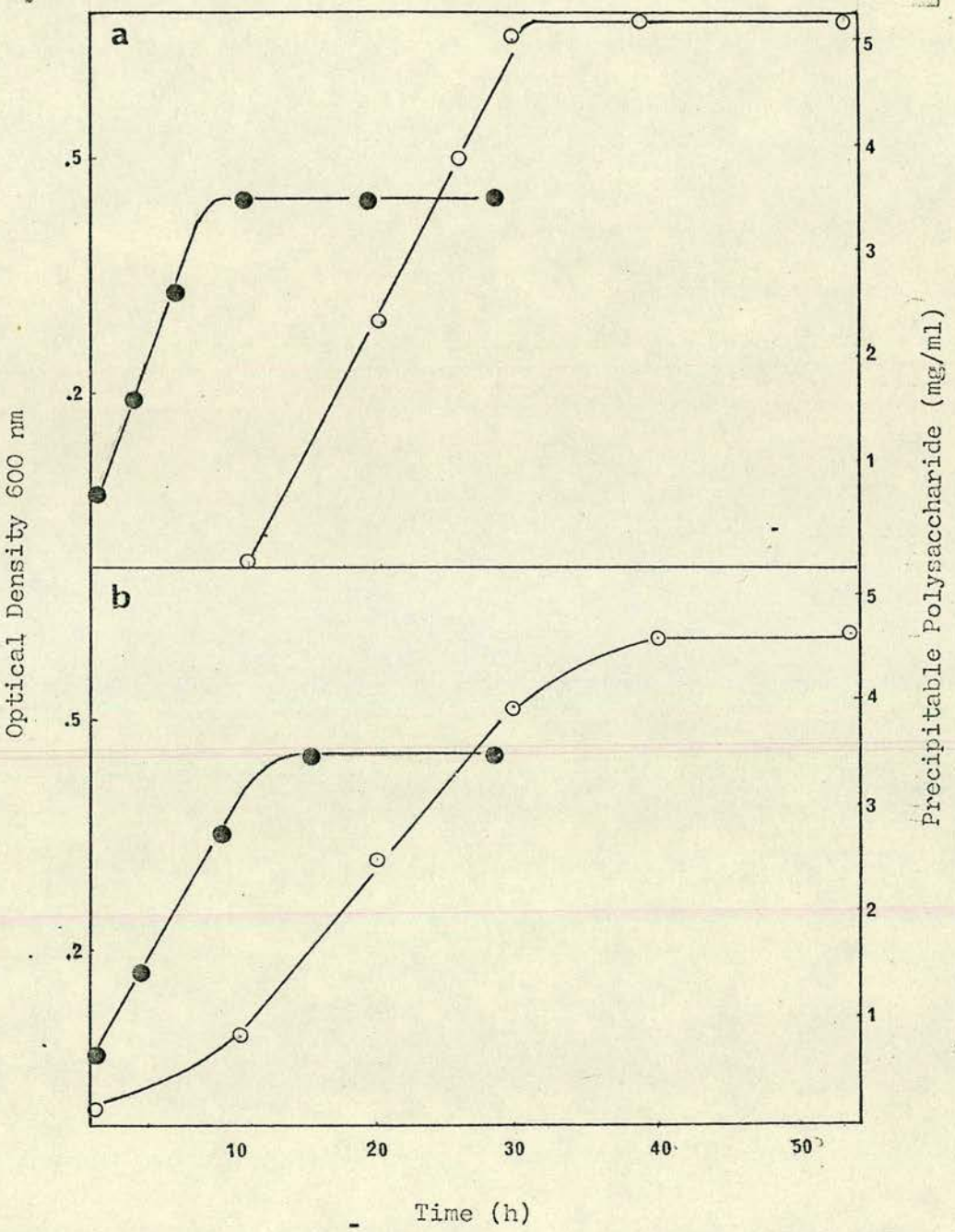
This work was performed using a 10% inoculum and due to the viscosity of the polymer difficulty in separating out the cells was encountered. Consequently a 3% inoculum was chosen for further work. Attempts to obtain a defined medium using the Davis and Mingoli (Cruikshank, 1965) medium with glucose as the carbon source failed due to poor growth. Other defined media, e.g. basal medium (BM) containing $(NH_4)_2SO_4$ and gluconate gave low yields of polymer. Using a modified version of a gluconate/glutamate(GG) medium (Goto et al, 1973) copious amounts of alginate were produced. This medium was used for all further batch work on the PsB strain.

The effect of inoculum size upon the levels of alginate and biomass were studied using this medium. Duplicate flasks were inoculated with 1 to 10% of an overnight culture and cell dry weight and alginate were estimated after 48h growth as previously described. The final pH of the medium was relatively constant varying from 7.7 to 7.8. The results indicated that a constant level of biomass and alginate occurred over the inoculum range 1 to 7.5%. At the 10% inoculum level a 50% increase in the concentration of alginate but only a slight increase, 6%, in cell dry weight occurred (Fig. 17).

Using the same GG medium the effect of using cells at different stages of their growth cycle were studied. If log phase or early stationary phase cells were used as an inoculum, precipitable levels of polysaccharide are only found when growth has finished. If late stationary phase cells are used as an inoculum (i.e. considerable alginate synthesis is already occurring), alginate synthesis becomes growth associated (Fig.18). When alginate is growth associated there is a decrease in the growth rate of the bacteria and also a decrease in the rate of synthesis of alginate. The former was reduced from a t_d of 3h to 5h synthesis was reduced from 0.80mg h^{-1}mg dry wt.$^{-1}$ to 0.33mg h^{-1}mg

FIG. 18

The effect of inoculum age upon the commencement of alginate synthesis. Bacteria were in GG medium pH 7, LOG or early stationary phase cells (A) and late stationary cells (B) showing biomass (●) Precipitable Polysaccharide (○).



dry wt.$^{-1}$.

For growth studies using mucoid PAO strains a number of media, both complex and defined, were used to determine the best medium for polysaccharide production. Initially one strain, 578, was chosen, this being grown overnight in Y.E.medium and a 3% (v/v) inoculum was added to duplicate flasks. Biomass and alginate levels were estimated in the usual manner after 48h growth at 30°C; the results are shown in Fig. 19. Polysaccharide was produced in all media and varied from 10.2g l^{-1} in yeast extract to 1.2 gl^{-1} in nutrient broth. Growth in McConkey medium gave 4.4gl^{-1} of polysaccharide but little alginate synthesis occurred in either of the defined media. Consequently yeast extract medium was chosen to determine the levels of alginate and biomass produced by these strains.

Two incubation temperatures were used 30°C and 37°C. A 3% inoculum of an overnight culture was added to 100ml medium and duplicate flasks were set up. The levels of biomass from the duplicate experiments were relatively constant but the levels of alginate varied considerably differing by $\pm$ 1 mg/ml. All cultures gave a fibrous precipitate on addition of IPA except PAO 581, which gave a powdery precipitate. The strains showed a range of polymer to biomass ratios varying from 3 to 8.4 (Fig. 20). Of the 6 strains studied 4 showed similar levels of alginate at the 2 temperatures tested, these were 585, 591, 579, 586. The other 2 strains showed a wide variation in the levels of alginate produced. PAO 579 produced similar levels of alginate at both 30°C and 37°C. Both 581 and 587 produced 35% and 50% less polysaccharide at the lower temperature. These latter 2 strains were chosen for further study.

FIG. 19 Growth (■)and precipitable polysaccharide levels (O) P.aeruginosa strain PAO 568 was grown in various media of pH 7 at 30°C.

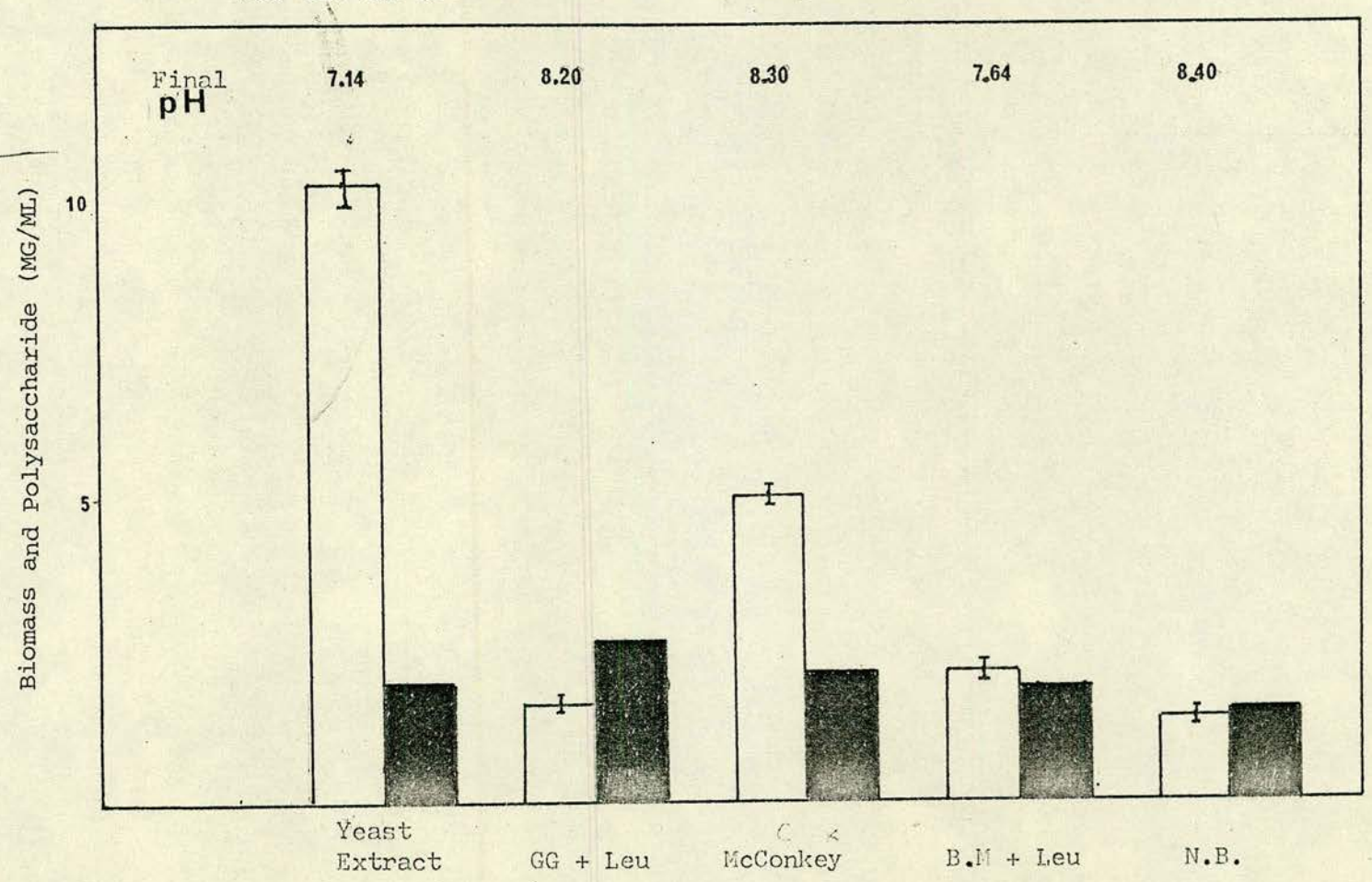
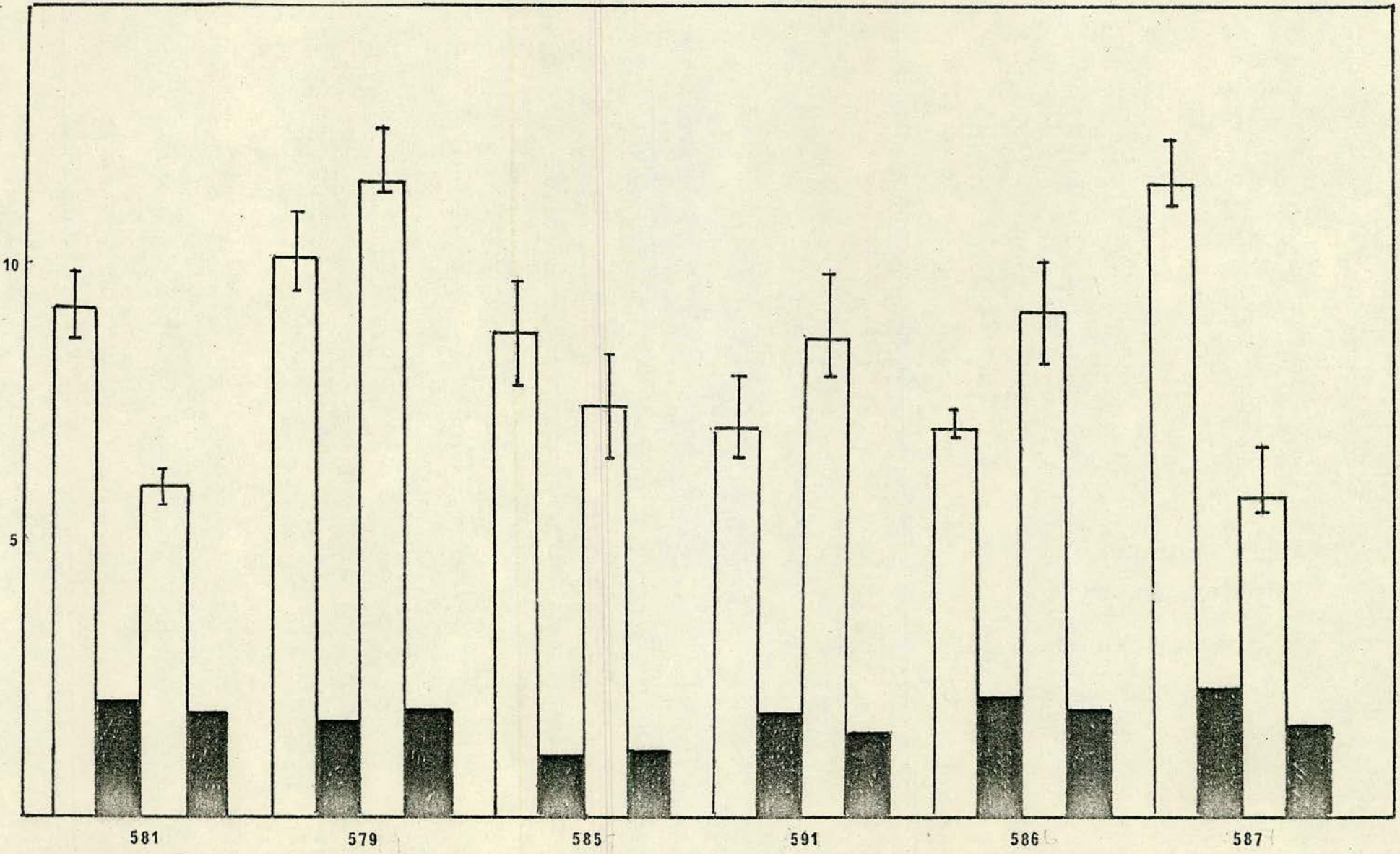


FIG. 20 Growth and polysaccharide production. Mucoid PAO strains showing precipitable polysaccharide (□) and biomass (■). The first pair of columns of cash strain are the levels produced at 37°C and the latter two at 30°C (The vertical lines show the range of values obtained from two expts.) IN Y.E. MEDIUM.



A growth curve of strain PAO 587 in yeast extract medium showed that alginate synthesis was growth associated (Fig. 21). Precipitable polysaccharide was produced during log phase and stopped after the cells had entered stationary phase. An increase in growth rate and alginate synthesis was observed at the higher temperature. However temperature had a greater effect on the rate of alginate synthesis (Table 5).

Examination of growth and alginate synthesis of strain PAO 581 showed no precipitatable levels of polysaccharide before 14h growth when the cells started to enter stationary phase. A linear rate of synthesis was observed over 14 to 24h followed by a declining rate of synthesis until its cessation at 40h (Fig. 21). In order to determine if alginate synthesis was growth associated a washed cell suspension experiment was performed. Periodically samples (150ml) were removed from a 1l flask of yeast extract medium at various stages of the bacterial growth cycle. The bacteria were collected and radioactivity from ^{14}C labelled gluconate into alginate was determined as in Methods (Fig. 23). The results indicated that synthesis of polymer occurred when the culture entered stationary phase, i.e. a switch on of synthesis occurred.

The polysaccharide produced by various strains was collected after 48h growth in yeast extract medium, purified and analysed as described in the Methods. The results are shown in Table 6.

The composition of the polymer produced by these strains varied although all of the samples had a high mannuronic acid content (Table 6). The viscosity of a 1% (w/v) solution and a 0.5% (w/v) solution in 50mM EDTA (this was used to exclude the effect of any ions which might be bound to the alginate) showed wide variations. The percentage acetylation also showed a similar variation, however a correlation between

TABLE 5 The effect of temperature on growth rate and rate of polysaccharide synthesis by strain PAO 587.

	30°C	37°C
t_d	5h	4h
Rate synthesis		
Alginate (mg alginate) (mg dry wt^{-1})	1.96	3.12

FIG. 21 Growth and polysaccharide production.

Time course of 11 batch fermentation of PAO 587 in yeast extract medium (containing 2% gluconate) pH 7 at 30°C showing biomass (●), precipitable polysaccharide, (O)

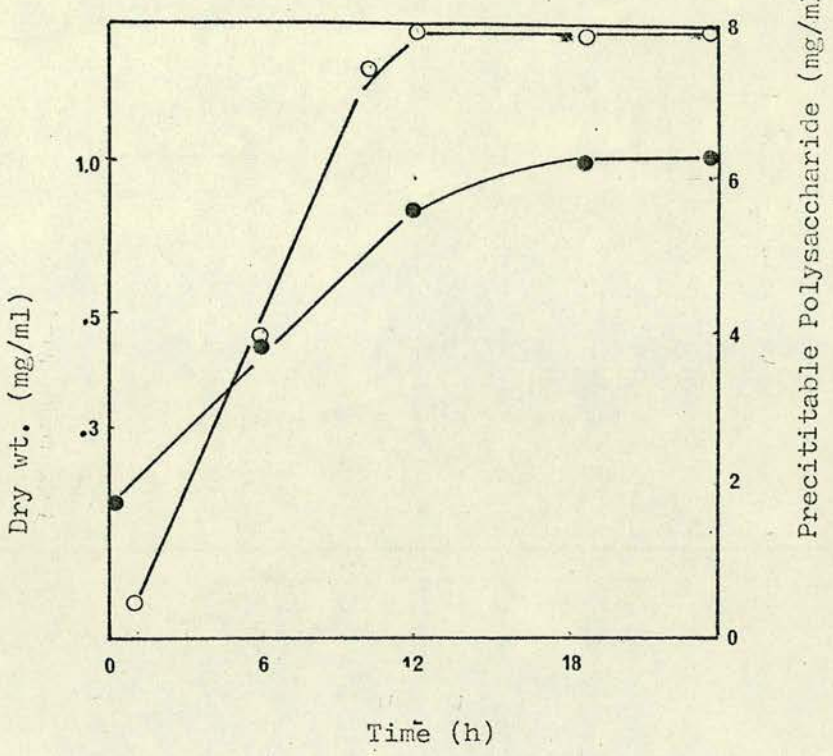


FIG. 22 Time course of 11 batch fermentation of PAO 581. Other details are as described in the legend to Fig. 21.

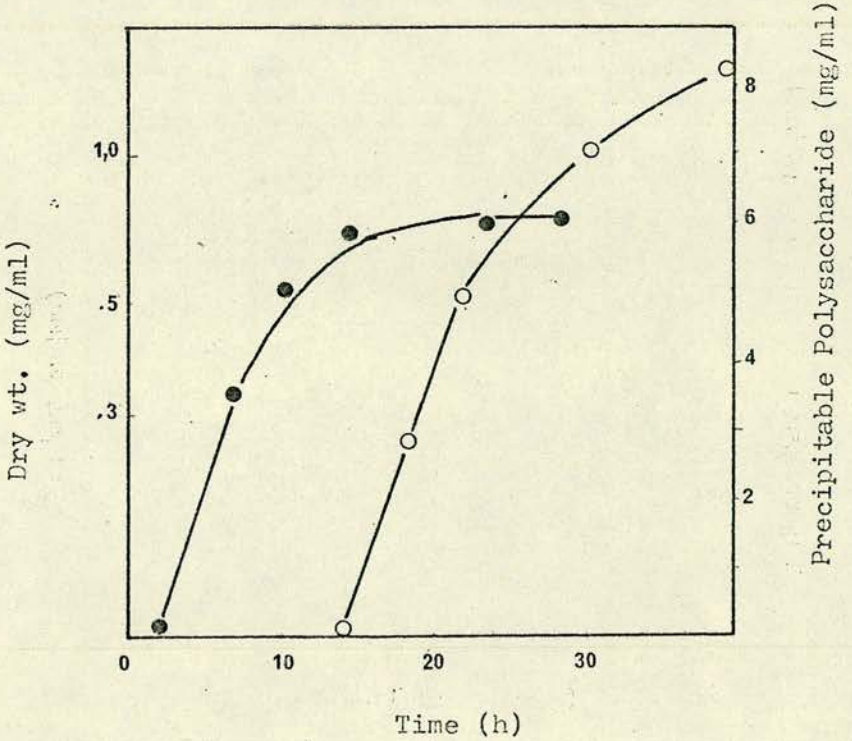


TABLE 6 Comparison of the Polysaccharide Produced by Mucoid PAO strains after 48h growth in yeast extract medium pH 7.

	578	568	579	587	566
% Mannuronic Acid[1]	75	80	85	95	89
% Acetate[2]	6.6	14.7	8.6	12.6	2.3
Viscosity (C_p) of a 1% (w/v) solution	4800	19000	10000	21000	150
Viscosity (Cp) of a 0.50% (w/v) solution in 50 mM EDTA	700	2100	1100	2900	22
% Poly M-G[3]	57	54	N.D.	N.D.	42
% Poly M Blocks	43	47	N.D.	N.D.	58

1 % total uronic acid

2 % (w/w) of polymer

3 % (w/w) of deacetylated polymer

N.D. - not determined

acetylation and viscosity was found (Fig. 24). A linear relationship between % acetylation and viscosity (either of a 1% (w/v) solution or a 0.5% (w/v) solution in 50mM EDTA) over the range 2.3-8.6% acetate content was observed (Fig. 24). After this point, increase in the acetate content of the polymer had no apparent effect on the viscosity of the polymer.

The effect of polysaccharide concentration on viscosity was determined for both Pseudomonas alginates, an Azotobacter sample (supplied by T.R.Jarman) and two algal samples, Mannutex RF and a BDH sample. The alginates were dried over P_2O_5 overnight and the dry weight calculated and a 1% (w/v) soln was made by dissolving the polymer in 99x volume of de-ionised water as in the Methods. A range of 0.25-1.0% (w/v) solutions of these alginates were made and the apparent viscosity determined using a Brookfield viscometer. The results are shown in Fig. 25. Of the five PAO alginates tested four were of a higher viscosity than the Mannutex RF and the Azotobacter sample and one was considerably lower. This sample of Azotobacter alginate should not be taken as representative as Deavin et al (1977) have shown that both the viscosity and M:G ratios of the polymer are dependant on the cultural conditions.

The effect of salts (NaCl and $MgCl_2$) on the viscosity of a 0.5% (w/v) solution of alginate (both PsB and PAO 568) was determined. To a 1% (w/v) solution of alginate prepared as above, an equal volume of salt solution was added and the apparent viscosity determined as stated previously. The presence of both salts led to a rapid decrease in the apparent viscosity (Fig. 26). A similar effect was seen in the case of the PAO alginate and these results are not shown.

Polyacrylamide gel electrophesis by the method of Bucke (1974) indicated that one discrete molecular size of alginate was

FIG. 23 Growth in gluconate medium. Growth of strain PAO 581 in yeast extract medium containing 2% gluconate, bacterial dry wt. (●), rate of incorporation of gluconate (O). Samples (150ml) were removed at intervals and the rate of incorporation of gluconate determined using washed cell suspensions.

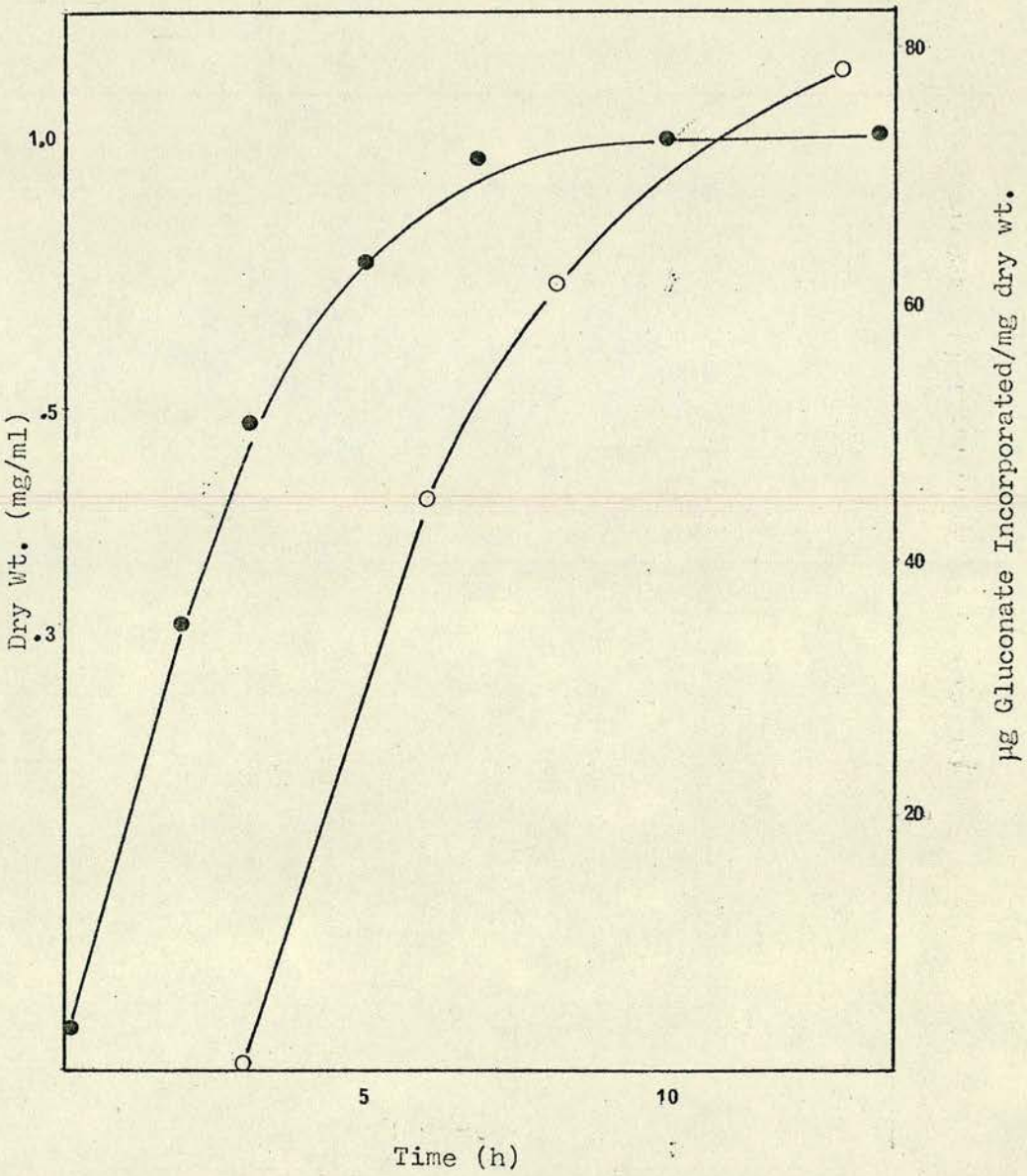


FIG. 24

The relationship between acetylation and viscosity of alginate produced by various PAO strains. Solutions were 1% (w/v).

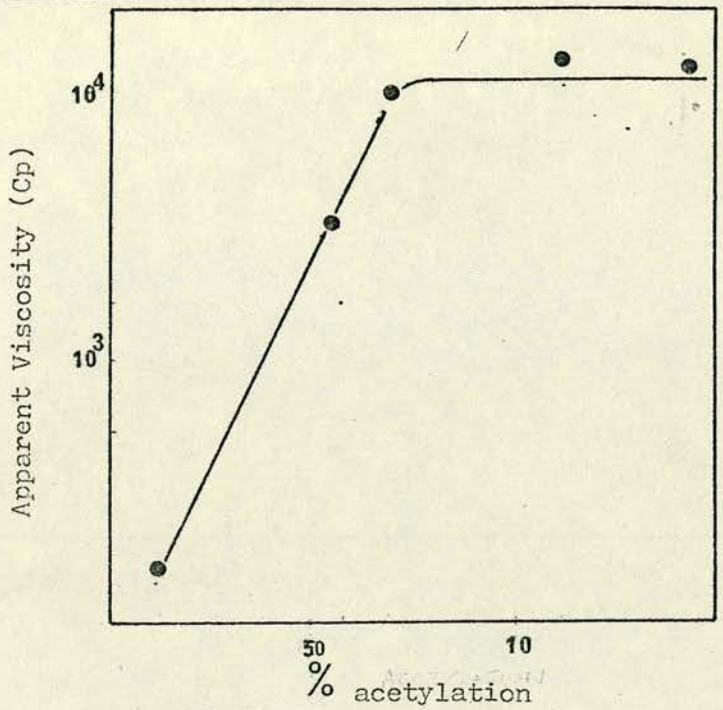


FIG. 25

The effect of concentration of alginate on viscosity for BDH (■), Mannutex RF (▲), Azotobacter (□), PAO strains 566 (○) 587 and PsB (▨), 578 (△) 568 (●).

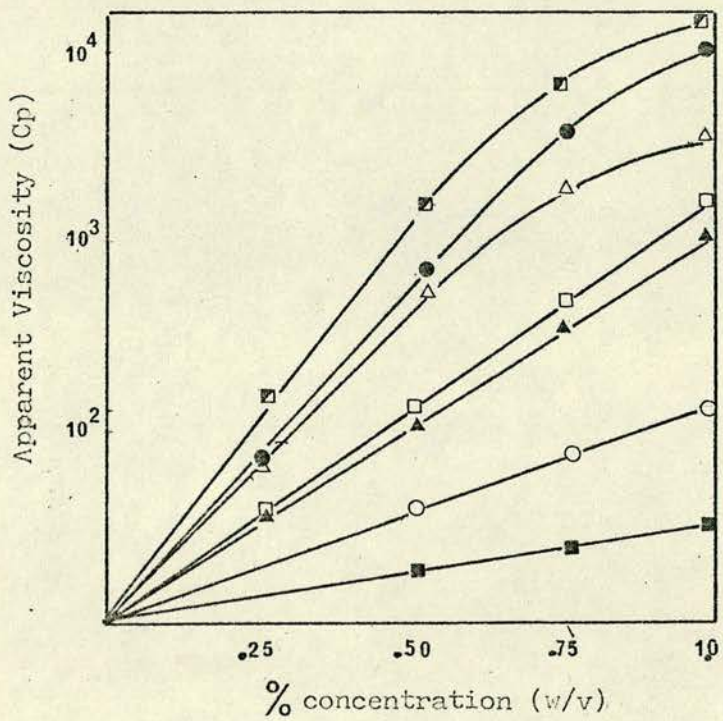
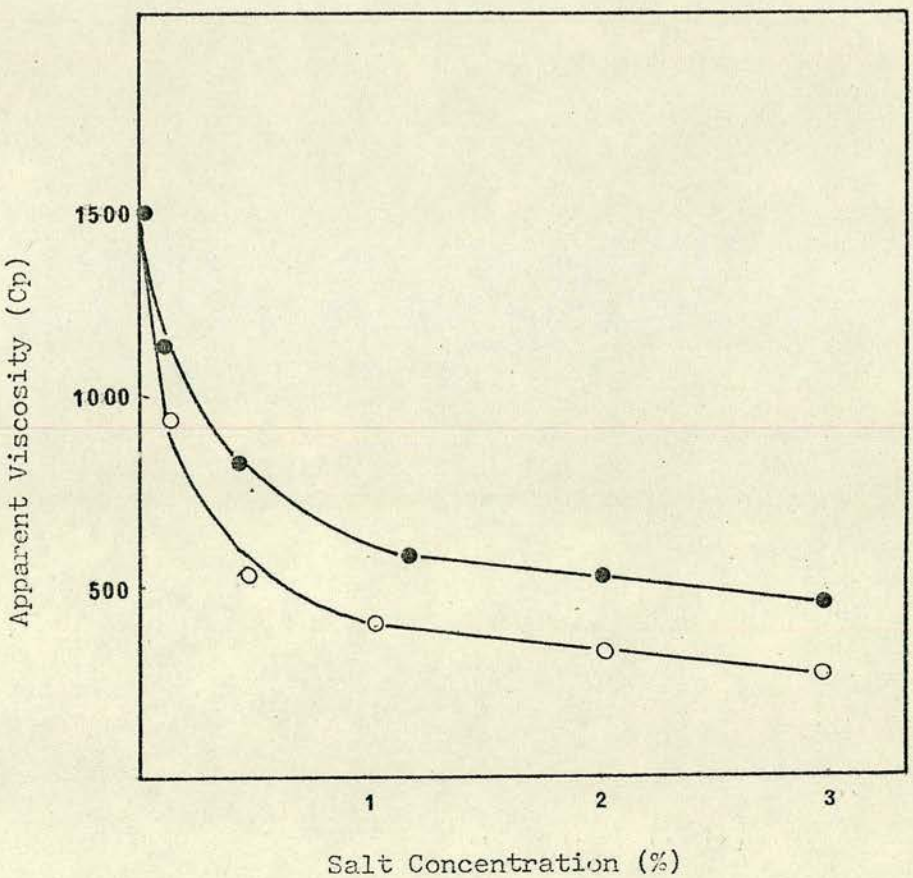


FIG. 26

The effect of NaCl (●) and $MgCl_2$ (○) on the apparent viscosity of a 0.5% (w/v) solution of PSB alginate



being synthesized by both the PsB and PAO strains. The Mannutex RF sample was shown to be polydisperse in relation to these samples (Fig. 27). Only the PsB alginate is shown in the figure. A similar pattern was seen for all the PAO strains tested, only the R_F values varied slightly.

From these samples of alginate three PAO and one PsB samples were deactylated and the block structure determined gravimetrically according to the method of Pennman and Sanderson (1972). A sample of Kelvin HV (obtained from T.R.Jarman) was also analysed as a comparison. These results are shown in Fig. 28. The algal sample (Kelvin HV) contained both poly M and poly G blocks, the latter were absent from both the PsB and PAO samples tested.

In order to determine the presence or absence of an alginase, cell extracts from all the mucoid PAO strains were prepared as previously described for the corresponding experiment with PsB. Late stationary phase cells were used and the release of TBA +ve material from a 1% (w/v) soln of alginate was tested over 2½h. There was no release of unsaturated uronic acids (TBA +ve material) either from a commercial sample (BDH) of alginate or a native Pseudomonas sample.

Thus the apparent absence of an alginate depolyerising enzyme was confirmed, as noted earlier for Pseudomonas aeruginosa strain B.

To determine the effect of inoculum size on the ratio of biomass to alginate yeast extract medium was inoculated from an overnight culture of PAO 579. Duplicate flasks were incubated for 48h and alginate and biomass levels were estimated. The results indicated that increasing the inoculum size over the range 1-10% lead to an increase in both alginate and biomass levels (Fig. 29).

FIG. 27

P.A.G.E. of Mannutex RF (—) and PsB alginate (-O-) according to the method of Bucke (1974).

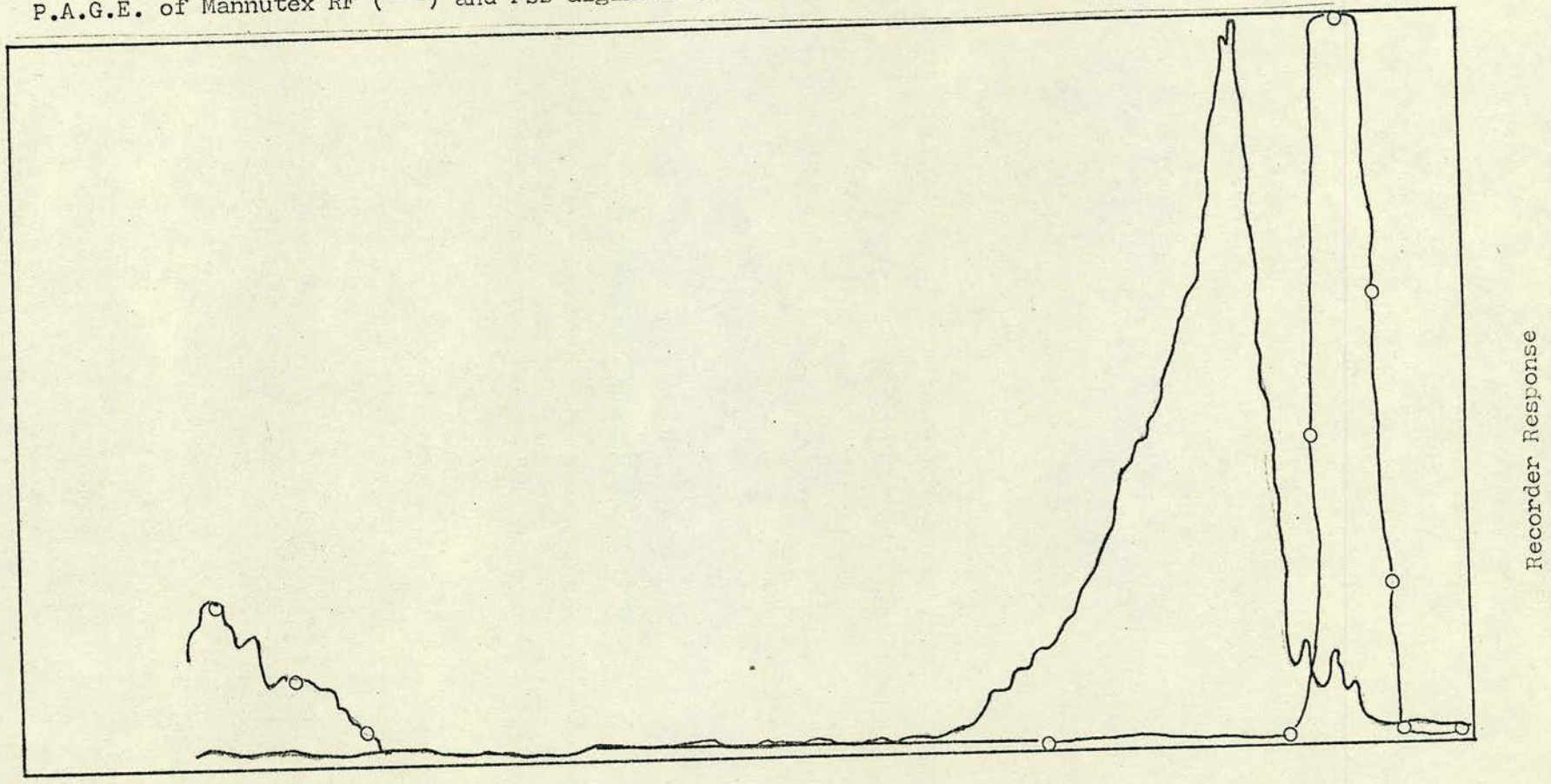


FIG. 28

FIG. 28

Proton magnetic spectrum of homopolymeric blocks obtained from Kelvin MV (A) and PAO 578 (B) alginate

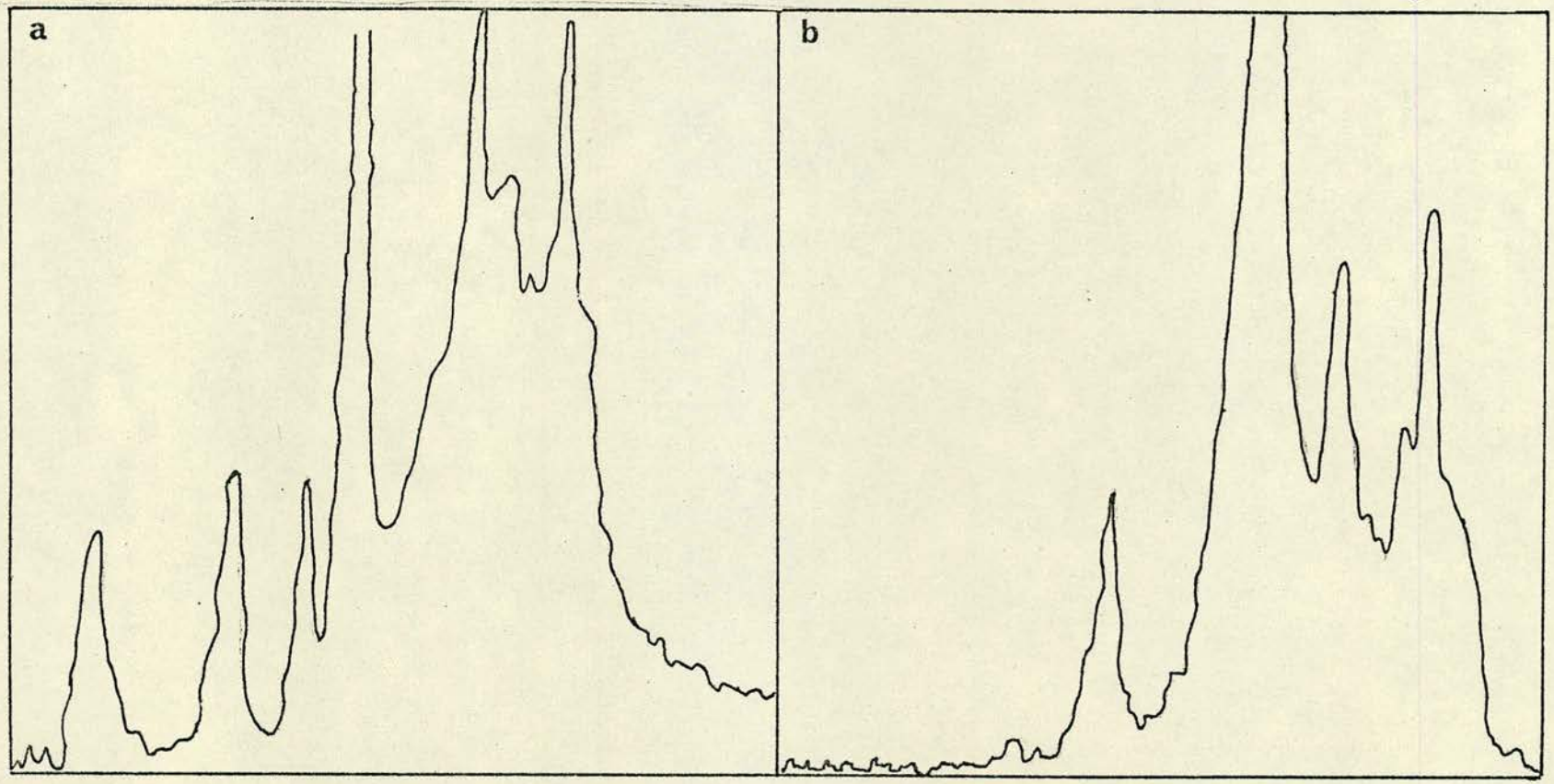


FIG. 29 Relationship of inoculum size to alginate and biomass production. The level of alginate (■) and biomass (□) produced by PAO 579 grown on yeast extract containing 2% gluconate, pH 7 and 30°C (vertical bars represent range of values obtained from duplicate expts.)

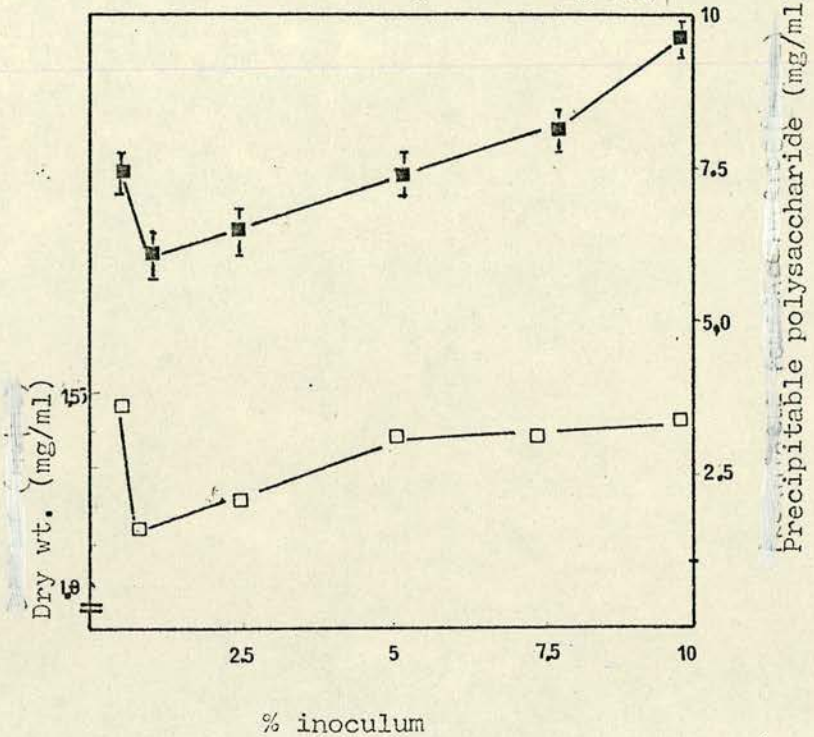


TABLE 7 Effect of Temp. and Inoculation Medium on PAO 581 Grown in (NH_4) SO_4/Gluconate Medium at 37°C.

	B.M. Grown Cells at 30°C	Y.E. Grown Cells	N.B. Grown Cells	B.M. Grown Cells
Biomass	0.88	0.80	0.62	0.80
Alg	1.33	3.57	1.98	3.04
Bio: Alg	1.50	4.50	3.20	3.80
pH	7.12	7.65	7.71	7.62

However the 0.5% inoculum gave the same level of alginate as the 7.5% inoculum and the highest dry weight, of the inoculum range studied. The pH stayed relatively constant varying from 7.10 to 7.35. The 10% inoculum gave a slight increase in biomass but a 28% increase in the alginate level over the 7.5% inoculum. The ratio of biomass:alginate was constant over the range 0.5-3% but showed a linear increase over 3.0-7.5% range.

Due to the problems involved in using a medium containing high phosphate levels, i.e. that of phosphate precipitating on addition of IPA, a chemically defined medium was used. The medium chosen (BM) contained gluconate and $(NH_4)_2SO_4$, but polysaccharide production was greatly reduced. Using PAO 581 the effect of temperature (30°C and 37°C) and the effect of the medium used to grow the inoculum was investigated. This strain was chosen as at the higher temperature a powdery precipitate of alginate was observed instead of the usual fibrous precipitate when IPA was added. The other five PAO strains gave a fibrous ppt at both temperatures and were not studied. When the BM was used to grow up the inoculum a 200% increase in alginate levels was observed at the higher temperature. The nature of the IPA precipitate also changed as previously described. However if at the higher temperature an inoculum was taken from a flask of yeast extract or nutrient broth which had been grown at 30°C a fibrous precipitate was observed. Additionally there was an increase in both the yield of alginate and biomass:alginate ratios (Table 7). Thus in some manner the cells are preadapted by growth in different media prior to inoculation.

CHAPTER II

CONTINUOUS FERMENTATION OF MUCOID STRAINS OF PS. AERUGINOSA

Continuous culture of mucoid strains was made difficult due to the appearance of non mucoid variants. These arose after 2 to 3 generation times and quickly displaced the original culture. Also at different times during the fermentation bacteria with different colonial morphologies were present. The initial culture was entirely mucoid which was of a similar size, the first colonial variant to arise were small mucoid colonies. These were displaced by small non mucoid colonies and by the end of the fermentation these non mucoid colonies were 3-4 times larger than the initial non mucoid type.

In all experiments using mucoid bacteria a single colony isolate was obtained by plating a freeze dried stock culture onto a N.B. plate and incubating for 24h at 30°C. For fermentation a 3% inoculum which had been grown overnight in a GG shake flask culture was used. This was checked for non mucoid or small colonies by plating onto N.B. plates which were examined after 24h growth at 30°C. The cultures were stored at 4°C in a cold room for 24h until inoculation.

Batch fermentation of PsB wild type over a 30h period in glucose minimal medium favoured both bacterial growth and polysaccharide synthesis (Table 8). After this time a sample (about 1l) was collected for polysaccharide analysis and the medium was then continuously added giving a dilution rate of $D = 0.050h^{-1}$. A reduction in the level of biomass occurred with a concomitant increase in the level of alginate until the culture reached a steady state after 90h (Fig. 30). On plating out the culture small colony variants became apparent after 8 generation times of which 4 were in continuous growth (Table 9). Prior to the

TABLE 8 Batch fermentation in glucose minimal medium pH 7.0 ± 0.2, 30°C air flow rate 3 l/min of Ps. aeruginosa B

Time	Biomass (mg/ml)	Polysaccharide (mg/ml)
0	0.26	-
10	0.49	2.7
23	1.04	6.38
30	2.60	7.39

TABLE 9 Presence of non mucoid variants during continuous culture

Time (h) of continuous culture operation	Non mucoid colonies % of total
0	0
24	0
50	0.25
74	1.53
96	20

Culture samples were plated on nutrient broth and mucoid and non-mucoid colonies were scored after 30h growth 30°C.

FIG. 30 Time Course of Continuous fermentation. PsB wild type in a nitrogen limited chemostat grown on a glucose minimal medium at D = 0.05 h^{-1}, pH 7 ± 0.02, 30°C, air flow rate of 31 min^{-1} showing alginate (▽) Biomass (▲) % Mucoid colonies (■).

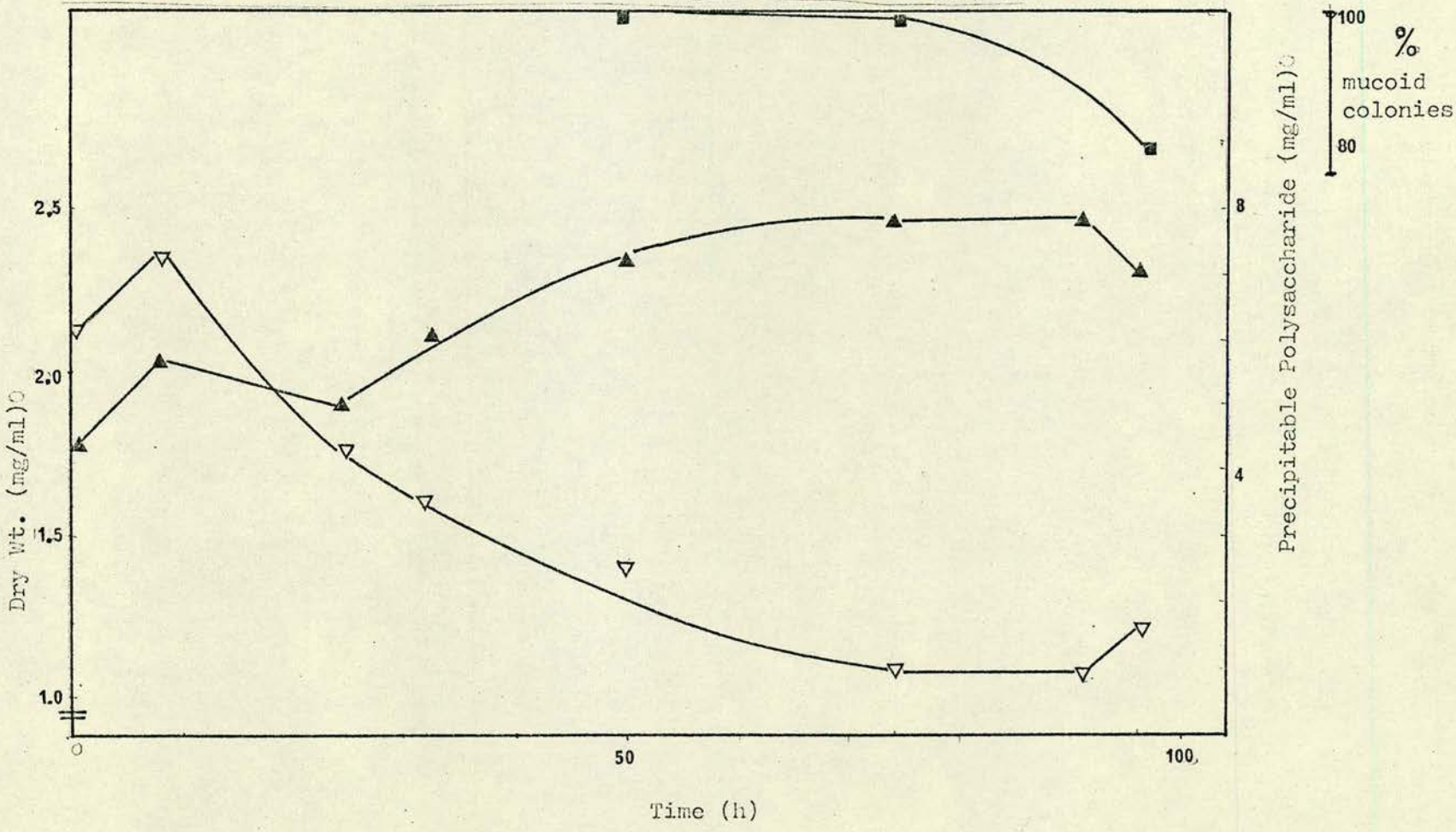


TABLE 10 Comparison of polysaccharide produced by PsB wild type grown in GG shake flask (48h polymer) and glucose minimal medium (96h steady state).

	Glucose minimal	GG shake flasks
Viscosity of a 1% (w/v) soln	21 000 cp	22 000 cp
Viscosity of a 0.5% (w/v) soln in 50mM EDTA	1700	1600
% acetylation (w/w)	13.9	7
% mannurunic acid	85	100
% poly M blocks	75	100

TABLE 11 Carbon balance for PsB wt grown on glucose minimal medium pH 7.0 $\pm$ 0.2, 30^{o}C airflow rate 3 l/min from initial continuous culture experiment

	MG/ML	MG carbon/ml
Initial glucose	20.0	8.4
Final glucose	1.59	0.74
Cell dry wt.	1.08	0.54
Polysaccharide	7.77	2.88
Respiration rate mM CO_2/l/hr	4.05	1.02

Assuming C in = C out

$$\text{C utilised} = (0.5 \times \text{dry wt}) + (0.37 \times \text{Ps}) + 12 \times \frac{CO_2 \text{ resp rate}}{D \times 1000}$$

$$7.36 = 0.54 + 2.88 + 1.02$$

$$\% \text{ recovered} = \frac{4.44}{7.36} = 60\%$$

appearance of these small colony variants the wild type bacterial colony was of a similar size. Once these variants appeared the size of the wild type colony became less uniform. A steady state sample was taken after 96h and the polysaccharide analysed and compared with that produced in GG shake flasks (Table 10). This showed that the polysaccharide produced in both cases were of a similar viscosity. The alginate isolated from this steady state showed an increase in the amount of guluronic acid present and percentage acetylation compared with that produced in GG shake flask. A carbon balance was constructed and the percentage recovered being 60% (Table 11).

In a second experiment culture degeneration was followed more closely. On plating out the culture onto N.B. plates after batch growth all the colonies appeared mucoid but smaller than those of the previous experiment. After 3 generations in continuous culture small colony variants appeared (Fig. 31) which were non mucoid after 24h growth at 30°C. After another 24h incubation at room temperature these small colonies became mucoid. This colony type increased linearly and the total number of bacteria were constant over this period (44-144h). The rate of appearance of these mutants can be calculated assuming that there is no selection occurring over this time period (44-144h).

Using

$$dM/_{dt} = Rn \quad \text{(Kubitshek, 1970)}$$

(where M = mutant concentration, n = total no. of bacteria, R = mutation rate).

After 144h growth

$$\frac{2.1 \times 10^9}{10^2} = R \times 3.63 \times 10^9$$

$$\underline{\underline{R = 5.8 \times 10^{-3}}} \text{ mutants } h^{-1} \text{ cell}^{-1}$$

FIG. 31 The No. of non mucoid colonies v time for continuous culture PsB wild type. Other fermentation details are described in the legend to Fig. 30.

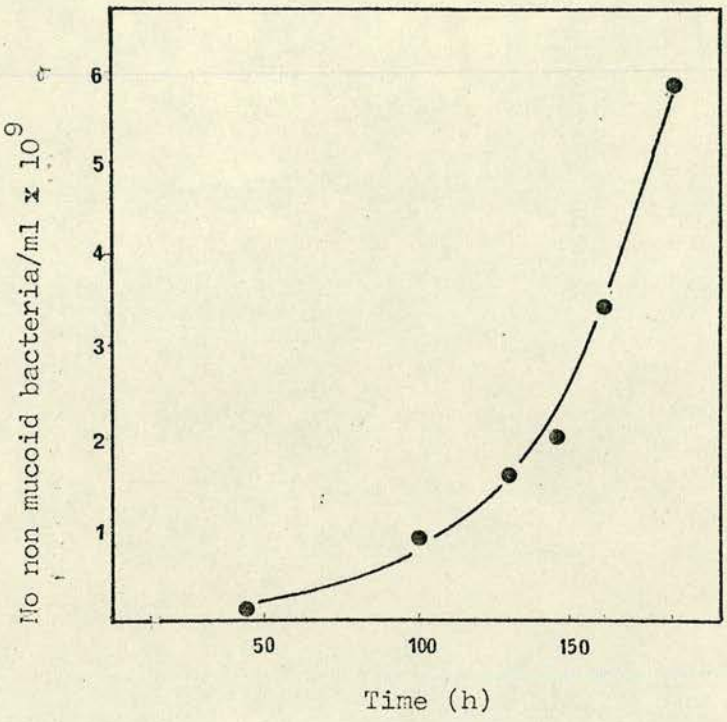
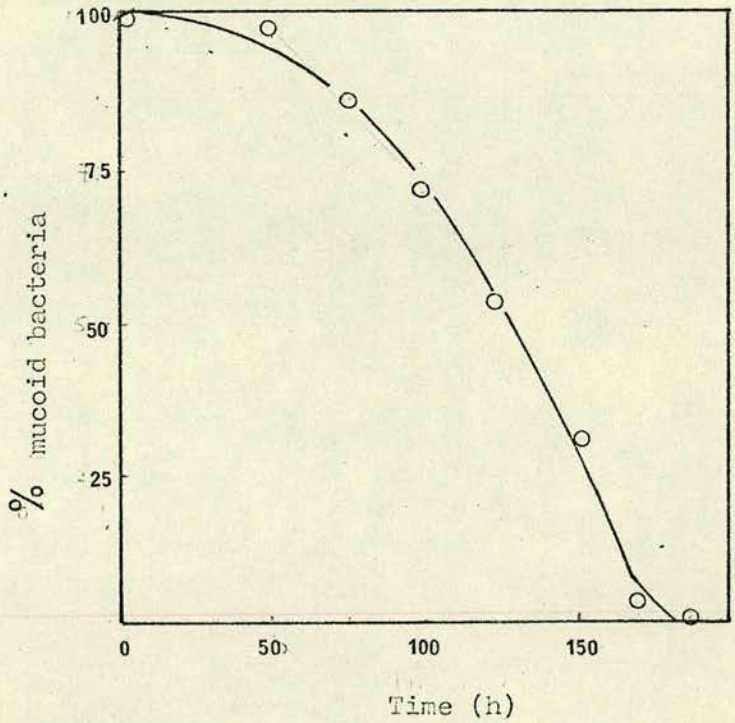


FIG. 33 The % mucoid colonies v time for continuousculture of PsB wild type. Other fermentation details are described in the legend to Fig. 30.



Associated with the presence of these small colonies was a 21% decrease compared with the level of alginate produced from the previous experiment, while the cell dry weight remained constant in this case. Despite the appearance of these small variants a "steady state" was obtained, i.e. the levels of biomass and alginate remained relatively constant over 80-120h after the onset of continuous culture. After 144h (10.5 generations) these small colonies did not become mucoid even after 4-5 days on N.B. plates at room temperature. There was a linear decrease in the level of alginate, concomitant with this was a linear increase in the level of biomass produced and a mutant with a higher yield on the growth limiting substrate was selected for(Fig. 32). At various stages during this degeneration samples (300 ml) were collected and the polysaccharide analysed. The results are shown in Table 12. The viscosity of 0.5% (w/v) solutions were very similar and the percentage acetylation was reduced slightly from the initial steady state. After 90h the viscosity was down to 300 cp and percentage acetylation down to 7.7%.

These small colonies were slow growing taking 48h to become 1 mm diameter. Plotting the number of small variants against time indicated that the mutants were under a positive selection pressure as they increased exponentially (Fig. 31). Ultimately the mutant population caused the displacement of the original culture (Fig. 33).

On picking a non mucoid colony from the end of this experiment and inoculating into shake flasks containing GG medium, copious amounts of polysaccharide were produced. The yield of alginate was similar to that of the wild type but the biomass produced was increased six fold (Table 13). This indicates that the non mucoid cells retain the genes coding for alginate synthesis but they are not expressed in the glucose minimal medium.

This "non mucoid" was inoculated into the fermenter after overnight

FIG. 32 Time Course of Contintous fermentation. PsB wild type in a nitrogen limited chemostat grown on a glucose minimal medium at D = 0.05 h^{-1}, pH 7 ± 0.02, 30°C, air flow rate of 31 min^{-1} showing alginate (▽) Biomass (●) % Mucoid colonies (■).

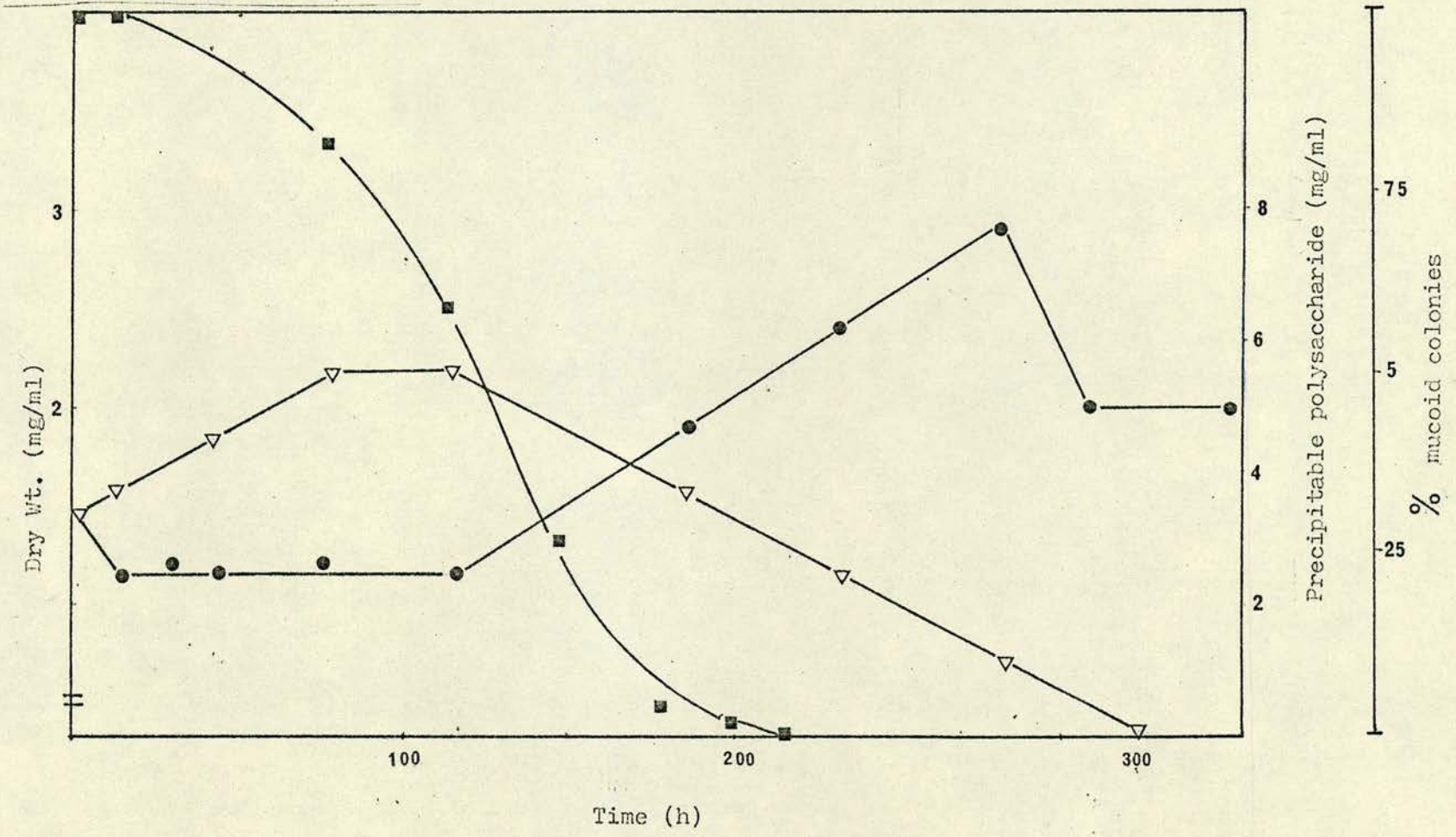


TABLE 12 Relation of the degeneration of PsB culture to alginate composition produced by PsB

	Initial s.s. (96h)	Time of onset continuous flow		
		50h	90h	250h
0.5% (w/v) viscosity in 50mM EDTA	1700	1900	2000	300
% mannuronic acid	85	80	83	89
% acetylation	13.9	11.8	11.3	7.7

TABLE 13 Comparison of growth (43h) in GG shake flasks of PsB wild type and PsB "rev".

	PsB wild type	PsB rev
Cell dry wt (mg/ml)	0.5	3.12
Alginate (mg/ml)	5.2	5.4
Ratio dry wt : alginate	1:10	1:1.62

TABLE 14 Comparison of steady states obtained with PsB under nitrogen limitation in a glucose minimal medium pH 7.0 $\pm$ 0.2, 30°C airflow rate 3 l/min at D = 0.05 $\pm$ 0.002.

	PsB wt	PsB 'rev'	PsB non mucoid
Cell dry wt (mg/ml)	1.08	1.95	2.02
Alginate (mg/ml)	7.77	5.80	0
Respiration rate	3.75	2.08	2.03
Residual glucose (q/l)	1.59	1.58	1.60
Viscosity of culture(cp)	520	8	1

TABLE 15 Biochemical tests carried out to differentiate between Pseudomonad sp.

	Ps aeruginosa	Ps wild type	Small variant
Growth on N.A. at 41°C	+	+	+
Dentrification (nitrate broth)	+	+	+
Pyocyn Prod^{n} on Kings A	+	+	+
Pyocyn Prod^{n} on Kings B	-	-	-
Tweed 80 hydrolysis	+	+	+
Growth on geraniol as C source	+	+	+

incubation in a GG shake flask. On continuous growth in the glucose minimal medium some alginate was produced although at a lower level than the wild type and the culture viscosity was similarly reduced (Table 14). The level of biomass was increased by 80% while the level of alginate was reduced by 25%.

A comparison of the steady states obtained from fermentation of the PsB strains in glucose minimal medium are summarised in Table 14. In the non mucoid strain 87% more carbon was incorporated into cellular material and the respiration rate fell by 43%. The level of residual glucose in all cases was very similar. Calculation of carbon balances gave low figures ranging from 61% - 57% - presumably due to a high conversion of glucose to gluconate and 2-oxo-gluconate.

The presence of gluconate could only be detected by the gluconate dehydrogenase method in medium where only small amounts of alginate were present as this interfered with the assay. Any attempt to remove the alginate by precipitation led to the precipitation of the gluconate as well. Using a similar method of determining 2-oxo-gluconate was impossible as the assay system was not sensitive enough.

To determine if inhibition of the mucoid bacteria by the non mucoid strain occurred a small percentage of the former was added to a mucoid culture in a GG shake flask. Inoculation with 15% mucoid bacteria gave 14% mucoid bacteria after 24h growth, thus inhibition of growth was discounted.

The small type variants were shown to be Ps. aeruginosa by performing seven biochemical tests. These are summarised in Table 15.

TABLE 16 Continuous fermentation of PAO 579 in glucose minimal medium pH 7.0 $\pm$ 0.02, 30°C airflow rate 3l/min and D = 0.05 $\pm$ 0.002

Polysaccharide conc.	$1.15 gl^{-1}$
Biomass	$1.18 gl^{-1}$
Culture viscosity	15 cp
Residual glucose	$1.30 gl^{-1}$
Residual gluconate	$6.77 gl^{-1}$
Residual 2-oxo-gluconate	n.d.

Carbon balance

$$C_{utilised} = C_{recovered}$$

$$= C_{cells} + C_{alginate} + C_{co_2} + C_{gluconate}$$

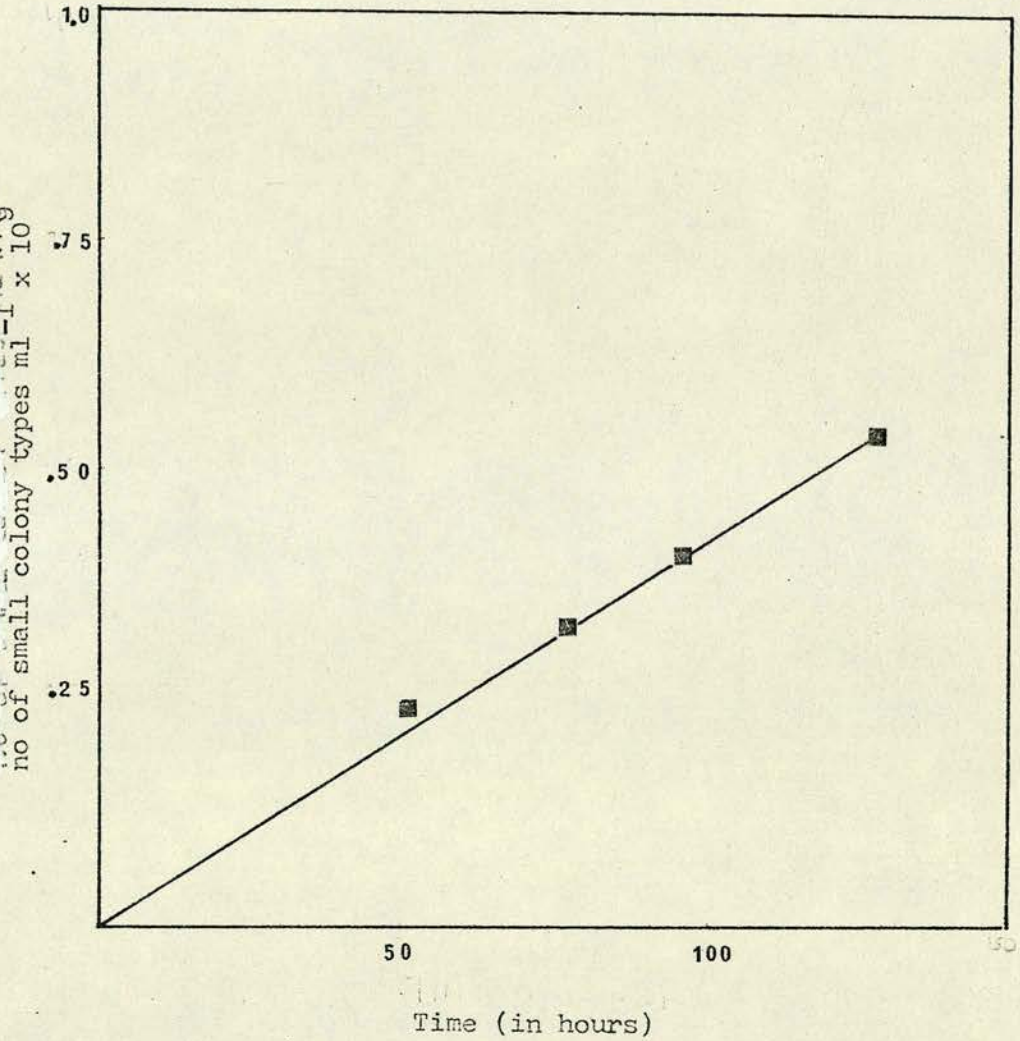
$$C_{utilised} = (0.5 \times 1.15) + (0.37 \times 1.18) + 1.02 + 2.28$$

Glucose utilised = 72/180 (20 - 1.30) = 7.56

$$\% C_{recovered} = \frac{4.31}{7.56}$$

$$= 57\%$$

FIG. 34 The number of small colony varients during continuous culture of PAO 579. Growth in a glucose minimal medium supplemented with 50 µg leucine/ml at D = 0.05 ± 0.002 h^{-1} pH 7 ± 0.02 air flow rate 3 l min^{-1}



Average no bacteria/ml = 5.47 x 10^9 ± 3%.

Culture generation time = 13.66h

R = 8.1 x 10^4 bacteria/ml

For the continuous cultivation of PAO 579 (leu^-) the glucose minimal medium was supplemented with 50 µg leucine/ml^{-1}. The culture was grown in batch for 24h and run at a dilution rate of $D = 0.05h^{-1}$. On plating out during batch growth and after 3 generation times in continuous culture colony sizes were similar. But after this time (40h) PAO 579 exhibited a similar pattern of colony sizes to that of the B strain after incubation on N.B. plates for 24h at 30°C and 24h at room temperature:-

(1) large - 3mm diameter

(2) medium - 2mm diameter

(3) small - 1mm diameter

After 11 generation times (150h), there was no production of non mucoid bacteria, but there was a linear accumulation of small colonies. A graph of the number of small colonies versus time indicated that these mutants were under no selection pressure i.e. their growth rates were identical (Fig. 34). The slope of which gave the mutation rate:

Since $\mu wt = \mu s$ (w_t = wild type

$ds/dt = Rn$ s = small colony

n = total number bacteria

t = time)

$$\frac{0.53 \times 10^8}{130} = R \times 5.47 \times 10^9$$

$$R = \underline{\underline{7.5 \times 10^{-4}}} \text{ bacteria } h^{-1} \text{ cell}^{-1}$$

Due to this instability of the mucoid strains it was decided to restrict the rest of the work to batch culture.

CHAPTER 3

ENZYME LEVELS IN MUCOID AND NON MUCOID BACTERIA

A) PAO strains

Due to the instability of mucoid bacteria when grown in continuous culture enzyme levels were estimated in bacteria grown in shake flask culture. Various enzymes were chosen for this study including those which are known to be present in the alginate synthetic pathway of Azotobacter vinelandii: phosphomannose isomerase, GDP-Mannose pyrophosphorylase, and GDP-Mannose dehydrogenase (Pindar and Bucke, 1975). Enzymes involved in carbohydrate metabolism were also analysed: gluconate dehydrogenase, glucose dehydrogenase, 2-oxo-gluconate kinase and 2-oxo-gluconate-6-P reductase, 6-P-gluconate dehydrogenase, glucose-6-P dehydrogenase and gluconokinase. Also two Krebs cycle enzymes were assayed: aconitase and isocitrate dehydrogenase.

A 2l flask containing 1l yeast extract medium pH 7 was inoculated with 3% of an overnight culture and stationary phase cells were collected by centrifugation. A whole cell extract was prepared as in the Methods by sonication and centrifugation twice at 7000 r.p.m. to remove unbroken cells. This extract was used to assay all enzymes except those utilising GDP-derivatives. A membrane-free extract for studying the latter was prepared by centrifugation at 100,000g for 45 min as a membrane bound nucleotide sugar hydrolase was present in the extracts first used.

Both wild type and mucoid strains were examined; the results obtained from PAO 381 and 579 are shown in Table 17. Of the enzymes not involved in alginate synthesis there was no significant change. The three enzymes thought to be involved in the biosynthesis of alginate all showed a significant increase in activity. This varied

TABLE 17 Comparison of enzyme levels in strains PAO 381 (non mucoid wild type) and PAO 579 (alginate synthesising)

The figures are an average of 2 expts. performed using 2 separate cultures, The results are expressed as μmoles formed mg protein^{-1} min^{-1}.

	PAO 381	PAO 579	Fold Derepression
Gluconate Dehydrogenase	240	400	0
Glucose dehydrogenase	60	20	0
2-oxo-gluconate kinase + 2-oxo-gluconate 6-P reductase	11	16	0
6-P-gluconate dehydrogenase	11	14	0
Glucose-6-P-dehydrogenase	63	51	0
Gluconokinase	5	4	0
Hexokinase	15	19	0
Aconitase	63	146	0
Isocitrate dehydrogenase	400	314	0
Phosphomannose isomerase	22	380	17
GDP-Mannose pyrophosphorylase	6	21	35
GDP-Mannose dehydrogenase	1	17	17

from a four fold increase for GDP-Mannose pyrophosphorylase, 17 fold for both GDP-Mannose dehydrogenase and phosphomannose isomerase.

To see if strain PAO 579 was typical of the mucoid strains three others were chosen and the same enzymes assayed. In the enzymes of carbohydrate metabolism a wide variation was observed from strain to strain. This is best exemplified by gluconate dehydrogenase in these four strains. Two strains had slightly elevated levels (60% higher than the wild type) and one strain (568) had intermediate levels between those of the wild type and 578 and 579. The other strain, 585, had the same level as the wild type. Aconitase and isocitrate dehydrogenase showed no significant increase in activity (Table 18).

Three strains showed a significant increase in activity of the alginate synthetic enzymes which utilise GDP-Mannose; the exception was strain 568 in which these enzymes were only slightly elevated. Addition of 0.5 μM flouride to the extract led to an increase in activity, indicating the probable presence of a nucleotide-hydrolysing enzyme. Such an enzyme appeared to be absent from the extracts of the other three strains.

As can be seen from Table 17 the wild type contains all the genetic information required to synthesise alginate but it is not expressed. This could be due to a mutation in a regulator gene or alternatively one of the enzymes of the biosynthetic pathway is subject to feedback inhibition. Fyfe and Govan (1978a) provided genetic evidence that exopolysaccharide in these strains of Ps.aeruginosa takes place following a chromosomal mutation. Consequently it was decided to pursue the former hypothesis i.e. that of a mutation in a regulator gene.

In studies by Kang and Markovitz (1967) concentrations of para-fluorophenylalanine (FPA) were found which caused the

TABLE 18 Enzyme levels in mucoid bacteria

The figures are an average of two expts. using two separate cultures, the results are expressed as μmoles product formed mg protein^{-1} min^{-1}.

Rate of synthesis (mg h^{-1} mg dry wt^{-1})	1.0	0.50	1.60	0.60
Strain number	578	568	579	585
Gluconate Dehydrogenase	400	316	395	224
Glucose Dehydrogenase	34	62	20	61
2-oxo-Gluconate Kinase 2-oxo-gluconate 6-P reductase	44	30	17	19
6-P gluconate dehydrogenase	6	3	14	N.D.
Glucose-6-P dehydrogenase	34	170	51	54
Gluconokinase	3	8	4	N.D.
Aconitase	75	68	146	92
Isocitrate dehydrogenase	423	337	314	263
Phosphomannose isomerase	360	280	380	41
GDP-Mannose pyrophosphorylase	23	9	21	33
GDP-Mannose dehydrogenase	6	2	17	55

N.D. - not detectable

repression of the enzymes involved in colanic acid synthesis. A loopful of the wild type strain was inoculated onto a basal medium plate (containing 2% gluconate and 0.6g $(NH_4)_2SO_4$) with varying concentrations of FPA from 10^{-5} to 10^{-7}M. The plates were incubated at 30°C for 24h and then at room temperature for 4 days, after which some colonies appeared slightly mucoid at 5×10^{-5}M, FPA. Subsequently the effects of FPA on the alginate synthetic enzymes were studied. A 3% inoculum of strain 381 grown overnight in basal medium was added to 1l basal medium containing 5×10^{-5}M FPA and incubated at 30°C for 24h. Both GDP-Mannose dehydrogenase and GDP-Mannose pyrophosphorylase were absent, although some nucleotide hydrolase activity was noted. The level of phosphomannose isomerase was reduced to 8 μM min^{-1} mg $protein^{-1}$ (see Table 17).

As Ps.aeruginosa is resistant to the effects of metabolic analogues and repression cannot be demonstrated by the methods which apply to E.coli this approach was abandoned. It would seem that repression will have to be demonstrated genetically.

ENZYME LEVELS IN NON-MUCOID STRAINS

For this study strains containing a supressor (sup^+) and those in which a supressor (sup^-) was unable to be demonstrated were chosen; two strains of each type were used. Enzyme levels were estimated in 16h stationary phase cells grown in yeast extract gluconate medium. Crude extracts were prepared as in the Methods. The results obtained from four such strains are shown in Table 19.

Of the two sup^+ strains (555,553) the levels of gluconate dehydrogenase were similar to that of the wild type strain, 381. One strain, 553 showed a two fold increase in the levels of the 2-oxo-gluconate metabolising enzymes, otherwise all other levels of enzymes involved in carbohydrate metabolism were similar to those of the parent mucoid strain. The levels of phosphomannose isomerase were the same as was found in the mucoid strain. However no activity of GDP-Mannose dehydrogenase or the pyrophosphorylase was detected.

Of the two sup^- strains (552,554) the levels of gluconate dehydrogenase were elevated 3 and 4.5 fold respectively. The levels of glucose dehydrogenase were also elevated, two fold for 552 and four fold for 554. Concomitant with the increase in the level of glucose dehydrogenase was an increase in the level of glucose-6-P dehydrogenase. The level of phosphomannose isomerase was the same as that found in the parent mucoid strain. Neither of the GDP-Mannose-utilising enzymes were detectable and there was no significant increase in the levels of the other enzymes tested.

To determine if these non mucoid strains were typical, another two sup^+ and two sup^- strains were analysed. The mucoid parent strain chosen was strain 568. Cell extracts were prepared by the standard procedures and the enzymes assayed. The results are shown in Table 20.

TABLE 19 Comparison of enzyme levels in non mucoid strains derived from strain PAO 579.

The figures are an average of two expts. using two separate cultures, the results are expressed as μmoles product formed mg protein^{-1} min^{-1}.

	555	553	552	554
Gluconate dehydrogenase	250	280	716	1050
Glucose dehydrogenase	37	46	30	170
2-oxo-gluconate kinase and 2-oxo-gluconate 6-P reductase	12	33	11	22
6-P gluconate dehydrogenase	7	2	2	7
Glucose-6-P dehydrogenase	46	17	76	204
Gluconokinase	5	8	2	7
Aconitase	88	78	87	87
Isocitrate dehydrogenase	336	408	464	485
Phosphomannose isomerase	405	400	350	580
GDP-Mannose Pyrophosphorylase	N.D.	N.D.	N.D.	N.D.
GDP-Mannose dehydrogenase	N.D.	N.D.	N.D.	N.D.

ND. - not detected.

TABLE 20 Comparison of enzyme levels in non mucoid strains derived from strain PAO 568 .

The figures are an average of two expts. using two separate cultures, the results are expressed as μmoles product formed mg protein^{-1} min^{-1}.

	557	558	556	575
Gluconate dehydrogenase	250	239	358	345
Glucose dehydrogenase	53	55	84	113
2-oxo-gluconate kinase 2-oxo-gluconate-6-P-reductase	2	3	7	7
6-P-gluconate dehydrogenase	2	3	4	4
Glucose-6-P dehydrogenase	88	68	94	150
Gluconokinase	2	1.6	N.D.	N.D.
Aconitase	75	35	56	36
Isocitrate dehydrogenase	303	431	265	517
Phosphomannose isomerase	20	2	4	N.D.
GDP Mannose pyrophosphorylase	33	16	16	31
GDP mannose dehydrogenase	N.D.	N.D.	N.D.	N.D.

ND - not detected.

In sup$^+$ strains gluconate dehydrogenase returned to the wild type level. There was also a reduction in the level of gluconokinase, but there was no significant change in any of the enzymes intermediary metabolism. Estimation of the GDP-metabolising enzymes showed the absence of GDP-Mannose dehydrogenase but high levels of the pryophosphorylase were present. The observed levels of the latter were increased 5.5 fold (557) and 2.6 fold (558) over the wild type. Unfortunately a comparison could not be made with the mucoid parent due to the presence of the nucleotide hydrolase in that extract. The levels of phosphomannose isomerase were either the same as the wild type or barely detectable.

The levels of gluconate dehydrogenase were not significantly elevated over the mucoid parental level in the two sup$^-$ stains tested. In one strain (575) there was a two fold increase in the level of glucose dehydrogenase and a two fold increase in the level of glucose-6-P-dehydrogenase. Gluconokinase was not detected in either of these strains and the level of the two 2-oxo-gluconate metabolising enzymes was increased two fold. Otherwise there was little difference in the levels of the other enzymes involved in carbohydrate metabolism. The levels of GDP-Mannose pyrophosphorylase were elevated 5 and 2.6 fold over the wild type for strains 575 and 556 respectively. Again no GDP-Mannose dehydrogenase was detected. The levels of phosphomannose isomeras were either barely detectable or absent.

B) Enzyme levels in Ps.B. strains

The Ps.B wild type strain (mucoid) was analysed for the same enzymes as the PAO strains. The wild type was grown in yeast extract medium pH 7 containing 2% gluconate and crude extracts prepared as in the Methods. The alginate synthetic enzymes were estimated in membrane-free extracts from both log phase (3h) and stationary phase (16h) cells (Table 21).

The alginate synthetic enzymes were present in very low levels, in 16h cells, below those found in the PAO 381 wild type strain. This indicated that most of the enzymic activity involved in alginate synthesis in this strain is membrane bound. The only difference between log and stationary phase cells was that GDP-Mannose dehydrogenase was elevated in the former.

Consequently it was decided to assay the enzymes involved in intermediary metabolism of both Ps.B wild type and the Ps.B non mucoid strain. These results are shown in Table 22. These indicated that all the enzymes measured except gluconokinase (which was elevated three fold) and isocitrate dehydrogenase (no change from the wild type) were significantly lowered. This indicated that the flow of carbon along the external pathway to 2-oxo-gluconate was repressed and gluconate was being transported directly into the cell.

It was decided to study the nucleotide hydrolase in more detail, the Ps.B wild type was used although the same results were found for the mucoid PAO 578 strain. A cell free extract was prepared as in the Methods (with membranes present) from 16h cells grown in yeast extract medium. Various attempts to inhibit the enzyme with NaF or to stimulate the back reaction by the addition of 40mM GMP or combinations of the two procedures. The results are shown in Fig. 35. Hydrolase activity was found to be firmly membrane bound; partial ibhibition with 0.5 μM fluoride occurred but increasing the concentration of NaF had no effect. The back reaction could not be stimulated as GMP was metabolised by the membranes even by the presence of fluoride.

TABLE 21 Enzyme levels in log phase (3h) and stationary phase (16h) cells grown in yeast extract medium pH 7

The results are expressed as μM product formed mg protein^{-1} min^{-1}

	Log phase cells	Stat. phase cells
Phosphomannose Isomerase	0.70	0.60
GDP mannose Pyrophosphorylase	0.90	0.70
GDP mannose Dehydrogenase	6.20	0.50

TABLE 22 Comparison of enzyme levels in Ps. aeruginosa strain B and its non mucoid mutant. The figures are an average of 2 expts. performed using two separate cultures, the results are expressed as μmoles product formed mg protein^{-1} min^{-1}.

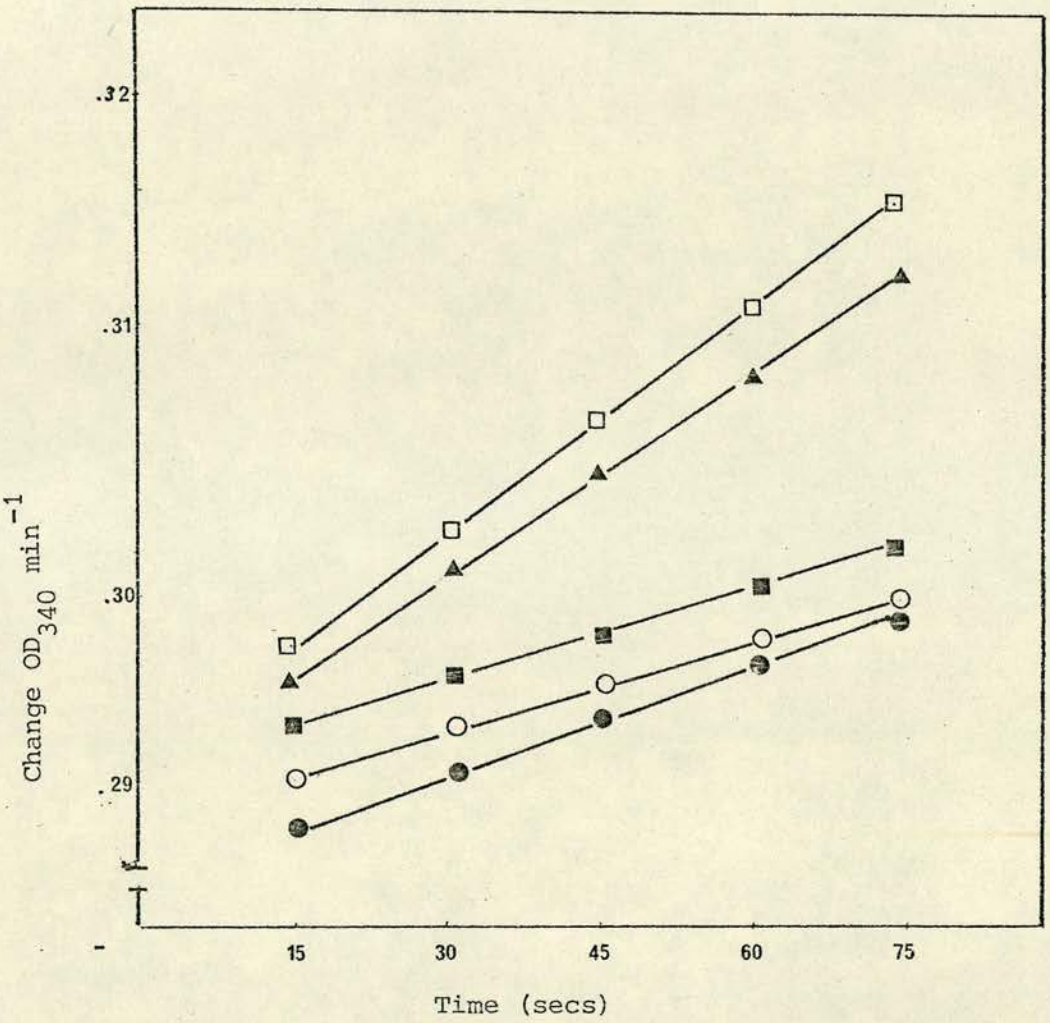
	PsB (alg^+)	PsB nm (alg^-)
Gluconate dehydrogenase	137	13
Glucose dehydrogenase	16	2
2-oxo-gluconate kinase 2-oxo-gluconate 6-P reductase	25	3
6-P gluconate dehydrogenase	N.D.	N.D.
Glucose-6-P dehydrogenase	141	11
Gluconokinase	5	16
Aconitase	165	23
Isocitrate dehydrogenase	308	245

N.D. - not detected.

Figure 35

The effect of NaF and GMP on GDP-Mannose dehydrogenase in membrane preparation of PsB. Reaction mixture contained 50mM Tris pH 8.3 (50µl), 100mM NAD (100µl) BSA (10mg/ml) (10µl) and enzyme preparation (control ○) to which was added:

- ■ control + GDP-Mannose
- ● Control + 40mM GMP
- □ 40mM GMP + 0.5µMF⁻ + 10mM GDP-Mannose
- ▲ 10mM GDP-Mannose + 0.5µMF⁻



C) Epimerase

Haug and Larsen (1971) first demonstrated the presence of an enzyme epimerising mannuronic to guluronic acid in Az. vinelandii. The enzyme was located externally to the cell and its activity was stimulated by Ca^{2+} ions (see p.14) for further details).

A cell free preparation of Ps.B alginate was prepared as in the Methods and to this varying concentrations of Ca^{2+} ions were added. Two controls were used, one in which no calcium was added and the other was a cell free preparation which had been heated at 100°C for 60 sec. to inactivate any degradative or epimerase activity that might be present. This was incubated for 24h at 30°C and the presence of guluronic acid in the polymer was detected using a guluronic acid specific enzyme, 2191. This enzyme was isolated from a marine bacterium Benekia neptunei 2191 and it showed specificity towards gul-gul or gul-man linkages (I.W.Sutherland, personal communication). The release of unsaturated uronic acid residues was followed after the addition of 2191 to the polymer. The results are shown in Fig. 36. There was no difference between the heated control and the control to which no calcium had been added indicating the absence of lyase activity. The addition of Ca^{2+} up to 1.30mM caused an increase in the guluronic acid content of the polymer. Attempts to increase the calcium levels failed due to precipitation.

In a second experiment a 20-50% $(NH_4)_2SO_4$ precipitate was prepared as in the Methods, an aliquot (0.2ml) of this preparation was added to a solution of poly M blocks containing 0.72mM Ca^{2+} and incubated for 3h at 30°C. The presence of guluronic acid was detected as stated previously. The result is shown in Fig. 37. This indicates the presence of an epimerase.

In the preparation of the $(NH_4)_2SO_4$ precipitate some alginate was

FIG. 36

The effect of $[Ca^{2+}]$ on the guluronic acid content of a cell free suspension of alginate.

Guluronic acid was detected in the polymer by treatment with a guluronic specific enzyme (2191) and following the release of unsaturated uronic acids. The enzyme showed specificity towards Gul- Gul or Gul-Man linkages.

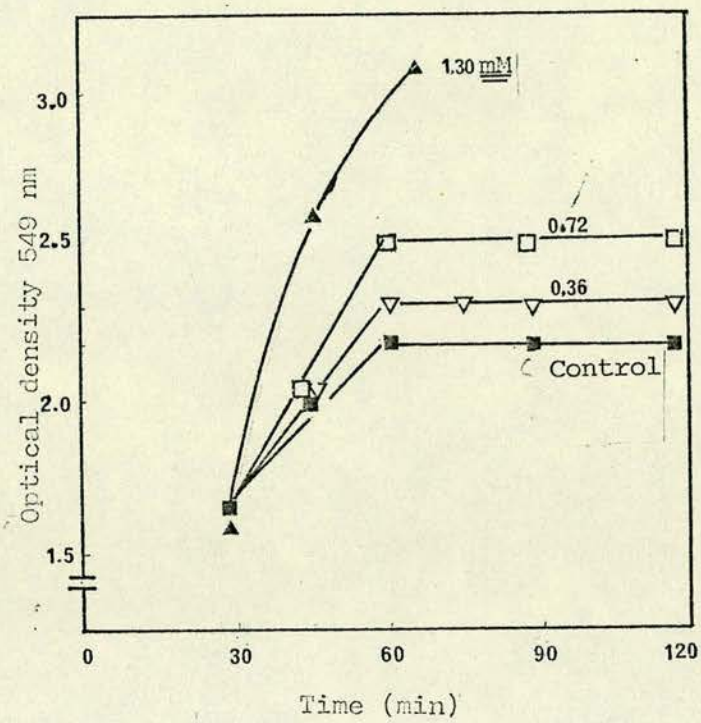
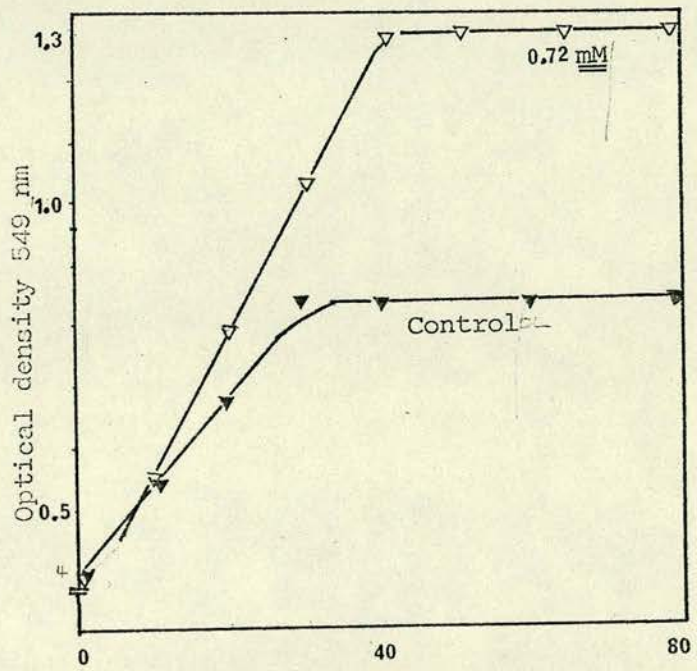


FIG. 37

Addition of epimerase preparation to Poly M Blocks

A 20-50% $(NH_4)_2 SO_4$ ppt was added to Poly M blocks and epimerisation to guluronic acid was followed as described in the legend to Fig. 36.



also precipitated and added to the incubation mixture causing the level of Ca^{2+} to be reduced due to precipitation. Consequently an attempt was made to try and separate the alginate from the epimerase.

In the attempts to separate the alginate from the epimerase no success was achieved. Partial precipitation of the alginate with Ca^{2+} or addition of the precipitation aid, Celite, were unsuccessful. Attempts were made to reduce alginate synthesis by ommiting the carbon source (Ps.B strain produced considerably less alginate when grown on glutamate alone) were unsuccessful as the alginate was again precipitated when $(NH_4)_2SO_4$ was added.

Attempts to isolate the epimerase from non-mucoid PAO strains were also tried but again they were unsuccessful.

Attempts to confirm these results obtained with the guluronic specific enzyme were also tried. Initially the carbazole/sulphuric acid method of Knutson and Jeanes (1968) was tried, both in the presence and absence of borate. Unfortunately the ammonium sulphate precipitate interfered with the colour formation. Attempts to purify the carbazole by recrystallisation from ethanol had no effect.

Another method tried was to scale up the reaction mixture 100 fold and use n.m.r. to determine if there were any changes in the poly M blocks. No detectable changes were found and in many cases no spectra could be obtained from the blocks following treatment with any epimerase preparation. Thus it was concluded that this method could not be used for detecting small changes in the poly M blocks.

As these methods failed to confirm the evidence obtained with 2191 it was decided to determine if Ca^{2+} stimulation of mannuronic to guluronic acid could be detected in a cell free preparation of Az.vinelandii NCIB 9608. A mutant strain (obtained from T.R. Jarman) which produced a polymer with a low guluronic acid content was used. The results are shown in Fig. 38. The addition of Ca^{2+} stimulated the conversion of mannuronic to guluronic acid.

FIG. 38

The effect of Ca^{2+} on the guluronic acid content of a cell free suspension of Azotobacter alginate. The presence of guluronate was detected as in the legend to Fig. 36.

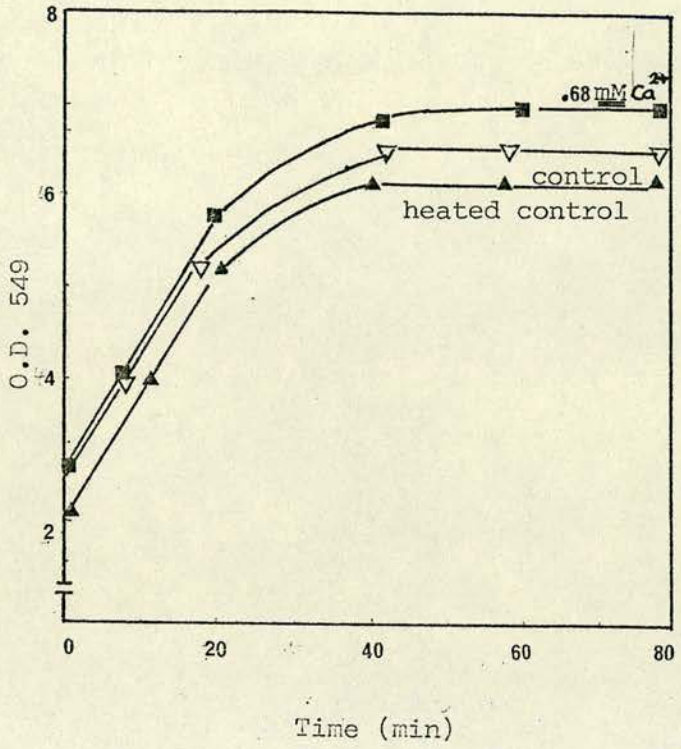
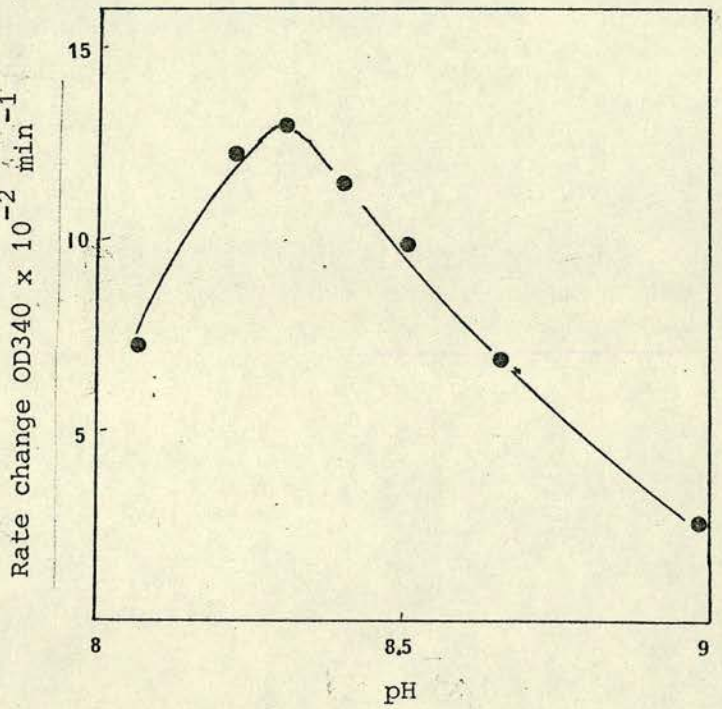


FIG. 39

The effect of pH on GDP-Mannose dehydrogenase activity.



D) GDP Mannose Dehydrogenase

Due to the low levels of GDP-Mannose dehydrogenase in the extracts of mucoid bacteria it was decided to try to partially purify the enzyme. Various attempts were made using the method of Preiss (1964).

Using strain PAO 579 51 of cells were obtained, collected by centrifugation at 15,000 r.p.m. for 20 minutes. The cells were broken by sonication and the membranes deposited by centrifugation at 100,000g for 60 minutes and the activity estimated. To the supernatant, a 1% protamine sulphate solution was added and allowed to stand for 10 minutes, centrifuged at 26,000g for 10 minutes and the supernatant was discarded. The precipitate was eluted twice with potassium phosphate buffer pH 7 containing 1% β-mercapto ethanol. The eluates were combined and made 50% saturated with $(NH_4)_2SO_4$, allowed to stand at 4°C for 3h. The precipitate was collected by centrifugation at 30,000g for 30 minutes. This was dialysed against a large volume of potassium phosphate buffer pH 7 overnight and this was assayed for GDP-Mannose activity. Unfortunately no activity could be detected.

Other attempts to partially purify the enzyme by omitting the protamine sulphate step and making the membrane free extract 50% saturated with $(NH_4)_2SO_4$ and dialysing against 50mM MOPS pH 7 for potassium phosphate pH 7 both in the presence or absence of β-mercaptoethanol failed. Although activity could be detected in the crude extract, after overnight dialysis at 4°C no activity could be found. Consequently it was decided not to continue with this.

However a pH curve of the enzyme in a crude extract was performed and the pH optima was found to be 8.3 (Fig. 39). On standing the crude extract at 4°C overnight or freezing and thawing the extract led to inactivation of the enzyme.

CHAPTER IV

CELL SURFACE CHANGES ASSOCIATED WITH ALGINATE SYNTHESIS

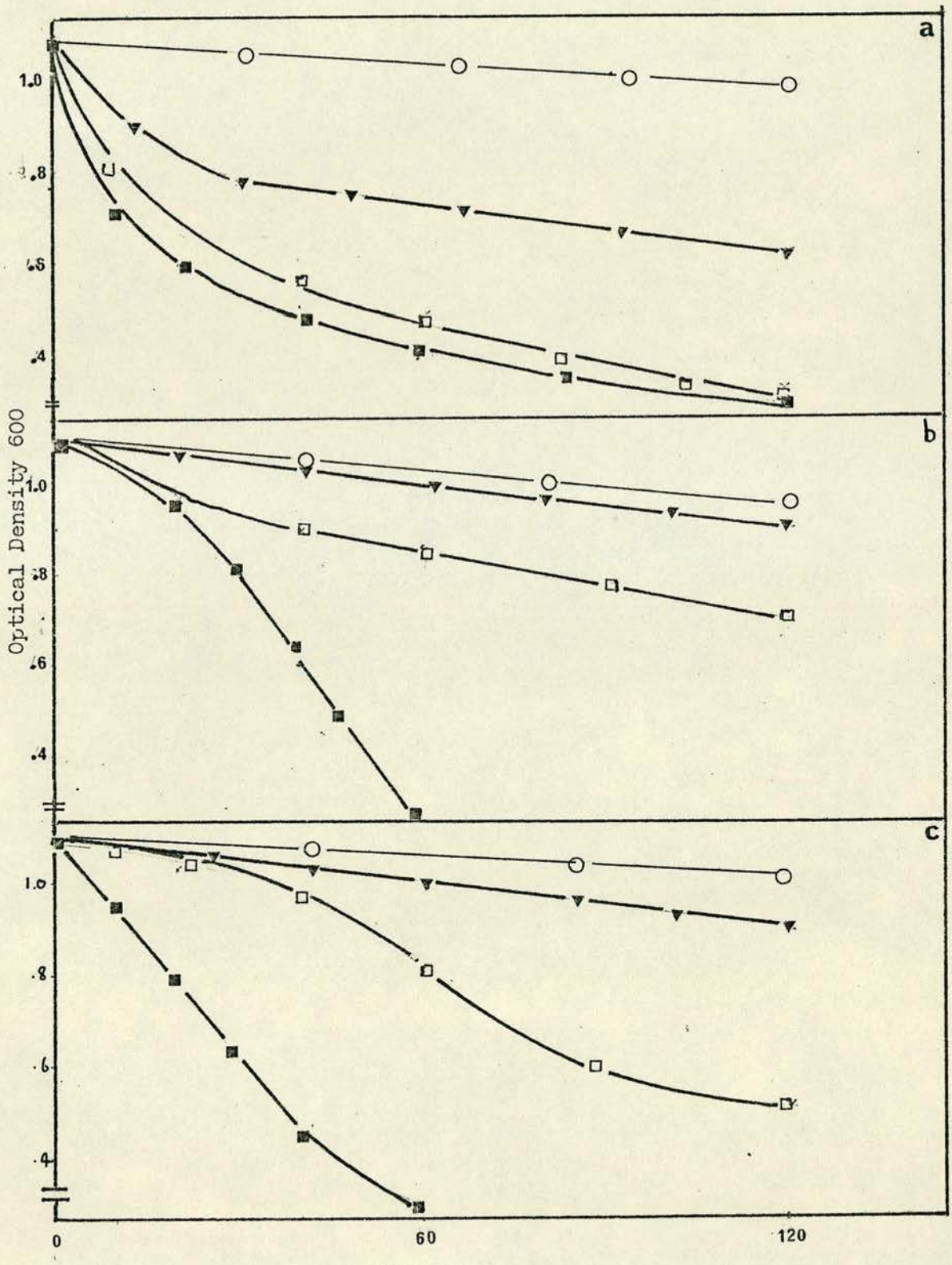
In conjunction with the ability of cells to produce alginate, the cell surface of both mucoid and non mucoid bacteria was investigated. Initially this was studied by determining sensitivity towards surface active agents, antibiotics and the ability of lectins to agglutinate cells. Changes in the sensitivity of the cells towards these agents was correlated with changes in both structure and composition of isolated cell wall components.

Three surface active agents were chosen, SDS, deoxycholate and EDTA. The former two were chosen because of the sensitivity of membrane mutants of Enterobacteriaceae (normally E. coli) towards them (Singh and Reithmeier, 1975). The latter was chosen because of the extreme sensitivity of Pseudomonads towards this agent (Gray and Wilkinson, 1965). The role of the LPS in determining both sensitivity and resistance towards antibiotics has been well documented for E. coli and Salmonella minnesota (e.g. Schlecht and Westphal, 1968). Similar studies with P. aeruginosa PACI have suggested similar behaviour for certain antibiotics (Koval and Meadow, 1977). Three antibiotics, carbenicillin, polymyxin B and tetracycline were used. Finally changes in the LPS were studied.

An overnight culture of bacteria was prepared as in the Methods and sensitivity was estimated by following loss of turbidity at 600 nm against time. Both PsB strains, various PAO strains, 381, 578, 579 and various non mucoid bacteria derived from the latter were studied.

Of the PAO strains studied the non mucoid parent 381 was the most susceptible towards SDS and deoxycholate (Fig. 40). With SDS induced lysis 381 was twice as susceptible as the mucoid strain. This

FIG. 40 The effect of EDTA (A) SDS (B) and Deoxycholate (C) on strain PAO 381 (■) 579 (□) 553 (▼) Controls(○) To cell suspensions in 50mM Tris pH 7.3 the surface active agent was added to a concentration of 5mM, 1mg/ml and 6mg/ml respectively and decrease absorbance of the culture was followed.



strain showed greater susceptibility towards the other surface active agent, deoxycholate, the rate of lysis being three times that of the mucoid derrivative. Sensitivity of the two mucoid strains tested, 578 and 579, showed very similar behaviour towards all 3 agents and only strain 579 is shown in the figures. With sensitivity towards 5mM EDTA there was no difference between 381 and the alginate-producing strains.

The non mucoid bacteria which were derived from mucoid bacteria showed a wide range of sensitivity towards all three agents. Of these six strains tested, five showed an increased resistance towards all 3 agents (Fig. 41). One strain, 549, showed a similar sensitivity towards both SDS and deoxycholate as did its parental strain (578). With SDS sensitivity these five strains showed a very similar rate of lysis, 8 to 10%. However with deoxycholate the rate of lysis varied; after 120 min , the degree of lysis was 11 to 16% compared with 36% for the mucoid strain (Fig. 42). Strain 552 (derived from 579) showed a similar sensitivity towards EDTA as its parent strain. The other five strains showed an increased resistance towards this agent (Fig. 43). This category included strain 549 despite it being as sensitive as its mucoid parent towards SDS and deoxycholate. The exact degree of resistance towards EDTA varied from 20% (551, 549) to 50% (553, 555) compared with 70% for the mucoid strains.

Of the PsB strains studied, the mucoid wild type was sensitive to 5mM EDTA although less than the PAO strains. The non mucoid strain derived from this was totally resistant to this concentration of EDTA (Fig. 44a). Increasing the concentration of EDTA tenfold caused a 10% lysis after 120 min . exposure.

With sensitivity towards SDS, the alginate producing strain was much more sensitive than the non mucoid strain, showing a similar

FIG.41

Effect of SDS on non mucoid PAO strains grown in nutrient broth. To cell suspensions in 50mM Tris pH 7.3, SDS was added to a final concentration of 1 mg ml^{-1} and decrease absorbance of the culture was followed. Strains tested were 554 (△) 553 (▼) 551 (□) 549 (□) 552 (▼) controls (○)

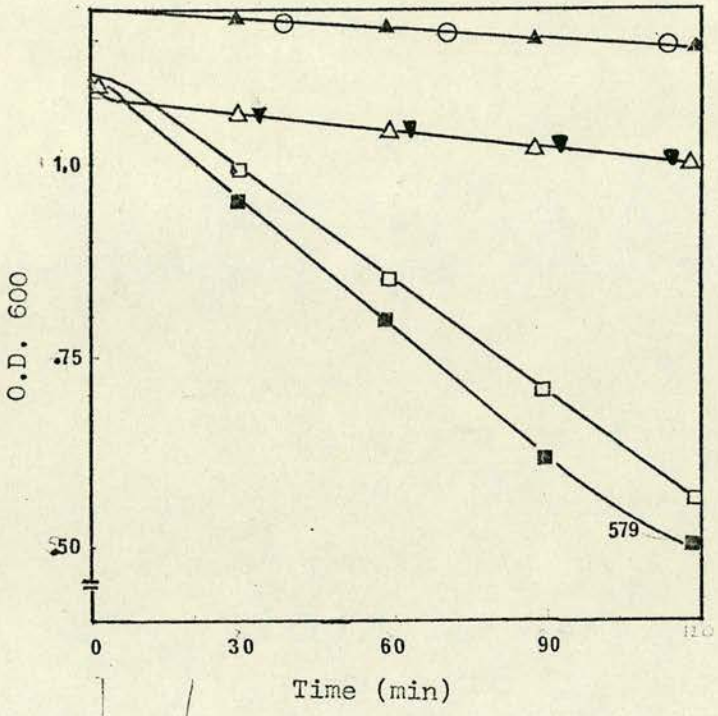


FIG. 42

Effect of Deoxycholate on non mucoid PAO strains grown in nutrient broth. Deoxycholate was added to final concentration of 6mg/ml other details are described in the legend to Fig. 41.

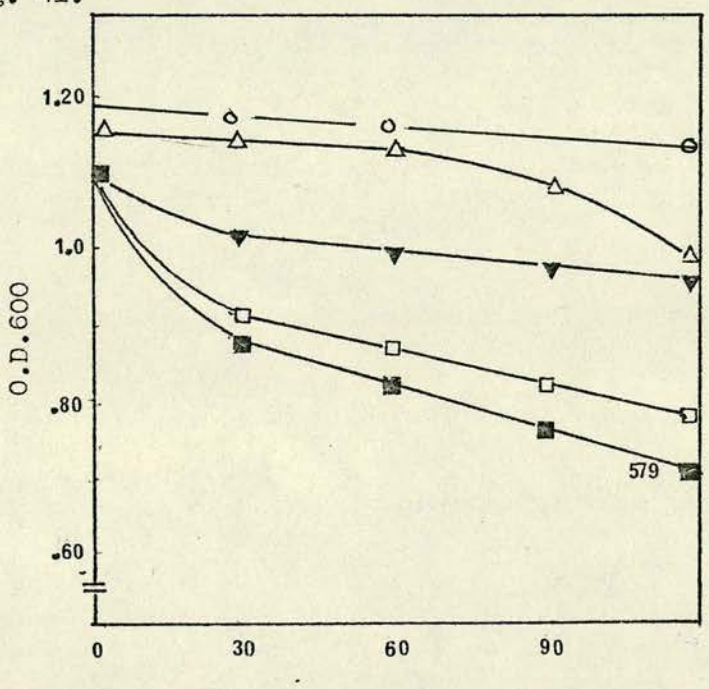
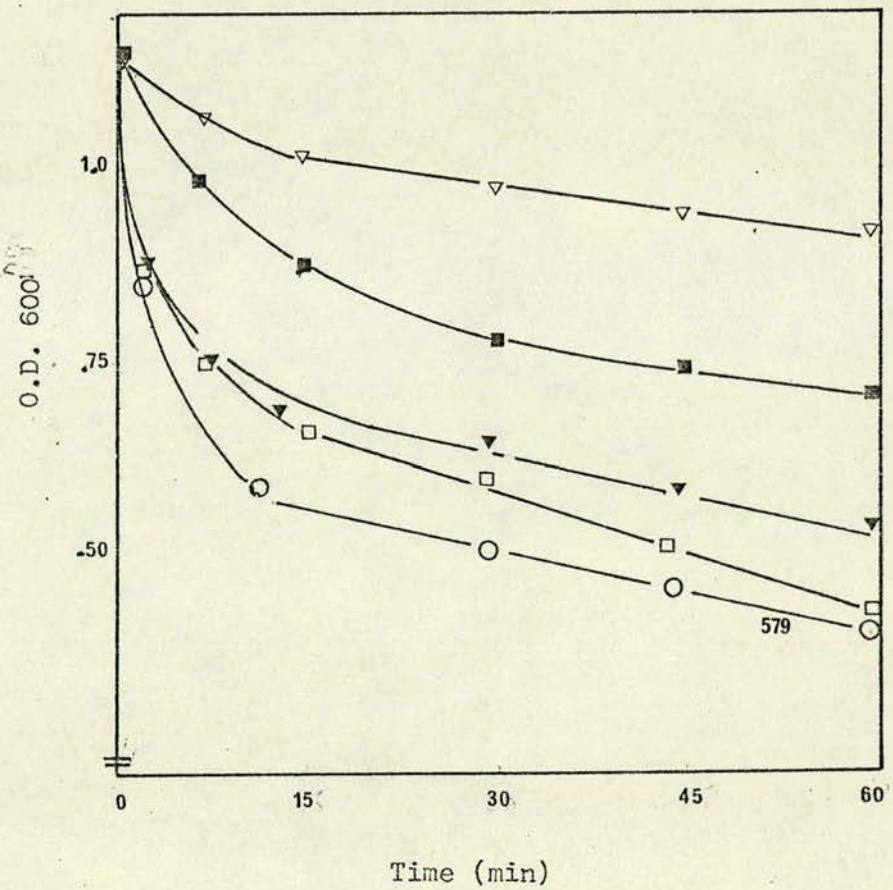


FIG.43 Effect EDTA on non mucoid PAO strains grown in nutrient broth. EDTA was added to a final concentration of 5mM other details are described in the legend to Fig. 41.



degree of sensitivity as the two PAO mucoid strains (Fig. 44b). With deoxycholate sensitivity both the non mucoid and the mucoid strains showed very similar and slight sensitivity (Fig. 44c).

In determining sensitivity of these strains to the surface active agents the cells were washed with 50mM TRIS buffer pH 7.3 prior to addition of the surface active agent. This could remove cations which are known to have a function in maintaining the integrity of the outer layers of Ps. aeruginosa (Fosberg, Costerton and MacLeod, 1970a, b). Consequently the cations Ca^{2+}, Mg^{2+}, Mn^{2+} were included in the washing buffer prior to suspension of the cells into 50mM TRIS pH 7.3 to which EDTA was added. The same experimental procedures were used as previously stated except that the culture was divided into four before washing with the buffers containing different concentrations of cations. Both PsB and PAO 579 were chosen for these experiments.

Using PsB both calcium and magnesium, at a concentration of 1mM afforded protection to the cell although the latter was more effective. The addition of ions to the control, in which there was no EDTA caused a 50% inhibition of lysis. Manganese even at a concentration of 3.5mM failed to protect the cell (Fig. 45).

With PAO 579, 1mM Mg^{2+} had no significant effect, so 2.5mM was chosen. Similar results to that seen for the PsB strain were found with Ca^{2+} and Mg^{2+} protecting the cell against lysis while Mn^{2+} did not do so. Additionally Cu^{2+} was chosen to see if it protected the cells against lysis. Lysis was only partically inhibited, being intermediate between Mn^{2+} and Mg^{2+} (Fig. 45). Thus the order of protection against lysis was

$$Ca^{2+} > Mg^{2+} > Cu^{2+} > Mn^{2+}$$

The effect of ions in determining the sensitivity cell envelope of Ps. aeruginosa towards EDTA and polymyxin has been described

FIG. 44 The effect of EDTA (A) SDS (B) and Deoxycholate (C) on PsB wild Type (▲) and PsB non mucoid (■) grown in nutrient broth. Other details are described in the legend to Fig. 40.

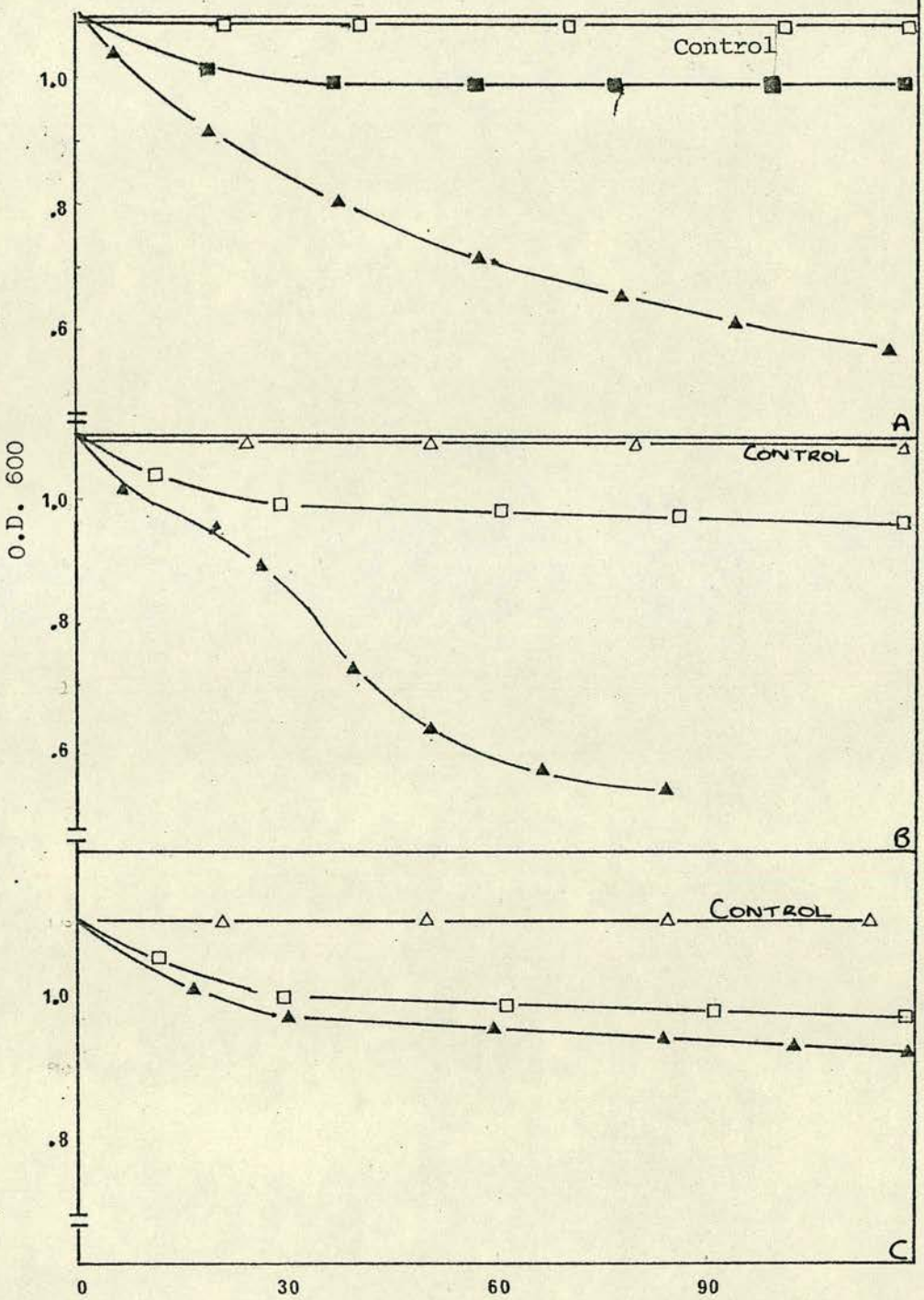


FIG. 45

Protection of PsB wild type by divalent cations against EDTA induced lysis grown in nutrient broth. The cations (1mM) Mg^{2+} (▼) Ca^{2+} (○) Mn^{2+} (△) were included in the washing buffer and EDTA added to a final concentration of 5mM EDTA and the decrease in absorbance of the culture followed.

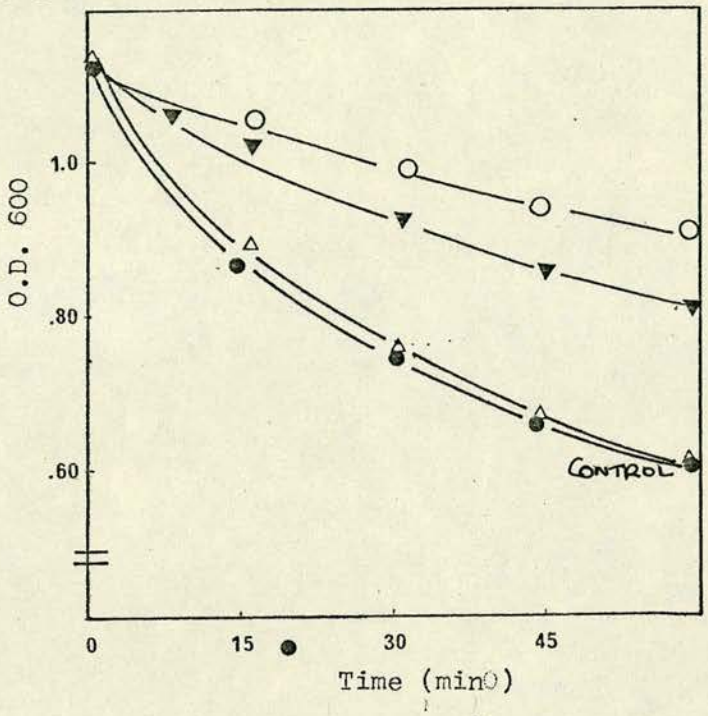
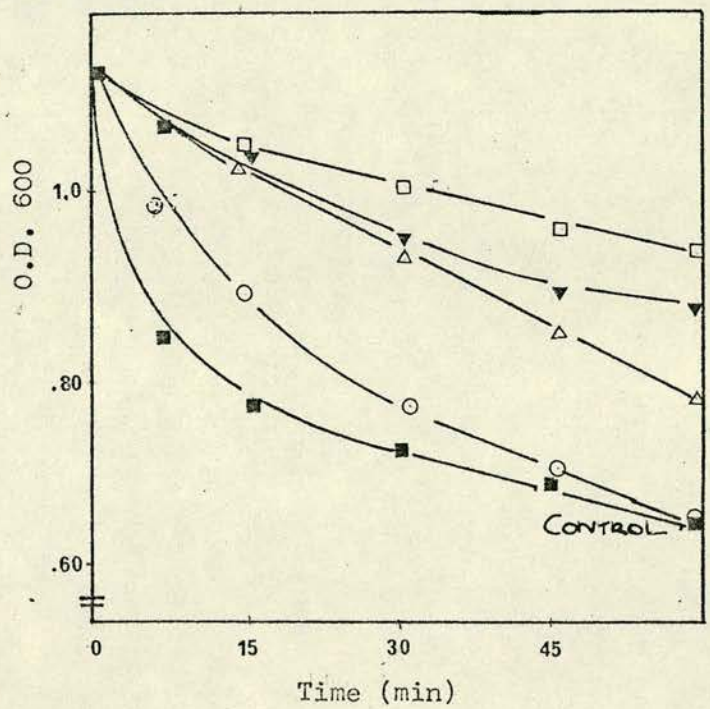


FIG. 46

Protection of strain PAO 579 by divalent ions against EDTA induced lysis grown in nutrient broth. The cations Mg^{2+} (▼) Ca^{2+} (□) Cu^{2+} (△) Mn^{2+} (○) were at 2.5mM. Other details are described in the legend to Fig. 45.



previously (Brown and Melling, 1969). Consequently it was decided to investigate this to determine if the sensitivity of these strains towards EDTA, SDS or deoxycholate was changed by growth in a medium containing divalent ions. The medium chosen was nutrient broth containing (gl^{-1})K_2HPO_4 2.2, $NaHPO_4$ 0.8, $MgSO_4$ 0.3, 1ml stock trace elements. The methods used were the same as stated previously, various mucoid and non mucoid strains were used.

With EDTA sensitivity the wild type, 381 was most sensitive with both mucoid and non mucoid strains showing a slight increase in resistance (Fig. 47). The only large increase in resistance to EDTA was shown by strain 553.

The effect of SDS on these strains is shown in Fig. 48. All strains tested (both mucoid and non mucoid) were more resistant than the wild type, although the exact degree of resistance varied.

With deoxycholate sensitivity the mucoid strains showed a similar degree of sensitivity to the wild type, the non mucoid strains 554 and 552 were more sensitive while 553 was more resistant (Fig. 49). In these strains the cell surface changes are probably relatively minor.

With the PsB strains the mucoid strain was highly sensitive to all three surface active agents while the non mucoid strain was totally insensitive (Fig. 50).

The role of the outer membrane as a selective barrier against antibiotics has been well documented (e.g. Gilleland, 1977). Consequently the sensitivity of mucoid and non mucoid strains towards various antibiotics was investigated.

An overnight culture of nutrient broth was diluted 1:100 and 0.05 ml was added to a quarter ounce vial containing 2 ml of nutrient broth containing varying concentrations of antibiotic. The vials were incubated at 30°C with shaking for 24h and the minimal inhibitory

FIG. 47

Effect of EDTA on PAO strains grown in salts medium. Both mucoid 566 (○) 579 (●) and non mucoid strains 381 (●) 552 (○) 553 (□) 554 (▲) controls (■) were tested. Other details are described in the legend to Fig. 40.

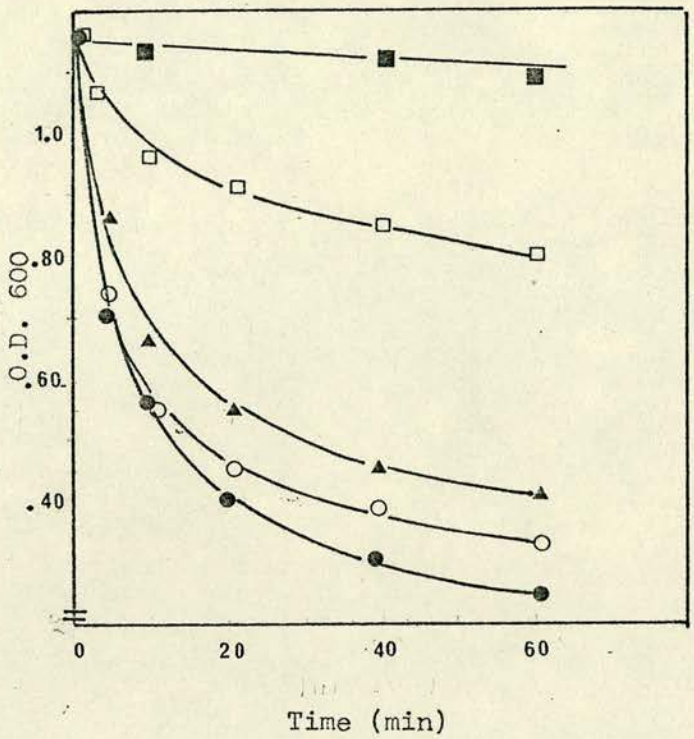


FIG. 48

The effect of SDS on PAO strains grown in salts medium. Both mucoid 566 (○) 579 (○) and non mucoid strains 381 (●) 552 (▲) 553 (□) 554 (▲) control (■) were tested. Other details are described in the legend to Fig. 40.

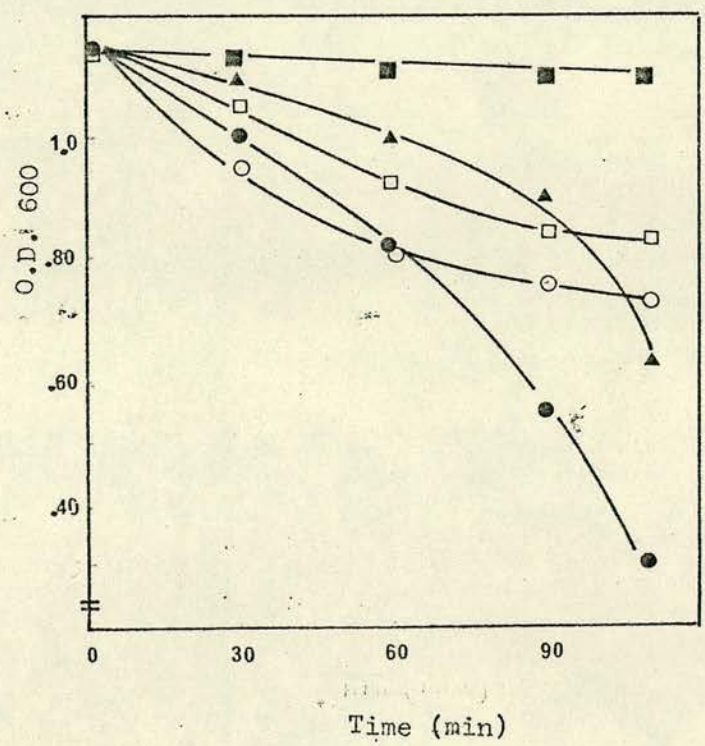


FIG..49

The effect of deoxycholate on PAO strains grown in salts medium. Both mucoid 566 (O), 579 (Ø) and non mucoid strains 381 (O) 552 (■) 533 (▲) and 554 (□) controls(◕) were tested. Other details are described in the legend to Fig. 40.

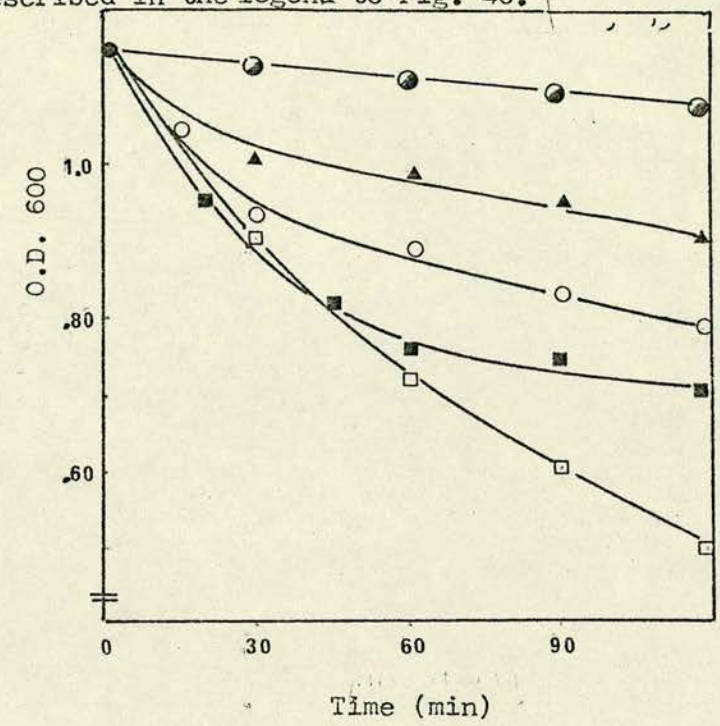


FIG. 50

The effect of EDTA, SDS and deoxycholate on PsB wild type and PsB non mucoid grown in salts medium. PsB non mucoid (■), PsB wild type with SDS (●) or deoxycholate (◕) and PsB wild type with EDTA (O). Other details are the same as the legend to Fig. 40.

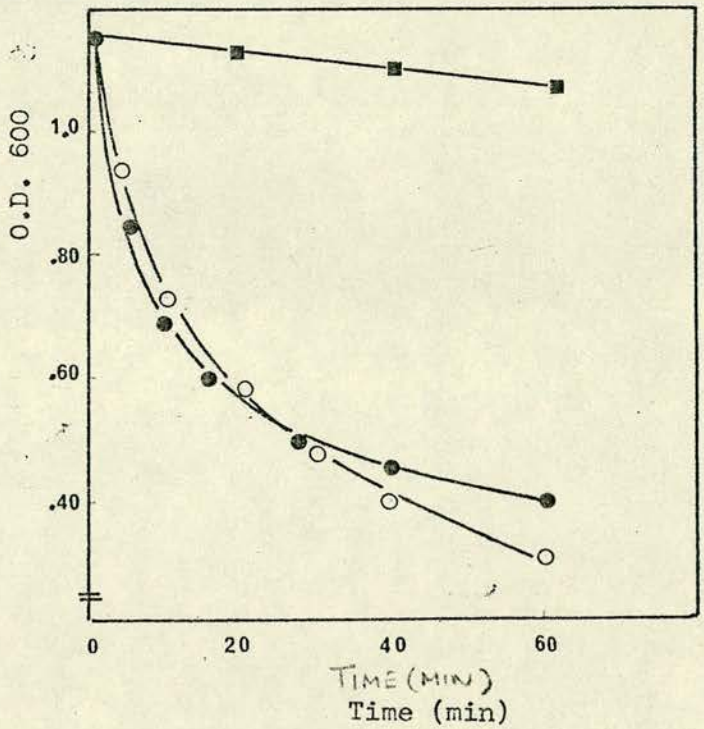


TABLE 23 Minimal inhibitory conc. of antibiotic required to totally inhibit bacterial growth of PsB strains and PAO strains 381 and 579. An overnight culture of bacteria was diluted X100 and 0.05 ml added to 2 ml N.B. + antibiotic and incubated at 30°C for 24h.

	PsB WILD TYPE	PsB NON MUCOID	PAO 381	PAO 579
CARBENICILLIN	15	100	60	100
POLYMYXIN B	4	20	4	5
TETRACYCLINE	10	30	8	6

TABLE 24 Minimal inhibitory conc. of antibiotic required to totally inhibit bacterial growth of non mucoid PAO strains. Other details are described in the legend to Table 23.

STRAIN	TETRACYCLINE	CARBENICILLIN	POLYMYXIN B
549	8	80	4
551	8	100	4
552	10	100	7
553	8	80	4
554	10	70	6
555	10	70	4

concentration (MIC) of antibiotic was taken as the lowest dilution of antibiotic which totally inhibited growth. Fyfe and Govan (1978) described an increase in resistance of the mucoid strains to three antibiotics and a decrease in resistance to tetracycline in studies using an agar dilution technique whereas this study used a liquid dilution technique.

The production of alginate by PAO 579 led to an increase in resistance to carbenicillin and a reduction in resistance to tetracycline, as previously noted by Fyfe and Govan (1978b). The effect of polymyxin on both the mucoid strain and its non mucoid parent was the same (Table 23). The non mucoid strains derived from the mucoid strains were studied next, these results are summarised in Table 24. For two strains losing the ability to synthesise alginate led to an increase in resistance towards carbenicillin over the mucoid strain (551, 552). The other 4 strains all became more sensitive than the mucoid strain although slightly higher than the wild type strain 381. With tetracycline there was a decrease in resistance back to the parental (non mucoid) level for three strains (549, 551, 553), while the other three strains showed an increased resistance. With polymyxin only a slight increase in resistance was found with two strains (552, 554). No clear pattern emerged from this study and only one strain 552 showed an increase in resistance towards all 3 antibiotics.

With the PsB strains the non mucoid derivative showed increased resistance to all three antibiotics, varying from seven fold for carbenicillin to five fold for polymyxin and to three fold for tetracycline (Table 23).

Another method chosen to distinguish differences in cell surface structure was the ability of lectins to agglutinate whole cells.

TABLE 25a Lectin agglutinability of mucoid bacteria.

1. Soya Bean agglutinin - SBA
2. Wheat germ agglutinin - WGA
3. Concanavalin A - Con A

Abbreviations:- 1. + agglutination in 5 min at 20°C (slide test)
2. ± weak agglutination in 5 min at 20°C
3. - no agglutination in 5 min at 20°C

STAIN	SBA[1]	WGA[2]	CON.A[3]
PsB	±	±	+
PAO 587	±	±	±
PAO 578	-	±	±
PAO 579	-	-	+

TABLE 25b Lectin agglutinability of non mucoid PAO strains

	SBA	WGA	CON A
PAO 381	-	-	+
549	-	±	+
551	±	-	+
552	±	-	+
553	-	-	+
554	+	+	+
555	+	+	+

Abbreviations are the same as in the legend to Table 25a.

Three lectins were chosen Conconavalin A (Con A, a protein obtained from the jack bean, Canavalia ensiformis) which reacts with glucosyl, manosyl or fructosyl residues. Soya bean agglutinin (SBA) binds to n-acetyl-D-galactosamine and to D-galactose, whereas wheat germ agglutinin (WGA) binds to linked n-acetyl-D-glucosamine. The lectins were used in slide agglutination tests at a concentration of 5 mg/ml (Tables 25A, B).

There was little difference in the behaviour of the mucoid strains tested towards all three lectins. The only lectin to cause a strong agglutination reaction was Con A and this only occurred with two of the four strains, PsB and PAO 579. Some of the weak agglutination responses could be due to a non specific interaction with the alginate and were not considered significant (Table 25a). However with the non mucoid strains this problem did not arise. Of the seven strains tested, five gave similar results i.e. only strongly agglutinating with Con A. Of these five strains, three did not agglutinate with SBA and only two strains weakly agglutinated with WGA. Strains 554 and 555 gave a strong reaction with all three lectins, suggesting that only these strains differ markedly from the others tested (Table 25b).

With these differences in sensitivity to surface active agents, antibiotics and lectins, it was thought necessary to examine the LPS, both structural and compositional analysis of the polysaccharide moeity (i.e. core and o-antigen region) was undertaken.

Lipopolysaccharides from PsB wild type and PsB non mucoid were prepared as described in Methods and totally hydrolysed. Paper chromatography in butanol:pyridine:water (6:4:3) of the neutralised hydrolysate indicated that the strains had the same component sugars. The major sugars (as estimated from the size of spots on a chromatogram) were glucose, another neutral sugar (possibly mannose), galactosamine and

TABLE 26 Analysis of sugar components of PsB non mucoid LPS
Sugars were identified by paper chromotography.

Presumed O-antigen	Core region
Rha	GLc
	Rha
Amino sugar	Hep
Glucose	$GalNH_2$
	Man

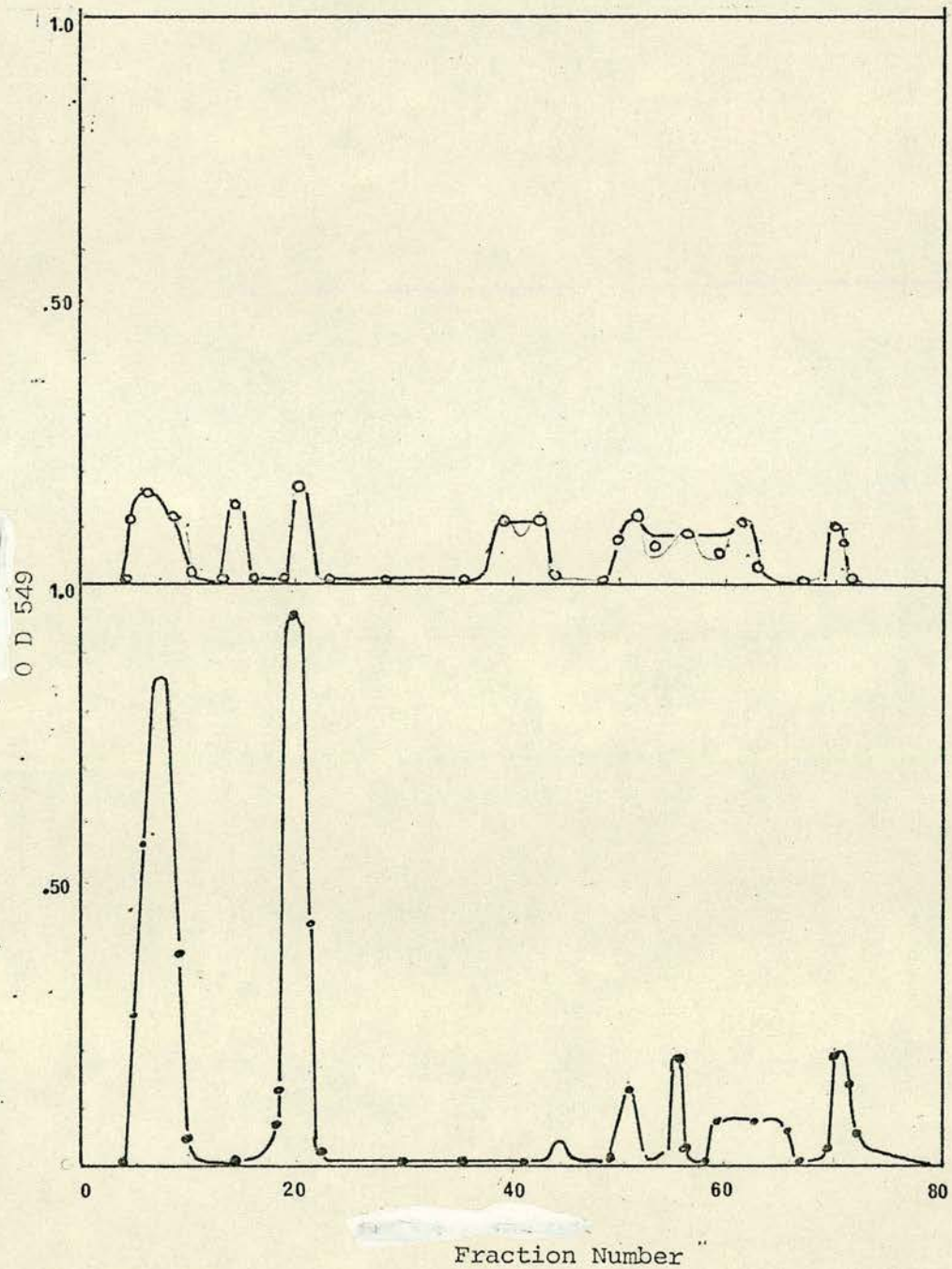
TABLE 27 Approximate compositional analysis of PsB wild type and non mucoid LPS

		Approximate Composition				
	% LPS (dry wt)	Amino	Glc	Man	Rha	$GalNH_2$
PsB wt	0.5	N.D.	2	2	2	1
PsB nm	2.0	3	3	2	3	1

a $GalNH_2$ was taken as being equal to 1.

N.D. - not detected.

FIG. 51 Fractionation of polysaccharide obtained from PsB wild type (A), PsB non mucoid (B) after hydrolysis with 1% acetic acid. The hydrolysate was eluted from a column (15 x 1.5 cm) with pyridine/acetic acid pH 5.4 on sephadex G-50. Fractions (4ml) were collected and 0.25 ml analysed for carbohydrate.



an unknown amino sugar with a small amount of rhamnose and KDO present. The component sugars of the PsB non mucoid strain was the same as the strain but in the wild type the unknown amino sugar was absent.

Partial hydrolysis of the LPS with 1% acetic acid separated the lipid A from the polysaccharide, the former was removed by centrifugation. Both PsB strains gave a clumpy precipitate of lipid A. The polysaccharide was obtained by freeze drying; the yield of polysaccharide obtained varied from 40-50% of the LPS.

In order to analyse the component sugars of the peaks the procedure was scaled up so that 5 mg of polysaccharide could be applied to a 25 x 1 cm column of Sephadex G-50. Four ml fractions were collected and 0.25 ml analysed for carbohydrate by the Dubois method (1955). The elution profiles are shown in Fig. 51.

The fractions corresponding to high molecular weight (presumed O-antigen) and low molecular weight (presumed core region) were pooled and freeze dried. The qualitative analysis of these peaks are shown in Table 26. The LPS isolated from both PsB strains contained glucose, a neutral sugar (mannose?), heptose, rhamnose, galactosamine, KDO and alanine. The non mucoid strain contained at least one additional amino sugar.

Chromatographic analysis of the polysaccharide fraction showed that the PsB wild type lacked most of the O-specific side chains. The component sugars of the core and O-antigen were similar to those previously studied by Chester et al (1973). In the study by these authors various other amino sugars were also present, e.g. glucosamine phosphate, amino-galacturonic acid and fucosamine. As these sugars were not estimated and the fact that they are labile during acid hydrolysis the compositional data is only very approximate (Table 27.)

DISCUSSION

BATCH CULTURE

Of the two species of Gram -ve bacteria which synthesise alginate most of the previous studies have been performed using Az. vinelandii. The alginate synthesising Pseudomonad strains used in previous studies were isolated from patients with cystic fibrosis or from patients with urinary tract infections. This was the case with Pseudomonas aeruginosa strain B but the mucoid strains isolated from PAO 381 were not from a clinical source. These were isolated from a non mucoid strain (Fyfe and Govan, 1978b) using a carbenicillin selection technique. Since a non clinical strain posesses the genes coding for alginate synthesis this implies that other strains of Pseudomonas including strains other than Ps. aeruginosa could also contain these genes.

The nature of the polymer produced by the strains tested was shown to be an alginate-like polysaccharide by I.R. spectroscopy and the action of an alginate lytic enzyme. Previous analysis of the polymer produced by clinical strains (Evans and Linker, 1973) showed a wide variation in the degree of acetylation, mannuronic to guluronic acid ratio and molecular weight. The O-acetyl content of the polymer was found to be proportional to the mannuronic acid content, from this the authors inferred that the mannuronic acid residues were acetylated. In the case of the alginates isolated from the PAO strains a wide variation in acetylation, viscosity occurred but there was a smaller variation in the mannuronic to guluronic acid content. When grown in batch culture the PsB strain produced a high viscosity, highly acetylated polymer which contained only mannuronic acid. Continuous fermentation in a glucose minimal medium led to an increase in the degree of acetylation and guluronic acid content but the viscosity of the alginate was similar to the batch produced polymer.

For the alginates isolated from the PAO strains a direct relationship

between the viscosity and the degree of acetylation was found which was linear over the range 2.3-8.6% and after this displayed a saturation effect. This could be explained by the O-acetyl moeity opening sites on the polysaccharide chain by which interactions (possibly ionic or hydrogen bonding) between adjacent chains could occur. The saturation effect could then be explained by only a limited number of sites being available for interaction.

No attempt was made to determine the viscosity of deacetylated alginates as the procedures available (either 0.3M NaOH at room temperature for 60 min. or ammonia solution at 60°C for 60 min) will effect the viscosity by removing ions or other impurities present.

An important characteristic of some of these Pseudomonas alginates is their high viscosity which is indicative of the absence of an alginate lyase. The presence of such an enzyme in Az.vinelandii was described by Haug and Larsen (1971a). The function of the lyase is unclear but it does not have a function in the assimilation of alginate by the bacteria as Az. vinelandii cannot grow using alginate as a sole carbon source (T. R. Jarman, personal communication).

The effect of ions on the rheological properties of aqueous polysaccharides is very important. The presence of salt (either NaCl or $MgCl_2$) lowering the viscosity of dilute solutions of polyelectrolytes is relatively common (e.g. Sandford, 1977). This effect is largely attributed to the polyelectrolyte effect, that is the ionic strength of the medium causes a conformational change in the polysaccharide chain. This is due to the ions reducing the repulsion between adjacent charges on the polymer chain and its conformation changing from an extended configuration to that of a random coil. In general salt solutions are poorer solvents for water soluble polysaccharides, consequently the viscosity of dilute solutions are lower in salt solutions.

Examination of the molecular size distribution of alginates produced by the PsB and PAO strains indicated that for individual strains the chain length was uniform for that particular strain. In contract to the commercial algal sample tested (Mannutex RF) and those algal samples described by Bucke (1974) which are polydisperse.

Batch culture of the PAO strains indicated that in all cases studied, except one, polymer production was not concommitant with growth. Polysaccharide production by these strains commenced during late exponential phase and continued maximally during the stationary phase of growth. Studies by Williams and Wimpenny (1977) using Pseudomonas NCIB 11264, Unz and Farrah (1976) using Zoogloea MP6 and Eagon (1956) with Ps. Fluorscens showed a similar type of behaviour. Dudman (1964) using Rhizobium melitoti showed that cell growth commenced more rapidly than polymer production, and that polymer was not produced at the same rate throughout the growth cycle.

In Xanthomonas campestris (Moraine and Rogovin, 1973), Az. vinelandii (Haug and Larsen, 1970) and Arthrobacter viscous (Cadmus et al, 1963) polymer production occurred throughout growth, this was observed with the PAO strain 587. The rates of synthesis of alginate were also estimated and these were found to vary from 500 μg mg dry $wt^{-1}h^{-1}$ to 1700 μg mg dry $wt^{-1}h^{-1}$ for PA0568 and 579 respectively. The strain which produced the highest levels of alginate, 579, also had the highest rate of synthesis.

The production of polysaccharide during late log and early stationary phase of growth has not been satisfactorily explained. However Sutherland (1977) has suggested that this could be explained by the availability of isoprenoid lipid. A considerable amount of indirect evidence obtained by numerous authors (see Sutherland, 1977) indicate that peptidoglycan synthesis has priority over LPS synthesis

which in turn has priority over exopolysaccharide synthesis. Direct evidence of this limitation other than by preparative studies is difficult to obtain due to the absence of a direct and accurate assay, for isoprenoid lipid. This is possible in Gram +ve bacteria as the isoprenoid lipid can be labelled using radioactive mevalonic acid but Gram -ve bacteria were unable to take up this compound.

Polysaccharide synthesis by the PsB strain is different from the PAO strains. If log or early stationary phase cells are used as an inoculum, polymer synthesis is not growth associated. However if late stationary phase cells are used as inoculum (where alginate synthesis is actively occurring) polymer synthesis is growth associated. This implies that some irreversible arrangement of components involved in alginate biosynthesis occurs in late stationary phase cells. If alginate synthesis is growth associated a decrease in the growth rate and in the rate of synthesis of alginate is observed. This result is expected as precursors would have to supply both the systems involved in cell wall synthesis and those for exopolysaccharide synthesis. No such comparison can be made with the PAO strains as the rate of synthesis of the polymer varies considerably. However, the growth rate of PAO 587 is reduced in comparison to those strains which produce alginate at the end of log phase.

Enzyme analysis of the PsB strain indicated that the enzymes involved in alginate biosynthesis are present in log phase cells. This implies that polymer production is controlled through regulatory mechanisms affecting precursors rather than through altered enzyme activities. One of the key enzymes in alginate synthesis, GDP-Mannose dehydrogenase, was significantly higher in log phase cells than in stationary phase cells. Since this enzyme was found in membrane free extracts this indicates that some rearrangement in the bacterial cell membrane before

polysaccharide synthesis can occur.

To determine the optimal medium for polysaccharide synthesis one strain, 568, was grown in a variety of different media. Polymer production occurred in all media tested and the optimal medium for polysaccharide formation was found to be yeast extract containing 2% gluconate. In the media studied, the final pH varied considerably from pH 5-8.5 which would affect polymer synthesis especially in the former case. This strain only produced a small amount of alginate when grown within GG medium (supplemented with 200 mg/l of leucine) in which the PsB strain produced copious amounts of polymer.

A similar type of behaviour was seen in another study using alginate-synthesising strains of Ps. aeruginosa (Evans and Linker, 1973). Using solid media, alginate was produced under all cultural conditions and the optimal medium for polysaccharide production was found to be Haynes potassium gluconate medium. In another study using Ps. aeruginosa IFO 3445 the nutritional requirements were investigated using a cellophane plate method (Goto et al, 1975). Polysaccharide was produced under all cultural conditions and the optimal medium was found to be 2% sodium glutamate and 2.5% glucose; using these conditions the pH did not fall in the manner noted for the strains tested here.

The control of pH near neutrality has been frequently shown to enhance polysaccharide production (Harada, 1969; Deavin, 1976). This was observed with the strains tested, as the pH infrequently rose above pH 7.6 when grown in 2% gluconate. Optimum conditions for polymer synthesis have been shown to be maintained in cultures when the pH was controlled using buffers, e.g. Tris-$CaCO_3$ (Ameura and Harada, 1971) or phosphate buffer (Cadmus et al, 1963). In contrast to this pullulan synthesis by Aureobassidium pullularia is highest at an acidic pH (Catley, 1971).

In another series of experiments carried out using the PAO strains the effect of growing the strains at 30°C and 37°C in yeast extract 2% gluconate was studied. A wide variation in the levels of alginate and in the alginate to biomass ratios were observed. Some strains produced significantly more polymer at 37°C than at 30°C and with other strains this was reversed; the reason for this behaviour is unknown. The strain which produced alginate concommitantly with cellular growth (587) also produced 50% more polysaccharide at the higher temperature than at 30°C.

Harada (1969) using Alcaligenes sp. found that polysaccharide production was enhanced at a temperature below the optimum for growth. Using Cryptococcus laurentii var. flavescens Cadmus et al (1961) found that the optimum yield of polymer was produced at 25°C or below but this decreased at 38°C and 32°C.

In the initial studies using PsB a 10% inoculum was used which was later reduced to 3%. Consequently the effect of inoculum size on polysaccharide production was investigated. The amount of alginate produced in GG medium was independant of the inoculum size over the range 1-7.5%. At the 10% inoculum level a significant increase in the level of alginate was observed.

With the PAO 579 strain a linear increase in the levels of alginate occurred over the inoculum range 1-7.5% with a slightly larger increase occurring at the 10% level. At the 0.5% level the same levels of alginate were observed as at the 7.5% level.

This type of behaviour is different from that observed by Cadmus et al (1961) using Cryptococcus laurentii var.flavescens who found that decreasing the inoculum size below 5% caused a drop in the yield of polymer, but increasing the level above 5% had no effect. Deavin (1976) found that with Az.vinelandii the largest alginate:biomass ratio could be

achieved using a low inoculum size in a low phosphate Burks medium.

In the experiment described above the PsB strain was grown in nutrient broth before inoculation into GG medium. The polysaccharide levels produced were not affected in any way but the level of biomass was elevated 3-fold. This indicated that the cells were pre-adapted in some fashion by growth in different media prior to inoculation. In order to determine if the PAO strains could be similarly pre-adapted a series of experiments were performed using different media to grow the inoculum in. The highest level of alginate were found in the BM flasks inoculated with cells which had been grown in yeast extract medium and the lowest level in cells which had been grown in nutrient broth. Not only did the levels of alginate change but the nature of the polysaccharide precipitate also changed, in some cases a fine powdery ppt was observed and in other cases a fibrous ppt of alginate was noted.

In summary the conditions for optimal polysaccharide vary from species to species and optimal conditions for one strain need not be the same for another strain. It seems that the only similarity between these PAO strains is that they produce an acetylated polyuronide. With every aspect studied different strains varied considerably although they were all derived from the same parental strain.

Continuous culture

When mucoid strains of Ps. aeruginosa were grown in an ammonia limited chemostat culture, small colony variants arose spontaneously. The time of appearance of these small colonies from the onset of continuous growth varied from 1.5-4 generations. These mutants appeared at a high rate which led to a rapid displacement of the parental

type indicating that some of these variants possessed a competitive advantage over the wild type.

This type of dissociation has been described since the early work on the species (Wahba and Darrell, 1965; Zeidt and Schmidt, 1964; [illegible] Many phenotypic changes may occur including colonial morphology, phage and antibiotic resistance, antigenic properties, aerogicin susceptibility and pigment production. These small colonies were also slow growing on nutrient agar plates and probably similar to dwarf colonies described by Shionoya and Homma (1968). The high frequency of these changes 5.8×10^{-3} mutants h^{-1} $cell^{-1}$ (mucoid to non-mucoid in the case of PsB) and 7.5×10^{-4} mutants h^{-1} $cell^{-1}$ (large mucoid to small mucoid in the case of PAO 579) suggests a genetic mechanism such as a deletion of a short region of chromosome or the loss of a plasmid has occurred, rather than a point mutation. This high frequency is 10^{2}-10^{3} higher than the spontaneous mutation rate of $10^{-6} - 10^{-7}$. These variants exhibited altered colonial morphology and were not tested for any changes in other phenotypic changes described earlier. The mucoid PAO 579 showed a similar high rate of dissociation to a small mucoid varient. However no non-mucoid colonies were isolated after 150h continuous growth. In this case little alginate synthesis was occurring and consequently the non mucoid strain would not have such a competitive advantage over the mucoid strain as seen in the PsB strain. It seems probable that the type of dissociation which occurs is dependant upon the physiological state of the cell.

In the ammonia limited chemostat culture of PsB wild type the ability to synthesise alginate was lost rapidly. A linear decrease in the level of alginate was observed, concomitant with this was a linear increase in the level of biomass. Ultimately a mutant with an increased yield of biomass was selected. The yield of biomass obtained from the

wild type was 1.08 gl^{-1} compared with 2.02 gl^{-1} from the non-mucoid, an increase of 87%. The maximum theoretical yield of biomass from the 0.6g $(NH_4)_2SO_4$ present is 1.26 gl^{-1}. As Pseudomonads do not produce any storage compound e.g. polyhydroxy β -butyrate or glycogen it seems likely that more cell wall material is being synthesised or that some alginate is produced and is closely bound to the cell surface. However using the India ink technique of Duguid (1951) the absence of capsular material was demonstrated.

Using batch culture changes in the cell surface were indicated by the difference in sensitivity of these two strains towards surface active agents and antibiotics and differences in LPS structure.

A non mucoid varient was isolated from the end of this experiment, grown overnight in GG medium and used as an inoculum for another fermentation and shake flask culture, alginate production occurred in both cases. In the chemostat culture a high yield of biomass was obtained at the steady state, 81% higher than the wild type but the viscosity of the culture and the yield of alginate was reduced. Batch culture of this isolate for 48h at 30°C gave the same yield of alginate as the wild type but the yield of biomass was greatly increased. This indicated that the phenomona was partially reversible, although when another non mucoid colony was isolated from the end of the fermentation there was no production of alginate even in GG medium. This indicated that this strain still contains the genes for alginate synthesis but they are not expressed under certain cultural conditions.

Genetic analysis of the PsB strains was impossible as clinical strains are only infrequently amenable to genetic studies. Also the presence of alginate inhibits conjugation in strains which can conjugate in its absence (J. Govan, personal communication). Consequently the non mucoid strain could not be classified into sup^+ or sup^- (as is

possible in the PAO strains) or to determine if the GDP-Mannose utilising enzymes were present (see the section on enzyme levels).

Growth of the PsB non mucoid strain in batch culture using yeast extract/gluconate medium indicated that of the enzymes involved in carbohydrate metabolism only gluconokinase showed an increase over the wild type level. All the other enzymes assayed showed a significant reduction in their levels. This indicated that in this case the flow of carbon through the extracellular pathway is repressed and carbon is being brought directly into the cell as gluconate.

Cultural degeneration of mucoid strains of various species of bacteria is by no means a rare phenomana. Small colony variants of Xanthomonas campestris NRRL B-1459 (Cadmus et al, 1976) and Xanthomonas phaesoli (Corey and Starr, 1957a, b) have been described.

Cadmus et al described variant colony types which were smaller in size than the wild type. The mutation rate of the wild type to the small variant was diminished when sufficient glucose was added to avoid depletion. When the variant was grown in yeast malt medium the cultural viscosity was reduced from 7000 cp to 4000 cp. The percentage yield was also reduced from 63% to 43% the small variant also produced polysaccharide with lower pyruvate and O-acetyl substituents.

Corey and Starr (1957a) described four colony variants of X.phaseoli XP 104, rough, smooth, semi-mucoid and mucoid. Both colony size and production of polysaccharide increased from rough to mucoid. No attempt was made to analyse the polysaccharide produced by these mutants. Growth in a glucose minimal medium which was supplemented with 0.5% yeast extract gave rise to 1% non mucoid colonies after 48-72h incubation at 30°C. The same authors (Corey and Starr, 1957b) were able to demonstrate that transformation of colony type occurred, indicating the genetic nature of these changes.

The genetic instability of Alcaligenes facaelis var. myxogenes 10C3 has been reported by Amemura (1977). This strain produces large quantities of succinoglycan with small amounts of curdlan being present. When stocked on nutrient agar slopes mutation occurred in the absence of cell division leading to the increased synthesis of curdlan, which became the major polymer synthesised. The different colonies were detected on plates by their ability of curdlan producing colonies to stain with analine blue. Treatment with mutagenic agents, e.g. NTG, EMS or UV increased the mutation rate. Mutant strains were stable and did not revert for up to 12 months when stocked on nutrient agar slopes.

Although the mutation rate observed for the PsB strain is high, a much greater frequency of segregation of variant colony types was described in Bacteroides thetaiotaomicron (Burt et al, 1978). The observed frequencies varied from 1.1×10^{-2} (from grey to white clone) to 1.4×10^{-2} (white to grey clone) at 37°C. At 42°C the segregation frequency decreased to 10^{-3} and 3×10^{-3} respectively. Burt also described a correlation between colony morphology, encapsulation, Giemsa staining and phage resistance.

Due to the instability of mucoid strains in continuous culture both biosynthesis and cell surface studies were restricted to batch culture.

Biosynthesis experiments

The pathway for the biosynthesis of alginate in Ps. aeruginosa appears to differ very little from the reaction sequences proposed in both Az.vinelandii (Pindar and Bucke, 1974) and Fucus gardneri (Lin and Hassid, 1966b). The specific activities of the enzymes which could be assayed in crude extracts were higher than those found by

Pindar and Bucke (1974) due to the higher rate of synthesis. The major difference is that, in common with Az. vinelandii, these strains probably possess an extracellular epimerase. This implies that the formation of guluronic acid occurs at the polymer level. In Fucus gardneri a nucleotide sugar GDP-Gulose was identified as a precursor of algina synthesis. This work was placed in doubt by the detection of an epimerase in another algal species, Pelvetia canaliculata (Madgwick et al, 1973). In common with the epimerase of Az. vinelandii (Haug and Larsen, 1971) the conversion of mannuronic to guluronic acid was stimulated by Ca^{2+} ions, although the activity was low in the strains studied. The polymer is modified by C5 epimerisation, which is probably irreversible as no epimerisation of guluronic acid residues has been detected (Haug and Larsen, 1971).

Another possible function of the epimerase in encystement of Az. vinelandii was described by Page and Sadoff (1975). They reported that the uronic acid composition of the cyst is dependant upon the Ca^{2+} levels in the cultural medium. In the intine (inner coat) of the cyst high mannuronic acid levels were found while in the exine (outer coat) higher levels of guluronic acid were detected. Epimerase activity was detected in the mature cyst body and the encystment culture fluid. As the enzymic activity is dependant upon the levels of Ca^{2+}, insufficient Ca^{2+} may be available for enzyme function in the intine.

As the epimerase has two probable functions in Az. vinelandii this may be the reason why the enzyme is present in greater quantities than in Ps. aeruginosa.

Epimerisation at the polymer level is unknown in other bacterial systems but it does occur in two eukaryotic systems. In dermatan sulphate synthesis L-ioduronic residues are epimerised to D-glucuronic acid residues (Frannson et al, 1973). In heparin formation D-glucuronic acid residues are epimerised to iduronic acid residues (Lindhal

et al, 1972).

The other difference between algal and bacterial alginate synthesis is the presence of acetyl groups in the latter. In Az. vinelandii the acetyl groups are found in association with the mannuronic acid residues (Davidson et al, (1977). It has been suggested in Ps. aeruginosa acetyl groups are similarly associated with mannuronic acid residues (Evans and Linker, 1973), however the exact site of acetylation in this polymer is unknown. The stage at which acetyl groups are added to the polymer, either at the monomeric (lipid intermediate) or at the polymeric level is also at present unknown. The exact mechanism of formation of the three block types of alginate is also at present unknown. However the speficity of the epimerase must involve the recognition of the nearest neighbour to the residue which is to undergo epimerisation.

A possible mechanism of control of epimerisation has been suggested by Davidson et al (1977). If aceylation precedes epimerisation, acetyl groups may play a role in controlling which portions of the molecule are susceptible to the action of the epimerase. If this hypothesis is correct polymers having a low acetate content would be expected to have a high guluronic acid content and vice versa. This is not observed as all the strains produced a polymer with a high mannuronic acid content. As the method used to estimate guluronic acid is semi-quantitative no firm conclusions can be drawn from the data other than no poly G blocks occur in the polymer produced by these strains.

In the study of enzymes involved in the biosynthesis of alginate by PAO strains similarities occurred to the biosynthesis of colanic acid by E.coli K12. Extensive studies by Markovitz and colleagues have

led to the elucidation of a number of the regulatory mechanisms in colanic acid synthesis (Markovitz, 1977).

Various sites on the chromosome of E.coli K12 were mapped in which a mutation gave rise to the mucoid phenotype. The wild type non mucoid allele was designated $capR^+$ and the mutant allele CapR. This site capR (lon) is linked to proC as determined by transduction with phage P1. Another site capS which is epistatic to $capR^+$, mapped near trp (Markovitz and Rosenbaum, 1965). A derivative of capR designated capR6 was also discovered. In the capR gene it displayed a different phenotypic expression in partial dipliods. Another mucoid locus was designated capT; strains carrying this mutation are mucoid on EMB glucose at 37°C whereas capR and capS are not mucoid. This site was not precisely mapped although it was not linked to proC by transduction or to trp using conjugation studies (Markovitz, 1977).

In a series of studies which assumed that $capR^+$ specified for a cytoplasmic repressor protein and $capR^-$ produced an inactive or no repressor protein, the enzymes involved in colanic acid synthesis were studied. The first mucoid strains tested by Markovitz (1964) in a glucose minimal medium contained derepressed levels of UDP-galactose-4-epimerase and the enzymes involved in conversion of GDP-Mannose to GDP-fucose. In further studies using capR strains, ten of the enzymes involved in colanic acid synthesis were found to be derepressed between two and twenty fold. The observed pattern of derepression were different for each mutation, capR, capS and capT. Two of the most widely studied strains were capR6 and capR9 which showed different phenotypic expression of enzyme levels in partial diploids. Derepression was greatest in the enzymes tested in capR9, capR9 also produces UDP-glucose pyrophosphorylase almost as well as 37°C as at 23°C, whereas capR6 produced much less enzyme at 37°C. CapR6 produced

colanic acid at 37°C than do capR9 strains. Both capS and capT were derepressed for UDP-glucose pyrophosphorylase but the effect of temperature on growth was reversed compared to capR9.

CapR9, capS and capT were shown to be involved in the regulation of UDP-Glucose pyrophosphorylase synthesis. The galactose operon was shown to be derepressed in capT although this was not required for colanic acid synthesis (capS) but does occur (capR and capT). Both capR and capT were shown to function along the same metabolic pathway and to affect a single target site on the galactose operon.

Previous genetic analysis of the Ps.aeruginosa PAO strains by Fyfe and Govan (1978) demonstrated that a mutational event occurring on the chromosome preceeds alginate synthesis. This would imply that a mutation occurs in a regulatory gene which controls the synthesis of key enzymes in alginate biosynthesis. Analysis of the enzymes levels in the wild type non mucoid strain indicated that the strain contained all the genetic information required to produce alginate but was not expressed.

Kang and Markovitz (1967) were able to find concentrations of FPA which caused the derepression of enzymes involved in colanic acid synthesis in the wild type strain. This provided further evidence for the protein nature of the repressor molecule. Further evidence to support this was provided by demonstrating that t_s mutants in the lac repressor or alkaline phosphortase phoS gene could be derepressed at low temperature with FPA. Attempts to derepress both the enzymes involved in alginate synthesis and β-galactosidase in the wild type were unsuccessful. This is not surprising as Pseudomonas sp. are notoriously resistant to both metabolic analogues and antibiotics. Also the methods which work in E.coli are not always applicable to

Ps.aeruginosa. These experiments also require the use of a minimal medium in which little alginate synthesis occurs. It would seem that repression will have to be demonstrated genetically.

The genetic study by Fyfe and Govan (1978a) indicated that a chromosomal mutation could occur at two different sites to give rise to the mucoid phenotypes which have been designated algA and algB. The exact site of the mutations is unknown at present but they map near the cis-his region of the chromosome (12 min) Analysis of the enzymes involved in alginate synthesis in mucoid strains which map at algA (PAO 579) and algB (566, 585) showed derepression. No clear pattern of enzyme levels emerged from the study. All strains tested showed an increase in the level of phosphomannose isomerase. Those strains in which a nucleotide hydrolase-free extract could be prepared showed a significant increase in both GDP-mannose utilising enzymes.

The attempted isolation of GDP-mannose dehydrogenase was unsuccessful due to the lability of the enzyme. Preiss (1964) isolated the corresponding enzyme from Arthrobacter which was stable for one month at $-10^{\circ}C$ if stored at a protein concentration > 1 mg/ml. Storage of the Pseudomonas aeruginosa enzyme at $-20^{\circ}C$, overnight dialysis at $4^{\circ}C$ or standing (as the crude extract) at $4^{\circ}C$ led to inactivation. Both enzymes had a similar pH optima at approximately pH 8.3.

In a study of enzyme levels involved in exopolysaccharide synthesis in Klebsiella aerogenes types 1 and 8 (Norval and Sutherland, 1973), the loss of the ability to produce exopolysaccharide had little effect on the specific activities of the enzymes studied. Some mucoid strains had reduced levels of enzymes involved in polymer production while specific activities of other enzymes were uneffected but in this strain there was no evidence for regulatory genes similar to those in E. coli or Ps. aeruginosa.

In another study of wild type and non mucoid strains of Xanthomonas campestris (C. Whitfield, personal communication) no significant differences in enzyme levels involved in xanthan synthesis were found under the conditions tested.

The presence of a nucleotide hydrolysing enzyme is another possible type of control by turnover of the nucleotide pool. The enzyme in Ps. aeruginosa appeared to be bound to the particulate cell fraction obtained after sonication and is thus similar to the nucleotide hydrolase found in Salmonella sp. (Glaser et al, 1967). Associated with this enzyme described by Glaser et al (1967) was 5' nucleotidase activity. A similar enzyme was described by Cercignani et al (1974) in Bacillus cereus.

Analysis of enzyme levels from non mucoid strains isolated from PAO 579 (algA) indicated that with respect to the enzymes involved in alginate synthesis both sup^+ and sup^- strains were the same. In these strains GDP-Mannose dehydrogenase and GDP-Mannose pyrophosphorylase were absent, although phosphomannose isomerase was still elevated. This would indicate that the sup^- are supressed but it cannot be demonstrated genetically. Sup^- strains also showed increased levels of certain enzymes involved in carbohydrate metabolism.

In the case of non mucoid strains from PAO 568 (algB) both sup^+ and sup^- strains were identical with respect to the enzymes of alginate synthesis. GDP-Mannose was again absent from these strains; phosphomannose isomerase was present at a similar level to the wild type strain or was absent. However GDP-Mannose pyrophosphorylase was elevated. Sup^- strains showed these changes in the levels of carbohydrate metabolism enzymes, although they were not as high as seen in the case of algA non mucoid strains. An increase in the flow of carbon along the extracellular pathway from gluconate to 2-oxo-gluconate and hence into the cell was suggested. The only similarity between these non mucoids from algA and

algB is the absence of GDP-Mannose dehydrogenase. The other enzymes of alginate synthesis could be regulated independently depending upon the initial site of mutation which gave rise to the mucoid phenotype.

Considerable evidence has been presented that catabolic flow through the cell is strongly influenced by the regulation of enzyme activity. The inducible glucose-6-P dehydrogenase which initiates the Entner-Dourdoroff pathway is significantly elevated in some algA non mucoid strains. Lessie and Neidhart (1967) found that high concentrations of GTP or ATP inhibited this enzyme by decreasing its ability to bind glucose-6-P. Another form of control of this enzyme was shown by Lessie and Vander Wyk (1972) in Ps. multivorans. Two forms of the enzyme were found, one form was active with NAD or NADP and inhibited by ATP and the other isoenzyme required NADP and was not inhibited by ATP.

Another form of regulation is by compounds which repress the synthesis of inducible enzymes and may act by repressing the synthesis of specific transport systems. This has been extensively studied by Dawes and colleagues using the inducible glucose transport system (see p. 22 for further details.) In sup$^-$ strains other enzymes which were derepressed were glucose dehydrogenase and glucose-6-P dehydrogenase. There appeared to be coordinate control between these two enzymes. The method by which the linkage of catabolic enzymes to sup$^-$ strains is achieved is unknown.

This type of behaviour is not consistent with the genes for alginate synthesis being present as an operon. This is not surprising as the genes coding for the enzymes of the biosynthetic pathways studied so far are more widely scattered than for the corresponding enzymes in E.coli (Fargie and Holloway, 1965). In the histidine biosynthetic pathway the genes coding for the enzymes occur in five separate trans-

duction groups in Ps. aeruginosa (Mee and Lee, 1969a). In E.coli these genes are present as a single operon.

The synthesis of biosynthetic enzymes in E.coli is normally repressed in the presence of the end product, while feedback inhibition provides the fine control which responds rapidly to changes in the concentration of the end product. Feedback inhibition of GDP-Mannose pyrophosphorylase by GDP-Mannose has been described previously in Salmonella sp. (Kornfeld and Ginsberg, 1966). This type of control appears to be absent, as in non mucoid strains derived from algA GDP-Mannose pyrophosphorylase is absent while in non mucoid strains from algB elevated levels are present. These results do not support the idea of sequential induction where the precursor substrate induces the synthesis of the first enzyme and its product induces the synthesis of the following enzyme (Stanier, 1947).

Repression of enzyme synthesis can be regarded as a coarse control which effects greater cell economy by conserving energy and substrates which would otherwise have been needed to make the enzymes. Thus alginate synthesis per se does not have the fine controls present in other biosynthetic pathways studied in Ps.aeruginosa, e.g. aromatic amino acids (Calhoun and Jensen, 1972). The absence of such controls might be a reflection of the infrequent synthesis as apparently only in a highly specialised environment (the CF lung or urinary tract infections) do strains naturally produce alginate. Despite the infrequent synthesis a positive selection pressure to retain these genes must be present. This lack of fine control could partially explain the wide variation seen in practically every aspect studied here. In theory, as alginate synthesis only requires the formation of one nucleotide sugar the system should be simpler than that observed in E.coli where four nucleotide sugars are required. The complexity observed is most likely a reflection of the genetics of the bacterium itself, e.g. the scattering of the

biosynthetic genes.

In summary there are several sites on the chromosome mutations in which lead to alginate synthesis. The regulation of the repression/derepression of the enzymes involved is complex but the pathway lacks the fine control mechanisms present in other biosynthetic pathways. Both sup^+ and sup^- non mucoid strains are similar with respect to the levels of alginate synthetic enzymes. The method by which sup^- non mucoid strains have elevated levels of certain carbohydrate enzymes is achieved is unknown.

Cell surface properties

The capR mutation has a number of pleiotrophic effects. It has been well established that capR and lon mutants are identical, i.e. mucoid and radiation sensitive (Markovitz and Rosenbaum, 1965) and arise from the same mutation. Reeve (1968) isolated mucoid and non mucoid strains which were resistant to low levels of tetracycline. The capR mutation also gives rise to increased resistance to puromycin and chloramphenicol. A second mucoid locus (not capS) increased sensitivity to puromycin. Hamelin and Chung (1975) isolated mucoid strains which were more sensitive to ozone. Other mucoid E.coli strains included those defective in the in vivo degradation of β-galactosidase polypeptide nonsense fragments (Bukhari and Zipser, 1973) and the ability of phages P1 and λ to replicate in the cytoplasm are impaired (Takamo, 1971). The reason for such diverse effects of a single mutation are at present unknown.

In the case of strain PAO 579 alginate synthesis led to an increase in resistance to carbenicillin and a decrease in resistance to tetracycline.

The sensitivity to antibiotics is more complex than stated here as mucoid strains have been isolated which have increased sensitivity to carbenicillin (J.Govan, personal communication). Mucoid strains were not more sensitive to uv radiation and are thus not similar to lon mutants (J.Fyfe, personal communication).

In general most PAO strains, whether mucoid or non mucoid, showed an increase in resistance to EDTA, SDS and deoxycholate when grown in salts deficient medium. Some exceptions to this did occur, notably the wild type strain which was more sensitive to these surface active agents. Thus these strains are similar to those previously studied by Brown and Melling (1969). The authors found that Mg^{2+} deficient medium caused cells to become more resistant to both EDTA and polymyxin EDTA causes the release of a complex of protein and LPS through a mechanism which involves the extraction of divalent ions which are bound to the cell surface. Roberts et al (1970) described this complex as being 60% protein, 30% LPS and 10% loosely bound lipid. Resistance to EDTA was correlated to an ultrastructural alteration in the outer membrane using freeze etching (Gilleland, 1977). It has been a consistant finding that cell envelopes resistant to both EDTA and polymyxin (Brown and Watkins, 1970) and carbenicillin (Thomas and Broadbridge, 1972) contain more lipid than sensitive envelopes.

The difference in antibiotic sensitivity together with changes in sensitivity to surface active agents (especially when grown in salts media) suggest that only slight changes in the cell surface of these PAO strains occur.

The PsB strains showed major differences in both antibiotic resistance and sensitivity to surface active agents. The mucoid strain showed an increase in resistance to surface active agents when grown in salts deficient medium. The non mucoid strain was only slightly

sensitive to SDS and deoxycholate in salts deficient media. In salts media this strain was totally resistant to these agents. The resistance of this strain to EDTA in both types of media suggests that when cations are present they are more firmly bound or are located at a deep site within the cell wall. Changes in the sensitivity towards these agents could be correlated with changes in LPS.

The selective binding of cations to cell suspensions of mucoid strains was demonstrated when the cells were treated with EDTA; this protected the cell against lysis. The binding of ions to the cell surface has been shown to be important by numerous workers. Sykes and Tempest (1965) showed that Mg^{2+}-limited cultures of Ps.putida were not permeable to glucose unless Mg^{2+} was present in the suspending media. This study with salt (especially Mg^{2+})-deficient media, the effect of osmotic shock and of EDTA suggest that Mg^{2+} is important for the integrity of the cell envelope. As well as membrane bound Mg^{2+}, much Mg^{2+} is loosely bound to the cell surface: in the case of Ps.putida this can be easily removed by washing with 0.9% NaCl (Tempest and Strange, 1966). The role of this loosely bound Mg^{2+} is unknown but the authors showed that in K.aerogenes polysaccharide synthesis was stimulated as well as an increase in resistance to starvation, heat accelerated death and cold shock. The function of both Mg^{2+} and Ca^{2+} is thought to provide a stiffening mechanism for lipoprotein membranes by forming salt bridges between COO^- groups (Brown, 1964).

One of the proposed functions of exopolysaccharides is the binding of cations (e.g. Harris and Mitchell, 1973). An important feature of ion binding properties of exopolysaccharides is their selectivity for certain ions. The ion exchange properties of algal alginates have been studied previously (Haug, 1959). Mannuronic acid was not selective

towards divalent cations whereas the guluronic acid residues were selective for cations in the order Cu > Ca > Sr > Mg. The difference in selectivity for cations shown by the PsB and PAO 579 strains could be due to the latter producing a polymer containing guluronic acid residues.

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