ALGINATE LYASES AND THEIR SUBSTRATES

Ian W. Davidson

A Thesis presented for the Degree of

Doctor of Philosophy

University of Edinburgh

1975

CONTENTS

INTRODUCTION

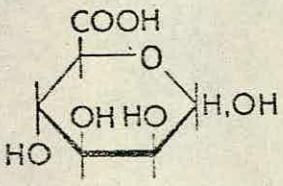
ALGINIC ACID

Alginic acid is the major constituent of the cell wall of the Phaeophycae, the algal family, which includes the familiar orders of seaweeds Fucales and Laminariales.
Located in the middle lamella and primary cell wall (Andersen, 1956) the alginic acid content of these algae is usually between 10 and 25% of the total dry weight. In general the alginic acid forms a smaller proportion of the total dry weight in periods of rapid growth than in colder months when little growth is taking place (Black, 1950).

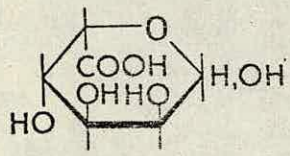
Alginic acid was first obtained from laminaria by Stanford (1883), a chemist concerned that better utilization could be made of seaweed than its burning for the recovery of iodine. More interested in the potential practical applications of alginate, the sodium salt of alginic acid, than its chemical make-up, Stanford considered the compound to be proteinaceous in nature. However, when pure, alginic acid was shown to be nitrogen free and to have the empirical formula of a polysaccharide (Hoagland and Lieb, 1915).

When a uronic acid containing polysaccharide is boiled with 12% hydrochloric acid the amount of carbon dioxide evolved can be stoichiometrically correlated with the amount of uronic acid present. Nelson and Cretcher (1929) showed that when alginic acid was treated in this way the carbon dioxide evolved indicated a uronic acid content of 100%.
Subsequently mannuronic acid was shown to be a constituent of the polysaccharide (Nelson and Cretcher, 1930).
Hirst, Jones and Jones (1939) confirmed the findings of Nelson and Cretcher by methylation studies. Treatment of a

FIGURE 1



D – Mannuronic Acid



L – Guluronic Acid

degraded sample of alginic acid with thallous hydroxide and methyl iodide, followed by methanolysis gave rise to the methyl ester of methyl 2,3 - di - O - methyl - D - mannoside, characterised by degradation to 2,3 - di - O - methyl - erytharic acid. Using a similar procedure on a less degraded sample of alginic acid these results were later confirmed (Chanda, Hirst, Percival and Ross 1952).

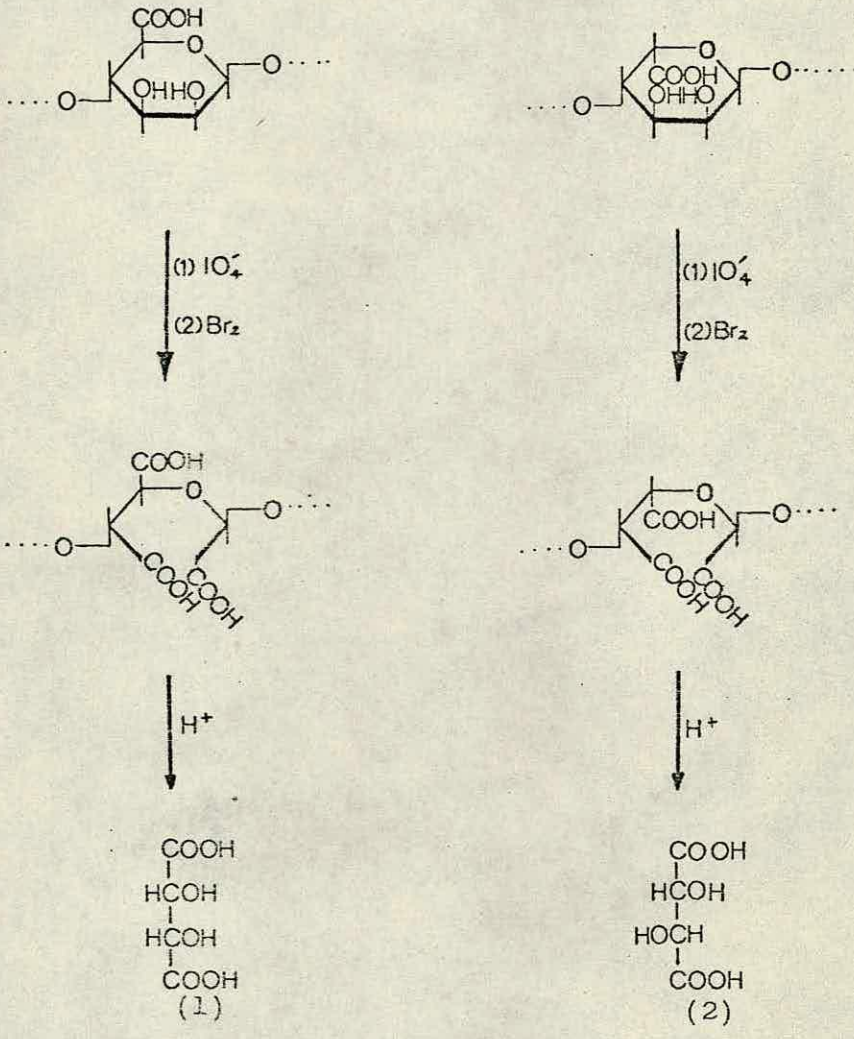
These results coupled with the high negative rotation of the molecule ($[\alpha]_D$ - 139°) led to the belief that alginic acid was composed of - 1,4 - linked - D - mannuronic acid residues.

However, in 1955 Fischer and Dörfel detected variable amounts of L - guluronic acid in samples of alginic acid from seventeen different genera of brown seaweed. See Figure 1.

Lucas and Stewart (1940) had isolated mesotartaric acid from alginic acid after periodate oxidation, bromine oxidation and hydrolysis, but Fischer and Dorfel using improved chromatographic techniques were able to show L (+) tartaric acid in addition to mesotartaric in the final hydrolysate. This also provided evidence that the uronic acid units were linked through C - 1 and C - 4. (See Fig. 2).

Although establishing some of the features of the structure of alginic acid several important problems remained, namely proof that alginate was a heteropolymer and not a mixture of two different polysaccharides composed entirely of one or other uronic acid; and unambiguous evidence for the nature of the glycosidic linkage.

FIGURE 2



Mesotartaric acid (1) and tartaric acid (2) from alginic acid.

Haug (1959a, 1959b) showed that alginate from L. digitata and L. hyperborea could be separated into two fractions; 30% of the alginate was soluble in 0.6 M KCl and the remainder insoluble. The soluble fraction was acid hydrolysed and shown to contain mannuronic and guluronic acids in approximately equal proportions, while after the same treatment the insoluble fraction was shown to contain a high proportion of mannuronic acid. In contrast, precipitation of guluronic acid-rich material was achieved by the addition of manganous sulphate and potassium chloride to solutions of sodium alginate (McDowell, 1958). Again, fractionation of alginate solutions with 0.1% calcium chloride in the presence of 0.08N - 0.5N magnesium chloride gave rise to precipitates of guluronic acid-rich molecules (Haug and Smidsrød, 1965). Nevertheless, repeated refractionation using any of these methods failed to yield a product composed solely of one uronic acid.

In 1960, Vincent separated a number of oligouronides produced by partial acid hydrolysis of alginic acid, including two diuronides and one triuronide, all of which contained both mannuronic and guluronic acids.

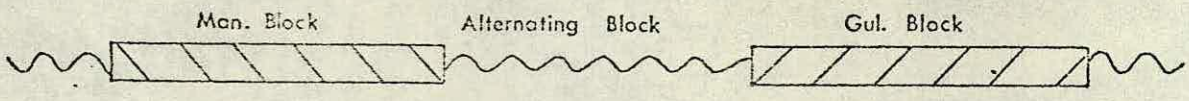
Confirmation that at least some alginate molecules contained both uronic acids came with the isolation and identification of 4 - O - β - D - mannosylgulose, from the final hydrolysate of partially acid hydrolysed and reduced alginic acid (Hirst, Percival and Wold 1964).

Haug and Larsen (1965) by hydrolysing alginic acid with 1 M oxalic found that about 30% of the material was readily brought into solution in the form of oligouronides which were

further hydrolysed to uronic acids. The precipitate remained insoluble even after prolonged hydrolysis. This resistant material had a number average degree of polymerization (D.P.) of about 25 and after dissolving in alkali could be separated into two fractions by adjusting its pH to 2.85. The insoluble portion was found to consist almost entirely of guluronic acid units and the soluble fraction of mannuronic acid units. On this evidence Haug and Larsen suggested that alginic acid consisted of acid-resistant, semi-crystalline portions with an average D.P. of 25, linked together with non-crystalline portions. The crystalline portions were thought to be composed of one type of uronic acid, the hydrolysable section having an alternating mannuronic acid, guluronic acid structure (Fig. 3).

Evidence for the conformation of the glycosidic linkage between mannuronic acid residues was reported by Hirst et al (1964), who isolated O - β - Man - p (1-4) - D - Man p. Rees and Samuel (1967) by three independent methods of gas-liquid chromatography showed that after reduction and hydrolysis of nine methylated alginates examined, only the 2,3 - di - O methyl ethers of mannose and gulose could be detected and concluded that these alginates and probably others, contained only 1,4 - linked units. Recent X - ray diffraction studies (Atkins, Mackie and Smolko, 1970) on samples of polymannuronic acid and polyguluronic acid, prepared by partial acid hydrolysis of alginic acid, have suggested that D - mannuronic acid residues are β- linked in the C1 conformation and the L - guluronic

FIGURE 3



Possible Structure for Alginic Acid

acid residues are α linked in the 1C conformation.

Using P.M.R. spectroscopy, Penman and Sanderson (1972) confirmed the results obtained by X - ray diffraction studies. The resulting spectra were compared to the model reference compounds methyl mannopyranosiduronic and gulopyranosiduronic acids.

The state of combination of alginic acid in the seaweed plant and other physiological properties of the polyuronide have been discussed elsewhere (Wasserman, 1949, Preston, 1974) and will not be considered here.

Bacteria in common with other micro-organisms and higher organisms are capable of synthesising a number of polysaccharides which represent the principal carbohydrate form found in the cell. Bacterial polysaccharides function either as structural components within the cell or as storage compounds. In addition many bacteria produce polysaccharides which occur on the outside of the cell wall or are secreted into the surrounding environment. Classification of such polymers is ultimately dependent upon the structural relationship to the cell, although the general term exopolysaccharide has been applied to all polysaccharides found outside the cell wall (See Fig. 4).

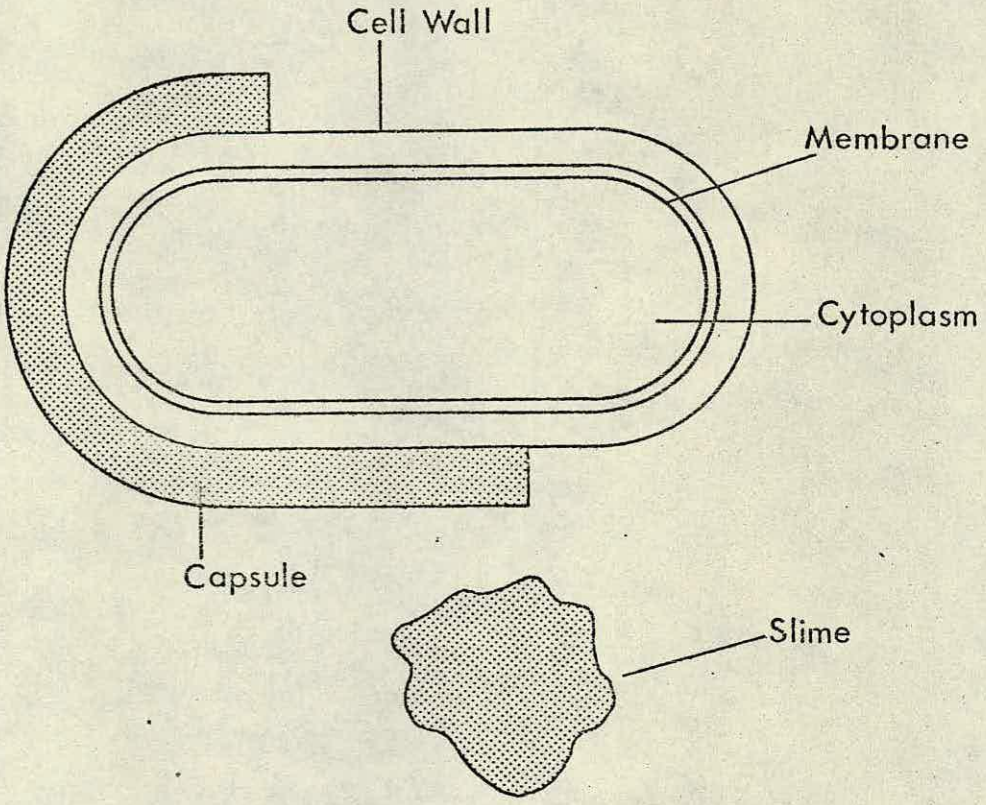
The amount of polysaccharide produced varies according to the bacterial species and its environment, but can in many cases be several times greater than the dry weight of the cells that produces it (Wilkinson, 1958).

The field of bacterial exopolysaccharides has been reviewed elsewhere, (Wilkinson, 1958, and Stacey and Barker, 1960) and more recently by Sutherland (1972). Table 1 illustrates the diversity of species which produce exopolysaccharides as well as the diversity of monomer unit found. Discussion here will be limited in detail to only two bacterial exopolysaccharides.

TABLE 1

MICRO-ORGANISM	MONOSACCHARIDE COMPOSITION	REFERENCE
Gram-ve Species		
Klebsiella aerogenes type 54 (A3(S1))	Glucose, Glucuronic acid, Fucose	1.
Rhizobium Meliloti	Glucose, Galactose, Glucuronic acid	2.
Agrobacterium tumefaciens	Glucose, Galactose, Pyruvate, Acetate	3.
Xanthomonas campestris	Glucose, Mannose, Glucuronic acid, Pyruvate, Acetate	4.
Alcaligenes faecalis var myxogenes	Glucose, Succunic acid	5.
Serratia marcescens	Glucose, Mannose, heptose, Fucose, Rhamnose	6.
Gram+ve Species		
Streptococci bovis	Glucose	7.
Bacillus circulans	Glucose, Mannose, uronic acid	8.
Lactobacillus bifidus	Glucose, Galactose, Fucose, pentose	9.
Clostridium perfringens	Glucose, Galactose, Mannose, Glucosamine, Galactosamine, Glucuronic acid	10.
Mycobacterium tuberculosis	Mannose, Glucose, Phosphate	11.
Yeast and Fungal Species		
Pullularia pullulans	Glucose	12.
Succharomyces rouxii	Mannose	13.
Black yeast spp	N-Acety glucosamine N-Acety glucosaminuronic acid	14.
Torulopsis rotundata	Glucose, Xylose	15.

FIGURE 4



Relationship of capsule and slime to bacterial cell (Sutherland, 1972).

Azotobacter vinelandii and certain strains of Pseudomonas aeruginosa, two completely different species of bacteria from dramatically different environments produce exopolysaccharides which resemble the algal polysaccharide alginic acid.

A vast amount of work has been published on the genus Azotobacter, due mainly to the ability of the bacterial group to assimilate molecular nitrogen. The general literature on Azotobacter has been reviewed in detail elsewhere (Mushustin and Shil'Nikova, 1970; and Rubenchik, 1963).

Young Azotobacter cells are Gram negative ovoid rods frequently occurring in pairs. They are motile by means of peritrichous flagella. When the cells age they lose their motility, shorten and assume an almost coccoid form. Endospores are not produced, but thick-walled cysts are formed. The micro-organism is common in soil and water (Johnstone, 1974).

The biosynthesis of copious amounts of capsular polysaccharide is a characteristic of the species and has been recognised for many years (Martin, 1945).

Parikh and Jones (1963) isolated an extracellular polysaccharide from Azotobacter indicum and suggested that the polysaccharide was a linear, high molecular weight molecule composed predominantly of the repeating trisaccharide: O - D - gluco - pyranouranosyl - (1 → 3) - O - D - gluco-pyranosyl - (1 → 2) - O - D - glycero - D - mannoheptopyranose. A similar structure for the capsular polysaccharide of Azotobacter chroococcum has been proposed by Lawson and Stacey (1954).

Kaufman and Repaske (1958) first described the production of large amounts of polysaccharide when Azotobacter vinelandii

was grown on a wide range of carbon sources in a nitrogen free medium. Kaufman and Repaske acid hydrolysed the polysaccharide and suggested that rhamnose and glucose were the sole components. Cohen and Johnstone (1963) observed a concurrent drop in the pH of the growth medium from 7.5 to 4.5 with an increase in the exopolysaccharide concentration when A. vinelandii was again grown in a nitrogen free mineral broth. Glucuronic acid and glucose as well as rhamnose were detected when the products of acid hydrolysis of the polysaccharide were analysed by paper chromatography. Cohen and Johnstone implied that the presence of glucuronic acid in the polymer was responsible for the drop in pH observed during the growth of the organism. Cohen and Johnstone (1964) subsequently reported that hydrolysis of A. vinelandii extracellular polysaccharide gave rise to galacturonic acid, smaller amounts of glucose, rhamnose and a hexuronic acid lactone, which was thought to be mannurono-lactone; in addition O - acetyl groups and some thiobarbituric acid (T.B.A.) positive material (see later) was also present. Claus (1965) considered 2 - keto - 3 - deoxy galactonic acid to be the T.B.A. positive material and that the keto acid was the main constituent of the capsular polysaccharide.

The first exhaustive chemical analyses of A. vinelandii slime were carried out by Gorin and Spencer (1966). Results of acid hydrolysis experiments suggested that the purified exopolysaccharide from the organism was composed exclusively of hexuronic acid residues which were shown to be mannuronic and

guluronic acids. Analysis of the reduced polysaccharide showed that mannose and gulose were present in the ratio of 11 to 1. The polymer had a saponification equivalent of 152 compared with 176 for any glycuronan, however, this discrepancy was explained by the fact that the polymer contained one acetyl group per 5.2 anhydro-hexuronic acid units. Larsen and Haug (1971a)using techniques perfected in the analysis of algal alginate showed that the sequence of guluronic and mannuronic acid in the exopolysaccharides form three strains of A. vinelandii was of the type observed for seaweed alginate i.e. that the monomers were distributed in a block-wise fashion along the chain. This observation was confirmed in an independent study by Penman and Sanderson (1972) and in a later study by Haug, Larsen and Smidsrød (1974).

Pseudomonas aeruginosa is type species of the fluorescent pseudomonads. Stanier, Palleroni and Doudoroff (1966) defined the aerobic pseudomonads as : "unicellular rods, with the long axis curved or straight but not helical. Motile by means of one or more polar flagella. Gram negative and do not form spores, stalks and sheaths. The energy yielding mechanism is respiratory, never fermentative or photosynthetic. All use molecular oxygen as terminal oxidant. All are chemo-organotrophs". Ps. aeruginosa is found in soil, water sewage and air.

Ps. aeruginosa possesses a wide variety of biochemical capabilities as well as the ability to cause a wide variety of infections in compromised hosts. The organism causes serious complications in patients undergoing major surgery or suffering from serious burning, due mainly to the failure of Ps. aeruginosa to respond to antibiotic treatment. According to Makley and Smallman (1968) the most common cause of death in man after thermal injury is now Gram negative, and especially Pseudomonas septicaemia. Rogers (1960) claimed Ps. aeruginosa was the most dangerous pathogen in a children's hospital in Birmingham. Ironically, outside hospitals Ps. aeruginosa rarely troubles humans, but the hospital provides an environment in which disinfectants have reduced the numbers of competing organisms.

The greatest single incidence of Pseudomonas infection is chronic respiratory infection associated with the disease cystic fibrosis (Lacocca, Sibinga and Babero, 1963).

Cystic fibrosis (C.F.) is an inherited disease which results in the production of abnormal body secretions including a highly tenacious mucus in the lungs. This has the effect of creating a static condition in the lungs and consequently C.F. patients are very vulnerable to pulmonary infections (5th International Cystic Fibrosis Conference, 1969).

Studies by Burns and May (1968), Doggett (1969) and Kilbourn (1968, 1970), have shown that Staphylococcus aureus is the primary pathogen in the lungs of children suffering from C.F. After prolonged treatment with antibiotics, Haemophilus influenza becomes established,

and is followed by Ps. aeruginosa as the sole pathogen. Høliby (1974) in a recent epidemiological investigation of the respiratory tract of patients suffering from C.F. showed that 90 per cent of the patients harboured Ps. aeruginosa amounting to 50 per cent of the total flora.

At first Ps. aeruginosa appears as a non-mucoid strain, however, with continued antiobiotic treatment a mucoid strain predominates and this is associated with a concomitant decline in the clinical condition of the patient. Pyocin typing of such mucoid strains isolated from sputum specimens from patients with C.F. suggested that mucoid strains occurred simultaneously and that these were variants of the same strain (Williams and Govan, 1973).

The slime polysaccharide produced by C.F. Pseudomonas strains has been examined by a number of workers. Doggett, Harrison and Wallis (1964) first isolated the polymer and on total acid hydrolysis, glucose, galactose, glucosamine and galactosamine were identified by paper chromatography using only more common sugars for reference. However, two sugars present in 'considerable quantity' remained unidentified. Linker and Jones (1964) first observed the similarity between algal alginic acid and the polysaccharide produced by the organism. Acid hydrolysis of the crude polysaccharide gave rise to sugars corresponding to mannuronic acid and mannurono-lactone and guluronic acid. No additional spots could be detected when chromatograms were developed using other reagents. In addition, comparison of the infra-red spectra of the sodium salt of Pseudomonas exopolysaccharide and sodium alginate showed no differences in either intensity or positions of peaks. Later in a

more detailed description of Pseudomonas alginate Linker and Jones (1966) established the presence of O- acetyl groups. Carlson and Matthews (1966) examined the polysaccharide produced by thirteen mucoid strains of Ps. aeruginosa isolated from the respiratory tract of patients with C.F. By a procedure of reduction and hydrolysis D - mannose and L - gulose were identified, the former by preparation of the phenylhydrazone derivative and the latter by conversion to sorbitol. One difference between Azotobacter and Pseudomonas alginates which has been observed is the lack of insoluble material remaining after partial acid hydrolysis of the latter, (Haug et al, 1974). The alginate from these Ps.aeruginosa strains is thus envisaged as having a high proportion of alternating blocks.

Slime-producing strains of Ps. aeruginosa from non C.F. environments are known, and these produce glucose and mannose containing polysaccharides (Brown, Foster and Clamp, 1969), bearing no similarity to bacterial alginate.

Stanford's belief that alginic acid had many properties worthy of commercial exploitation has now been well justified. The total world alginate production has been estimated at greater than 10,000 tons per annum, (McNeely and Pettitt, 1974).

The economic importance of alginic acid, and its salts, is based on its interesting physical and chemical properties:

(a) the formation of viscous solutions at relatively low concentrations

(b) their behaviour as polyelectrolytes in solution

(c) the formation of gels by chemical reaction

(d) the formation of films on surfaces

(e) the formation of films on fibres

(f) base exchange properties

(Percival and McDowell,1967)

The theoretical consideration of these properties has been dealt with elsewhere (Rees, 1972, Reid et al, 1974, Segern, et al 1974, Smidsrød, 1974).

The main uses of alginic acid are as: emulsifiers, thickeners, stabilising agents, gelling agents, surfacing agents and synthetic films and fibres. In the pharmaceutical industry it is used to suspend drugs and antibiotics in emulsion preparations and in a large number of creams and ointments. Alginic acid, and its salts and the propylene glycol ester, are approved food additives in most countries of the world, (F.A.O. Nutrition Meetings Report Series, 1974).

Some of the manufactured products in which alginates are used are shown in Table 2. The applications of alginates have been described in detail in a number of publications (McDowell, 1960, McNeely and Pettitt, 1974 Ciba Review, 1969).

TABLE 2

Pharmaceutical
- Aureomycin tablets
- Anti-acid tablets
- Aspirin tablets
- Calamine lotion
- Dental impression compounds
- Toothpaste
- Surgical jellies
- Mineral oil emulsions

Foods
- Bakery icings
- Salad dressings and creams
- Frozen foods
- Fruit syrups and concentrates
- Candy
- Milk puddings
- Ice cream
- Sherbet
- Chocolate drinks
- Sterilised cream
- Cheese

Rubber
- Latex creaming and thickening
- Foam rubber, cushions, etc.
- Rubber coatings
- Tyres
- Electrical insulation

Textiles
- Size for cotton and rayon
- Textile print pastes
- Plastic laundry starch

Paper
- Milk containers
- Insulation board
- Food wrappers
- Greaseproof paper
- Acoustic tiles

Miscellaneous
- Paints
- Polishes
- Ceramic glazes
- Leather ware
- Boiler compounds
- Battery plate separators
- Waxes
- Jointing cements

Some of the uses of Alginates (Johnston, 1968).

ENZYMIC HYDROLYSIS OF ALGINIC ACID

The physical properties of alginates which have found application in industrial processes depend to a great extent on the mannuronic acid to guluronic acid ratio and the molecular weight of the polysaccharide. It is clear that if successful production of alginate by Azotobacter vinelandii is to be achieved on a commercial scale, an understanding of any alginate depolymerizing enzymes is essential. Such enzymes could arise from either bacteriophage or microbial contamination of the Azotobacter culture and could lead to the loss of a valuable product.

In general polysaccharides can be degraded in two ways: in a proton- rich or in a proton-poor environment. Degradation in a proton-rich medium leads to oligosaccharide or monosaccharide fragments in which no important structural changes in the sugar units derived from the polymer can be observed. However, degradation in a proton-poor environment causes greater change in the structure of the monosaccharide unit directly involved in the degradation mechanism.

A large number of enzymes obtained from micro-organisms, and other sources, have the property of degrading uronic-acid-containing polysaccharides by an "eliminative" pathway.

The phenomenon was first observed during an investigation into the structure of sodium hyaluronate. Hyaluronic acid is a polysaccharide composed of equimolar quantities of D-glucuronic acid and 2-acetamido-2-deoxy-D-glucose. Hyaluronic acid is widely distributed in connective tissue, vitreous humour of eyes, umbilical cord and joint fluids.

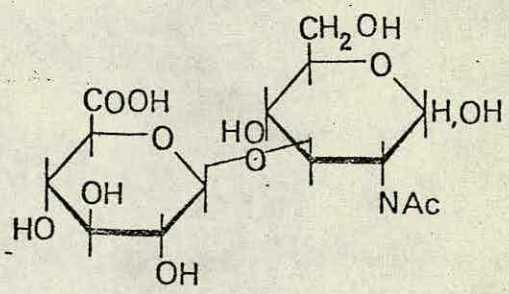
Streptococcus faecalis and certain strains of Pseudomonas aeruginosa are believed to synthesise hyaluronic acid (Bergan and Hovig, 1969; Brown, Foster and Clamp, 1969). For review, see Brimacombe and Webber (1964).

Linker, Meyer and Hoffman (1956) showed that hyaluronidase preparations from Clostridium welchii, Staphylococcus aureus, Group C haemolytic streptoccocci and type III pneumococci all gave the same degradation product which was shown not to be identical to N-acetyl hyalo-biuronic acid, (N-acetyl HBA) the disaccharide isolated by Weissman and Meyer (1954) and suggested as the repeating unit of the molecule.

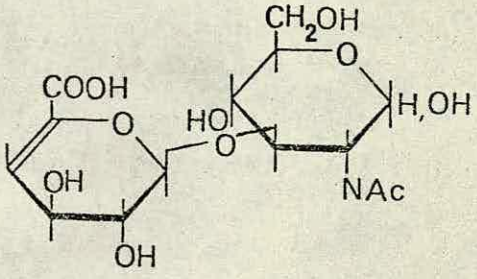
This 'novel' degradation product absorbed strongly in the ultraviolet spectrum at 232 nm, indicating the presence of an α, β unsaturated carboxylic acid. The presence of a double bond in the molecule was confirmed by the decolourization of bromine. Acid hydrolysis of the disaccharide yielded glucosamine as the only identifiable product, unlike acid hydrolysis of N-acetyl-HBA which yielded glucuronic acid, glucurono-lactone and glucosamine. The disaccharide gave a positive thiobarbituric acid test. On ozanolysis the disaccharide gave oxalic acid. These facts suggest a compound with the structure shown in Figure 5. was the product of hyaluronidase activity.

Since then, a number of reports describing the release of unsaturated products by bacterial hyaluronic acid lyases have appeared (Abramson and Friedman, 1968; 1969; Linker, 1966 and Abramson and Rautela, 1971). The term lyase was introduced by the International Union of Biochemistry (1961) and is used to describe eliminative-type enzymes.

FIGURE 5



Repeating unit of Hyaluronic acid



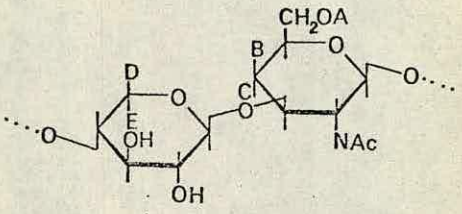
Product of Bacterial Hyaluronidase Activity

The diversity of specificity shown by lyase-type enzymes reflects the diversity and abundance of uronic acid containing polysaccharides. The medical, as well as theoretical, interest in many of these polysaccharides (see table 3) has meant that a considerable amount of enzymological study has been directed against them.

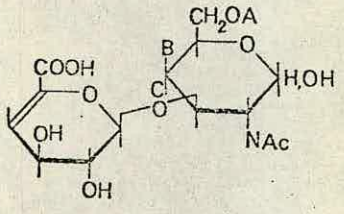
Among the sulphated mucopolysaccharides of connective tissue, three distinctive isomers containing acetylgalactosamine, uronic acid and sulphate ester in equimolar proportions are collectively designated chondroitin sulphate and are differentiated by the appended letters A, B and C. Chondroitin sulphate A and chondroitin sulphate C contain acetyl galactosamine - 4 - O - sulphate and 6 - O - sulphate respectively, and on desulphation are converted to the identical polysaccharide, chondroitin, which is a sterioisomer of hyaluronic acid due to C-4 epimerism in the acetyl hexosamine group. Chondroitin sulphate A, and chondroitin sulphate C, like hyaluronic acid form their repeating sequences with alternating β-1,3-D-glucopyranosyl-uronic acid and β-1,4-acetyl hexosaminidic units. Chondroitin sulphate B differs from chondroitin sulphate C in that the D-glucuronic acid moiety is replaced by its C-5 epimer L-idopyranosyl-uronic acid.

The structural relationship between the polysaccharides was confirmed by the isolation of the unsaturated aldobiuronic acid, 2-acetamido-2-deoxy-3-O- (4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid-D-galactose), (see figure 6) from all four polymers. The disaccharide was the product of the

FIGURE 6



	A	B	C	D	E
1.	H	OH	OH	CO_2H	H
2.	H	OSO_3H	H	CO_2H	H
3.	H	OSO_3H	H	H	CO_2H
4.	SO_3H	OH	H	CO_2H	H



Action of chondroitinases

1. Hyaluronic acid
2. Chondroitin sulphate A
3. Chondroitin sulphate B
4. Chondroitin sulphate C

TABLE 3

Mucopolysaccharide	Component monosaccharides	Approximate mol. wt.	Occurrence
Hyaluronic acid	D-Glucuronic acid, 2-acetamido-2-deoxy-D-glucose	1,500,000	Vitreous humour, ground substance of connective tissues, umbilical cord, plasma, synovial fluids, cardiac valves
Chondroitin	D-Glucuronic acid, 2-acetamido-2-deoxy-D-galactose	50,000	Bovine cornea, septal cartilage
Chondroitin sulphate A (chondroitin 4-sulphate)	D-Glucuronic acid, 2-acetamido-2-deoxy-4-O-sulpho-D-galactose	50,000	Aortic tissue, cartilage, adult bone, ligamentum nuchae, cornea
Chondroitin sulphate B (dermatan sulphate)	L-Iduronic acid, 2-acetamido-2-deoxy-4-O-sulpho-D-galactose	50,000	Cardiac valves, pig skin, bovine lung tissue, bovine sclera
Chondroitin sulphate C (chondroitin 6-sulphate)	D-Glucuronic acid, 2-acetamido-2-deoxy-6-O-sulpho-D-galactose	5000	Aortic tissue, cartilage, adult bone, cardiac valves, umbilical cord
Keratosulphate (keratan sulphate)	D-Galactose, 2-acetamido-2-deoxy-6-O-sulpho-D-glucose	?10,000	Nucleus pulposus, costal cartilage, corneal tissue, aortic tissue
Heparin	D-Glucuronic acid, 2-deoxy-2-sulphoamino-D-glucose with additional O-sulphate groups	20,000	Liver, mast cells, spleen, muscle, lung, thymus, blood, heart

Properties of some common mucopolysaccharides

(Passmore & Robson 1968).

action of both eliminase and sulphatase activities produced by several strains of Flavobacterium heparinium and Proteus vulgaris (Hoffman et al, 1960; Yamagata et al, 1968; Saito et al, 1968 Suzuki et al, 1968).

Heparin has a more complex composition than other mucopolysaccharides as it contains N-acetylglucosamine, glucosamine N-sulphate, glucosamine N, O-disulphate, D-glucuronic acid and L-iduronic acid (Linker and Hovingh, 1973). It is found mainly in blood-vessel walls and may be associated with various disorders in the metabolism of mucopolysaccharides. Recently the isolation and purification of two enzymes from Flavobacterium, heparin lyase (EC 4.2.2.7) and heparan sulphate lyase (EC 4.2.2.8) (Hovingh and Linker, 1970) and the subsequent characterization of five unsaturated disaccharides produced by these lyases has led to a clearer understanding of the structure of the polysaccharide.

Further lyase type enzymes have been identified. A species of Bacillus isolated from soil was found to elaborate an inducible, extracellular enzyme capable of degrading the exopolysaccharide of Xanthomonas phaesoli, a polymer containing equal proportions of D-glucose, D-mannose and D-glucuronic acid. Examination of the polysaccharide degradation products revealed that the glucuronic acid moiety of the polymer was altered to a 4,5-unsaturated form as a result of enzyme action (Lesley, 1961).

The acidic capsular polysaccharide of Type III pneumonococcus (S3) contains alternating 3-O-substituted β-D-glucuronic acid and 4-O-substituted β-D-glucopyranose residues. Type VIII (S8) contains D-glucuronic acid, D-glucose and D-galactose in the ratio 1:2:1 and the arrangement

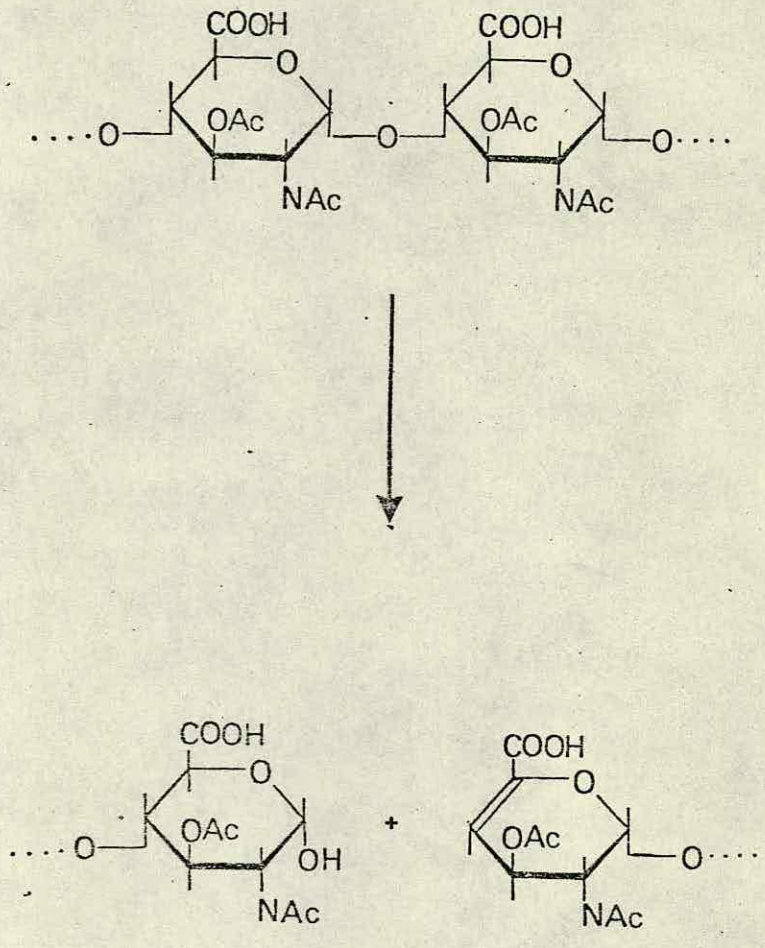
4-β-glucuronosyl-4-β-glucosyl-4-α-glucosyl galactose. Torriani and Pappenheimer (1962) induced the formation of pneumonococcal capsule depolymerases by Bacillus palustris. Both S3 and S8 depolymerases were shown to attack 1, 4 β linkages in the polysaccharide chains. In contrast to S3-depolymerase which was shown to be a hydrolytic enzyme, S8 depolymerase caused degradation of S8 polysaccharide by formation of products containing terminal 4,5-unsaturated glucuronic acid residues (Becker and Pappenheimer, 1966).

Another enzyme obtained from Bacillus palustris has been shown to degrade the surface polysaccharide formed by Salmonella typhosa, Vi antigen, to a series of 4,5 unsaturated oligomers. (Baker and Whiteside, 1965 and Nicol and Baker, 1970). Vi antigen is an acidic polysaccharide containing O and N-acetylated α-1-4-linked 2-amino-2-deoxy-D-galacturonic acid (See Figure 7).

It was of interest that the enzyme induced by B.palustris was capable of degrading polygalacturonic acid, while a polygalacturonate lyase induced from B.polymyoxa by growth on pectin released ultra-violet-absorbing material from Vi antigen. A commercial pectinase with a hydrolytic mechanism was ineffectual against Vi antigen.

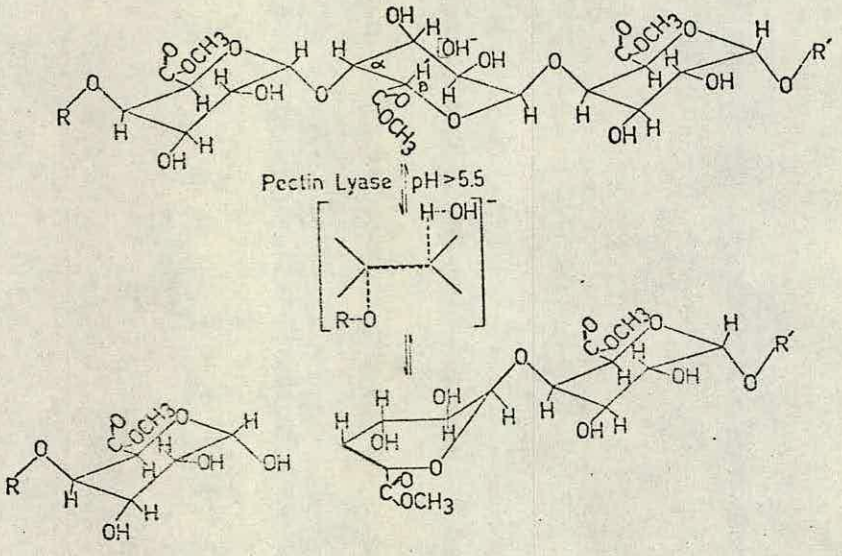
A considerable amount of work has been carried out on trans-eliminative enzymes from various micro-organisms capable of cleaving pectic substances and this has been reviewed elsewhere (Fogarty and Ward 1972, 1974; Rombouts, 1972 and Voragen, 1972).

FIGURE 7



Enzymatic Hydrolysis of Vi Antigen

FIGURE 8



Transeliminative degradation of pectin fragment

The non-enzymatic degradation of pectin in neutral or alkaline conditions involves a β-trans-elimination, resulting in the formation of oligomers with a β-4-5-unsaturated galacturonide unit at the non-reducing end in a similar way to pectin depolymerization by lytic enzymes. The chemical mechanism of this reaction has been studied (Albershiem, Neukom and Deuel, 1960; Bemiller and Kumari, 1972 and Keijbets, and Pilnick, 1974) and is believed to proceed by an E_2 mechanism as illustrated in Figure 8 . The field of β-elimination degradation in carbohydrates containing uronic acid residues has been reviewed by Kiss (1974).

Alginic acid, unlike many other hetero-polysaccharides described, has not been shown to possess a regularly repeating sub-structure. In addition the ratio of mannuronic acid to guluronic acid and the block structure of the polymer is highly variable (see Table 4). Consequently alginic acid provides an interesting and perhaps unique type of substrate for an enzymatic study.

Although alginate-degrading enzymes have been described from bacterial as well as other sources few of the properties of these enzyme systems have been studied in detail.

Oshima (1931) reported that no higher animals were capable of digesting alginate but when incubated with the juice from the gut of Haliotis giganteus (a marine gastropod) and of Sphaerochinus pulcherrimus (an echinoderm) alginate solutions rapidly showed a drop in viscosity.

TABLE 4

BLOCK DISTRIBUTION OF ALGINATES FROM DIFFERENT SPECIES OF BROWN ALGAE

Order	*Species (day/month of collection)*	*M/G ratio*		*Partial hydrolysis*[a]		
		Found	*Calc.*	*Soluble (%)*	*Insoluble (%)*	
					Soluble at pH 2.9	*Insoluble at pH 2.9*
Ectocarpales	*Pylaiella*	0.75	0.60	40	18	42
Chordariales	*Spermatochnus paradoxus* (15/8)	1.3	0.98	35	32	33
	Cordaria flagelliformis (15/8)	0.9	0.63	21	28	51
Dictyosiphonales	*Dictyosiphon foeniculaceus* (9/6)	0.85	0.60	25	25	50
	Scytosiphon lomentaria (19/6)	1.15	0.90	25	35	40
Desmarestiales	*Desmarestia aculeata* (9/6)	0.85	0.58	27	23	50
Fucales	*Pelvetia canaliculata* (9/4)	1.5	1.28	38	37	25
	Fucus serratus (26/6)	1.3	1.06	35	34	31
	Ascophyllum nodosum (3/5)	1.85	1.56	52	35	13
Laminariales	*Laminaria digitata* (15/1)	1.45	1.50	34	43	23
	Laminaria hyperborea, fronds (16/2)	1.35	1.28	26	43	31
	Laminaria hyperborea, stipes (16/2)	0.65	0.38	25	15	60

BLOCK COMPOSITION OF EXTRACELLULAR ALGINATES PRODUCED BY BACTERIA

Azotobacter vinelandii	2.0	32	42	26
Azotobacter vinelandii	0.5	23	11	66
Pseudomonas aeruginosa	2.0	100		

[a]For 2 h with 0.3M HCl at 100°.

Three years later Waskman, Carey and Allen (1934) isolated a number of bacteria from sea water, phytoplankton, the sea bottom and soil, capable of utilizing alginate as sole carbon source. Waskman et al were unable to culture alginate-decomposing fungi.

Since then a number of reports dealing with the bacterial decomposition of alginate have appeared. However, many of these have merely been concerned with the microbiological phenomenon as an aid either in taxonomic or ecological studies (Adams, Williams and Payne, 1961; Billy, 1965, 1966 and Lewin and Lounsbery, 1968). In this field the most comprehensive study was carried out by Meland (1962) in which fifty-seven strains of alginate-decomposing bacteria were described.

The advantages of cultivating bacteria adapted to produce enzymes which may be of use in the structural analyses of polysaccharides are clear from work already described. Such an adapted bacterium can conveniently provide as much enzyme as required. However, much information on the enzymatic hydrolysis of alginic acid in particular has been obtained using enzymes derived from non-bacterial systems. Both bacterial and non-bacterial alginase systems will be discussed here.

Kooiman (1954) was first to examine the biochemical nature of alginate depolymerization. A series of oligouronides were released from an alginate solution by an "alginase" preparation obtained from an unidentified bacterium. Mannuronic acid was thought to be the ultimate product of this digestion, detected by paper chromatography after the alginate solution had been incubated for four weeks with

the enzyme preparation. Similarly Ellen and Payne (1960) considered mannuronic acid to be the final product of enzymatic hydrolysis of alginate.

During work on alginate hydrolysates Tsujino and Saito (1961) observed the similarity between the enzymatic hydrolysis of alginate and the enzymatic hydrolysis of hyaluronic acid. An alginase preparation from homogenized liver of the abalone, Haliotis discus hannai liberated a diuronide as the main product. This disaccharide gave a positive TBA test, showed a strong absorption peak at 232 nm and on acid hydrolysis yielded mannuronic acid. It was concluded that the enzyme split glycosidic bonds by an elimination reaction.

Preiss and Ashwell (1962a) studied in some detail sodium alginate metabolism in an unidentified bacterium. The organism of this study was described as a yellow pigmented pseudomonad originally isolated from estuarine mud. The cell bound alginase system was partially purified and shown to degrade alginate into a series of oligosaccharides containing a 4,5-unsaturated uronic acid on the non-reducing end of the oligouronide chain. The rapid formation of a series of unsaturated oligosaccharides as demonstrated by paper chromatography, indicated that the polymer was cleaved by an endolyase. The enzyme exhibited maximal activity in pH range 7.0 to 8.0 and showed a striking increase in activity in the presence of 0.05M kcl. Preiss and Ashwell concluded that 4-deoxy-L-erythro-5-hexoseulose uronic acid was the ultimate product of alginate lyase activity. The fate of this compound was subsequently reported (Preiss and Ashwell, 1962 b). The enzyme, 2-keto-3-deoxy-D-gluconic acid

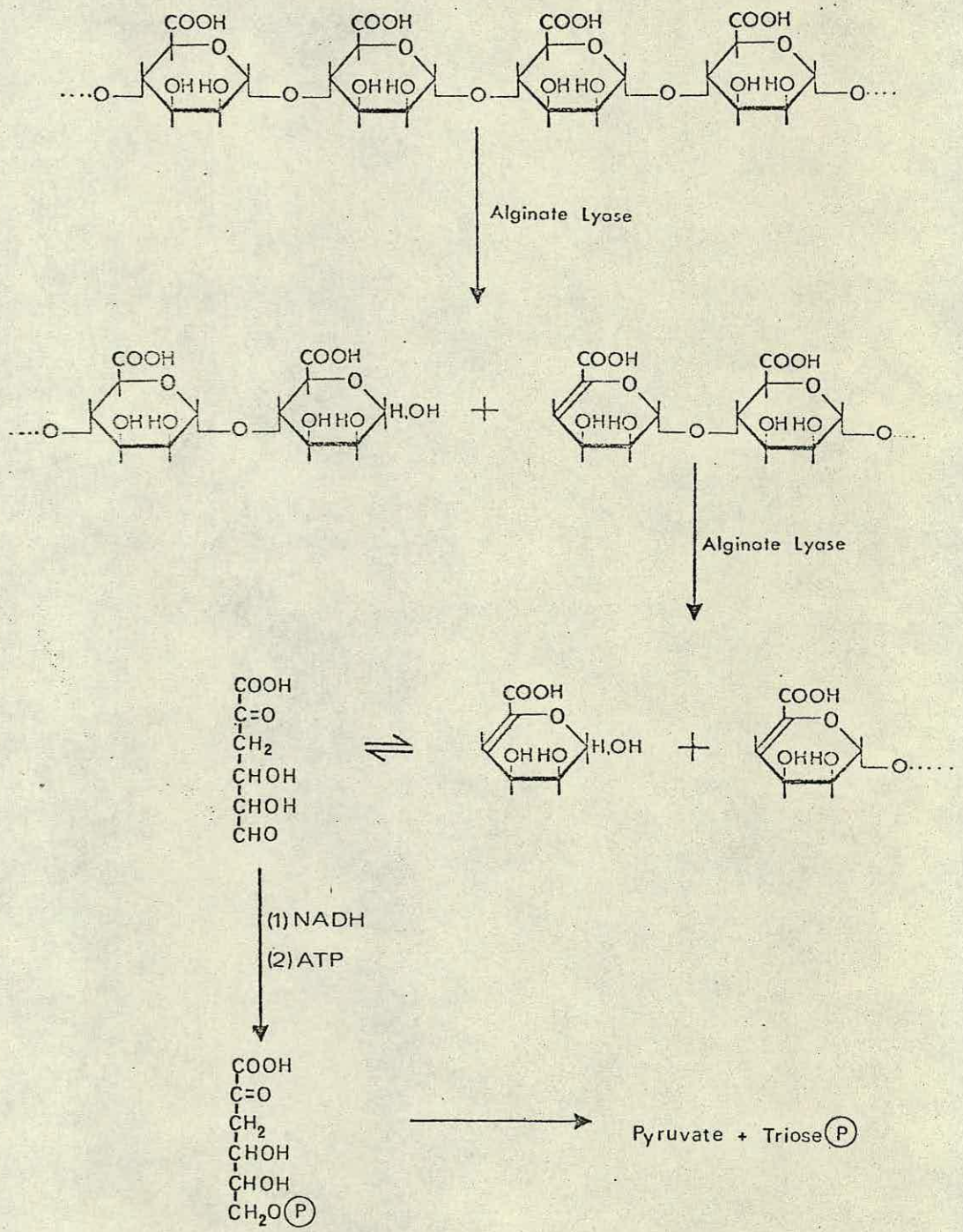
dehydrogenase was isolated from the alginic acid-adapted pseudomonad. This NADH-linked enzyme catalysed the formation of 2-keto-3-deoxy-D-gluconic acid from 4-deoxy-L-erythro-5-hexoseulose. The pathway of alginic acid degradation as envisaged by Preiss and Ashwell is shown in Figure 9 .

Nakada and Sweeny (1967) first suggested that alginate lyases might show certain specificities for linkages within the polysaccharide. Two eliminative type alginases were separated from abalone heptopancreas (Haliotis rufescens and Haliotus corrugata). The alginases, designated I and II exhibited different properties. Alginase I caused a rapid drop in the viscosity of alginate solutions and appeared to be specific for β-1,4 bonds as indicated by the high affinity the enzyme had for mannuronic acid-rich substrates. The enzyme was active over the pH range 6.5 - 8.5, showing maximal activity at pH 7.5. Increasing concentrations of the chlorides of k^+, Na^+, Mg^{++}, and Mn^{++} enhanced enzyme activity four-fold up to 0.05M after which with Mg^{++} and Mn^{++} an inhibitory effect was observed. Alginase II caused almost no drop in the viscosity of alginate solutions and was therefore thought to be an exo-lyase. The enzyme showed an increased affinity for 1,4-glycosidic linkages involving guluronic acid, was active over the pH range 3.5 - 4.5 and again activity was stimulated by the addition of Mg^{++} or Mn^{++} to the reaction mixture. Neither enzyme preparation showed activity towards chondroitin sulphate, hyaluronic acid, pectin or fucoidan.

The heptopancreas of another marine organism, the mollusc, Dolabella auricula Solander proved a useful source of alginate-depolymerizing enzymes.

FIGURE 9

Microbial degradation of Alginic acid



Two lyases which exhibited high activities towards mannuronic acid-rich alginates were examined by Nisizawa, Fujibayashi and Kashiwabara (1968). The degradation of alginates from various brown algae catalysed by these lyases was incomplete and the extent of this degradation seemed roughly proportional to the mannuronic acid content of the substrate. The difference between the two enzymes appeared to be in the specificity shown towards substrates of different molecular weight. One lyase degraded mannuronic acid-rich alginates while the other caused depolymerization in short chain polymannuronides. The crude enzyme system showed maximal activity at pH 8.0 in Tris-HCl buffer and yielded 4-O-β-D- 4,5-mannoseen pyran-uronosyl-D-mannuronic acid as the final product of alginate hydrolysis.

In a further study by the Japanese group, Kashiwabara, Suzuki, and Nisizawa (1969) examined two cell bound lyases from two unidentified pseudomonads. One of the bacterial enzymes studied in detail showed activity only towards short chain polyguluronides. The final product of this enzymatic action was an unsaturated triuronide. Both alginase systems were active in the pH range 6 - 11 showing maximal activity at pH 7.5.

Recently Elyakova and Favorov (1974) have suggested that the hepatopancreas of the mollusc Littorina sp. may contain as many as six alginate lyases. One of these enzymes, designated alginate lyase VI, was purified 360 times and shown to have a molecular weight of 40,000. The enzyme showed maximal activity at pH 5.6 and appeared to be specific for glycosidic linkages involving mannuronic acid.

Madgwick, Haug and Larsen (1973) presented evidence for the occurrence of an alginate lyase in the brown alga Laminaria digitata. An extract of homogenised algal stipes effected a rapid reduction in the viscosity of sodium alginate with a concomitant rise in TBA positive material.

No detailed study has been carried out on enzymes capable of depolymerizing alginates from non-algal sources. Ekland and Wyss (1962) described a capsule-digesting enzyme which was formed when Azotobacter vinelandii cells were infected with bacteriophage. The enzyme produced a rapid drop in the viscosity of alginate solutions but the mechanism of the depolymerization was not studied.

Finally, an observation by Haug and Larsen (1971), made during an investigation into the biosynthesis of Azotobacter alginate, indicated alginate lyase activity in cultures of Azotobacter vinelandii. These workers noted that during stationary phase of growth the viscosity of the culture medium fell and the presence of a significant amount of TBA positive material was detected.

The aim of this work was to investigate the properties of microbial alginases with particular reference to their action against bacterial alginates and to evaluate their possible use in the analysis of alginate structure.

MATERIALS AND METHODS

Isolation and Growth of Alginase Producing Bacteria

Alginolytic bacteria were isolated from various environments (see Results Section), using standard enrichment techniques. These bacteria were grown on a defined medium with the following composition: (g/l) sodium alginate, 2.0; K_2HPO_4, 7.0; KH_2PO_4, 3.0; $MgSO_4.7H_2O$, 0.1; $(NH_4)_2SO_4$, 1.0, (pH 7.2). 23.5g NaCl were added to 1 litre of the above medium for growth of bacteria isolated from marine environments. When required the medium was solidified by the addition of 1.5% (w/v) agar.

Sterilization was by autoclaving for fifteen minutes at fifteen pounds per square inch.

Liquid cultures were routinely grown in 1 litre volumes contained in 2l Erlenmeyer flasks, and incubated at 30°C (or 22°C for marine organisms) on an orbital shaker.

Smaller volumes were usually grown in 100 ml amounts contained in 250 ml Erlenmeyer flasks. Larger volumes were grown in a batch fermenter (L.H. Engineering Ltd., Stoke Poges, England) in 15l amounts of the described medium. Incubation was at the required temperature, with automatic antifoam addition, stirring (350 r.p.m.) and forced aeration (3-5l/min). Bacteria were grown without pH control.

Cultures were maintained on nutrient agar slopes (or nutrient agar with 2.35%(w/v) NaCl slopes) in screw capped vials at room temperature and were subcultured at six monthly intervals.

Growth of Azotobacter vinelandii

Azotobacter vinelandii wyss, a strain received by Dr. I.W. Sutherland from Dr. O. Wyss (University of Texas), was used throughout this study.

A. vinelandii was grown on Burk's modified nitrogen-free medium (Duff and Wyss, 1960). The medium contained (g/l): Na_2HPO_4, 0.189; KH_2PO_4, 0.011; $MgSO_4.7H_2O$, 0.200; $FeSO_4.7H_2O$, 0.006; MoO_3, 0.0005; $CaSO_4.2H_2O$, 0.02; $SrCl_2.6H_2O$, 0.01; NaCl, 0.01; $NaHCO_3$, 0.05; sucrose, 5.0 (added after separate sterilization).

A. vinelandii was routinely grown in 1 litre amounts as before on a shaker at 30°C for four days.

Growth of Pseudomonas aeruginosa

The organism used in this study was obtained from Dr. L. Evans, University of Utah, College of Medicine, Salt Lake City.

Ps. aeruginosa was grown at 30°C in yeast extract medium. This medium has been described by Sutherland and Wilkinson (1965). It contained (g/l): casamino acids, 1.0; Na_2HPO_4, 10.0; KH_2PO_4, 3.0; K_2SO_4, 1.0; NaCl, 1.0; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.01; $FeSO_4$, 0.001; yeast extract, 1.0; and glucose, 20.0 (added after separate sterilization).

Production of Extracellular Polysaccharides

A. vinelandii and Ps. aeruginosa were grown on the media described. Growth was carried out in sterile enamel

trays, containing 500 ml of medium, for 2 days in the case of Ps. aeruginosa and 4 days in the case of A. vinelandii. After incubation the mucoid bacteria were scraped from the trays with a glass spreader and suspended in 0.85% NaCl (200 ml/tray) containing 1% v/v formaldehyde. Suspensions were then mixed vigorously in a blender (John Oster, MFC Co., Wisconsin, U.S.A.), for 90 sec and where necessary diluted to facilitate the removal of bacterial cells. The suspensions were centrifuged at 23,000 g for 45 min.

Polysaccharide was precipitated from the supernatant by addition of 4-5 vols of cold acetone (-18°C). The gelatinous polysaccharide was isolated from the solvent by sedimentation, washed 3 times in acetone and lyophilised. Further purification was carried out by precipitating the alginate with $CaCl_2$ and dialysing the gel against 0.4% (w/v) EDTA and 0.4% (w/v) NaCl. When the gel was resolubilized the alginate solution was dialysed against 3 changes of distilled water and finally freeze dried.

Cell breakage and Preparation of Alginases

Cells were harvested by centrifugation at 10,000 r.p.m. for 20 min at 0°C in a Measuring and Scientific Equipment Ltd., London "High Speed" centrifuge (M.S.E.) and resuspended in cold Mops pH7.5 (see later) buffer.

Small volumes of cell suspensions (1-20 ml) were routinely broken in an M.S.E. 100 watt ultrasonic disintegrator set at maximum amplitude for 3 x 30 sec. The cell suspension

(up to 5 ml) was contained in a 25 x 50 mm glass tube cooled in an ice/ethanol mixture.

Larger volumes (>20 ml) were broken by passage through a pre-cooled French pressure cell (Aminco, Inc., Silver Spring, Md., U.S.A.) at 3-4000 p.s.i.

After breakage, cell debris was removed by centrifugation at 10,000 r.p.m. at 0°C for 30 min.

Supernatant solutions were then dialysed against a large volume of cold Mops buffer pH7.5 for 16 h. The non-diffusible material was used as starting material for further enzyme purification, if required.

Enzyme solutions were stored at -20°C.

Preparation of Azotobacter-Phage Alginate-Depolymerase

The Azotobacter vinelandii wyss bacteriophage used in this study was obtained from Dr. I.W. Sutherland. It was maintained as a high titre suspension in filter-sterilized Burk's medium at 4°C.

The lysate from a phage-bacterium mixture served as the crude enzyme preparation after removal of cell debris and phage particles. Bacteriophage particles were propagated by the sloppy agar over-lay method (as described by Clowes and Hayes, 1968). After 24h incubation at 30°C, agar was removed by slow speed centrifugation (10,000 r.p.m. for 10 min). Phage particles were then removed from the supernate by centrifugation at 45,000 g for 2.5 h. The resultant solution and harvested bacteriophage were examined for enzymatic activity.

Preparation of Sphaeroplasts

This was carried out using the procedure of Osborn, Gander, Parisi and Carson (1972). The cells from 1 litre of a mid-log phase culture of the marine bacterium SA1 were harvested by centrifugation at 15,000 g for 10 min at 4°C in an M.S.E. "High Speed" centrifuge. The pellet was drained of excess medium and rapidly suspended in 50 ml 0.75M sucrose in 10 mM Tris-HCl buffer at pH7.8. Immediately 2.5 ml lysozyme (20 mg/ml) was added and the mixture incubated in ice for 2 min. The suspension was slowly diluted with 100 ml 1.5 mM EDTA (Na^+), pH7.5 over a period of 8-10 min. Sphaeroplast formation was checked by phase contrast microscopy. Sphaeroplasts were harvested by centrifugation at 10,000 r.p.m. for 15 min at 4°C. Both supernate and sphaeroplast preparations were examined for alginase activity.

Chemicals and Biochemicals

All chemicals and biochemicals were of the purest grade commercially available and were purchased when available from B.D.H. Chemicals Ltd., Poole, England, or from Koch-Light Laboratories Ltd., Colnbrook, Bucks., England.

Mannuronic acid, guluronic acid and gulose were kindly given by Dr. C.J. Lawson, Tate and Lyle Ltd., Reading, England.

The sources of alginate samples used in this study are detailed in the Results Section.

Buffers

Buffers were prepared according to the methods outlined in "Medical Microbiology" (Cruickshank, 1965).

MOPS (3 - (N - morpholino) propanesulphonic acid)-NaOH buffer was used at a concentration of 10mM, with respect to MOPS.

Buffers were stored at 4^{o}C.

Chromatography

(i) Paper chromatography was carried out in a descending manner on Whatman No. 1 or No. 4 paper. Whatman 3MM paper was used for preparative work. The following solvent systems were used:

(A) ethylacetate: pyridine: acetic acid: water, 5:5:1:3 (v/v) (Fischer and Dorfel, 1955).

(B) ethylacetate: acetic acid: formic acid: water, 18:3:1:4 (v/v) (Feather and Whistler, 1962).

(C) butan-1-ol: acetic acid: water, 50:12:25, (v/v) (Preiss and Ashwell, 1963).

(D) ethyl methyl ketone: acetic acid: saturated boric acid, 9:1:1 (v/v) Rees and Reynolds (1958).

(ii) Column Chromatography. The products of enzyme hydrolysis of alginates were separated by gel filtration in columns of Sephadex (Pharmacia, Uppsals, Sweden) or Bio-gel (Bio-Rad Laboratories, Bromley, Kent, England).

Details of column dimensions and particular fractionating

medium employed are given in the Results Section.

The eluting buffer used was pyridinium-acetate (4 ml pyridine and 10 ml acetic acid diluted to 1 litre with distilled water).

Proteins were fractionated on Sephadex G-100 using Mops pH 7.5 buffer as eluant. Ion exchange chromatography was carried outusing Whatman (Whatman Biochemicals Ltd., Maidstone, Kent, England) diethyl-aminoethyl cellulose (DE32) and Sephadex C-50 (carboxymethyl). Again column dimensions and details of elution buffersare given in the Results Section.

Electrophoresis

Electrophoresis was performed on Whatman No 1 paper in pyridinium acetate buffer (pH5.3) using a current of 80-100 mA at 3.5 KV for periods of up to 1.5 h on Locarte (London) equipment with 75 cm x 20 cm cooled plate area.

Detection of sugars on paper

(i) Sugars were detected by dipping in the alkaline-silver nitrate reagent of Trevelyan, Procter and Harrison (1950).

(ii) Unsaturated uronides were detected by spraying the paper with the following reagents:

(A) 0.05M sodium periodate in 0.05M H_2SO_4

(B) ethylene glycol: acetone: H_2SO_4 (50:50:0.3)

(C) 6% sodium thiobarbiturate

The paper was sprayed lightly with reagent A. After 15 min reagent B was applied. Finally after a further

6 min reagent C was applied and the paper heated at 100 ^{o}C for 5 - 10 min (Warren, 1960).

Quantitative Analytical Procedures

(i) Protein was determined by the method of Lowry, Rosebrough, Farr and Randall (1951) using bovine serum albumen as standard.

(ii) Total carbohydrate was determined by the phenol/sulphuric acid procedure of Dubois, Gillies, Hamilton, Rebers and Smith (1956).

(iii) O-acetyl groups were estimated by the method of Hestrin (1949) using acetyl choline as standard.

(iv) Concentration of guluronic acid 'blocks' was determined by the carbazole/sulphuric acid method of Knutson and Jeanes, 1968. Six ml of concentrated H_2SO_4 was added slowly to 1 ml of sample cooled in ice, after heating at 55^{o}C for 20 min, 1 ml of 0.1% carbazole in ethanol was added. After mixing the colour was allowed to develop at room temperature for 3 h.

(v) Reducing power was measured by the method of Nelson (1944).

Glassware used for analytical procedures was cleaned with chromic acid and repeatedly rinsed with tap, then glass distilled water.

Spectrophotometry

All measurements were carried out using a Unicam (Unicam Instruments Ltd., Cambridge, England) SP 500, SP 600 or SP 800, spectrophotometer, or Zeiss (model PMQ - II) spectrophotometer (Carl Zeiss, Oberkochen, W. Germany).

Enzymatic reactions studied spectrophotometrically were carried out in an SP 800 fitted with an SP 870 constant temperature cell.

Estimation of Enzyme Activity

The amount of unsaturated uronic acid material produced by lyase action can conveniently be estimated using the Periodate-Thiobarbituric acid test.

The test used in this study was that of Weissbach and Hurwitz (1959). See Figure 10.

The following reagents were prepared:

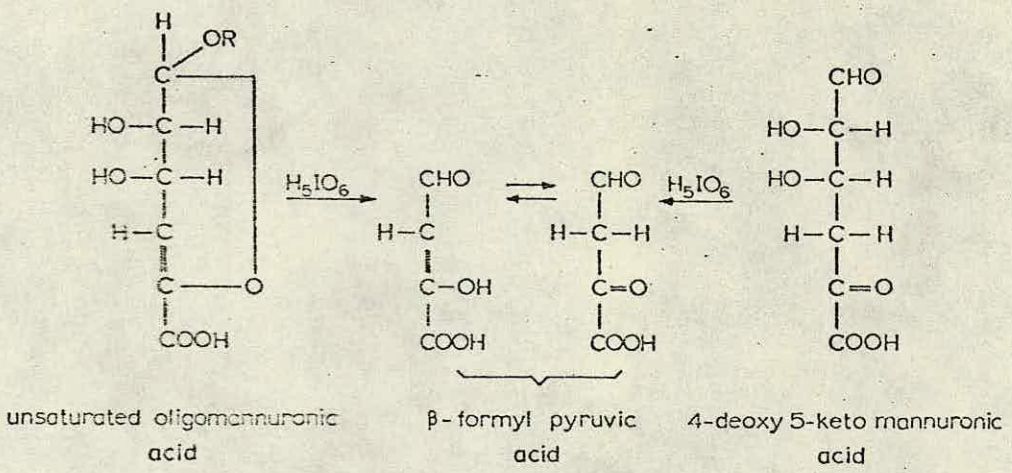
(A) 0.025M periodic acid in 0.125N H_2SO_4

(B) 2% (w/v) sodium arsenite in 0.5N HCl

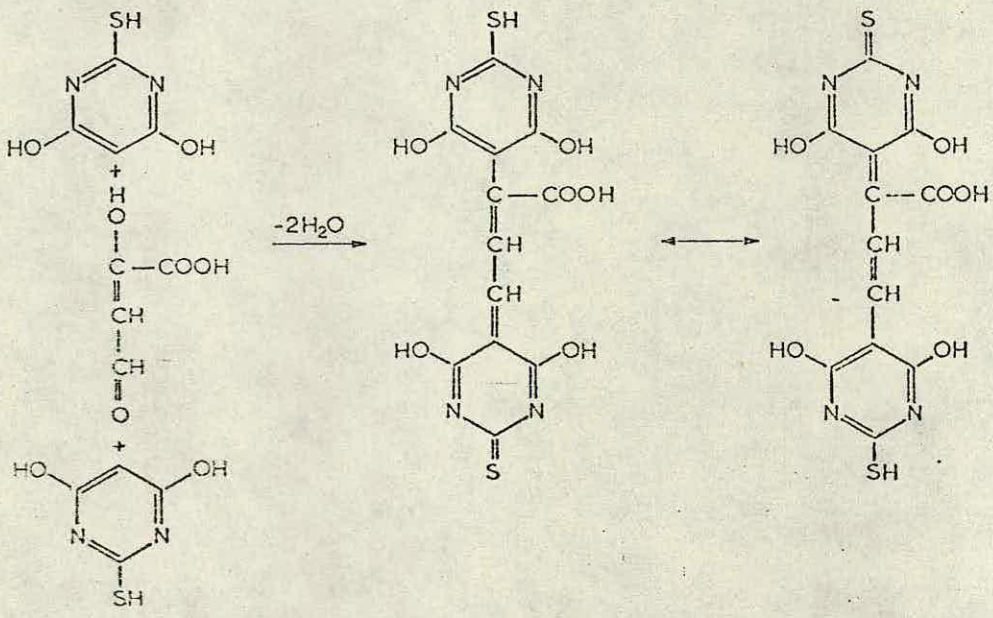
(C) 0.3% (w/v) thiobarbituric acid (TBA), pH 2.0

The test was carried out by mixing 0.20 ml of test sample with 0.25 ml of reagent A. After 20 min 0.5 ml of reagent B was added. The mixture was gently shaken and allowed to stand for 2 min, during which time the brown iodine colour appeared and disappeared. After the addition of 2 ml of reagent C the mixture was heated for 10 min in a

FIGURE 10



Action of periodate on unsaturated oligouronides and deoxyketouronic acid.



Reaction of β-formylpyruvic acid with thiobarbituric acid.

boiling water bath. The tubes were quickly cooled and the resulting red colour measured against a reagent blank in a spectrophotometer at 549 nm.

A unit of enzyme activity was defined as: the amount of enzyme required to liberate the equivalent of 1μ mole of β-formyl pyruvic acid per min. 0.01μ moles of β-formyl pyruvic acid produces an optical density reading of 0.290 at 549 nm (Preiss and Ashwell, 1962).

Details of enzyme incubation mixtures are given in the Results Section.

Hydrolysis of Oligouronides

Oligouronides were hydrolysed by heating with 2M HCl for 2 hours at 100°. The hydrolysate was evaporated to dryness under reduced pressure re-dissolved in distilled water, then re-evaporated before finally concentrating to a small volume.

Partial acid hydrolysis of alginate and preparation of homopolymeric blocks

Alginate samples were hydrolysed using the procedure of Penman and Sanderson (1972). Alginate solutions (10 mg/ml) were boiled under reflux for 5 h with sufficient 3.0 M HCl to give a final concentration of 0.3 M. After hydrolysis, insoluble material was removed by centrifugation. The supernatant was retained.

The precipitate was washed with 0.3M HCl, suspended in water and solubilized by the addition of sodium hydroxide. The pH of this solution was then carefully adjusted to 2.85 with 0.05M HCl, insoluble material was collected by centrifugation. The amount of carbohydrate in these three fractions was estimated by the phenol/sulphuric reaction, using mannurono-lactone as standard; the carbazole/sulphuric method, using guluronic acid blocks as standard; and the phenol sulphuric reaction, using mannuronic acid blocks as standard respectively.

The authenticity of 'blocks'was shown by N.M.R. spectroscopy. The homopolymeric blocks prepared by partial acid hydrolysis were deuterated by evaporation (3 times) with D_2O under reduced pressure. The deuterated sample was taken up in D_2O and examined in a Varian HA - 100 spectrometer (California, U.S.A.) using tert-butyl alchol (2-methylpropan-2-ol) as internal standard.

Alkaline Deacetylation

Polysaccharides were dissolved in water at 5 mg/ml. An equal volume of 2M NH_4OH was added and the mixture heated at 60°C for 60 min. After dialysis against 30 volumes of distilled water for 24 h the non-dialyzable material was lyopholized.

Polyacrylamide Gel Electrophoresis (PAGE) of Proteins modified from Weber and Osborn (1969)

(i) Preparation of Samples

Proteins were solubilized in gel buffer with 1% sodium dodecyl sulphate (SDS) and 1% 2-mercaptoethanol for 4 hr. at 37°C; or overnight at 20°C with vigorous shaking. For application to gels, 10µg of protein standard or 100-200µg of unknown, solubilized protein were mixed with 1 drop 10% sucrose (w/v) and 1µl tracking dye (0.05% bromophenol blue).

(ii) Preparation of Gels

Double strength gel buffer stock solution (7.8g $NaH_2PO_4.2H_2O$ + 51.0g $Na_2HPO_4.12H_2O$ + 2g SDS dissolved in 1 litre distilled water) and 10% acrylamide stock solution (22.2g acrylamide, recrystallized from chloroform + 0.6g methylene bis acrylamide) were prepared. The latter was stored in the dark at 4°C. 15 ml double strength buffer was de-aerated, mixed with 13.5 ml acrylamide solution and again de-aerated. 1.5 ml of freshly prepared ammonium persulphate (15 mg/ml) and 0.04 ml TEMED (N, N, N', N' - tetra methyl - 1,2 - diaminoethane).

Aliquots (1 ml) were quickly transferred from the bulk to stoppered tubes (65 x 5 mm). Before gelling, water was layered on top of each gel solution.

(iii) Electrophoresis

Electrophoresis was performed at a constant current of 8 mA per gel, with the positive electrode in the lower chamber using Quickfit PAGE equipment (Quickfit and Quartz Ltd., Stone, Staffs., England). Under these conditions the marker dye

moved three quarters through the length of the gel in approximately 3 h. The length of the gel and the distance moved by the dye were measured.

(iv) Staining and Destaining

After electrophoresis gels were removed from the tubes. Staining and destaining was carried out by placing the gels in the following solutions for the times stated (D.F.H. Wallach, Max-Planck-Institut fur Immunbiologie, Freiburg, Germany).

(a) 1.25 g Coomassie brilliant blue R (R.A. Lamb, Alperton, Middx., England), 1625 ml distilled water; 625 ml propan-2-ol; 250 ml acetic acid, for 6 - 12 h.

(b) 125 mg Coomassie blue; 2000 ml distilled water; 250 ml propan-2-ol; 250 ml acetic acid, for 6 - 12 h.

(c) 63 mg Coomassie blue; 2250 ml distilled water; 250 ml acetic acid, for 6 - 12 h.

(d) 1000 ml distilled water; 800 ml methanol; 200 ml acetic acid for 4 - 6 h.

(e) 10% acetic acid 12 h. and also to store stained gels.

To compensate for gels swelling during staining the mobility was calculated as:

$$\text{Mobility} = \frac{\text{Distance of protein migration}}{\text{length after destaining}} \times \frac{\text{length before staining}}{\text{distance of dye migration}}$$

PAGE of Alginates

Electrophoresis of alginate samples was carried out using the procedure described by Bucke (1974). The polyacrylamide gel consisted of 6% total monomer with N, N' - methylene bis acrylamide constituting 2% of the monomer. Monomers were dissolved in 0.25 M Tris-HCl, pH 8.3 containing 0.05% TEMED and sufficient ammonium persulphate to give a final concentration of 0.08%. Gel cylinders were prepared

as before.

Alginate samples (20 - 60µg in water) were mixed with 100µl of a viscous sample gel (6% w/v acrylamide polymerized without cross linker in 0.02 M Mops, pH 7.3) and 1µl of tracking dye. The running buffer was 0.1M glycine-Tris, pH 9.0. Electrophoresis was allowed to proceed for approximately 1.5h at 4mA per tube. Gels were stained overnight in 0.08% Alcian Blue in 7% (v/v) acetic acid. Excess Alcian Blue was removed electrophoretically. Again gels were stored in 10% acetic acid.

RESULTS

SECTION A

Bacterial Alginates

1. Cultural characteristics of organisms.

The appearance of Azotobacter vinelandii and Pseudomonas aeruginosa on solid medium is illustrated in plates 1 and 2 respectively. The colonies of both organisms exhibited typical mucoid characteristics being domed in shape and of smooth, glistening appearance. Microscopic examination by the India ink method of Duguid (1951) showed the exopolysaccharide produced by Ps. aeruginosa was less tightly associated with the cell than that of A. vinelandii which appeared capsulate.

2. Analysis of exopolysaccharides.

The polysaccharides of Ps. aeruginosa and A. vinelandii were prepared and purified, as described in 'Materials and Methods'. The polysaccharides (10 mg) were dissolved in 88% formic acid at a concentration of 5 mg/ml and heated at 100°C for 5 h in sealed tubes (Linker and Jones, 1964). After hydrolysis the products were analysed by paper chromatography (solvent C) and electrophoresis. Sugars were identified on the basis of their mobilities compared to standard sugars. In addition comparison was made to the products from a sample of algal alginate treated in a similar manner. The results are shown in Table 5.

Plate 1 — *Azotobacter vinelandii* after 48 h incubation at 30°C on Burk's medium.

Plate 2 — *Pseudomonas aeruginosa* (C.F. strain) after 24 h incubation at 30°C on yeast extract medium.

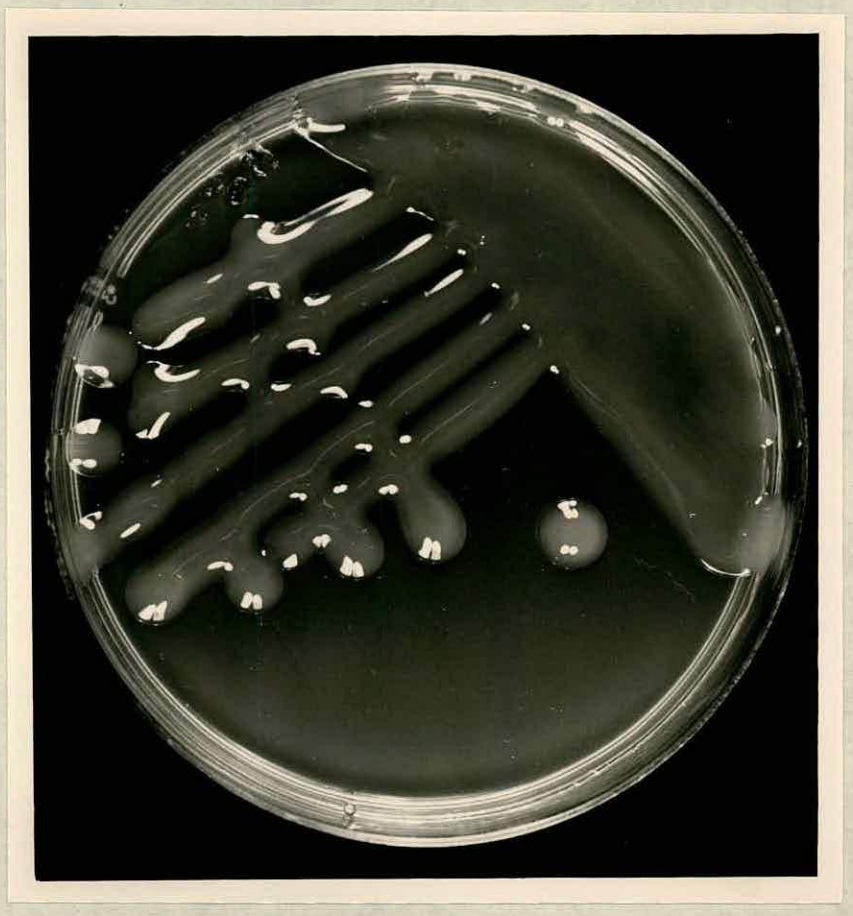


FIGURE 11

Infra-red spectra of algal and bacterial alginates.

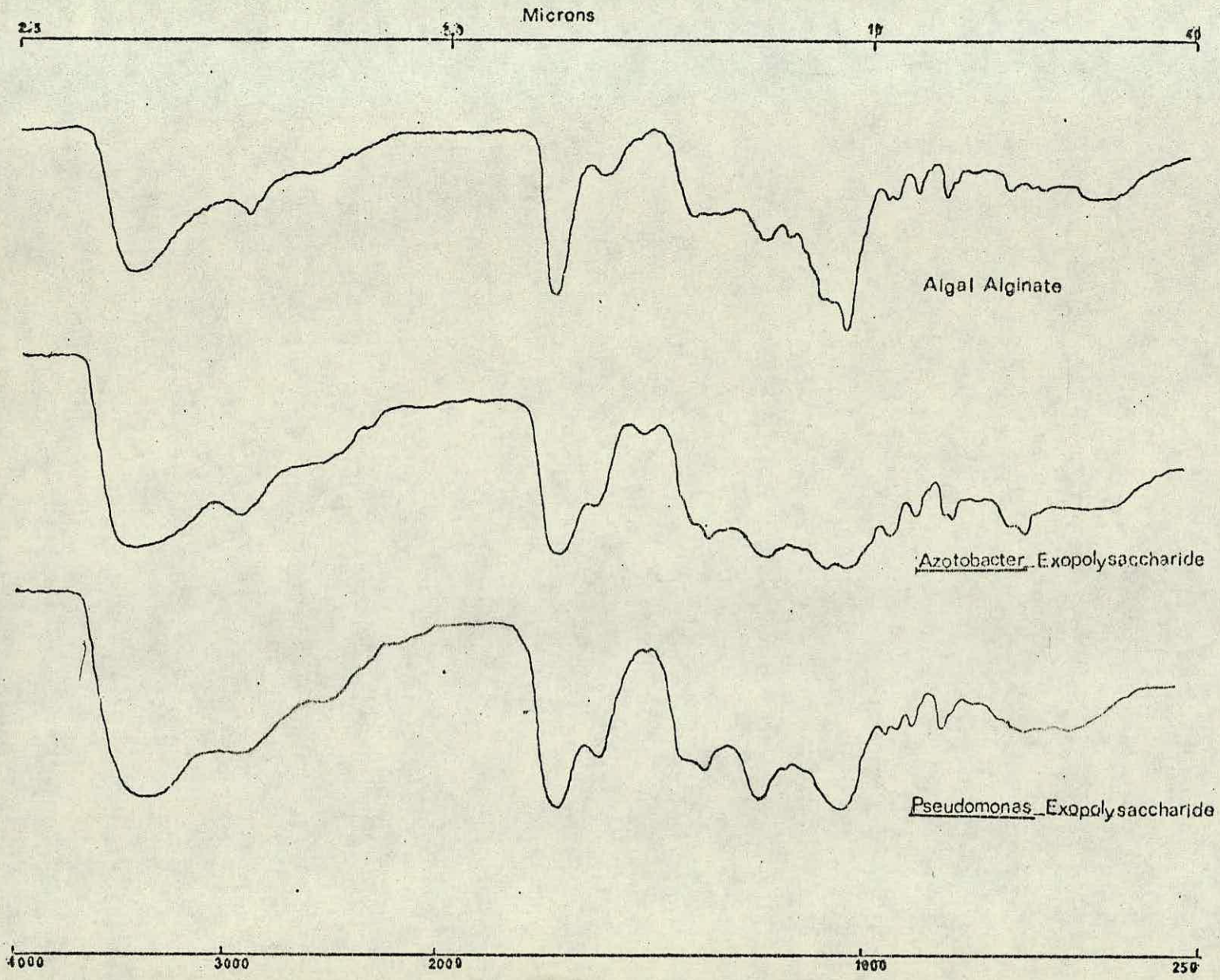


Table 5

Observed $^{R}_{Glc}$ values and mobilities (cm) of sugars found in Pseudomonas and Azotobacter exopolysaccharides and alginic acid.

	$^{R}_{Glc}$			Mobilities	
Alginic acid	1.03	1.30	0	10.5	13.1
Azotobacter exopolysaccharide	0.97	1.30	0	10.9	12.9
Pseudomonas exopolysaccharide	1.02	1.36	0	10.7	12.8
Mannurono-lactone/acid		1.27	0		13.0
Guluronic acid	0.96			10.8	

Dried films of the bacterial polysaccharides were prepared and the infra-red spectra obtained compared to the spectrum obtained from a sample of algal alginate (see Figure 11).

SECTION B

1. Alginate decomposing micro-organisms

The micro-organisms isolated, which were capable of growth on alginate as sole carbon source, were grown in the media described and harvested by centrifugation. Cell free supernates, prepared as described in 'Materials and Methods' were examined for alginase activity. The crude enzyme (0.5 ml) solution was added to 0.5 ml 1% alginate solution in 4.5 ml Mops buffer pH 7.5 and the mixture incubated at 30°C. At intervals 0.5 ml aliquots were removed and examined for TBA positive and reducing sugars. The results are shown in Table 6.

Many of these bacteria grow too slowly to be convenient for detailed study and as can be seen, several produced only low amounts of enzymatic activity. On the basis of these results, SA1, mixed culture 1, Azotobacter phage and A. vinelandii were chosen for detailed study.

It seems likely, that those organisms which grew on alginate medium, but failed to produce significant amounts of alginolytic activity, were utilizing a carbon source present as an impurity in the sodium alginate.

TABLE 6

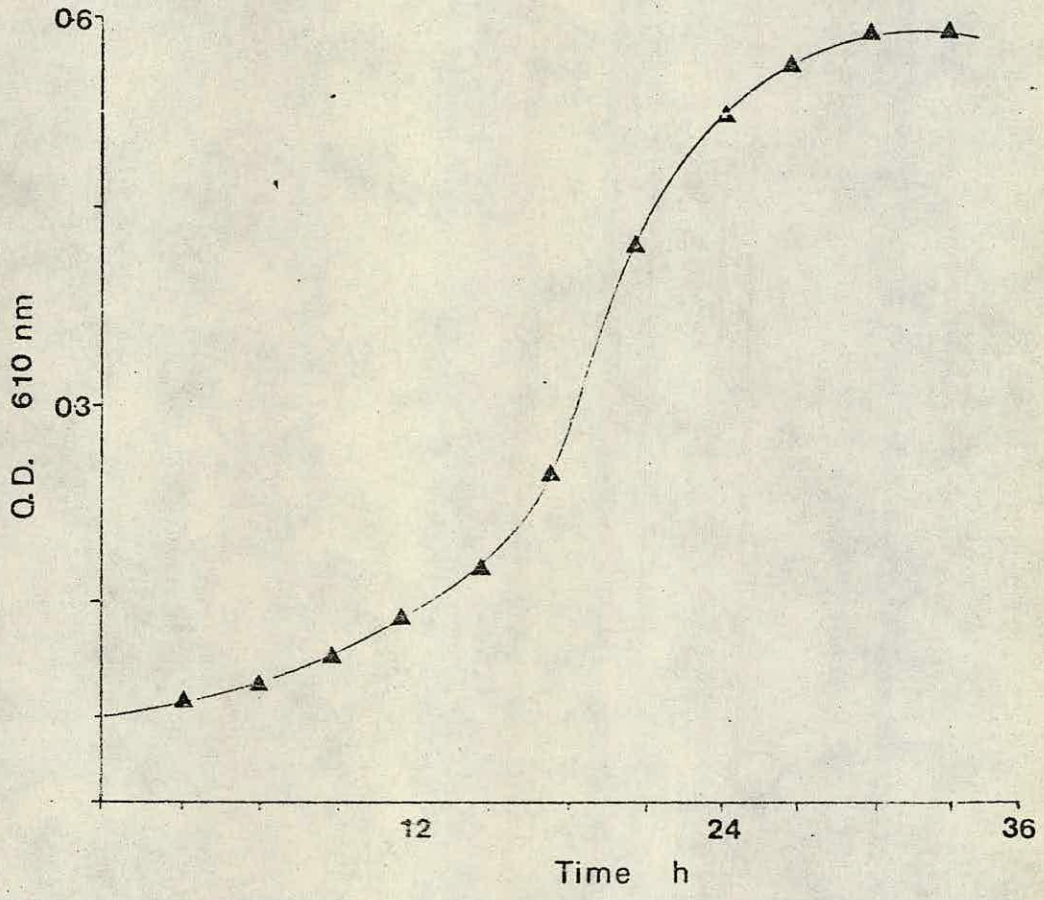
Alginate Decomposing Bacteria

Organism	Source	Alginase Activity 24h incubation	
		T.B.A.	Reducing power
P25	Fresh water	0.16	0.03
P26	Fresh water	0.21	0.05
A1	Soil	0.37	0.082
A2	Soil	0.43	0.10
A3	Soil	0.27	0.05
A5	Soil	0.22	0.06
A6	Soil	0.13	0.07
A7	Soil	0.14	0.04
SA1	Decomposing seaweed	> 3.0	
SA4	Decomposing seaweed	0.58	0.20
PA	1.	0.36	0.09
Mixed culture 1	2.	> 3.0	
Mixed culture 2	3.	0.30	0.04
A. vinelandii	-	> 3.0	
Azotobacter phage	-	> 3.0	

1. This organism was obtained from Dr. C. Bucke, Tate & Lyle Ltd.
2. This preparation was obtained by growing together the organisms A1, A2 and A3.
3. This preparation was obtained by growing together the organisms P25, A6 and A7.

FIGURE 12

Typical time course of growth of the isolate SA1 in synthetic alginate medium.



2. Isolate SA1

On agar this organism gave rise to smooth, off-white, convex colonies. SA1 grew well in the basic alginate broth described, when supplemented with NaCl. Some of the morphological and physiological properties of the organism are shown in Table 7.

Table 7

Cell shape	rod
Gram stain	-ve
motility	+ve
spore stain	-ve
pigment	-ve
fluorescence (U.V.)	-ve
catalase	+ve
oxidase	-ve
methyl red	-ve
Voges Proskeur	+ve

The preliminary diagnostic tests suggest SA1 may be classified as a pseudomonad (Cowan and Steel, 1961).

3. Purification of SA1 alginate depolymerase

Step 1. Preparation of crude extract. Fifteen litres of cells were harvested in mid-logarithmic (see Figure 12) phase of growth, disrupted and cell free supernates prepared as described in 'Materials and Methods'.

Step 2. Ammonium sulphate precipitation. Solid ammonium sulphate was added to the crude enzyme solution to obtain 40% saturation (preliminary, small scale experiments determined saturation concentrations at which the alginase was precipitated). The precipitate was removed by centrifugation at 10,000 rpm for 20 min, and discarded. Additional ammonium sulphate was

added to obtain 80% saturation and the suspension centrifuged as above. The precipitate was dissolved in 150 ml of Mops buffer pH 7.5 and dialysed overnight against 2l of the same buffer with two changes.

Step 3. Sephadex G-100 chromatography. The sample obtained from step 2 was divided into four portions, and each was chromatographed on a G-100 Sephadex column (51 cm x 2 cm) equilibrated with Mops buffer pH 7.5. The sample was applied to the column and eluted with Mops buffer. The fractions showing alginase activity were pooled and dialysed overnight against 0.05M Tris-HCl buffer pH 7.8(it was necessary to replace the zwitterionic Mops buffer to facilitate efficient use of ion-exchange materials; the buffers recommended by the manufacturers were employed).

Step 4 DEAE-cellulose chromatography. A DEAE-cellulose column (55 cm x 3 cm) was equilibrated with 0.05M Tris-HCl buffer pH 7.8. The dialysed sample from step 3 was divided into two equal portions. The loaded column was eluted with 500 ml of a linear gradient from 0-0.5M NaCl (in Tris buffer). Five millilitre fractions were collected and those showing alginase activity were pooled and dialysed overnight against 0.05M phosphate buffer pH 7.8.

Step 5. CM Sephadex chromatography. A CM (C-50) Sephadex column (40 cm X 1.6 cm) was equilibrated with 0.05M phosphate buffer pH 7.8. The sample from step 4 was applied to the column and eluted with a linear gradient as above. The fractions showing alginase activity were pooled and dialysed against Mops buffer pH 7.5.

The results of the purification procedure are tabulated below (Table 8). The elution profiles obtained for step 3, 4 and 5 are illustrated in figures 13A, B and C. The SDS

TABLE 8

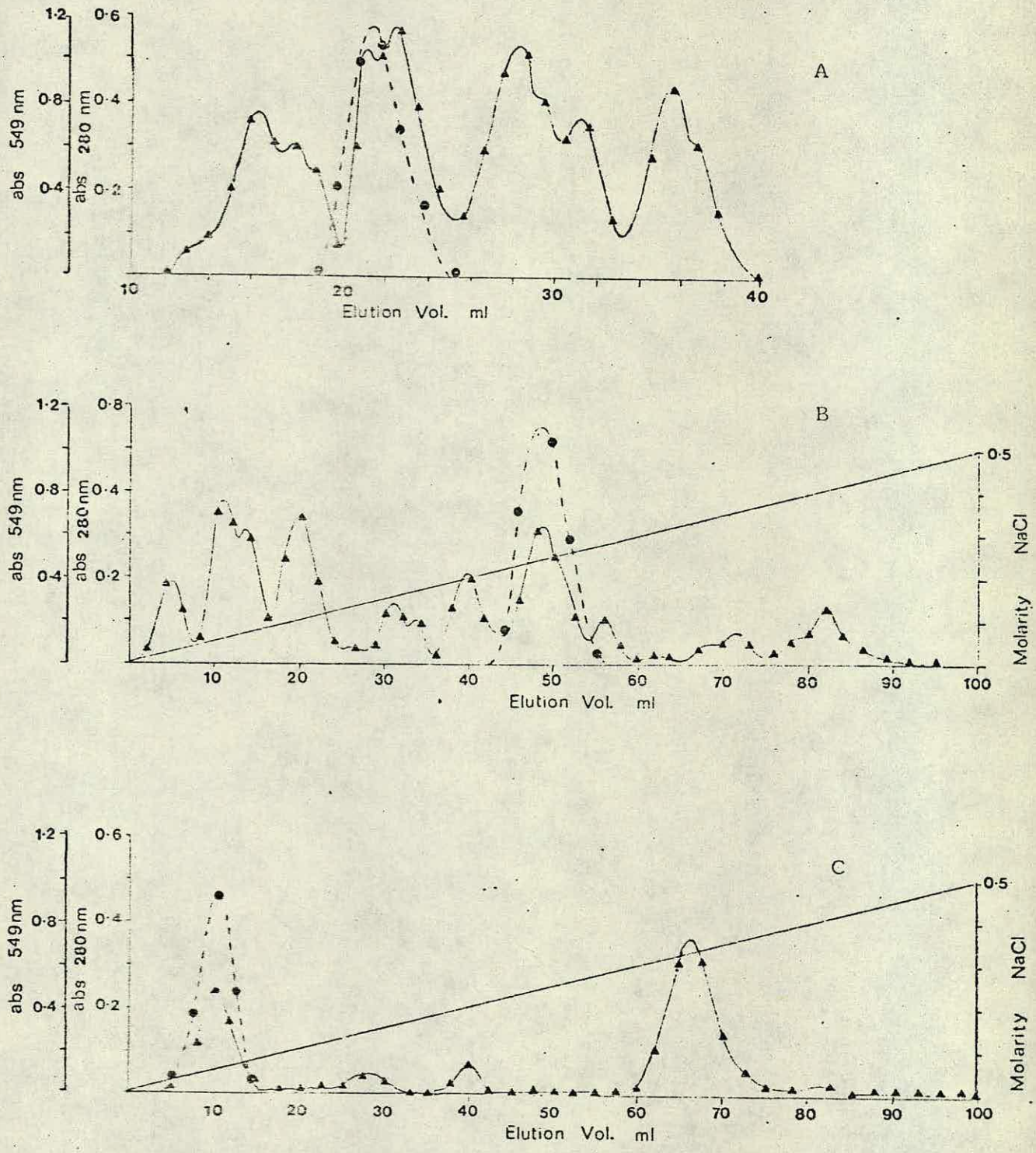
PURIFICATION OF ALGINATE LYASE (E.C. 4.2.2.3.)

Description	Vol (ml)	Protein (mg/ml)	Units /ml	Total Activity	Specific Activity	Yield	Purification
Crude	250	5.5	3.2	800	0.6	100	1
Amm. Sul.	150	0.62	4.3	640	7.0	80	11.7
Batch DE32	100	0.41	3.8	380	9.2	47	15.3
G100	50	0.11	3.8	352	33.0	44	55.0
Column DE32	30	0.075	4.1	184	55.0	23	92.0
Column CM50	24	0.05	3.3	80	73.0	10	120.0

FIGURE 13

Purification of SA1 alginate lyase.

A, B, C correspond to steps 3, 4, and 5 respectively.



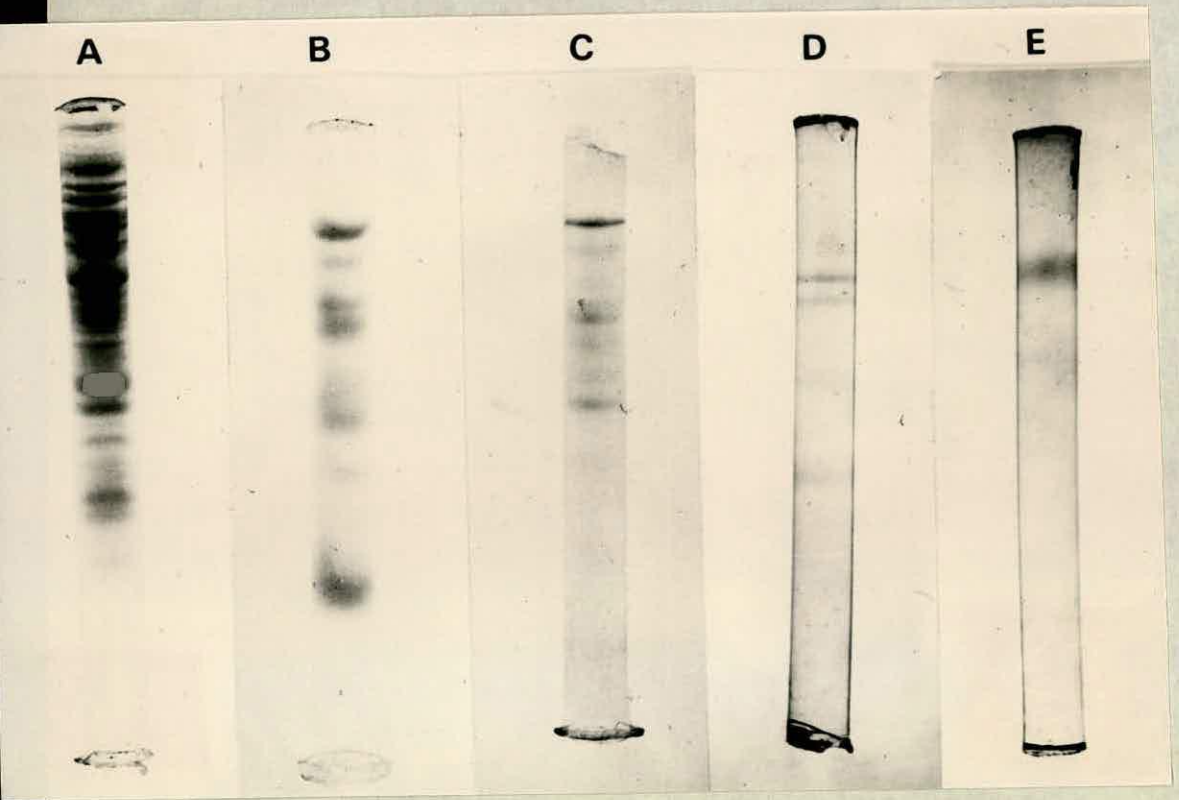
----- Enzymatic activity

——— Protein estimation

Plate 3

Acrylamide disc gel electrophoretograms; showing stages of SAl lyase purification.

A; crude preparation, B, C, D, and E correspond to stages of purification, see text.


A
B
C
D
E

page pattern obtained from the enzyme solutions at the various stages of purification is shown in plate 3.

4. Localisation of SA1 lyase

To facilitate maximum recovery of the enzyme, methods to investigate the localization of alginase activity within the bacterial cell were examined. In addition culture supernates were also examined for enzymatic action

Cells harvested from mid-logarithmetic phase of growth were harvested by centrifugation and washed twice with various surfactants. The washings were retained and the amount of enzyme released by these treatments (see Table 9) estimated. Culture supernates were concentrated by either (a) dialysis against polyethylene glycol or (b) lyophilization of (c) passage through an Amicon pressure cell fitted with a UM-2 membrane (10,000 molecular weight cut-off).

The results are shown in Table 9. The results indicated that up to 40% of the alginase activity could be recovered by treating the cells with Triton X 100, however, in practice it was difficult to remove the detergent from the enzyme solution and SA1 lyase was routinely prepared by the disruption of cells harvested in mid-logarithmic phase of growth.

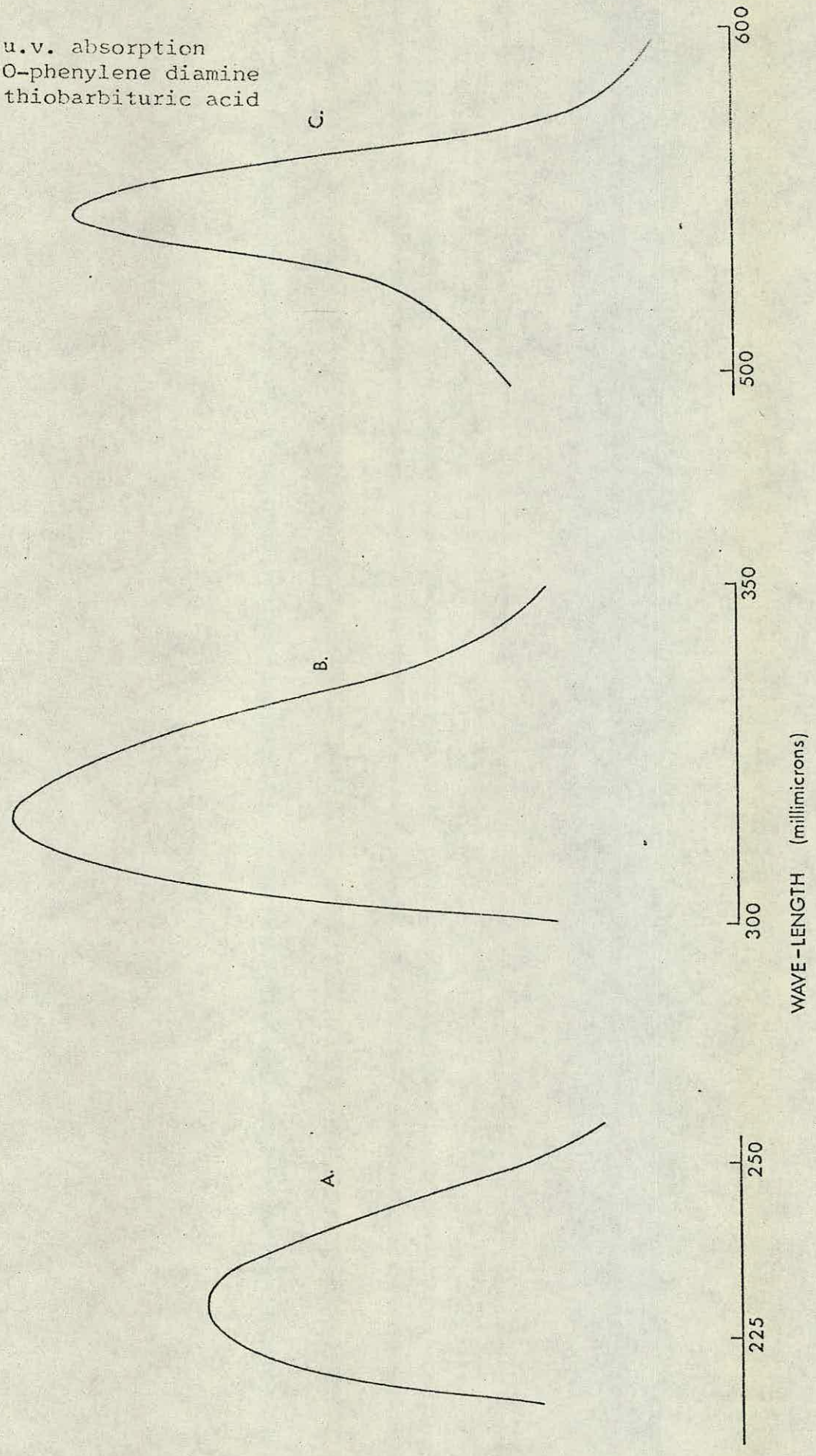
TABLE 9

Treatment	Total Units	Specific Activity	% of Crude
Crude (disrupted cells)	350	0.6	100
Triton x 100 (1%)	150	2.6	43
EDTA (10mM)	70	3.0	20
kCl (0.5M)	110	2.4	31
Lysozyme	130	2.1	37
Culture Supernate	40	1.8	12

FIGURE 14

Absorption Spectra of the products of Alginase activity, based on

A u.v. absorption
B O-phenylene diamine
C thiobarbituric acid



5. Properties of SA1 alginate depolymerase

5.1 The enzyme synthesised by SA1 released products from alginate solutions which gave a positive TBA test, reacted with O-phenylene diamine (Lanning & Cohen, 1951) to give a golden brown colour and absorbed strongly in the ultra violet region (See Figure 14). The results indicate that the enzyme produced by the pseudomonad was of the lyase type (EC 4.2.2.3) described earlier.

5.2 Molecular weight. The molecular weight of the enzyme was determined to be 50,000 as estimated from the S.D.S. polyacrylamide electrophoretic pattern shown in plate 3 (E) (See Figure 15).

5.3 Rate of release of products. Reaction mixtures containing 0.1 ml 1% alginate solution, 0.9 ml Mops pH 7.5 and 25µl enzyme solution (step 3, standard incubation mixture) were incubated at 30°C for increasing intervals of time. After incubation the enzymatic reaction was stopped by heating the mixture to 100°C for 5 min. The amount of TBA positive material present was measured. The result is shown in Figure 16.

5.4 Effect of increasing protein concentration. The linearity of the TBA assay with respect to protein concentration is shown in Figure 17. Increasing volumes of enzyme solution were added to the standard incubation mixture described in 5.3 and the mixture incubated for 60 min at 30°C. After incubation the reaction was stopped and TBA material measured.

5.5 Effect of pH on enzyme activity. The effect of pH on the activity of SA1 lyase is shown in Figure 18. Standard incubation mixtures, using the appropriate buffering system

FIGURE 15

Estimation of the Molecular weight of alginate lyase from isolate SA1.

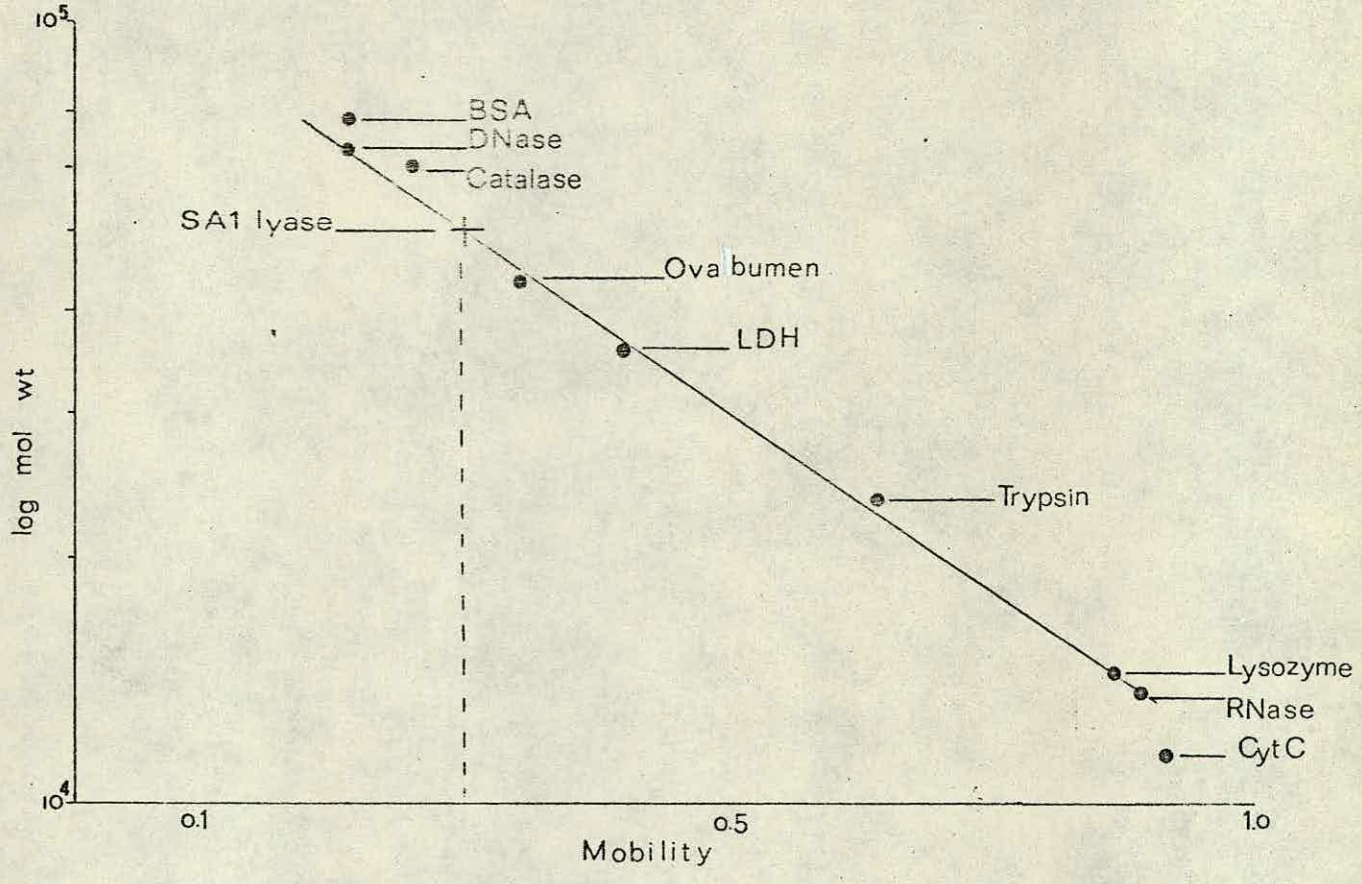


FIGURE 17

Effect of increasing enzyme concentration on release of TBA positive material from alginate.

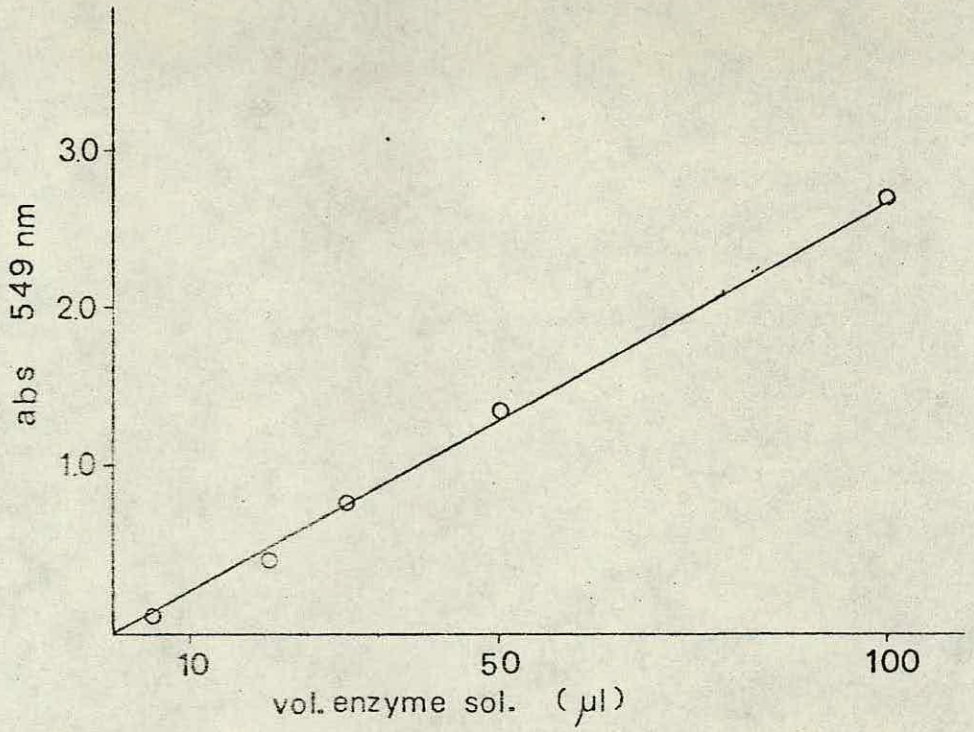


FIGURE 16

Release of TBA positive material from Algal Alginate by SA1 lyase.

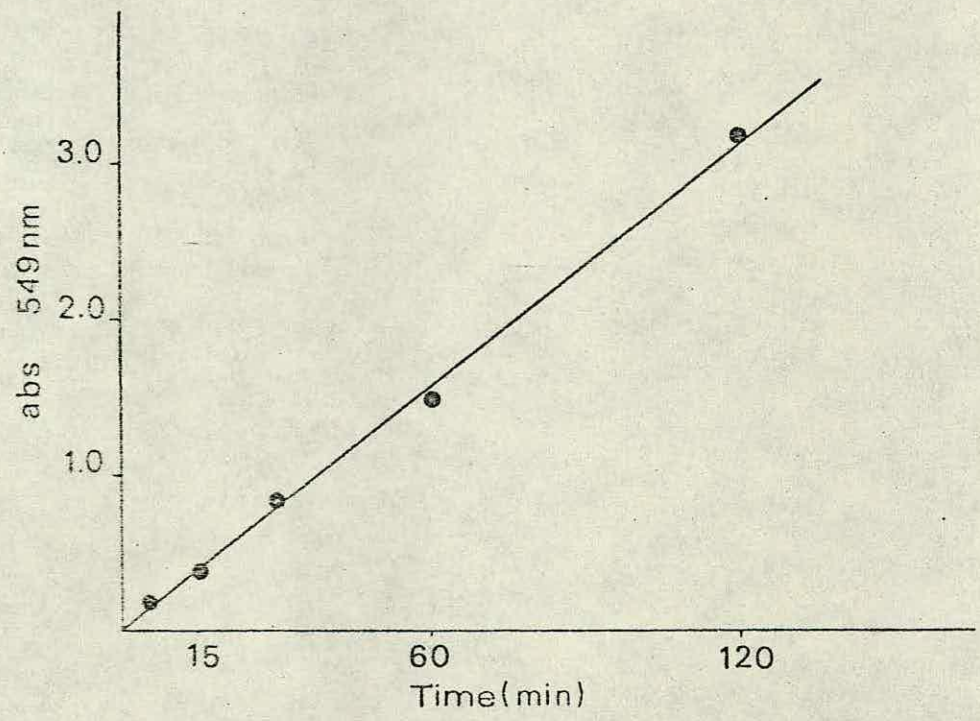


FIGURE 18

Effect of pH on SA1 lyase activity

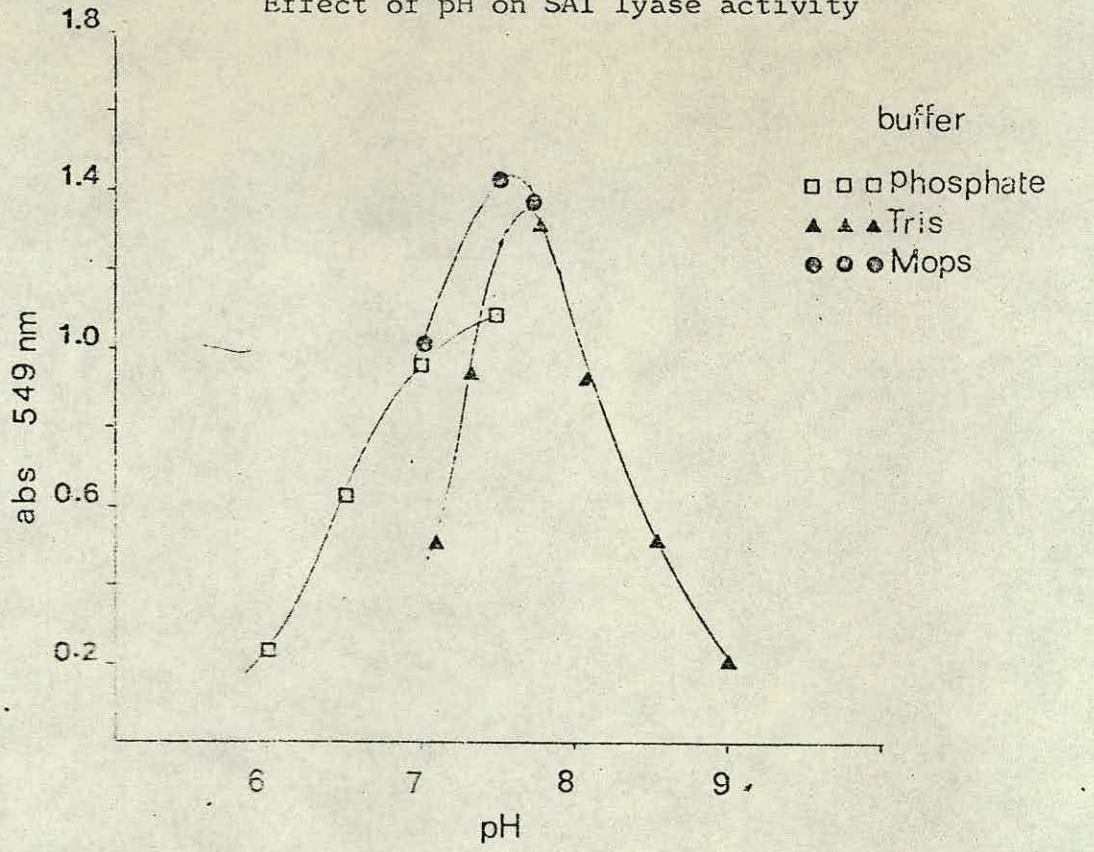


FIGURE 19

Effect of temperature on SA1 lyase activity

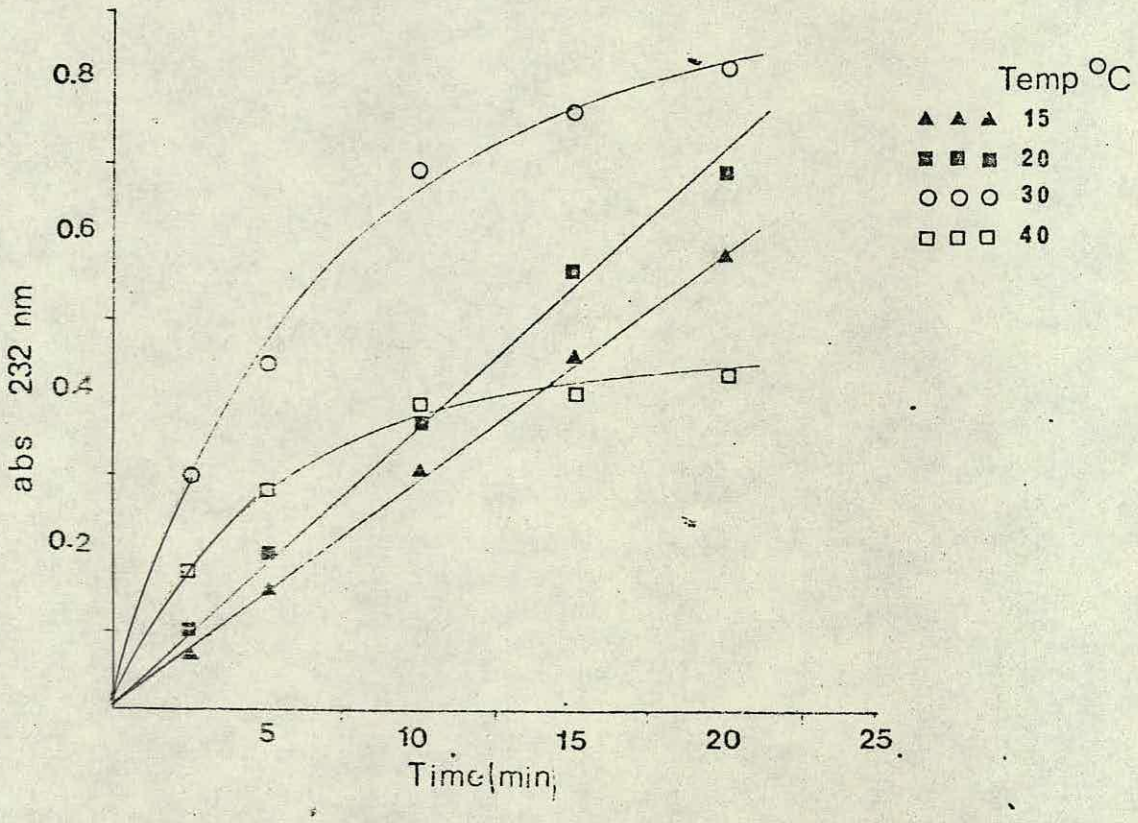
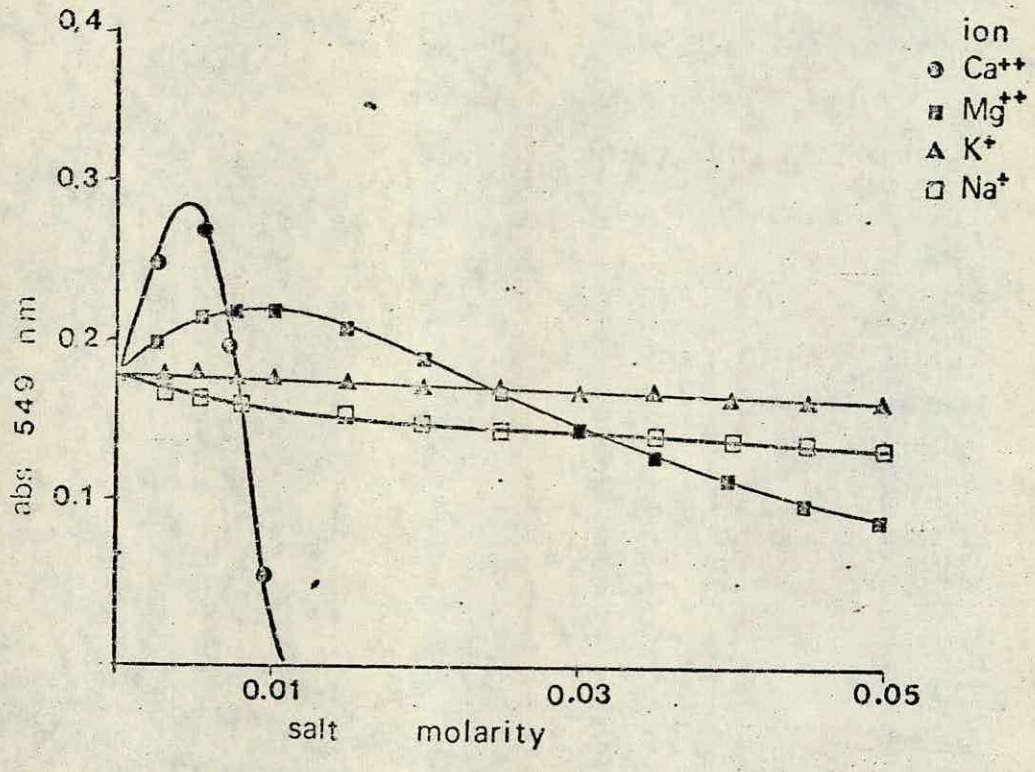


FIGURE 20

Effect of metal ions on alginase activity.



were incubated at 30°C for 60 min, after which time the amount of TBA positive material present was estimated.

5.6 Effect of temperature on enzyme activity. The rate of release of U.V. absorbing material from alginate solutions by SAI lyase action at various temperatures is shown in Figure 19. The reaction was studied spectrophotometrically. The reaction mixture was composed of 0.1 ml 0.01% alginate solution in 0.9 ml Mops pH 7.5 and 30 µl enzyme solution. The reference cuvette contained alginate solution, buffer and enzyme solution which had been heated to 100°C for 15 min.

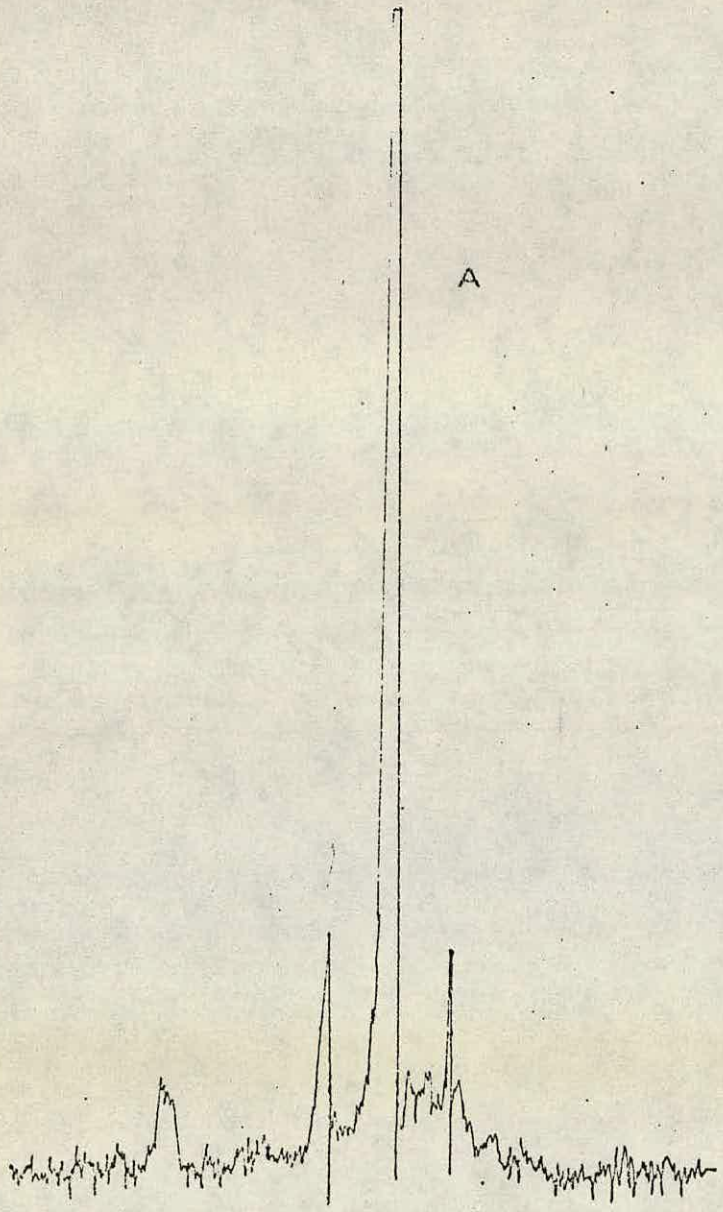
5.7 Effects of salts. Figure 20 shows the effects of increasing concentrations of the chlorides of k^+, Na^+, Mg^{++}, and Ca^{++} on the standard incubation mixture. Table 10 shows the effect of other salts on the reaction (concentration 50 mM).

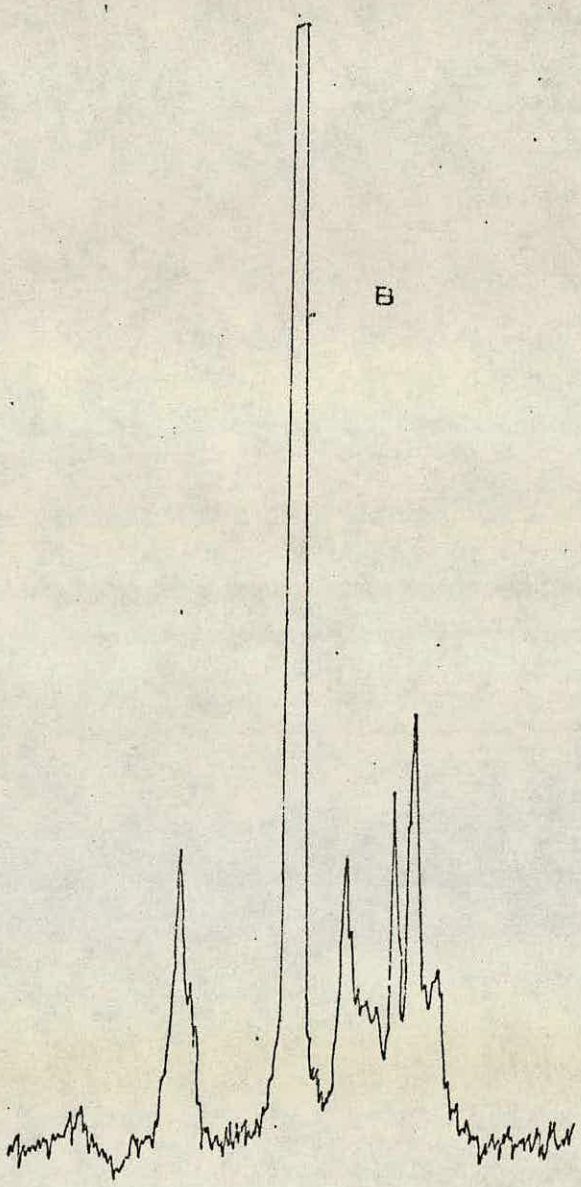
Table 10

without addition	100
$Cu\ Cl_2$	0
$Hg\ Cl_2$	44
EDTA	103
$Pb\ (NO_3)_2$	22
$Ba\ Cl_2$	18.5
$Cs\ Cl$	93
$Sr\ Cl_2$	32
urea	94

In general divalent ions caused inhibition of the enzymatic reaction, the increase in activity observed when low concentrations of Ca^{++} and Mg^{++} ions were added to the incubation mixture may be indicative of a comformational change occurring in the substrate and a consequent quaternary structure which was more accessible to enzymatic hydrolysis.

FIGURE 21





P.M.R. spectra of

(A) Polymannuronic acid

(B) Polyguluronic acid

5.8 Specificity of SA1 lyase. Alginates with approximately known mannuronic acid contents were used as substrates in the standard incubation mixture. The results shown in Table 11 provided preliminary evidence which indicated that the enzyme may have been specific for linkages involving guluronic acid. In order to establish this fact further experiments were undertaken.

Table 11

Alginate	% Mannuronic acid	TBA colour $E_{549\ nm}$/hour
SS/DJ [1]	28	2.10
71/- [2]	33	1.83
68/- [2]	42	0.50
F387 [1]	48	1.60
69/- [2]	55	0.96
62/- [2]	67	0.96
47/- [2]	73	0.76

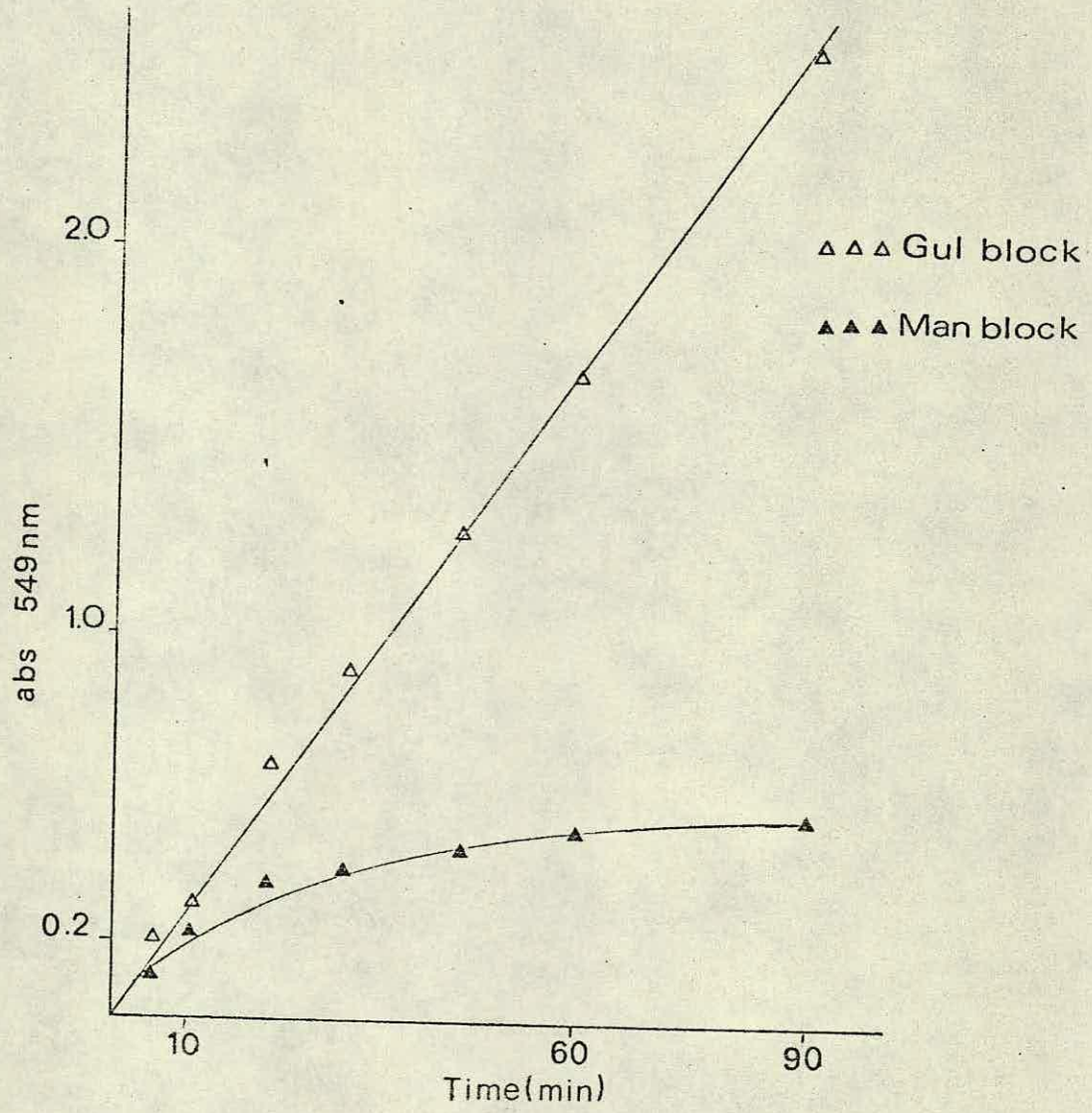
1 Algal Alginate

2 Azotobacter Alginate (Alginate samples were kindly provided by Dr. C.J. Lawson, Tate & Lyle Ltd.)

5.9 Action of SA1 lyase on homopolymeric substrates. Homopolymeric "blocks" were prepared from algal alginate as described and their authenticity confirmed by P.M.R. analysis (see Figure 21). The rate of release of TBA positive material from these substrates, using the conditions described earlier (5.3), is shown in Figure 22.

FIGURE 22

Rate of release of TBA positive material from Homopolymeric substrates



5.10 km and Vm determinations. The km and Vm constants were determined for the enzyme with various alginates as substrates. The constants were obtained by the method of Lineweaver and Burk (1934). The assay solutions contained 0.9 ml Mops buffer pH 7.5 and the following amounts of alginate (µg) contained in 0.1 ml water: 200, 100, 50, 33.3, 25, 10 and 5. Fifty micro litres of enzyme solution was added and the reaction allowed to proceed for 60 min at 30°C before being stopped by heating to 100°C. The results are shown in Table 12 and figure 23.

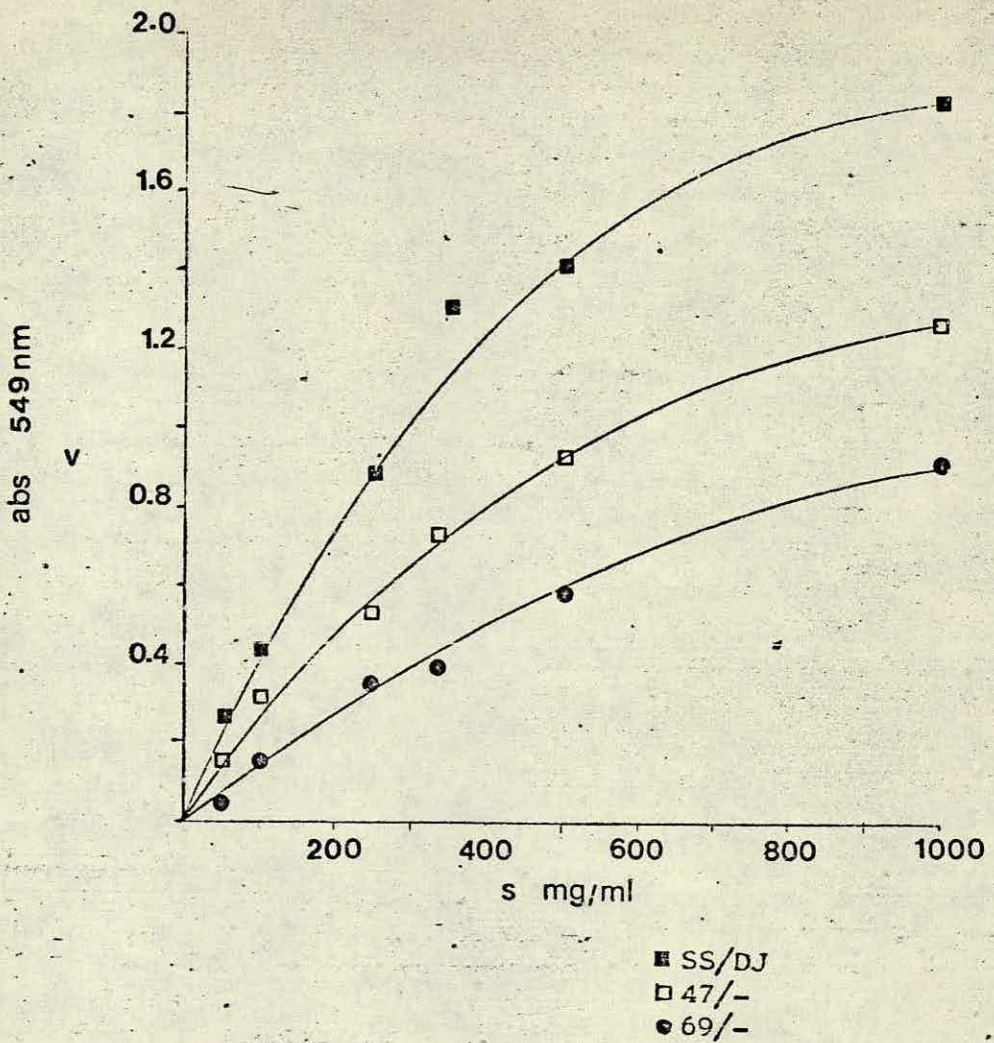
TABLE 12

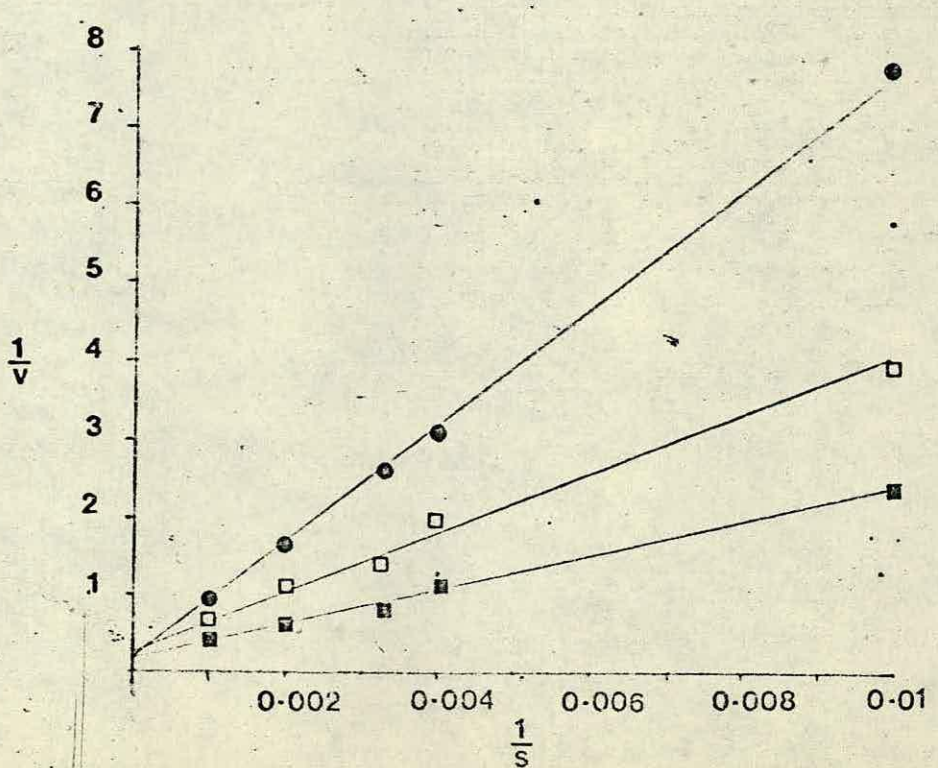
Alginate	% Man	km (mg/l)	km GulA (mM)
SS/DJ	28	1500	6.13
BDH	48	1850	5.51
F387	48	2460	7.27
69/-	55	2180	5.57
47/-	73	4400	6.74

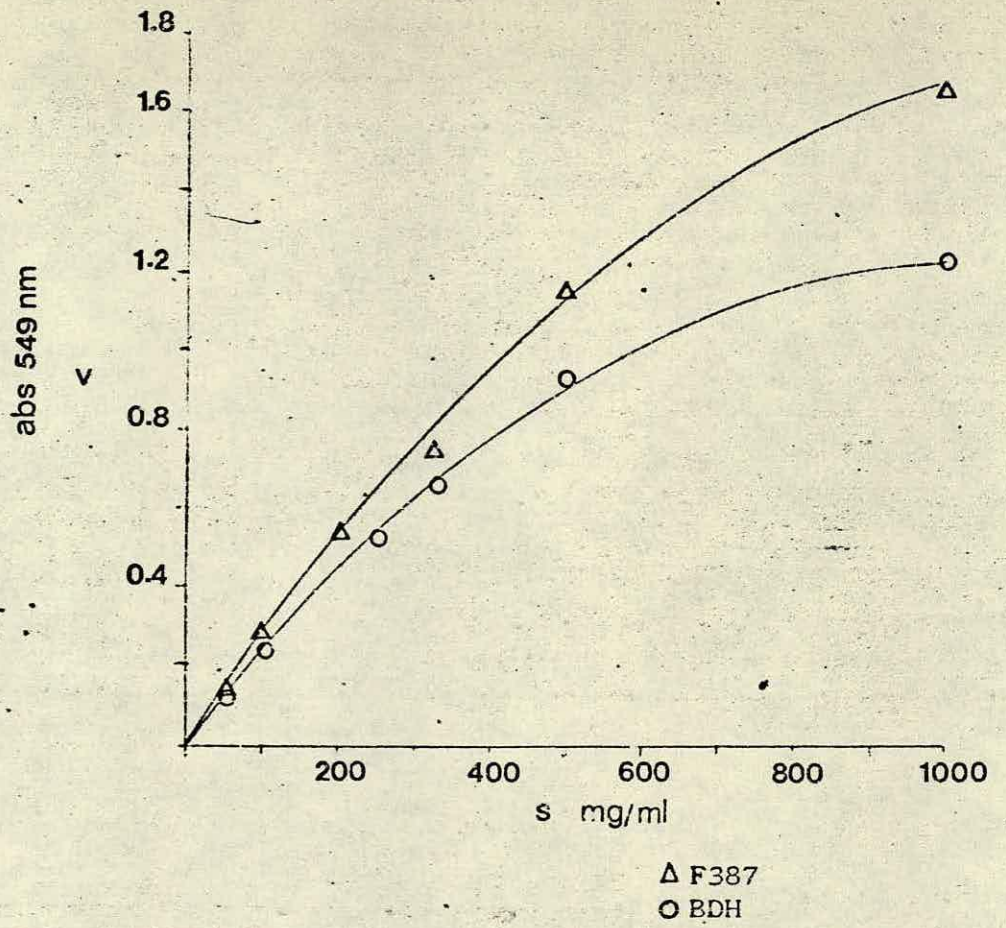
5.11 Action of SA1 lyase on substrates other than alginate. SA1 lyase showed no eliminative activity towards, polygalacturonic acid, hyaluronic acid, chondroitin sulphate or heparin, as estimated using the procedures described above, 5.1.

FIGURE 23

Kinetics of SA1 lyase action.






1.8
1.6
1.2
0.8
0.4
abs 549 nm
v
200
400
600
800
1000
s mg/ml
Δ F387
O BDH

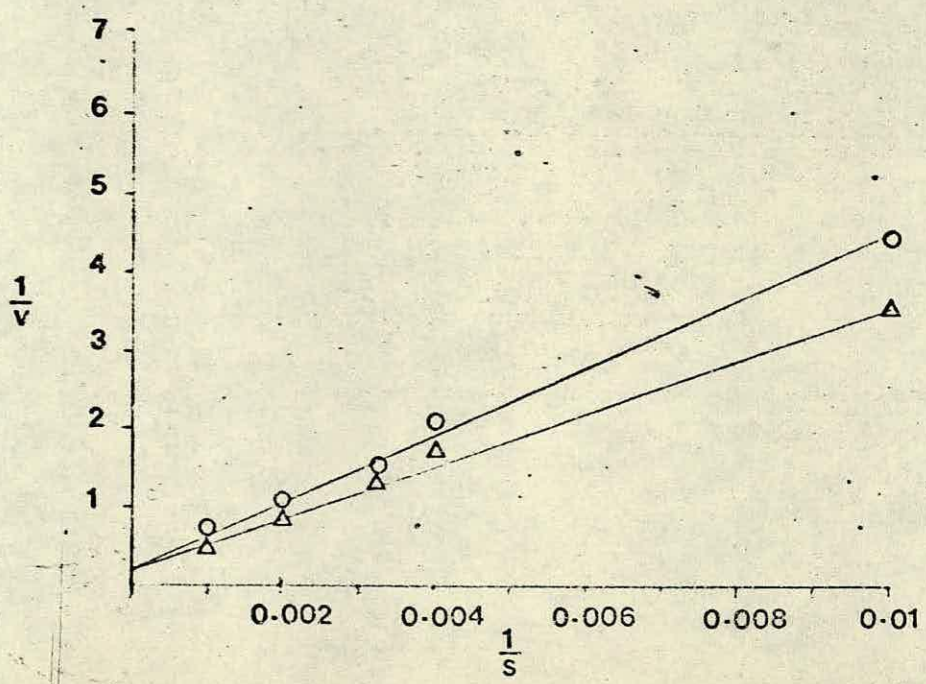
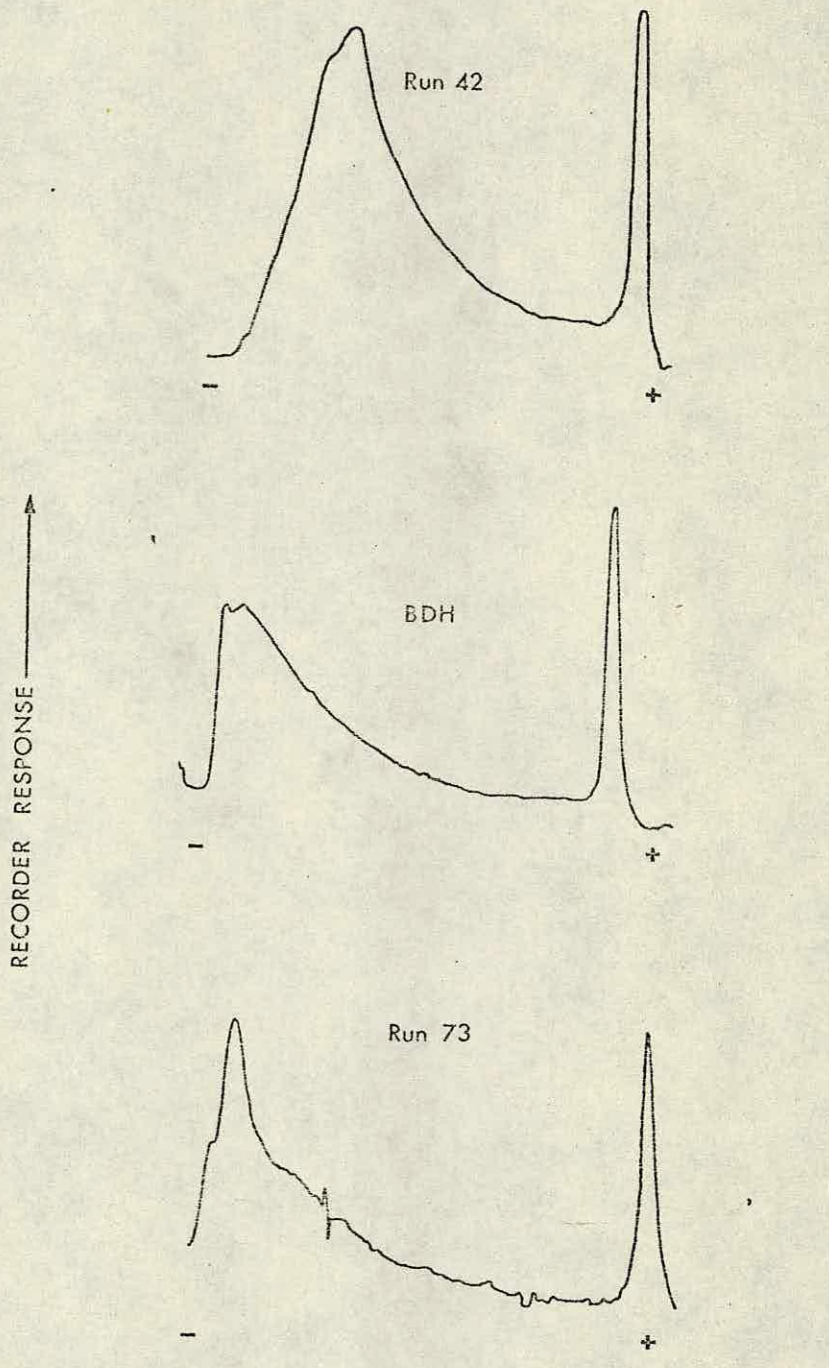

7
6
5
4
3
2
1
1/v
0·002
0·004
0·006
0·008
0·01
1/s

FIGURE 24

P A G E of Alginate Samples



6.0 Isolation and Characterization of Products of SA1 lyase action.

BDH an algal alginate, and two samples of Azotobacter alginate, RUNS 42 and 73 were used as substrates in the study of the products produced by SA1 lyase activity. Both Azotobacter alginates were supplied by Dr. C. Lawson, Tate and Lyle Ltd., and were available in sufficient quantity to avoid any substrate variability which may have been caused by batch preparation of Azotobacter exopolysaccharide.

All three alginates were partially acid hydrolysed using the procedure described in 'Materials and Methods' and the carbohydrate present in the resultant three fractions estimated by the colourimetric methods described. The result of the partial acid hydrolysis procedure is shown in Table 13.
In addition these alginates were examined by PAGE; records of the gels made by scanning in a U.V. Chromoscan densitometer (Joyce, Loebl & Co.,) are illustrated in Figure 24.
These alginates illustrate the diversity of alginate structure reported elsewhere (See Table 4).

Table 13

Alginate	% soluble	% insoluble pH 2.85	% insoluble pH 2.85	yield
BDH	51	20	18	89
RUN 42	42	23	30	94
RUN 73	32	9	48	88

6.1 The products released from alginate solutions by the action of SA1 lyase were examined by descending paper chromatography. Plate 4 shows the mobility of the products relative to guluronic acid. No other components were detected when chromatograms were stained with alkaline silver nitrate. Prolongation of the incubation time, beyond 12 h, failed to produce a qualitative change in the chromatographic pattern; similarly alginates with different Man:Gul ratios gave identical patterns. R Gul U values obtained from chromatographic analysis are shown in Table 14.

Table 14

Compound	Solvent System				
	A	A*	B	C	C*
dimer	0.63	0.50	0.94	0.92	0.89
trimer	0.32		0.64	0.72	
tetramer	0.27		0.46	0.52	
pentamer	0.12		0.21	0.43	
hexamer	0.05		0.09	0.20	

*Rombouts (1972), R Gal U values for unsaturated digalacturonide.

From the paper chromatography evidence shown, the degree of polymerization of the fastest moving compound was estimated as two. To confirm this the products of alginase activity were fractionated in a, previously calibrated, column of Sephadex G-15 (38 cm x 1.2 cm) using pyridine-acetic acid buffer as eluent. Fractions (1 ml) were collected and analysed for carbohydrate. The result is shown in Figure 25.

Plate 4

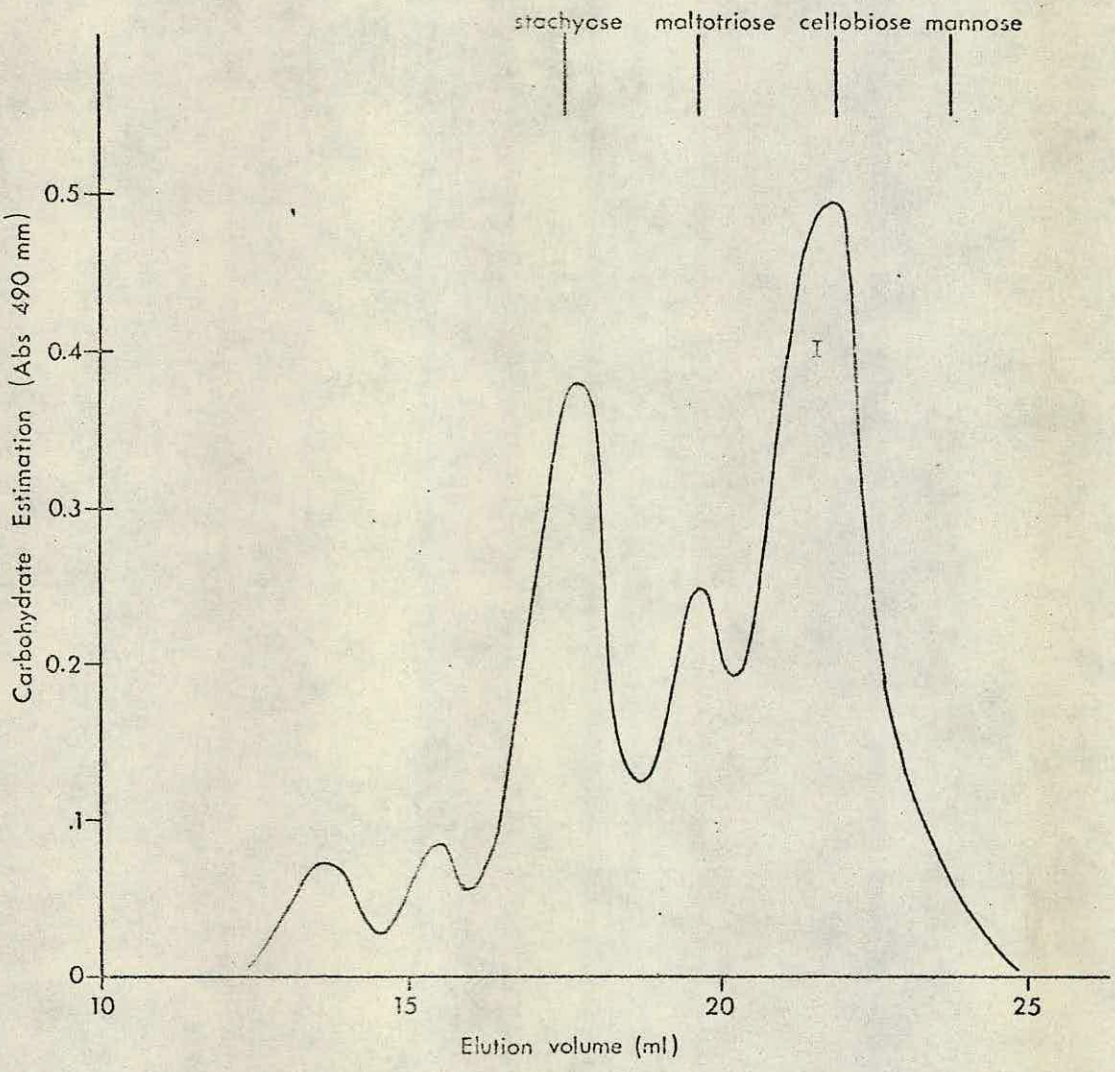
Chromatogram showing the mobility of low molecular weight products released from alginate solutions by the action of SA1 lyase relative to guluronic acid (A):

B: products from algal alginate: 24 h incubation
C: products from Azotobacter alginate; 6 h incubation
D: products from Azotobacter alginate; 24 h incubation


A
B
D
G

FIGURE 25

Fractionation of products of alginase activity on Sephadex G-15. Calibration points are shown.



Peak I (see Figure 25), was re-examined by descending paper chromatography and shown to have ^{R} Gul U values of 0.90 and 0.64 respectively in solvents A and C.

The oligouronides released by SA1 lyase activity were accumulated by preparative paper chromatography. Guide strips were stained to locate the oligosaccharides which were then eluted from the paper with distilled water. Each component was shown to be chromatographically pure in solvents A and B, then hydrolysed with 2 M HCl as described in "Materials and Methods". The hydrolysates were examined by descending paper chromatography (solvent C) and high voltage electrophoresis, after staining with alkaline silver nitrate, the approximate ratio of mannuronic and guluronic acids present in each hydrolysate was estimated by scanning the paper in a Chromoscan (Joyce Loebl Ltd.). The results are shown in Table 15.

Table 15

Component	Algal Alginate		Azotobacter Alginate	
	Man	Gul	Man	Gul
dimer	-	+++	-	+++
trimer	-	+++	-	+++
tetramer	+	+++	-	+++
pentamer	+	+++	+	+++

To confirm in part the result obtained by acid hydrolysis of the unsaturated oligosaccharides, the dimer released from BDH alginate was examined further. The diuronide (2 mg) was dissolved in 0.5 ml NN-dimethyl formamide, silver oxide (0.2g) was added with iodomethane (0.5 ml), and the mixture stirred at room temperature in the dark (Wallenfels, et al, 1963). After 18 h the suspension was filtered, and the filtrate washed

three times with chloroform. The supernate was evaporated to dryness under reduced pressure, dissolved in 0.5 ml benzene and treated with sodium dihydro-bis-(2-methoxy ethoxy) aluminate (0.5 ml). The reaction was stopped after 10 min by the addition of 10 ml of distilled water. Sodium aluminate was removed by centrifugation, the supernate evaporated to dryness and the residue hydrolysed with HCl as before. Finally the hydrolysate was passed through an ion-exchange column (Amberlite IR-120, H^+ form and Dowex 1X2, CO^-_3 form), the solution and washings reduced to a small volume and examined by descending paper chromatography (solvent D).

Reduction and hydrolysis of the unsaturated diuronide yielded a sugar with the mobility of gulose and a trace amount of a sugar with the mobility of glucose.

Evidence derived from both analysis of the products and kinetics of SA1 lyase activity indicated that the enzyme was polyguluronide specific. The Haug and Larsen 'model' for the structure of alginate (See Figure 3) implies that sections of the molecule would be resistant to the action of a poly-guluronide specific enzyme. To investigate this, alginates were hydrolysed with SA1 lyase and any enzyme-resistant material present after treatment examined.

6.2 BDH and Azotobacter (Run 42) alginates (0.5 g) were dissolved in 50 ml Mops buffer (pH 7.5) and incubated at 30°C with 500 µl SA1 lyase solution. After 0.5, 2, 6, 24 and 48 h aliquots (5 ml) were removed from the incubation mixture and the enzymatic reaction stopped by heating the sample to 100°C for 10 min. Microbial growth in the incubation mixture was inhibited by the addition of toluene.

The samples, removed at the appropriate intervals, were lyophilized and redissolved in pyridine-acetic acid buffer (5 ml). One millilitre of this solution was fractionated by chromatography in a column (1.6 cm x 30 cm) of Sephadex G-50, using pyridine-acetic acid buffer as eluent. Fractions (1 ml) were collected and analysed for carbohydrate, in addition fractions collected from Azotobacter hydrolysates were examined for O-acetyl groups.

Figures 26 a, b, and c show the elution profiles obtained when BDH alginate was incubated for 6, 24 and 48 h. The results obtained when Azotobacter alginate was treated in a similar manner are shown in Figures 27 a, b and c.

These experiments were repeated using other algal and Azotobacter alginates as substrates, and similar elution profiles to those illustrated were obtained.

6.3 Examination of High-molecular-weight material (Peak I)

Acid hydrolysis of the material excluded from Sephadex G-50 (Peak I, see Figure 27b) showed mannuronic acid was the only detectable product.

6.4 Action of SA1 lyase on de-acetylated Azotobacter alginate.

The results obtained from the experiments described above, indicated a portion of Azotobacter alginate was resistant to SA1 lyase action and that this material was associated with all the recoverable O-acetyl groups. To investigate the possibility that these O-acetyl groups were conferring enzyme-resistance on these regions, Run 42 was de-acetylated and examined as a substrate for SA1 lyase. Using the conditions described above (6.2), the

FIGURE 26

Fractionation of products from algal alginate on Sephadex G-50, 6h, 24h, 48h, times of incubation with alginase.

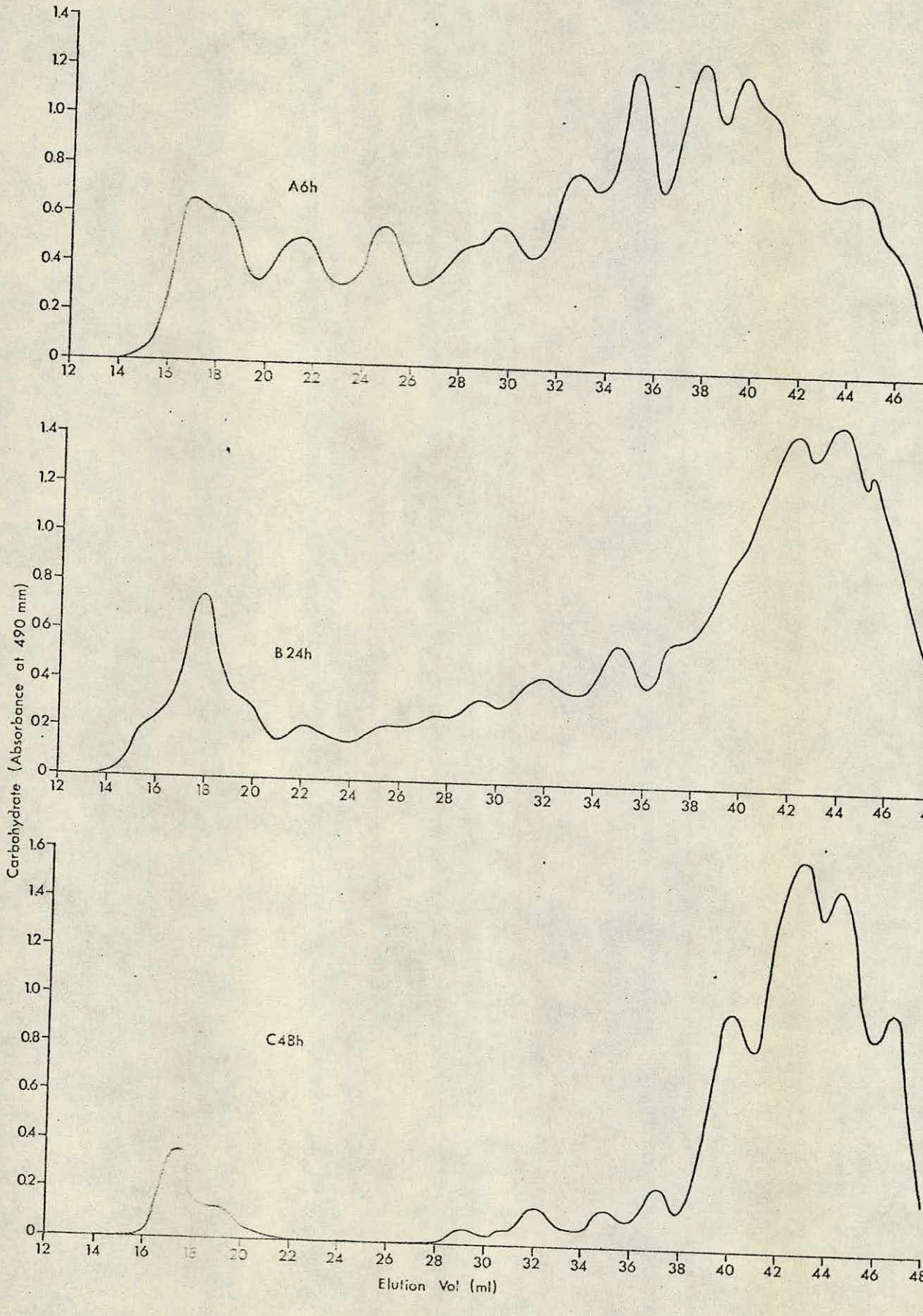


FIGURE 27

Fractionation of products from Azotobacter alginate on Sephadex G-50, 6h, 24h, 48h, times of incubation with alginase.

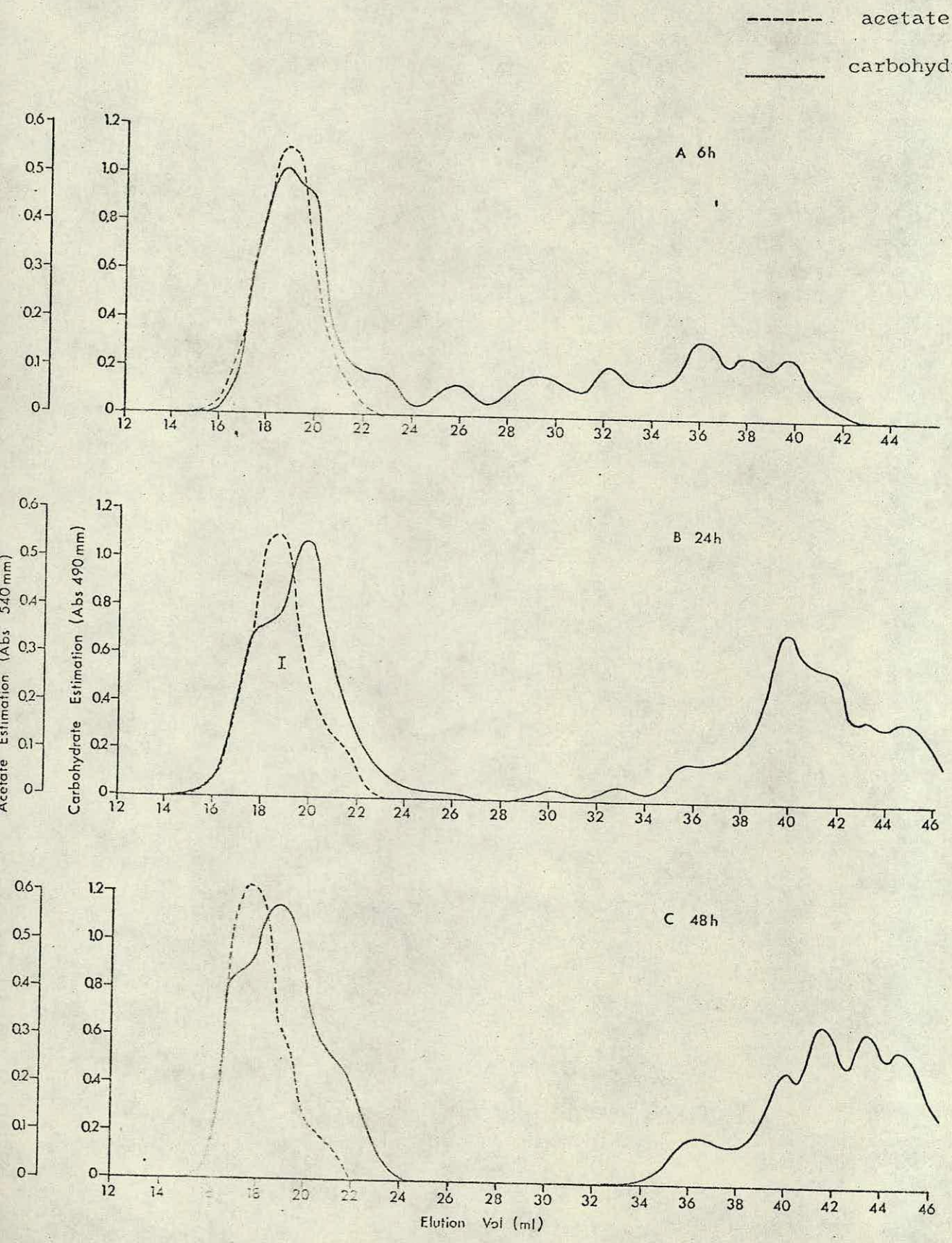
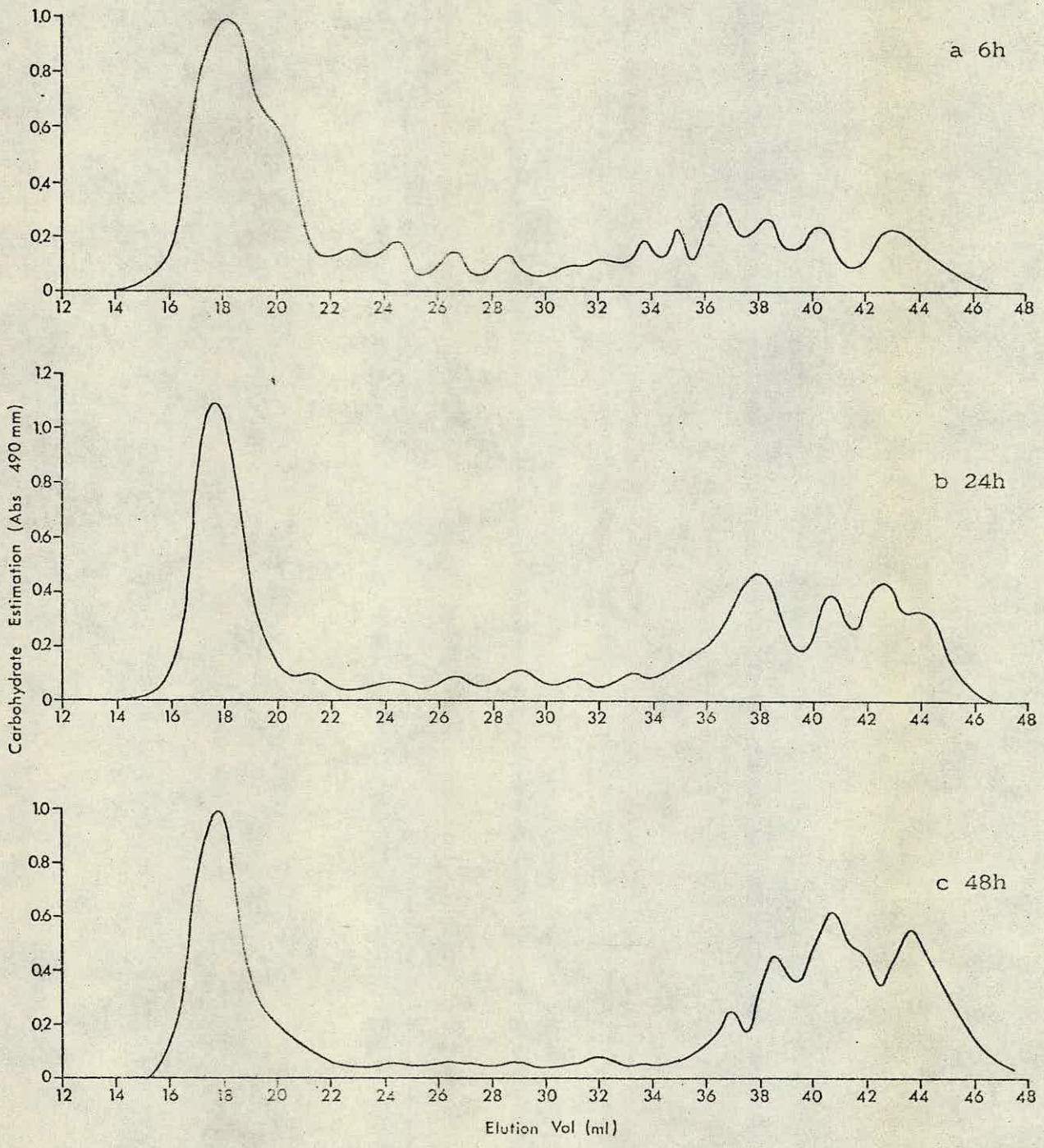


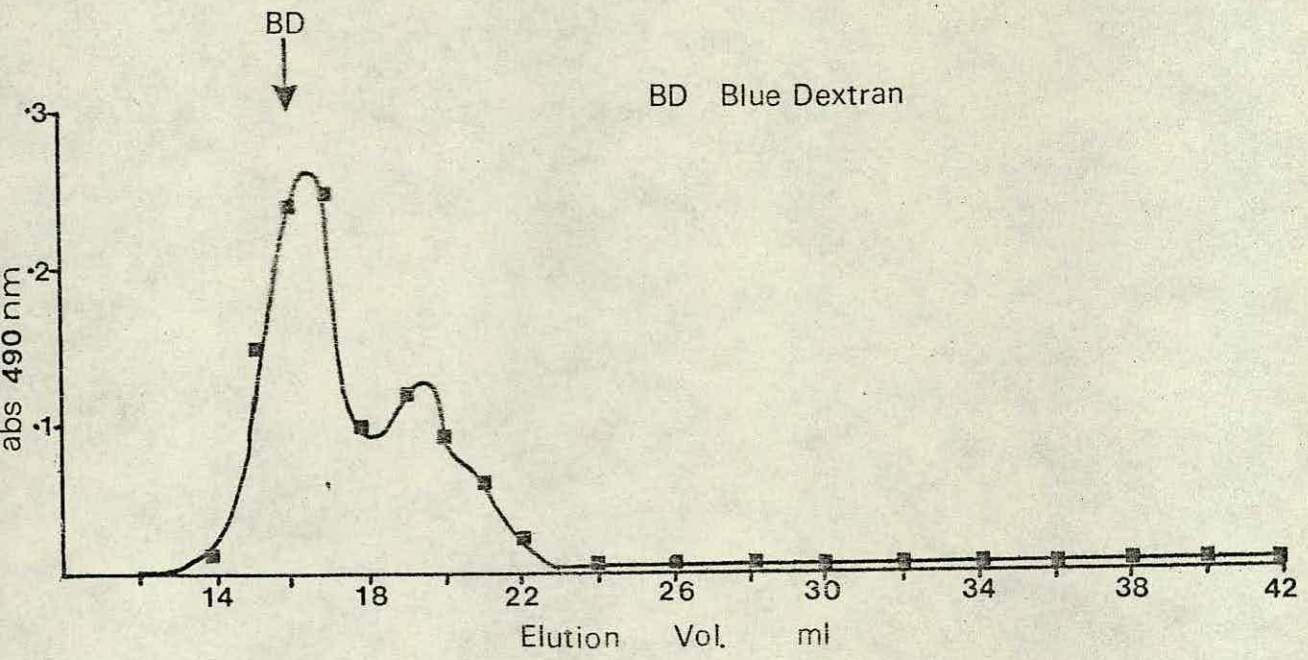
FIGURE 28

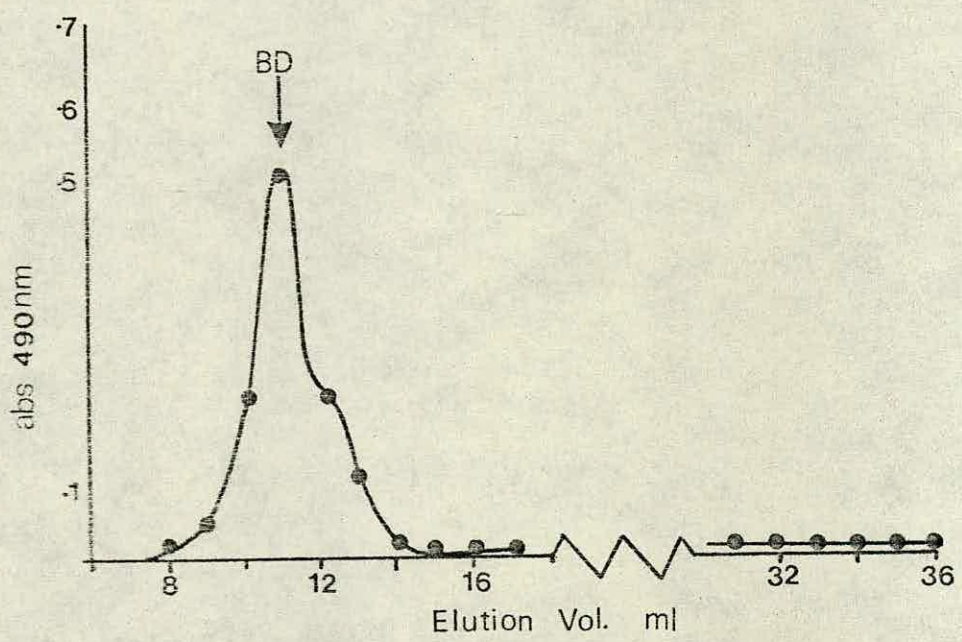
Fractionation of products from de-acetylated Azotobacter alginate, 6h, 24h, 48h, times of incubation with alginase.



FIGURES 29 and 30

Fractionation of peak I (see Figure 27b) on Sephadex G-100 and Bio-Gel P30 respectively.





de-acetylated polysaccharide was hydrolysed with SA1 lyase. The hydrolysates were fractionated in Sephadex G-50 and the elution profiles obtained are shown in Figures 28a, b and c.

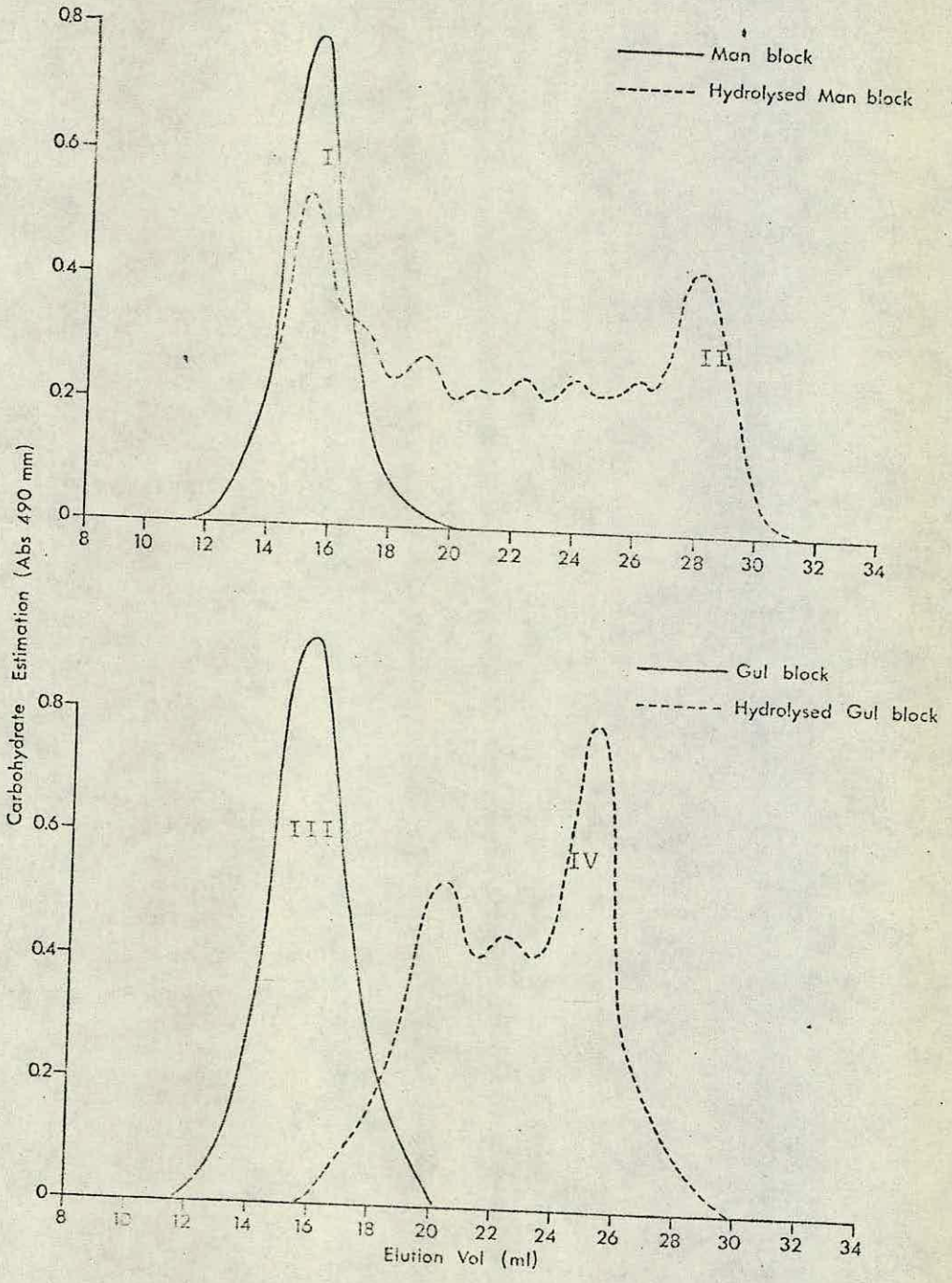
The high molecular weight material excluded from Sephadex G-50 was retained and a portion applied to column (1.5 cm x 27 cm) of Sephadex G-100. The column was eluted with pyridine-acetic acid buffer and fractions analysed for carbohydrate. The result is illustrated in Figure 29. To ensure the result shown in Figure 29 was not an artefact produced by the fractionating medium employed, a second portion of the material excluded from Sephadex G-50 was fractionated on a column (1.2 cm x 34 cm) of Bio-Gel P30. The column was eluted with distilled water and again fractions examined for carbohydrate. The result is shown in Figure 30.

6.5 Action of SA1 lyase on homopolymeric substrates

To investigate the marked difference observed between the enzymatic hydrolysis of algal and Azotobacter alginates, the action of SA1 lyase on homopolymeric substrates was examined. Polymannuronic acid and polyguluronic acid were prepared as described earlier (5.9). Both substrates were then incubated with SA1 lyase. Two milligrammes of each was dissolved in 1 ml Mops buffer, pH 7.5 and 25 µl enzyme solution added. After 18 h at 30°C the reaction was stopped by heating the mixture to 100°C. The hydrolysates were then fractionated in a column of Sephadex G-25 (1.6 cm x 30 cm) which had been equilibrated with pyridine-acetic acid buffer. The results are shown in Figures 31 and 32. Peaks I and II (Figure 31) and peaks III and IV (Figure 32) were hydrolysed with 2 M HCl as before. The hydrolysates were examined by descending paper chromatography and high voltage electrophoresis.

FIGURES 31 and 32

Fractionation of products released from homopolymeric substrates on Sephadex G-25.



The results are tabulated below (Table 16).

Table 16

	I	II	III	IV
Mannuronic acid	+++	-	+	-
Guluronic acid	-	+++	+++	+++

These results may indicate that 'blocks' prepared by partial acid hydrolysis of alginic acid are not truly homopolymeric structures and suggest that such structures could be 'punctuated' by the alternative uronic acid at a point along the homopolymeric chain.

7.0 Azotobacter bacteriophage alginate-depolymerase

Preparation and partial purification. The bacteriophage enzyme was prepared from cultures of A. vinelandii wyss infected with the phage, as described in "Materials and Methods". The crude enzyme solution was fractionated in a column (2.0 cm x 55 cm) of Sephadex G100 equilibrated with Mops buffer (pH 7.5). Fractions showing alginase activity were pooled and the enzyme stored at -20°C. This procedure resulted in an increase in specific activity from 3.5 to 18.6. The enzymatic activity was eluted from the column as a single peak.

The partially purified alginase solution was examined by SDS PAGE. After electrophoresis the gel cylinder was cut into 5 mm transverse slices and each section incubated overnight in 1% sodium alginate in 10 mM Mops buffer. The gel slice showing alginase activity indicated that the phage enzyme had a molecular weight of between 30,000 and 35,000 daltons. This is in close agreement with Pike and Wyss (1975) who quoted a molecular weight of 35,000 to 42,000 daltons for the lyase produced by Azotophage 310.

Properties of phage alginase

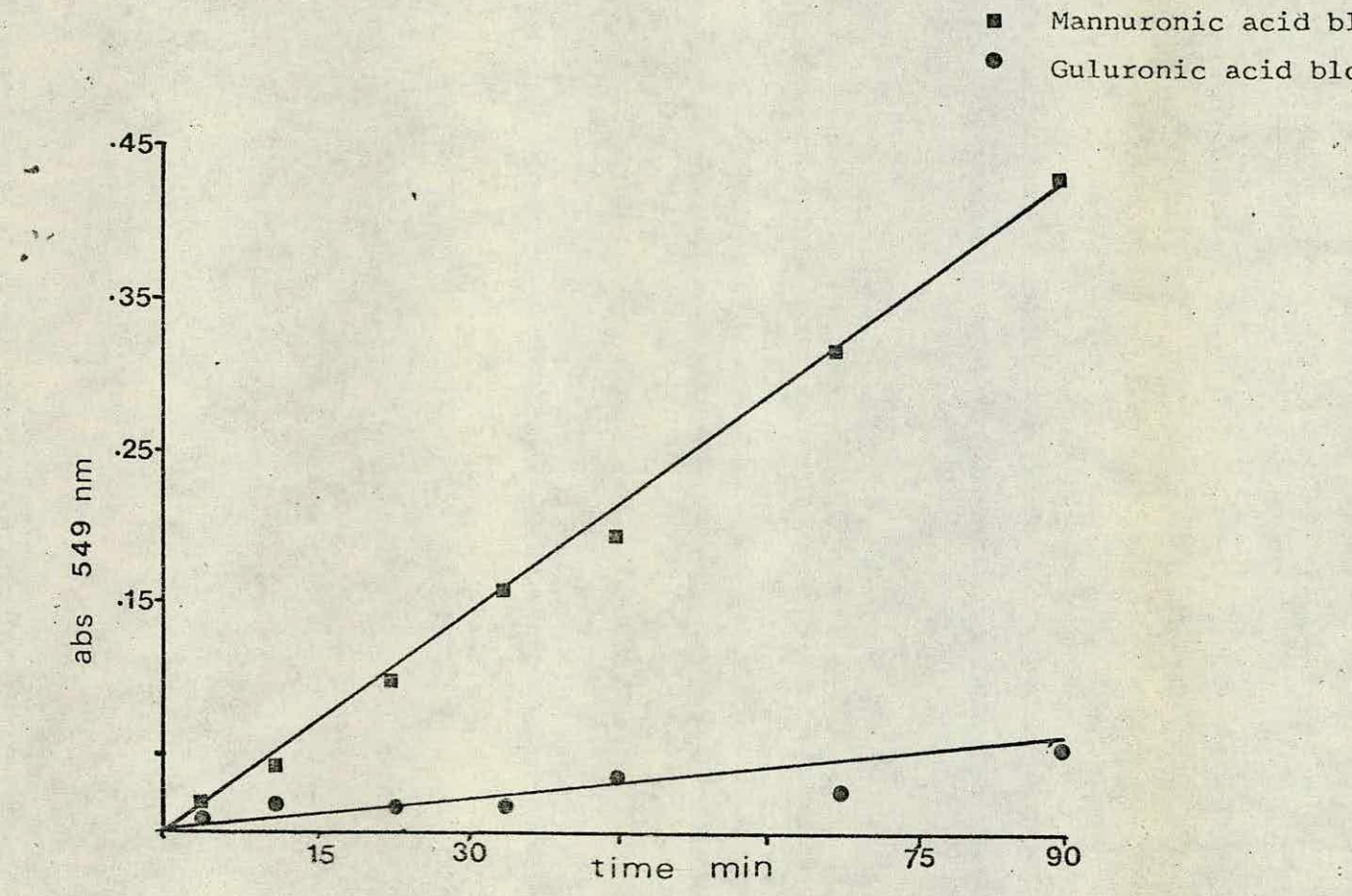
7.1 The phage-enzyme, like SA1 lyase gave rise to products which gave positive TBA, and phenylene diamine tests and absorbed strongly at 235 mn.

7.2 Effect of pH on enzyme activity. The enzyme was active over the pH range 7.0 to 8.5 and showed maximal activity at pH 7.7 as determined by the procedure described earlier (5.5).

7.3 Specificity of phage-lyase. The enzyme showed no activity towards hyaluronic acid, heparin, chondroitin sulphate or polygalacturonic acid, even after considerable incubation times.

FIGURE 33

Release of TBA positive material from homopolymeric substrates by Azotobacter bacteriophage lyase.



A series of alginates with approximately known mannuronic acid contents were incubated with phage lyase as described earlier (5.8). The results are shown below in Table 17.

Table 17

Alginate	% Man	Absorbance 549 nm
L98B	26.5	0.084
SSLD2	31.5	0.110
Lamitex	48.0	0.185
BDH	48.0	0.200
SSLH	55.0	0.240

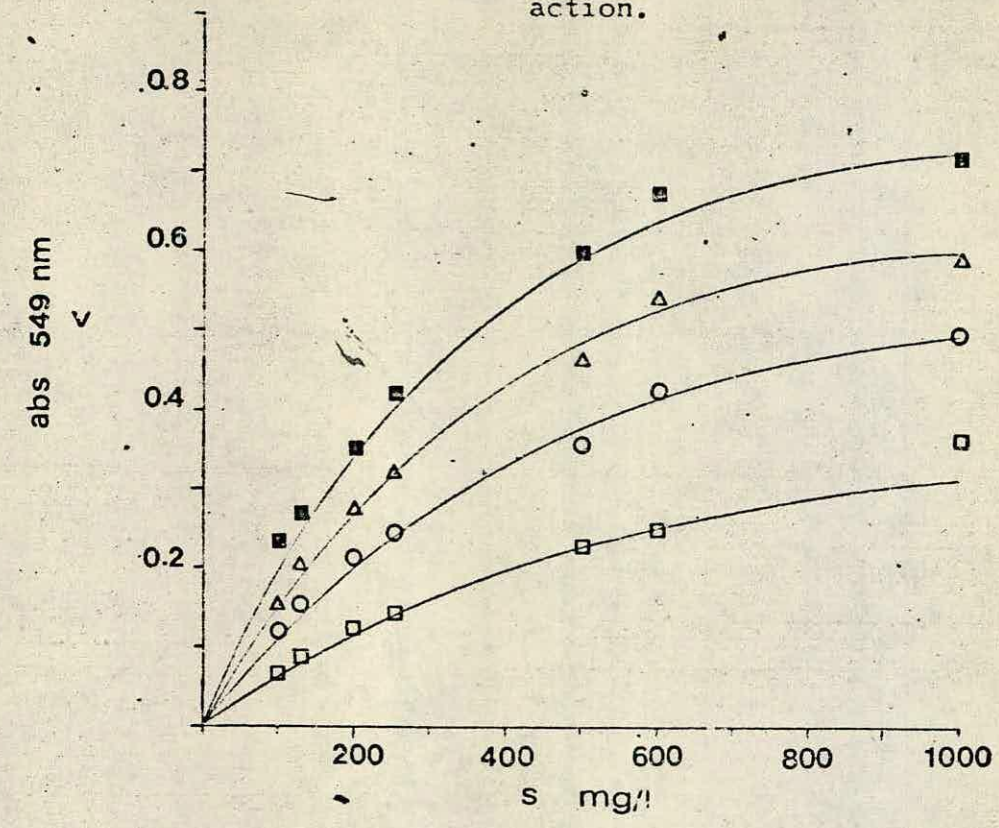
7.4 Action of phage lyase on homopolymeric blocks. The rate of release of TBA positive material from homopolymeric substrates, using the conditions described earlier (5.3) is shown in Figure 33.

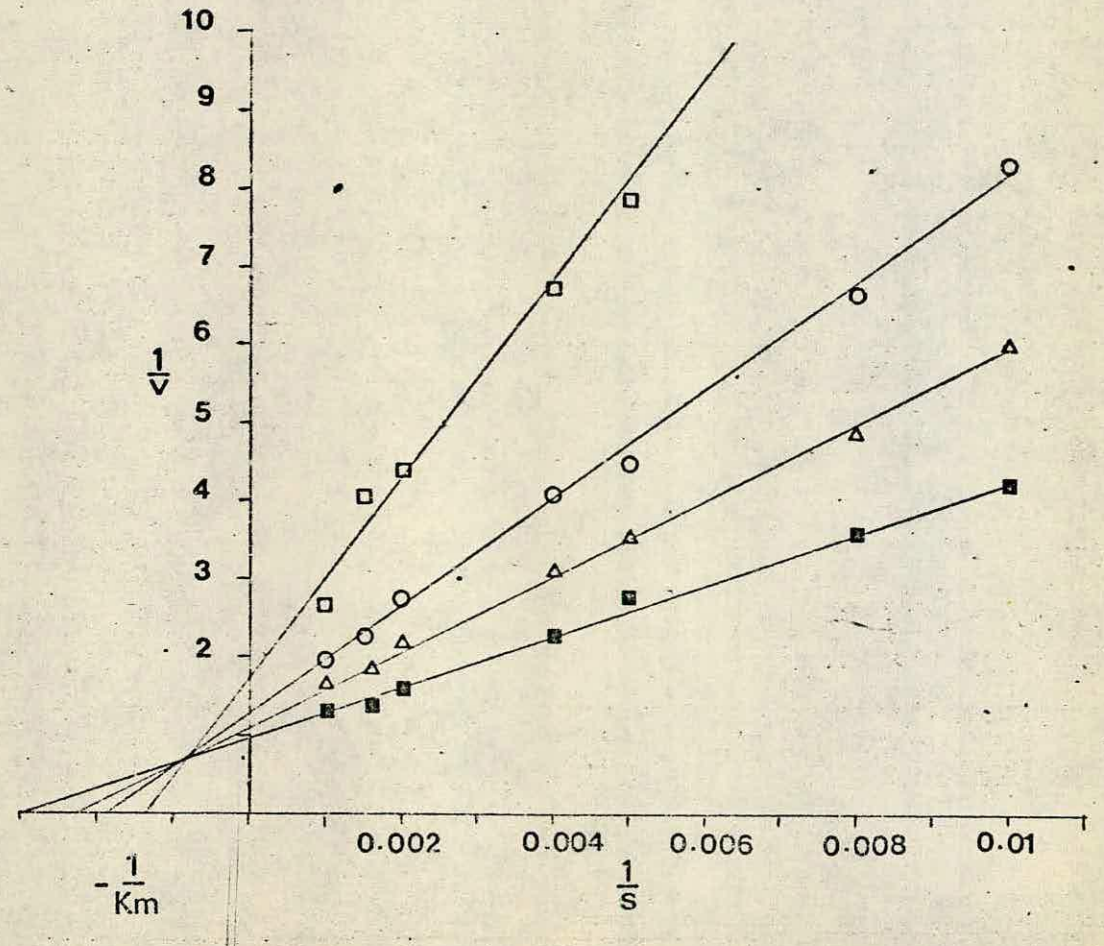
7.3 and 7.4 indicate the enzyme elaborated by Azotobacter bacteriophage is polymannuronide specific.

7.5 Km and Vm determinations. The Km and Vm constants were determined for the enzyme with various alginates as substrates, using the procedure described earlier (5.10). The assay solutions contained 0.9 ml Mops buffer pH 7.5 and the following amounts of sodium alginate (µg) contained in 0.1 ml water: 100, 60, 50, 25, 20, 12.5 and 10. Enzyme solution, (50 µl),

FIGURE 34

Kinetics of Azotobacter bacteriophage lyase action.





was added, the reaction allowed to proceed for 60 min then stopped by heating to 100°C. The results are shown in Table 18 and Figures 34a and b.

Table 18

Alginate	% Man	Km (mg/l)
L98B	26	715
SS/LD/2	31	530
BDH	48	470
SS/LH	55	350

7.6 Isolation and partial Characterization of Products released from Alginate solutions by phage-lyase.

The chromatographic mobilities of the products released by phage-lyase activity relative to guluronic acid are shown in Table 19. All compounds could be visualized by spraying the paper with TBA, and no additional compounds were detected when papers were developed with alkaline silver nitrate.

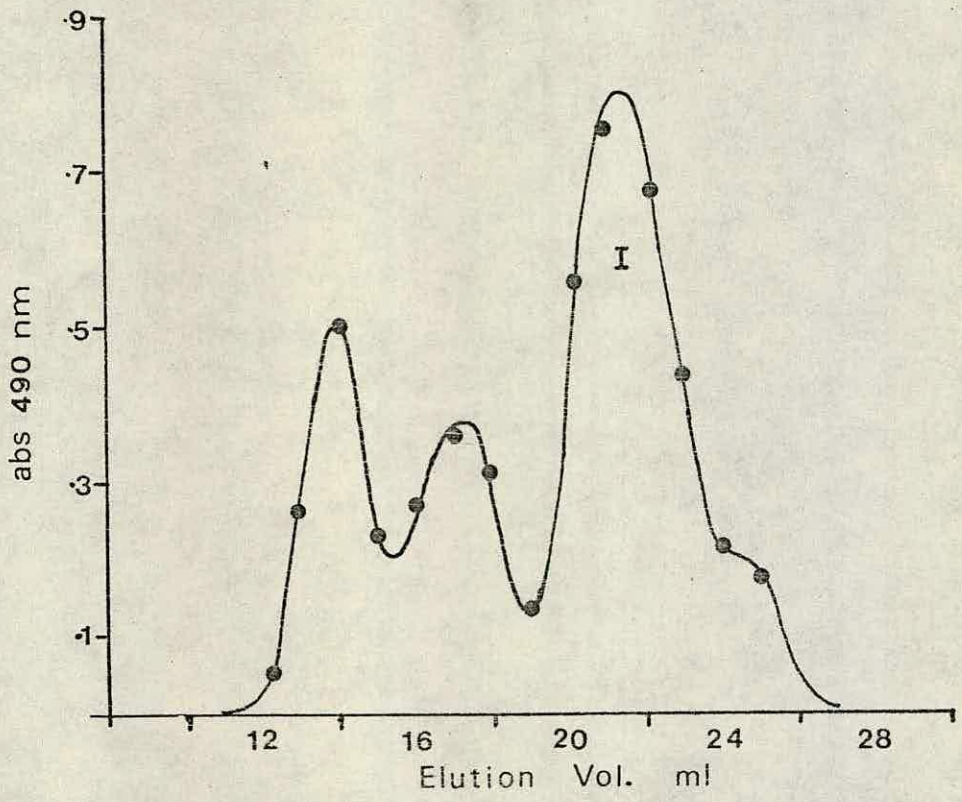
Table 19

Solvent System	
A	B
0.71	0.99
0.63	0.56
0.37	0.26
0.32	0.14
0.16	

Other solvent systems failed to produce satisfactory separation of the products of phage-lyase action.

FIGURE 35

Fractionation of products from alginate by the action of Azotobacter bacteriophage lyase on Sephadex G-15.



The R_{GulU} values obtained from chromatographic analysis differs from those obtained from the products released by SA1 lyase. However, when the oligouronides produced by the action of phage lyase were fractionated in Sephadex G-15, using the procedure described previously (6.1), a similar pattern to that shown in Figure 25 was obtained, see Figure 35, indicating that again the final product of phage lyase activity was an unsaturated diuronide.

Peak I, Figure 35, could be further fractionated by paper chromatography, solvent A, into two components with R_{GulU} values 0.70 and 0.65.

The oligouronides released by the action of phage lyase were accumulated by preparative paper chromatography, as described earlier. Each component was acid hydrolysed and the hydrolysates examined as before. The results are shown in Table 20.

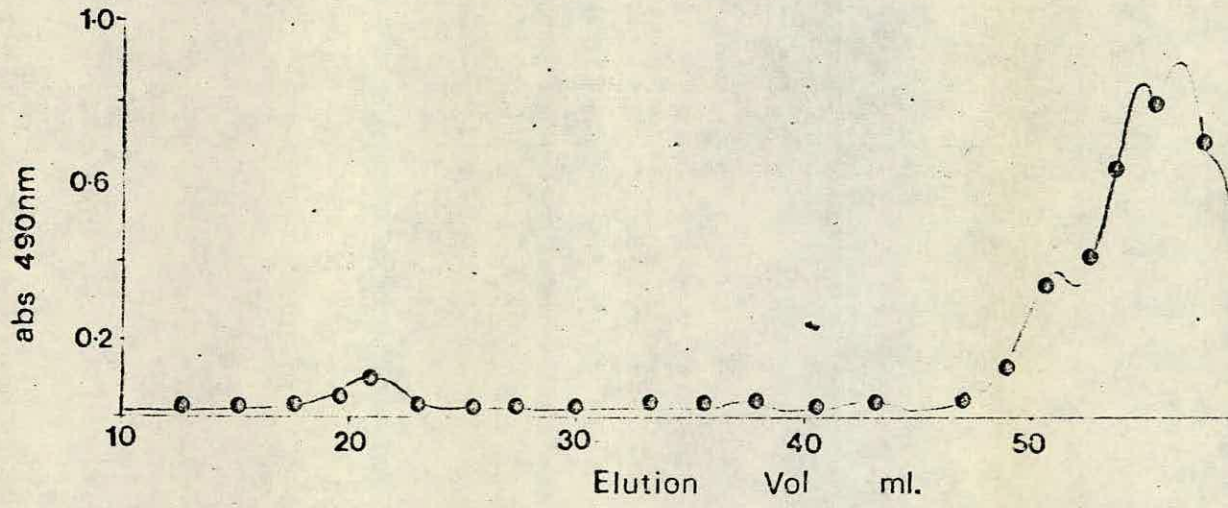
Table 20

R_{GulU} (Solvent A)	Result of Acid Hydrolysis	
	Man	Gul
0.71	+++	-
0.63	-	+++
0.37	+++	-
0.32	+++	-
0.16	++	+

It would appear from the results of Sephadex filtration and acid hydrolysis that the fastest moving product was an

FIGURE 36

Fractionation of products from Azotobacter alginate on Sephadex G-50 by the action of Azotobacter bacteriophage lyase.



unsaturated diuronide containing mannuronic acid. The unsaturated diuronide containing guluronic acid may have arisen from the reducing end of the alginate molecule.

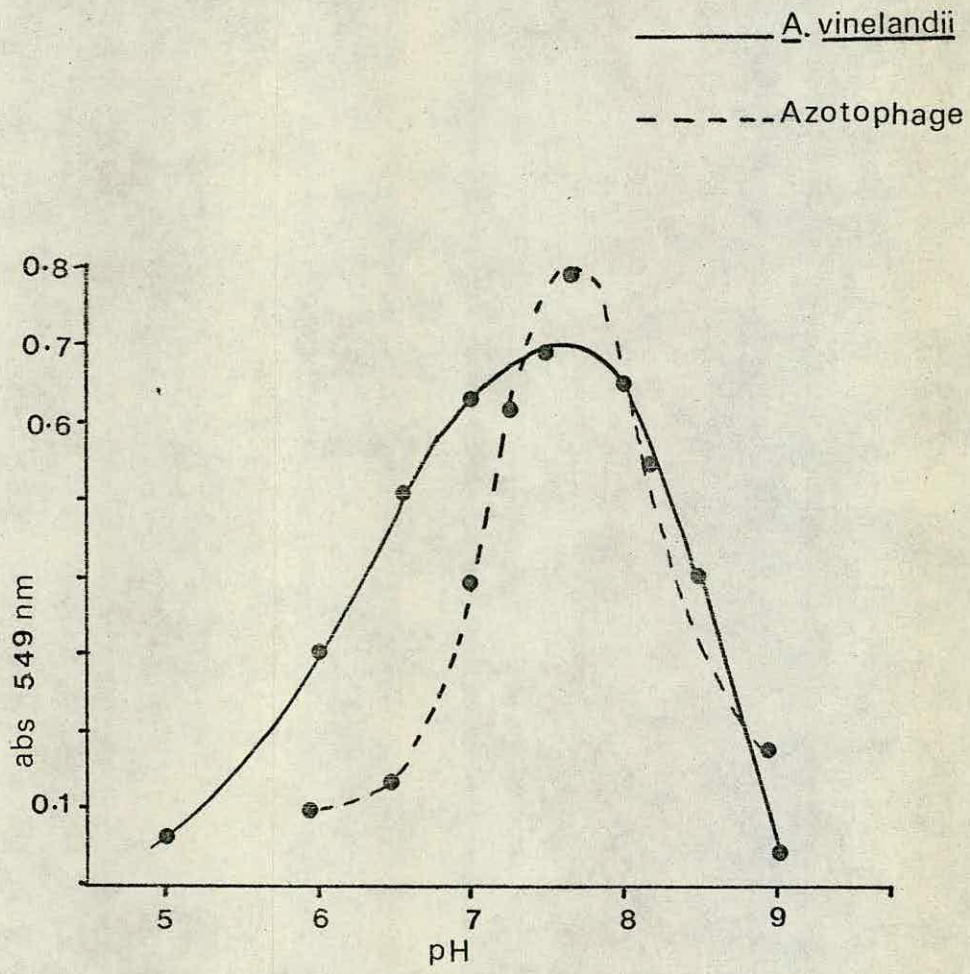
7.6 On the basis of the results obtained from kinetic studies and hydrolysis of the oligouronides released, the alginate depolymerase elaborated by Azotobacter bacteriophage appeared to be polymannuronide specific.

Azotobacter alginate, from the work described earlier, appeared to be composed in part of high molecular weight polymannuronic acid 'block'. If this was the case the enzyme produced by A. vinelandii exopolysaccharide. The experiments described earlier (6.2) were repeated using this polymannuronide specific enzyme. The result illustrated in Figure 36 shows the elution pattern from Sephadex G-50 of the products released from Run 42 after 24 h incubation with phage lyase.

Figure 36 shows Azotobacter alginate was hydrolysed more fully by the action of the polymannuronide lyase than by the action of SA1 lyase (compare Figure 27), and may confirm the presence of a high molecular weight"block" of mannuronic acid associated with bacterial alginate.

FIGURE 37

Effect of pH on Azotobacter endogenous lyase and Azotobacter bacteriophage lyase.



A. vinelandii wyss alginase

8.0 Preliminary experiments indicated that A. vinelandii produced considerable amounts of alginate lyase and consequently appeared to be a convenient system for study. In practice, however, the enzyme proved difficult to handle, rapidly losing activity even when stored at -20°C. In addition the enzyme preparation was associated with a high "background" level of non-diffusible TBA positive material which presented certain difficulties when estimating enzyme activity. It was decided to compare the properties of the Azotobacter enzyme to those of the Azotobacter bacteriophage lyase, in order to establish that the phage depolymerase was a product of phage genes, since phage infection may have resulted in the stimulation of the host cell to synthesis of bacterial alginate lyase. (For comparison see Mathews and Sutherland, 1965).

(a) Both enzymes were active in the pH range 7.0 - 8.5. However, the shape of the pH-activity curves were different (see Figure 37). The A. vinelandii enzyme showed a rapid loss of activity at higher pH values.

(b) The Km values for both enzymes were determined using Run 42 as substrate. These were shown to be 510 mg/l for the bacteriophage lyase and 910 mg/l for the Azotobacter endogenous lyase.

(c) Both enzymes appeared to be polymannuronide specific, as judged by the release of TBA positive material from various alginate samples with approximately known mannuronic acid to guluronic acid ratios (see Table 21).

Table 21

The release of TBA positive material from various alginates by A. vinelandii lyase

Alginate	% Man	TBA
L98B	26.5	0.167
SSLD2	31.5	0.170
Lamitex	48.0	0.200
BDH	48.0	0.240
SSLH	55.0	0.265

(Compare results shown in Table 17)

(d) The molecular weight of the phage enzyme was estimated to be in the region of 30,000 - 35,000 daltons. No accurate estimation of the molecular weight of the bacterial enzyme was achieved. When a crude solution of Azotobacter alginase was fractionated in a column of Sephadex G-50, the fractions showing lyase activity were eluted in the void volume of the column. These fractions were still associated with TBA positive material; the phenomenon of a "glyco-alginase" complex has been observed in another strain of A. vinelandii (C. Bucke, personal communication).

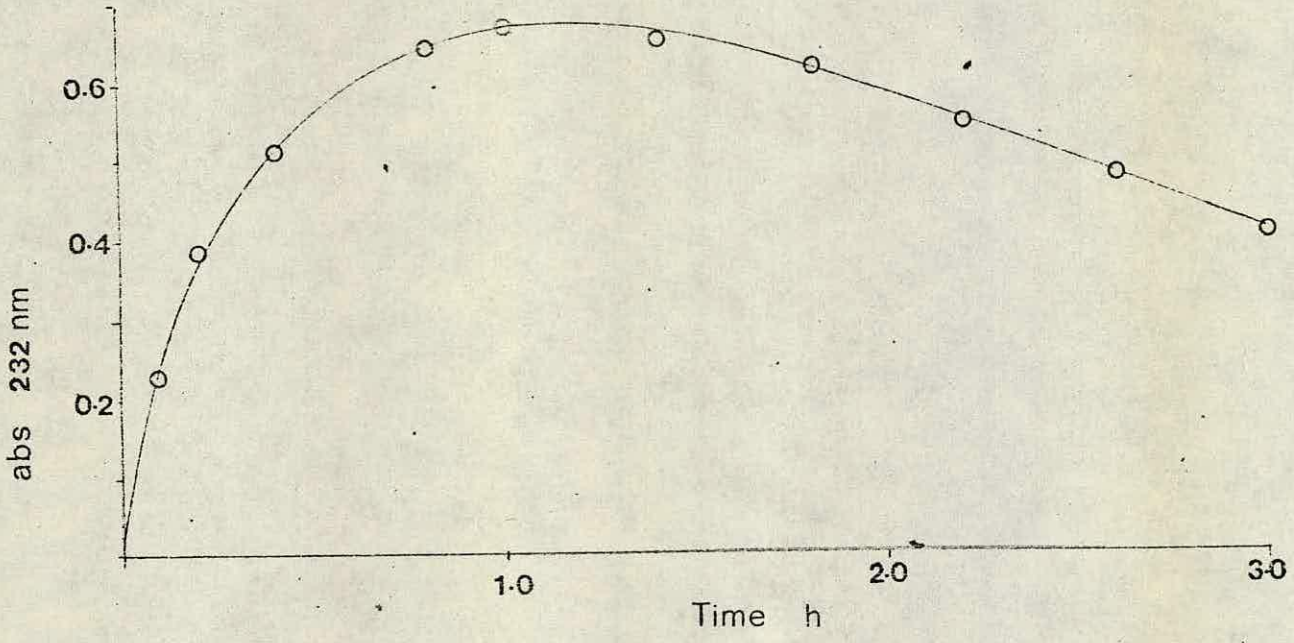
(e) Phage particles, propogated by the sloppy-agar overlay procedure (see Materials & Methods), were harvested by centrifugation and resuspended in 10 mM Mops buffer (pH 7.5). When sterilized by passage through a Millipore filter, this

suspension caused the formation of phage-plaques on lawns of A. vinelandii. In addition 22% of the total enzymatic activity recovered (supernate + bacteriophage suspension) was associated with the phage particles.

(f) Finally, the observation that the lyase produced by A. vinelandii rapidly lost its catalytic activity, while the bacteriophage enzyme retained activity even after three months storage, further indicated that the two enzymes were different.

FIGURE 38

Release of TBA positive material from alginate solutions by the action of 'crude' mixed culture alginase preparation.



Mixed Culture Alginase

9.0 Many of the organisms isolated (see Table 6) which grew on solid alginate medium, produced only limited amounts of alginate degrading action when grown in alginate broth. It was suggested (I.W. Sutherland, personal communication) that these organisms may, in the natural environment exist in such close proximity as to be mutually dependent.

When in the case of mixture 1, the three organisms together produced substantial amounts of alginate decomposing enzyme, as initially estimated by the rapid drop in viscosity caused when the crude cell free extract was incubated with alginate solutions. The solution contained 5.6 units per ml.

Properties of mixed culture enzyme. The crude enzyme preparation gave rise to products which, like the systems already described indicated the enzyme was again of the lyase type, i.e. a positive TBA test and UV. absorption at 235 nm.

9.1 Effect of pH. The enzyme was active over the pH range 5.0 - 9.0 and showed two peaks of activity, the first at 6.0 and a second at pH 7.5

9.2 Rate of release of products. The rate of release of UV. absorbing material from alginate solutions was monitored spectrophotometrically, as described in "Materials and Methods". The result is shown in Figure 38. The decrease in UV. absorbance observed after prolonged incubation, indicated the formation of unsaturated monomer (4-deoxy-5-keto-uronic acid). This was confirmed by chromatographic examination of the products released by mixed-lyase activity. Paper chromatography revealed a compound with a mobility relative to guluronic acid of

1.05 and 1.54 (solvents A and B respectively) and an electrophoretic mobility of 10.4 cm (guluronic acid 7.8, mannuronic acid 9.8) was the major product of this activity after prolonged incubation.

9.3 Specificity. The crude enzyme preparation showed no eliminative activity towards heparin, chondroitin sulphate or hyaluronic acid and only insignificant activity towards polygalacturonic acid as estimated by the TBA test after two days incubation at 30°C. When incubated with a series of alginates of various mannuronic acid contents little obvious specificity was observed, see Table 22.

Table 22

Alginate	% Man	TBA
L98B	26.5	0.53
SSLD2	31.5	0.75
Lamitex	48.0	0.63
BDH	48.0	0.57
SSLH	55.0	0.60
Guluronic blocks	-	0.64
Mannuronic blocks	-	0.67

9.4 Partial Purification. The preliminary results obtained from the mixed culture preparation were in conflict with the results obtained from other alginase systems, in exhibiting low specificity and releasing substantial amounts of unsaturated monomer as the final product of enzymatic activity. To confirm that the mixed-culture preparation was a single enzyme and not a complex system, the crude solution was fractionated in a column

of DEAE celluslose (DE32) using the procedure described earlier, (3. step 4). The enzyme was eluted as a single peak. The fractions showing alginase activity were pooled and the properties of this enzyme solution examined.

9.5 Effect of pH. The partially purified enzyme was again active over the pH range 5.0 - 9.0 and showed the same two peaks of activity as described earlier (9.1)

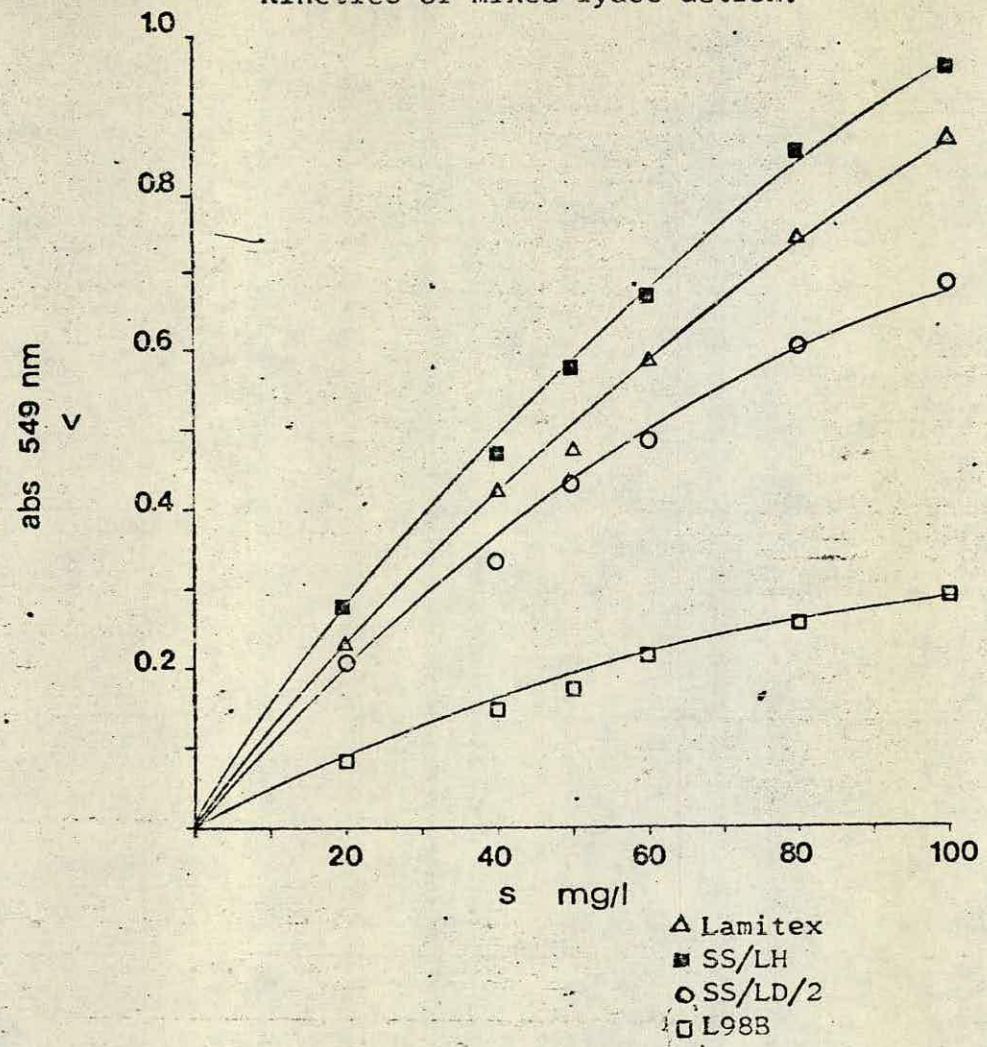
9.6 Rate of release of products. The rate reaction was re-examined (see 9.2) and initially the formation of UV. absorbing material was linear with time. After continued incubation, the "falling off" described earlier was not observed. Similarly the chromatographic pattern of the products in the incubation mixture was like that described earlier (Table 19). No compound with the mobility of 4-deoxy-5-keto-uronic acid was observed.

The implication derived from these results is the presence of a second enzyme capable of hydrolyzing unsaturated oligouronides to 4-deoxy-5-keto-uronic acid. The failure to detect this oligouronide lyase in the fractions of the eluant of the DE32 column must be due to the inability of the enzyme to hydrolyse native sodium alginate.

9.7 Specificity of partially purified enzyme. Km and Vm determinations on the crude enzyme solution were unsuccessful; plotting v against s resulted in sigmoidal curves, further indicating the presence of at least two enzymes. Km and Vm determinations for the partially purified preparation were carried out using the procedure described earlier. The results are shown in Figure 39 and Table 23.

FIGURE 39

Kinetics of mixed lyase action.



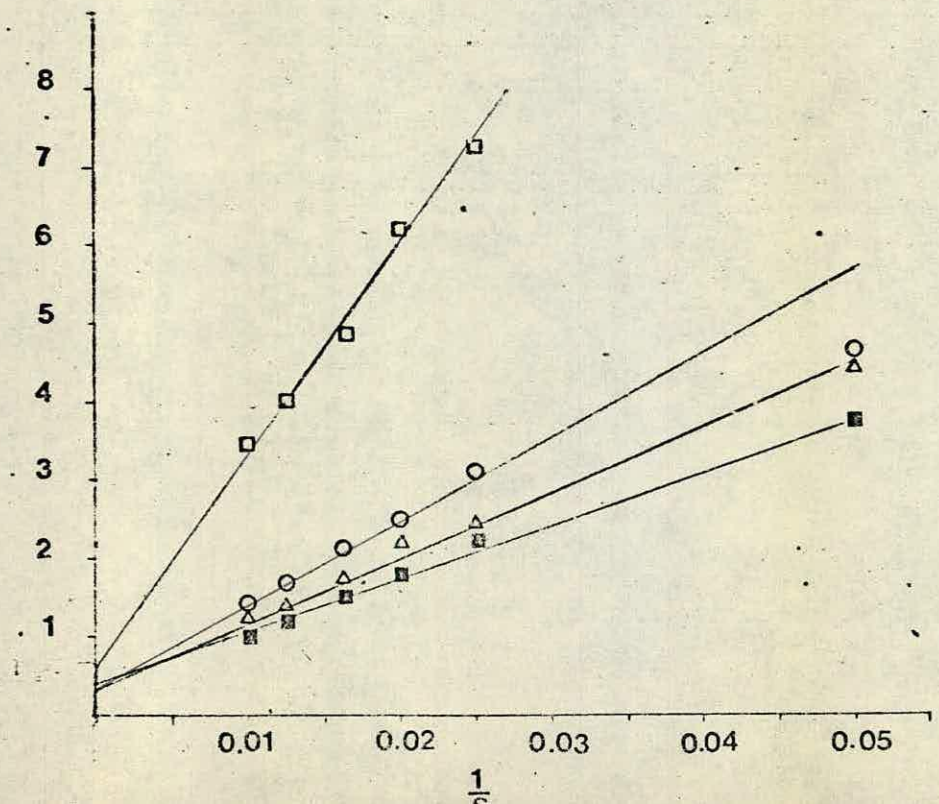


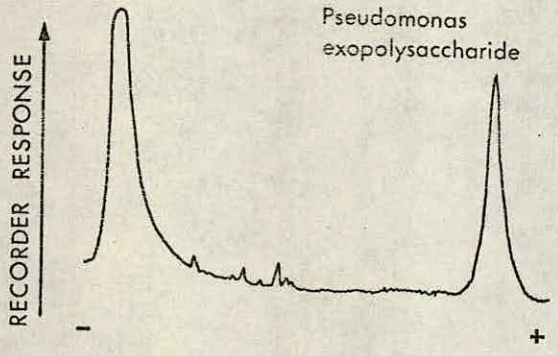
Table 23

Alginate	% Man	Km(mg/l)
L98B	26.5	500
SS/LD/2	32.0	400
Lamitex	48.0	250
SS/LH	55.0	170

The results shown above suggest that the partially purified preparation contains polymannuronide lyase.

FIGURE 40

P A G E of Pseudomonas alginate



Enzymatic Hydrolysis of Pseudomonas aeruginosa exopolysaccharide.

10.0 The exopolysaccharide produced by Ps. aeruginosa was partially acid hydrolysed and analysed by PAGE, as described earlier (6.0). Partial acid hydrolysis caused solubilization of 75% of the polysaccharide: mannuronic acid and guluronic blocks were present in the ratio of 4:1. The result of PAGE is shown in Figure 40.

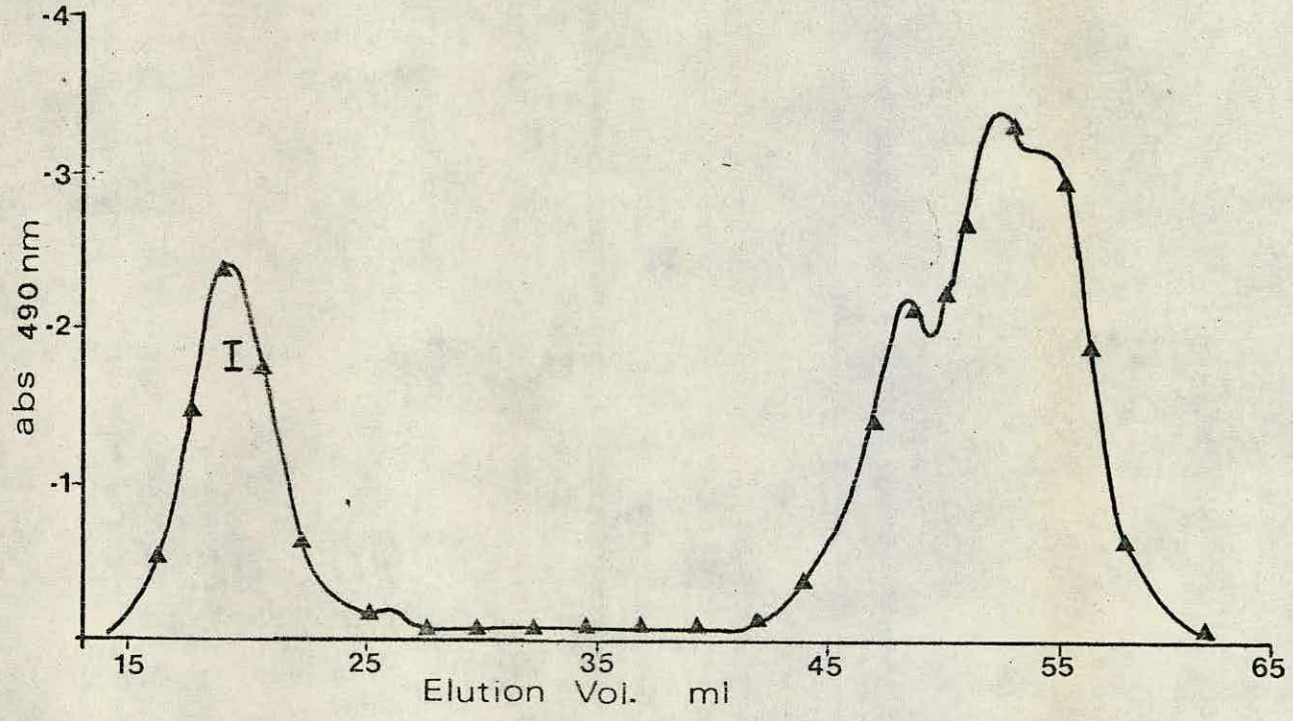
Release of TBA positive and UV. absorbing material from solutions of the polysaccharide by SA1 and phage alginate lyases, indicated the alginate nature of the polysaccharide.

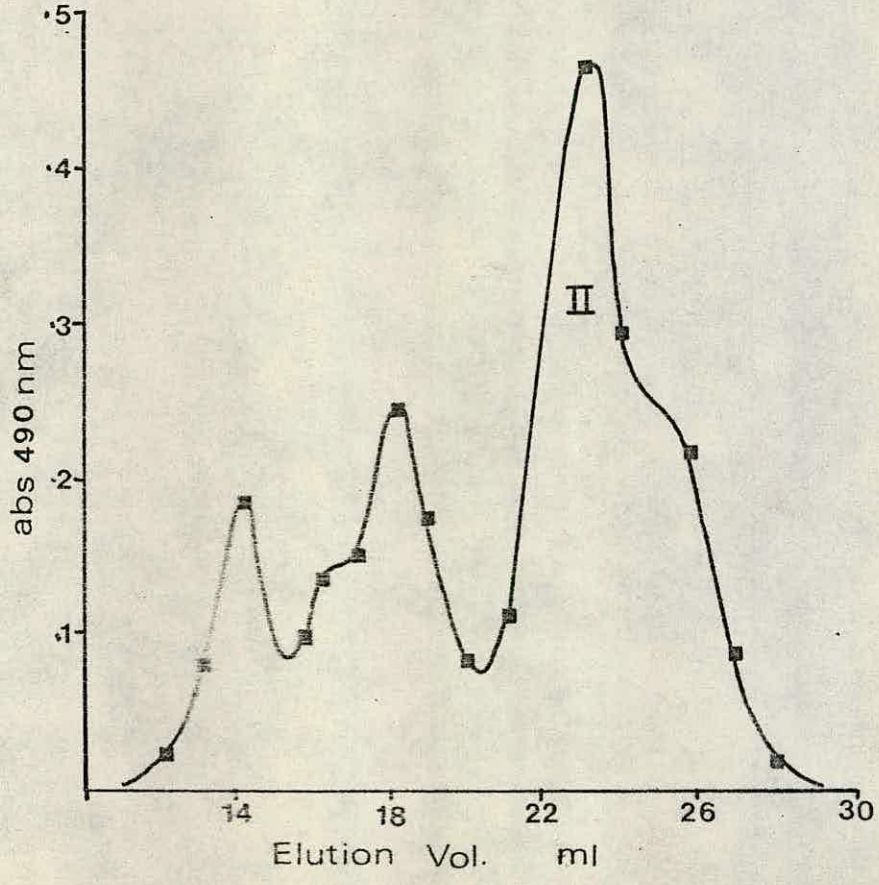
Km constants of 2300 mg/l and 530 mg/l for SA1 and phage lyases respectively further suggested the polysaccharide had mannuronic acid to guluronic acid ratio in the region of 1:1.

A solution of Pseudomonas alginate (25 mg in 5 ml Mops buffer, pH 7.5) was incubated with 250 µl SA1 lyase solution. After 18 h at 30°C the reaction was stopped in the usual way and the hydrolysate treated as described earlier (6.2). Portions of the hydrolysate were fractionated by gel filtration. 500 µg in 0.5 ml pyridine-acetic acid buffer was applied to a column (1.6 cm x 30 cm) of Sephadex G-50, while a second (250 µg in 0.5 ml of the same buffer) was separated in a column (1.2 cm x 38 cm) of Sephadex G-15. The elution profiles obtained are shown in Figures 41 and 42. Peak I (Figure 41) and Peak II (Figure 42) were hydrolysed using 2 M HCl. Analyses of these hydrolysates indicated that high molecular weight material (Peak I) was composed of mannuronic acid while the diuronide (Peak II) was composed of guluronic acid. Comparison of Figures 27 and 42 would indicate a similarity in the structure of Azotobacter and Pseudomonas alginates.

FIGURES 41 and 42

Fractionation of products from Pseudomonas alginate on Sephadex G-50 and Sephadex G-15 respectively.





The action of phage lyase on Pseudomonas alginate released as the major product an unsaturated diuronide which on total acid hydrolysis yielded mannuronic acid, further confirming the high proportion of alternating sequences found in this preparation of Pseudomonas alginate.

DISCUSSION

The work contained in this thesis has been concerned with the characterization of alginate-decomposing enzymes; with the aim of assessing their potential value in the structural analysis of alginic acid.

Before discussing the mode of action and possible application of these enzymes, aspects of enzyme recovery and any implications of microbial physiology which can be drawn from the results will be examined.

SA1 and Mixed Culture Alginase systems.

One of the simplest ways of finding organisms producing enzymes capable of degrading specific substrates is by enrichment culture. Usually the micro-organisms are from such natural sources as decaying vegatable matter, soil or sources rich in potential substrate. Bacteria and other micro-organisms isolated in this way are therefore convenient and important biochemical tools, and were chosen as sources of alginolytic enzymes in preference to non-microbial sources described earlier (see Introduction page 23). The importance of enrichment procedures per se in microbiology have been discussed elsewhere (Schlegeland Jannasch, 1967).

The organism designated SA1, isolated from decomposing seaweed, was classified as a marine pseudomonad on the basis of its morphological and physiological characteristics. (Results section 2B). Isolate SA1 elaborated considerable alginolytic activity, as judged by the analytical procedures described earlier (Results section 5.1), when grown in a simple salt medium containing sodium alginate as sole carbon source. The alginase system was readily purified using standard procedures (Results section 3.0) without dramatic loss of enzymatic activity.

In addition the enzyme retained activity for periods of up to one year when stored as a solution in 80% ammonium sulphate. These are valuable properties, worthy of consideration if routine enzymatic analyses are to be carried out.

The importance of maximal catalytic recovery to an enzymatic study, led to the examination of the cellular localization of the alginase system of SA1. In addition, it was of interest that in the case of this organism, only insignificant alginase activity could be recovered from culture supernates; since the macromolecule is of the type which would normally be considered a substrate for an extracellular enzyme.

The cell surface of Gram negative bacteria is a highly complex structure, for review see Costerton, Ingram and Cheng (1974). The treatments to which SA1 was exposed (Results Section 4.0)were likely to disrupt the cell envelope of the bacterium and indicated that up to 43% of the recoverable alginase activity was associated with the outer regions of the cell. Additionally, preparation of sphaeroplasts, complete removal of the cell envelope, released little if any further activity than when cells were treated with 0.5M kCl. Forsberg, Costerton and Macleod (1970) have examined in some detail the cell surface of a marine pseudomonad and have concluded that treating such bacteria with 0.5M salt solutions releases a loosely bound, outer membrane from the bacterial cells. It would appear that on the basis of this extraction technique, the alginase system of SA1 is associated with a similar outer

membranous structure. It would be advantageous to the organism to retain an alginolytic enzymatic system on the cell surface, yet not free in a continually shifting environment, since the enzyme may assist the bacterium in colonization of the algal plant.

Enzyme location studies may prove more valuable if it becomes necessary to produce the enzyme on a larger scale, for routine analysis of alginate samples: the recovery of 50% of enzymatic activity at a specific activity 5 to 6 times higher than that of a broken cell preparation is of practical appeal. Again in this laboratory scale study, enzyme was produced at a sufficiently high level of activity and in sufficient quantity, merely by growth of SA1 in the medium described. However, procedures for increasing enzyme production by genetic and environmental manipulations are available and these have been reviewed elsewhere (Demain, 1971).

The second alginase system examined in this study obtained using enrichment techniques was that produced by a mixed culture.

The use of a number of bacteria grown together to provide any enzymatic system which is not readily obtainable from a single organism is biochemically justified. In nature numerous bacterial types can be expected to be present in association with a given substrate, utilising it, or its degradation products. However, microbiologically, the definition of such a system gives rise to a number of problems. In this laboratory it proved difficult to effectively reproduce the system which gave rise to the vast amount of alginase activity elaborated by mixed culture 1.

The use of mixed culture preparations may be limited particularly because of difficulties in obtaining reproducable mixtures, but could provide an approach which might prove successful in a particular laboratory as an aid to the solution of a particular problem.

Mixed cultures probably reflect the natural bacterial population of any environment at any given time and for this reason such systems have been studied closely by microbial ecologists (see Jannasch, 1965).

Azotobacter Phage-Induced and Endogenous Azotobacter Alginases

Azotobacter phage-induced and endogenous Azotobacter systems were studied because, not only did they provide convenient sources of enzymes, they are of particular importance to any commercial scale production of Azotobacter alginic acid, since both could lead to the degradation and loss of the final product.

Exopolysaccharides from several species of bacteria are substrates for enzymes isolated from bacteriophage-infected bacterial cultures, the enzymes being normally present in phage bound and soluble forms (Ekland and Wyss, 1962; Sutherland, 1971; Thurow et al, 1975).

When both a host bacterium and a bacteriophage synthesise enzymes with the same catalytic properties, it is necessary to establish that the enzyme recovered from phage-infected cultures of host organism is a product of phage genes. In this study much effort was directed to this end, and five major points of difference between the bacteriophage and host enzymes were observed.

Function of Bacterial Exopolysaccharides

Various hypothetical functions have been suggested for bacterial exopolysaccharides (see Sutherland, 1972). Most of these have implied a protective function, such as against desiccation, against phagocytosis or against bacteriophage infection.

The polysaccharide from a phage-resistant strain of Azotobacter vinelandii was not studied in detail here, but it was of interest that a sample of capsular material from a phage resistant strain of A. vinelandii was depolymerized by both phage-enzyme and SA1 lyase, indicating the alginate-like nature of the polysaccharide. Since the exopolysaccharide synthesised by the phage resistant strain of A. vinelandii appeared to be qualitatively similar to that produced by a strain suceptible to phage infection, the mechanism of phage resistance and the modification of any phage receptor sites associated with the bacterial capsule remains in doubt. The nature of such a modification might usefully be studied, since a clearer understanding of exopolysaccharide function might be gained. Secondly the production of bacterial alginate from phage-resistant strains of Azotobacter is obviously advantageous.

As shown in this study the capsule of Azotobacter vinelandii presented no real barrier to phage infection, however, this classical explanation for the role of bacterial exopolysaccharide may be applicable to C.F. strains of

Pseudomonas aeruginosa. Numerous attempts to isolate bacteriophages specific for slime producing C.F. strains of Ps. aeruginosa were unsuccessful. On subculturing, mucoid C.F. pseudomonads revert to non-mucoid forms (see also Evans and Linker, 1973). Bacteriophage able to infect these non-mucoid forms were readily isolated from the same sources which had previously proved unsuccessful.

Further questions of exopolysaccharide functions are raised by the observation that Azotobacter elaborated an alginate lyase system.

Procter (1959) suggested that the capsule of Azotobacter functioned as a carbon source and thus could be regarded as a true storage material. Such a situation may, however, exist only under certain physiological conditions and the nature of the polysaccharide synthesised by Azotobacter and the wide variation in the physical and chemical properties of such a structure suggests that in nature the alginate exopolysaccharide is likely to function as an ion-exchange material, or as an ion-trap Lin and Sadoff (1969) have shown that cysts of Azotobacter vinelandii contain an abundance of calcium and lesser amounts of magnesium. The endogenous alginate lyase of Azotobacter may therefore facilitate modification of the properties of the capsule by altering its structure, since the enzyme appears to be specific for polymannuronic acid regions of the molecule. Further, the in vitro studies carried out here, which indicated that the endogenous enzyme was relatively unstable might suggest that any structural modification made to the exopolysaccharide could be executed without substantial digestion of the capsule; which may also render the bacterium resistant to phagocytosis or desiccation.

Properties and Potential Uses of Alginolytic Enzymes

The overall conclusion which can be drawn from this study is that, in agreement with the work described earlier (see Introduction pages 22-27), alginate depolymerases can be classified as endo-trans eliminases (lyases), see plate 4, showing specificities towards certain portions of the alginate macromolecule: i.e. poly (1,4-β-D-mannuronide) lyase (4.2.2.3a) and poly (1,4-α-L-guluronide) lyase (4.2.2.3b).

Illustrated below is a uronic acid sequence which might be cleaved by alginate lyases, and further clarifies the definitions given above for such enzymes.

$$-\,M\overset{1}{\blacktriangledown}M\overset{1}{\blacktriangledown}G\,- \qquad\qquad -\,G\overset{2}{\blacktriangledown}G\overset{2}{\blacktriangledown}M\,-$$

1. action of polymannuronide lyase
2. action of polyguluronide lyase

The failure to detect mannuronic acid in the unsaturated oligouronides released from alginate, by the action of SA1 poly-guluronide-lyase indicated that the enzyme cleaved the uronic acid sequence: -guluronosyl-(1- 4)-mannuronosyl-, where the oxygen-mannuronosyl bond of this glycosidic linkage underwent transelimination.

The above sequence and the hypothesised action of the enzymes is of course idealised and the influence of a uronic acid residue several residues displaced from the point of cleavage remains unknown. Sutherland (1971) has shown that conversion of the glucuronic acid residues of Klebsiella

type 2 and other exopolysaccharides to glucose renders the polysaccharide resistant to enzymatic hydrolysis by a phage-induced enzyme, even though the glucuronic acid residue was not directly in the hydrolytic process.

Both poly-mannurono and poly-gulurono-lyases failed to hydrolyse unsaturated oliguronides to 4-deoxy-5-keto-uronic acid (Results section 6.1,7.6), indicating that the oligosaccharides were not suitable substrates for these enzymes; and suggesting a probable molecular size specificity for poly-alginate-lyases in contrast to earlier studies (see Preiss and Ashwell, 1962).

Although the effects of divalent ions on the enzymatic process were investigated only briefly (Results section 5.7) it was of interest that Ca^{2+} ions in particular produced a marked stimulation on the degradative action of SA1 lyase. The tertiary and quaternary structures of polysaccharides have been studied recently (Dea, McKinnon and Rees, 1972; Kirkwood, 1974) and it would be of interest to develop these studies to encompass the involvement of tertiary and quaternary structures and the action of polysaccharidases.

In general before an enzyme can be of use in the elucidation of polysaccharide structure, its specificity must be ascertained, since there is little point in reacting together enzymes of unknown specificities and polysaccharides of unknown structure. In the case of the present study the specificities of the enzymes examined were determined by studying both the kinetics of enzymatic action and partial characterization of the products released from a series of alginate samples of known mannuronic acid: guluronic acid ratio.

That enzymes provide extremely quick and accurate information for the characterization of polysaccharides is now accepted (see Marshall, 1975). With an understanding of the specificities of alginate lyases, the tools for an enzymatic study of alginate structure were available; SA1 providing a polyguluronide lyase, while the complementary enzyme was synthesised by Azotobacter bacteriophage and the partially purified mixed culture preparation.

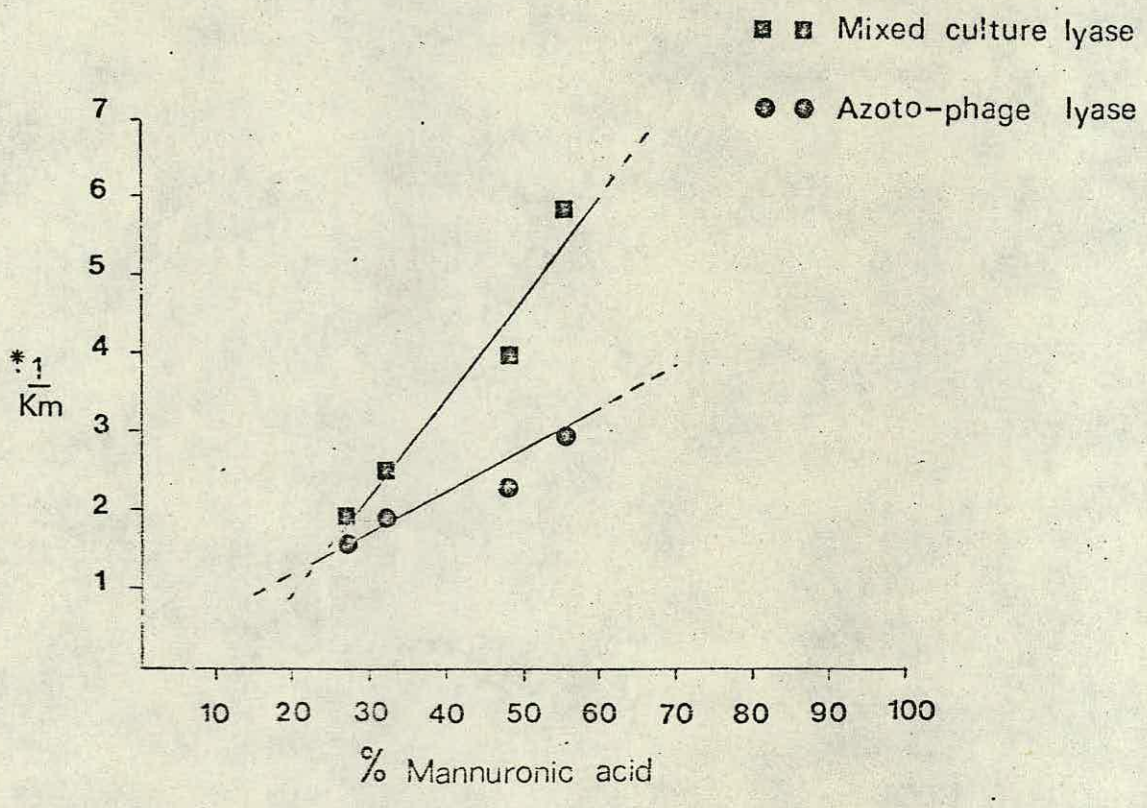
In the appraisal of the possible role alginate depolymerizing enzymes might play in the routine analysis of alginate samples two criteria must be judged; firstly their use in providing an indication of the primary structure of the molecule, and secondly their role in the elucidation of further details of the micro-structure of the polymer.

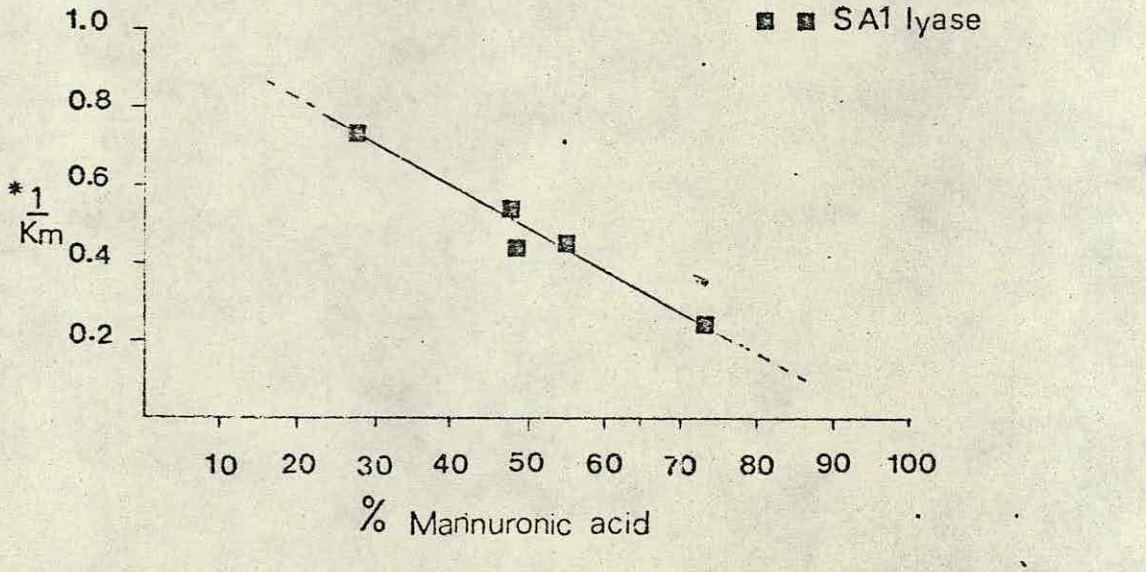
The results obtained from kinetic studies (Results section 5.10) indicated that an estimation of mannuronic acid to guluronic acid ratio in a sample of alginic acid was possible. The variations in Vm and Km observed for particular enzyme/substrate combinations reflect the concentration of specific substrate within the molecule. Additionally, Figure 43 illustrates the linear relationship between the percentage of particular uronic acid residue for which an enzyme is specific and the inverse of the Michaelis constant found for a particular substrate. Such lyase-type enzymes, therefore provide a rapid and accurate procedure for defining the primary structure of any alginate sample.

An alternative approach to the routine study of the determination of mannuronic acid to guluronic acid ratios of alginates might be to correlate decrease in molecular weight of the sample with the increase in the concentration of TBA

FIGURE 43

Use of Alginate lyases in the estimation of Mannuronic acid: guluronic acid ratios of alginic acid.





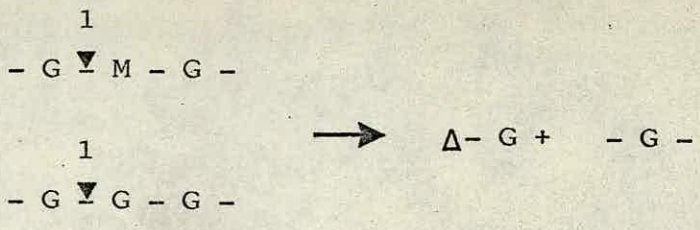
*ordinate $\times 10^{-2}$

positive material produced by the action of a specific alginase. Such an approach was attempted in this laboratory. The results obtained were initially difficult to interpret, for although rapid appearance of TBA positive material was readily estimated, decrease in molecular weight, as monitored by light scattering apparatus, did not follow the dramatic course suggested by the colourometric procedure. Gel filtration studies (Results section 6.2, 6.3), subsequently indicated a possible reason for these results. It might therefore be profitable to again attempt such studies to determine the percentage of linkages within the polymer which are cleaved by specific alginases, and thus develop a quantitative analytical procedure.

By virtue of the nature of alginic acid, namely its highly irregular structure, the preliminary enzymatic studies presented here play only a minor role in the elucidation of the detailed features of the polysaccharide. Even after comprehensive studies to determine the specificities of alginate depolymerases had been carried out, the lyase-type enzymes examined present problems not encountered in the analysis of other uronic-acid containing polysaccharides.

Both mannuronic acid and guluronic acid give rise to the same unsaturated product (4-deoxy-5-keto-uronic acid) irrespective of the specificity of the alginate-lyase or the nature of the glycosidic linkage which has undergone cleavage. This is again demonstrated by the failure to detect mannuronic acid in the low molecular weight products released from alginate by SA1 poly-gulurono-lyase. Illustrated below are uronic acid sequences which could give

rise to the same products.



Action of poly-gulurono-lyase

The converse would then apply to a poly-mannurono-lyase, such as that elaborated by Azotobacter bacteriophage.

In a study of any polysaccharidases and the analysis of the subsequent products produced by its action, the non-reducing end of the oligosaccharides are of particular interest. In the case of alginate lyases it is only by inference that the origin of the non-reducing end of the oligouronide can be deduced.

Microbial Catabolism of Alginic Acid

Although the scheme for the metabolism of alginic acid, illustrated in Figure 9, has not been of direct concern here, certain modifications to the pathway can be made as a result of this study. Firstly the high molecular weight polymer is cleaved by two specific enzymes, as described, and secondly evidence obtained from examination of the crude mixed culture alginase preparation indicated the presence of further enzymes which were capable of degrading to 4-deoxy-5-keto-uronic acid the oligouronides produced by "poly-lyase" activity.

Nisizawa et al (1968) described an enzyme which depolymerized

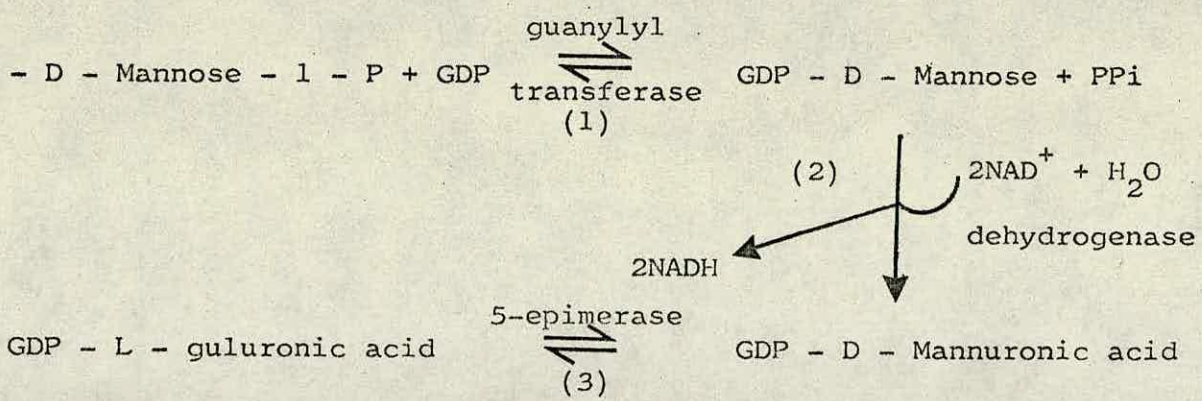
short chain polymannuronides, and it must be assumed that a second enzyme capable of degrading short chain poly-guluronides exists in some bacteria.

In all fifteen microbial, alginolytic enzyme systems were examined and without exception each system gave rise to products which gave a positive TBA test, indicating that each degraded sodium alginate via an eliminative route (Results section Table 6) ; further underlining the metabolic importance of such degradative pathways in the microbial world (see Introduction pages 15 - 27).

In general the biochemical degradation of polysaccharides follows the most energetically favourable pathway, for example, Sutherland (1967) showed that the phage-induced polysaccharidase which hydrolysed Klebsiella aerogenes type 54 (A3 (S1)) was a fucosidase. Interestingly, Conrad et al (1968) had shown that partial acid hydrolysis of the polysaccharide yielded oligosaccharides all of which had L-fucose at the reducing end.

The comparatively slow hydrolysis, by acid, of glycosides containing glucuronic acid has been described by Rees (1967); Nature, in lyase-type enzymes, has perhaps developed an alternative mechanism to overcome this energetically demanding step.

Enzymes which catalyse the random hydrolysis of -1,4 glycosidic links in polygalacturonic acid are known (Fogarty and Ward, 1972), but there does not seem to be any correlation, based on evolutionary status, between hydrolytic cleavage and organisms which degrade pectinic acid by transelimination. Additionally, (see Introduction page 22), lower orders from



GDP - D - mannuronate
GDP - L - guluronate
glycosyl transferase
acceptor (4)
alginic acid

Figure 44

Possible route for the biosynthesis of alginic acid in F.gardneri (Lin and Hassid, 1966).

the animal kingdom cause the breakdown of alginic acid via an eliminative pathway: a hydrolytic enzyme capable of degrading alginic acid and comparable to those for pectinic acid remains to be discovered.

Biosynthesis of Alginic Acid

Often in the examination of the bio-degradation of any natural-high-molecular weight polymer, some indication of its anabolic mechanism is achieved. Some circumstantial evidence was obtained during this work which may aid in the understanding of the routes of biosynthesis for algal and bacterial alginates.

Only a limited amount of biochemical data regarding the biosynthesis of alginic acid is available, even so, two possible biosynthetic pathways have emerged.

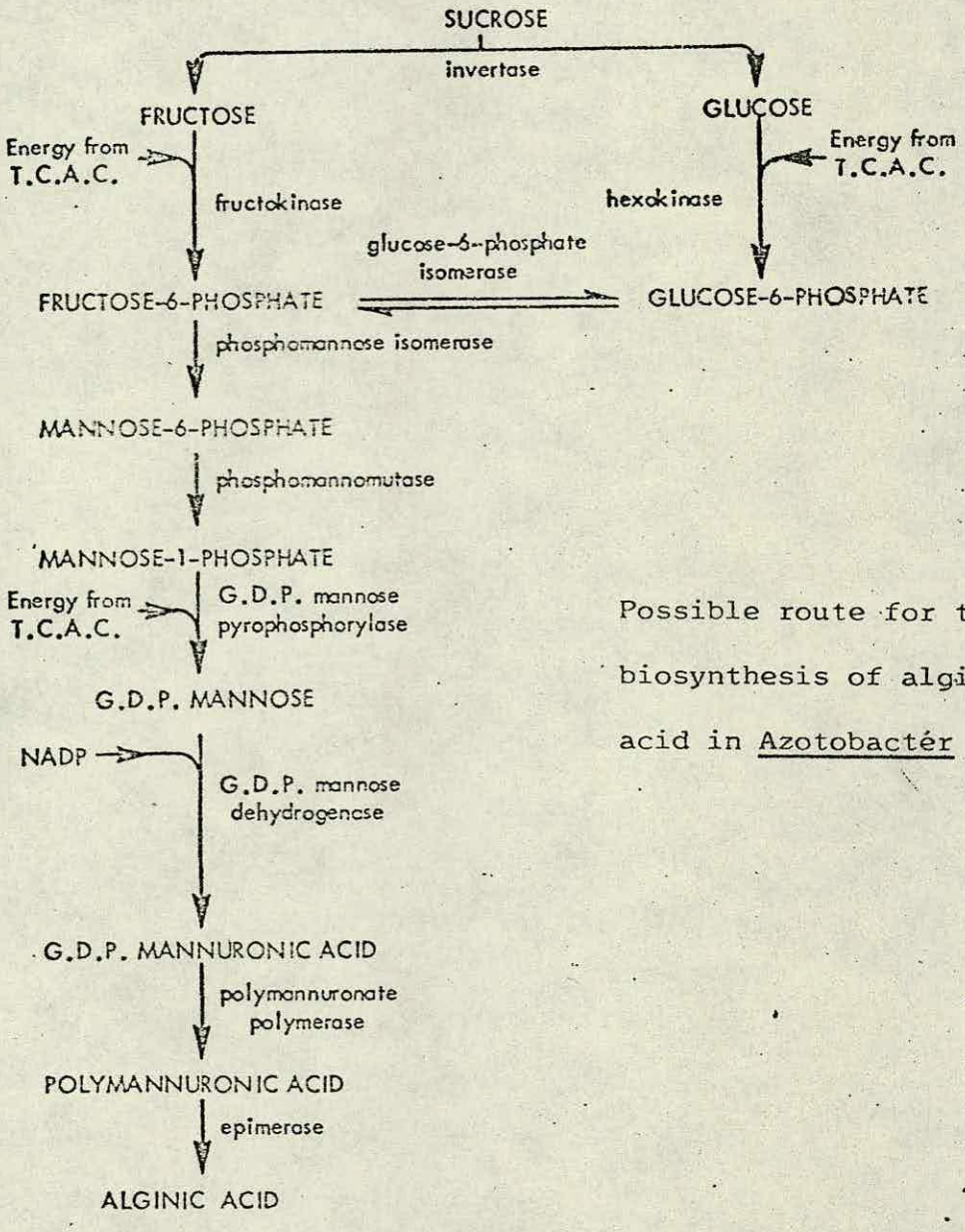
Lin and Hassid (1966a) isolated from the marine brown alga, Fucus gardneri Silva, the sugar nucleotides guanosine diphosphate (GDP)-D-mannuronic acid and GDP-L-guluronic acid. The former amounted to about 80% of the uronic acid nucleotides examined, while the latter was present only as a minor component of the sugar nucleotide pool. The scheme of alginic acid biosynthesis proposed by Lin and Hassid (1966b) is shown in Figure 44. The nature of the specificity of the final glycosyl transferase (reaction 4) and the overall control of such a biosynthetic route which would lead to a molecule of the structure proposed by Haug and Larsen (see Figure 3) is unclear. Further the interconversion of GDP-D-mannuronic acid and GDP-L-guluronic acid has yet to be confirmed, although

the conversion of UDP-D-glucuronic acid to UDP-L-iduronic acid has been shown to occur in rabbit skin (Jacobson and Davidson, 1962).

An alternative pathway for the biosynthesis of alginic acid was originally proposed by Haug and Larsen (1969). In a brief report the epimerization of D-mannuronic acid to L-guluronic acid residues in the polymer chain was described. The enzyme which catalysed this reaction was isolated from the culture supernate of A. vinelandii. In subsequent and more detailed studies Larsen and Haug (1971a, 1971b) showed that the partially purified enzyme was dependent on Ca^{2+} ions for full activity. Their replacement by Na^{+} or Mg^{2+} had little effect in restoring enzyme activity, but partial re-activation was obtained in the presence of Sr^{2+} ions. The reaction is believed to be uni-directional since no epimerization of L-guluronic acid to D-mannuronic acid was detected. Larsen and Haug examined the enzymatic reaction by measuring the increase in guluronic acid content of an alginate sample after incubation with the epimerase preparation. It should be pointed out that a similar relative increase in guluronic acid content could be produced by the action of a polymannuronide lyase, which has been shown in this study to be synthesised by A. vinelandii. However, the work of Haug and Larsen has been independently confirmed by Bucke and Pindar (Personal communication) and the biosynthetic pathway for the production of alginic acid from sucrose, suggested by these workers is shown in Figure 45.

No information on the biosynthesis of alginic acid produced by C.F. strains of Pseudomonas aeruginosa is available.

Figure 45



Possible route for the biosynthesis of alginic acid in Azotobactér vinelandii.

If algal and Azotobacter alginate are formed by the alternative pathways described, inevitably the final structure of the two polymers would reflect the bio-synthetic route adopted. Figures 26 and 27 may underline the differences between the polysaccharides. The high molecular weight material present after Azotobacter alginate had been digested with SA1 lyase was shown to be composed of D- mannuronic acid. This material could correspond to the original product of biosynthesis: that part of the alginate molecule which remained unaltered after the action of the L-5 epimerase system described above. The variable amount of high molecular weight material present after Azotobacter alginates, with different mannuronic acid to guluronic acid ratios, had been treated with SA1 lyase may be indicative of the epimerase activity which gave rise to the ultimate structure of the molecule.

The more complete hydrolysis of algal alginate which was observed may therefore reflect the absence of any high-molecular-homopolymeric portion of the molecule, as indicated by its biosynthetic route.

It must be emphasized that the biosynthesis of alginic acid remains uncertain and any hypothesis which might have been suggested to explain the different routes reported in eukaryotic and prokaryotic organisms has been further thrown into doubt by the recent work of Madjwick et al (1973) in which a C-5 mannurono-epimerase from the marine alga Pelevitia canaliculata is described and by Hook et al (1974) suggesting the iduronic acid residues of heparin are formed by C-5 epimerization at the polymer level.

Function of O-acetyl Groups in Azotobacter Alginates

It was observed that the O-acetyl groups associated with Azotobacter alginates were associated with mannuronic acid results (see Figure 27a). Removal of these groups had no significant effect on the degradative process (Figure 28a), of SA1 poly-gulurono-lyase. In addition acetylated Azotobacter alginates were completely hydrolysed by a specific polymannuronide lyase (Figure 36). The role of acetyl groups in Azotobacter alginate is not therefore to confer enzymatic resistance on the polymer.

On the contrary, the role of O-acetyl groups may be operative during the biosynthesis of bacterial alginate. O-acetylated D-mannuronic acid residues may be resistant to C-5 epimerization and would therefore not undergo conversion to L-guluronic acid. The control of acetylation of the polymer and the stage at which acetylation occurs, remains unanswered.

One further implication which can be drawn from this work is that epimerization does not play a part in the degradation of alginates, since Azotobacter exopolysaccharide could not be completely hydrolysed by a crude sample of SA1 lyase, even after removal of O-acetyl groups.

Finally, confirmation of the alginate-like nature of the exopolysaccharide of Azotobacter vinelandii is indicated by the failure to detect sugars other than L-guluronic acid and D-mannuronic acid (see Introduction pages 7-8), during either examination of the products of lyase action, or in the examination of acid hydrolysates of whole polysaccharides, and the

inability of the enzymes described, to cleave other uronic-acid containing polysaccharides. The alternative sugars which have been reported to be components of the polysaccharide must have arisen from the bacterial cell wall and not the bacterial capsule.

ACKNOWLEDGEMENTS

I wish to express my sincere thanks to Dr. I.W. Sutherland for his supervision and invaluable comment and discussion throughout the period of this research.

I wish to thank Professor J.F. Wilkinson for providing the facilities for research in his department.

I am indebted to Dr. C.J. Lawson and Dr. C. Bucke (Tate & Lyle, Ltd.) for their advice and encouragement.

Finally I thank the Science Research Council for financial support for the duration of this work.

REFERENCES

References to Table 1

1. Sutherland, I.W. and Wilkinson, J.F. (1968). The Exopolysaccharide of *Klebsiella aerogenes* A3(s1) (Type 54). Biochemical Journal, 110, 749-754.

2. Bailey, R.W., Greenwood, R.M. and Craig, A. (1971). Extracellular Polysaccharides of Rhizobium Strains Associated with Lotus Species. Journal of General Microbiology, 65, 315-324.

3. Zevenhuizen, L.P.T.M. (1971). Chemical composition of Exopolysaccharides of Rhizobium and Agrobacterium. Journal of General Microbiology, 68, 239-243.

4. Sloneker, J.H. and Jeanes, A. (1962). Exocellular Bacterial Polysaccharide from *Xanthomonas Campestris* NRRL B-1459. Canadian Journal of Chemistry, 40, 2066-2071.

5. Harada, T. (1965). Succinoglucan 10C3: A New Acidic Polysaccharide of *Algcaligenes Faecalis* var. myxogenes. Archives of Biochemistry and Biophysics, 112, 65-69.

6. Adams, G.A. and Martin, S.M. (1964). Extracellular polysaccharides of *Serratia marcescens*. Canadian Journal of Biochemistry, 42, 1403-1413.

7. Niven, C.F., Smiley, K.L. and Sherman, J.M. (1941). The Polysaccharides synthesized by *Streptococcus salivarius* and *Streptococcus bovis*. Journal of Biological Chemistry, 140, 105-109.

8. Forsyth, W.G.C. and Webley, D.M. (1949). Polysaccharides Synthesized by aerobic mesophilic spore-forming Bacteria. Biochemical Journal, 44, 455-459.

9. Norris, R.F., Despin, M., Zilliken, F.W., Harvey, T.S., and Gyorgy, P. (1954). Occurrence of mucoid variants of Lactobacillus bifidus. Journal of Bacteriology, 67, 159-166.

10. Baine, H.and Cherniak, R. (1971). Capsular Polysaccharides of Clostridium perfringens Hobbs 5. Biochemistry, 10, 2948-2952.

11. Narvmi, K. and Tsumita, T. (1964). The Isolation and Identification of α,α - Trehalose 6, 6' - diphosphate, a Subunit of the Phosphorylated Polysaccharide of Mycobacterium tuberculosis. Journal of Biological Chemistry, 240, 2271-2276.

12. Bouveng, H.O., Kiessling, H., Lindberg, B. and McKay, J. (1962). Polysaccharides Elaborated by Pullularia pullulans. Acta Chemica Scandinavica, 16, 615-622.

13. Gorin, P.A.J., and Perlin, A.S. (1956). A mannan produced by Saccharomyces rouxii. Canadian Journal of Chemistry, 34, 1796-1803.

14. Jeanes, A., Burton, K.A., Camdus, M.C., Knutson, C.A., Rowin, G.L. and Stanford, P.A. (1971). Extracellular Black yeast Polysaccharide composed of N-acetyl glucosamine and N-acetyl glucosaminuronic acid. Nature, New Biology, 233, 259-260.

15. Mager, J. (1947) Studies on the Polysaccharides of Capsulated Yeasts. Biochemical Journal, 41, 603-609.

GENERAL REFERENCES

Abramson, C. and Friedman, H. (1968). Staphylococcal Hyaluronate Lyase: Purification and Characterization Studies. Journal of Bacteriology, 96, 886-892.

Abramson, C. and Friedman, H. (1969). Electrophoretic Characteristics of Staphylococcus Hyaluronate Lyase. Journal of Bacteriology, 97, 715-718.

Abramson, C. and Rautela, G.S. (1971). Purification and Crystallization of Hyaluronate Lyase. Bacteriological Proceedings, 81.

Adams , J.N., Williams, J. and Payne, W.J. (1961) Agarbacterium alginicum: the appropriate taxonomic designation for Alginomonas alginica. Journal of Bacteriology, 80, 193-199.

Albersheim, P., Neukom, H. and Deuel, H. (1960). Splitting of Pectin Chain Molecules in Neutral Solutions, Archives of Biochemistry and Biophysics, 90, 46-51.

Andersen, G. (1956). On the Detection of Alginic Acid in Tissues by means of Birefringence. Second International Seaweed Symposium. Eds. T. Braarud and N.A. Sørensen, Pergamon Press, London and New York.

Atkins, E.O.T., Mackie, W. and Smolko, E.D. (1970). Crystalline Structure of Alginic Acid. Nature, 225, 626-628.

Baker, E.D. and Whiteside, (1965). Preparation and Properties of a Vi Antigen - Degrading Enzyme. Journal of Bacteriology, 89, 1217-1224.

Becker, G.E. and Pappenheimer, A.M. (1966). Lyase Activity of Inducible S8 - Depolymerases from Bacillus palustris. Biochimica et Biophsica Acta, 121, 343-348.

Be Miller, J.N. and Kumari, G.V., (1972). beta-Elimination in Uronic Acids: evidence for an ElcB mechanism Carbohydrate Research, 25, 419-428.

Bergan, T. and Hovig, B. (1969) Hyaluronic Acid Capsule in a Streptococcus faecalis var. zymogenes. Acta Patologica et Microbiologica Scandinavica, 75, 97-103.

Billy, C. (1965). Isolation in Pure culture of a Marine Clostridium, agent of anaerobic symbiotic alginolyses. Annales de L'Institut Pasteur, 108, 260-261.

Billy, C. (1966). Studies on an anaerobic alginolytic bacterium Clostridium alginolyticum. Annales de L'Institut Pasteur, 109, 147-151.

Black, W.A.P. (1948). The Seasonal Variation in Chemical Composition of Some of the Littoral Seaweeds Common to Scotland. Part I. Ascophyllum Nodosum. Journal of the Society of Chemical Industry, 67; 355-357.

Brimacombe, J.S. and Webber, J.M. (1964). Mucopolysaccharides. B.B.A. Library 6, Elserier Publishing Company, Amsterdam.

Brown, M.R.W., Scott Foster, J.H. and Clamp, J.R. (1969) Composition of Pseudomonas aeruginosa Slime. Biochemical Journal, 112, 521-525.

Bucke, C. (1974). Polyacrylamide gel electrophoresis of alginic acid. Journal of Chromatography, 89, 99-102.

Burns, M.W. and May, J.R. (1968). Bacterial Precipitins in Serum of Patients with Cystic Fibrosis. Lancet I 270-272.

Carlson, D.M. and Matthews, L.W. (1966). Polyuronic Acids Produced by Pseudomonas aeruginosa. Biochemistry, 5, 2817-2822.

Chanda, S.K., Hirst, E.L., Percival, E.G.V., and Ross, A.G. (1952). The Structure of Alginic Acid II. Journal of the Chemical Society, 1833-1837.

Ciba Review, (1969). Alginates.

Claus, D. (1965). 2 Keto-3-Deoxygalacturonic Acid as a constituent of an Extracellular polysaccharide of Azotobacter vinelandii. Biochemical and Biophysical Research Communications, 20, 745-751.

Clowes, R.C. and Hayes, W. (1968). Experiments in Microbial Genetics, Blackwell Scientific Publications, Oxford.

Cohen, G. and Johnstone, D.B. (1963). The Influence of the Extracellular Polysaccharide Slime of Azotobacter vinelandii on pH. Bacteriological Proceedings, 11.

Cohen, G. and Johnstone, D.B. (1964). Extracellular Polysaccharide of Azotobacter vinelandii. Journal of Bacteriology, 88, 329-338.

Conrad, M.E. Bamburg, J.R., Epley, J.D. and Kindt, T.J. (1966). The Structure of the Aerobacter aerogenes A3 (S1). Polysaccharide, II. Sequence Analysis and Hydrolysis Studies. Biochemistry, 5, 2808-2817.

Costerton, J.W. Ingram, J.M. and Cheng, K.J. (1974). Structure and Function of the Cell Envelope of Gram-Negative Bacteria. Bacteriological Reviews, 38, 87-110.

Cowan, S.T. and Steel, K.J. (1961). Diagnostic tables for the common medical bacteria. Journal of Hygiene, 59, 357-372.

Dea, I.C.M., McKinnon, A.A., and Rees, D.A. (1972). Tertiary and Quaternary Structures in Aqueous Polysaccharide Systems which Model Cell Wall Cohesion: Reversible Changes in Confirmation and Association of Agarose, Carrageenan and Galactomannans. Journal of Molecular Biology, 68, 153-172.

Demain, A.L. (1971). Increasing Enzyme Production by Genetic and Environmental Manipulations. Methods in Enzymology (ed Jakoby), 22, 86-95.

Doggett, R.G., Harrison, G.M. and Wallis, E.S. (1964). Comparison of Some Properties of Pseudomonas aeruginosa isolated from Infection in Persons with and without Cystic Fibrosis. Journal of Bacteriology, 87, 426-436.

Doggett, R.G. (1969). Incidence of Mucoid Pseudomonas aeruginosa from Clinical Sources. Applied Microbiology, 18, 936-937.

Dubois, M., Gillies, K.A., Hamilton, J.K. Rebers, P.A. and Smith, F. (1956). Colorimetric method for determination of Sugars and Related Substances. Analytical Chemistry, 28, 350-356.

Duguid, J.P. (1951). The demonstration of bacterial capsules and slime. Journal of Pathological Bacteriology, 63, 673-685.

Duff, J.F. and Wyss, O. (1961). Isolation and classification of a new series of Azotobacter bacteriophages. Journal of General Microbiology, 24, 273-289.

Eklund, C. and Wyss, O. (1962). Enzyme Associated with Bacteriophage Infection. Journal of Bacteriology, 84, 1209-1215.

Eller, J. and Payne, W.J. (1960). Studies on bacterial Utilization of Uronic Acids. IV. Alginolytic and Mannuronic acid Oxidizing isolates. Journal of Bacteriology, 80, 193-199.

Elyakova, L.A. and Favorov, V.V. (1974). Isolation and Certain Properties and Alginate Lyase VI from the Mollusc Littorina sp. Biochemica et Biophysica Act, 358, 341-354.

Evans, L.R. and Linker, A. (1973). Production and Characterization of the Slime Polysaccharide of Pseudomonas aeruginosa. Jouronal of Bacteriology, 116, 915-924.

Feather, M.S. and Whistler, R.L. (1962). Isolation and characterization of the principal hemicellulase from Corn Germ. Archives of Biochemistry and Biophysics, 98, 111-115.

Fischer, F.G. and Dorfel, H. (1955). Die Polyuronsauren der Braun algen. Zeitschrift fur Physiologische Chemie Hoppe-Seyler's, 302, 186-203.

Fogarty, W.M. and Ward, O.P. (1972). Pectic Substances and Pectinolytic Enzymes. Process Biochemistry 13-17.

Fogarty, W.M. and Ward, O.P. (1974). Pectinases and Pectic Polysaccharides in Progress in Industrial Microbiology 13, 59-120, Churchill-Livingstone, Edinburgh.

Food and Agriculture Organisation of the United Nations (1974). Toxicological Evaluation of Some Food Additives. No. 53 A, 381-385.

Gorin, P.A.J. and Spencer, J.F.T. (1966). Exocellular Alginic Acid From Azotobacter vinelandii. Canadian Journal of Chemistry, 44, 993-998.

Haug, A. (1959a). Fractionation of Alginic Acid. Acta Chemica Scandinavica, 13, 601-603.

Haug, A. (1959b). Ion exchange properties of Alginate Fractions. Acta Chemica Scandinavica, 13, 1250-1251.

Haug, A. and Smidsrød, O. (1965). Fractionation of Alginates by Precipitation with Calcium and Magnesium Ions. Acta Chemica Scandinavica, 19, 1221-1226.

Haug, A. and Larsen, B. (1965). A Study Constitution of Alginic Acid by Partial Acid Hydrolysis. Proceedings of the Fifth International Seaweed Symposium Pergamon Press, Oxford.

Haug, A. and Larsen, B. (1969). Biosynthesis of alginate. Epimerization of D-mannuronic to L-guluronic acid residues in the polymer chain. Biochimica et Biophysica Acta, 192, 557-559.

Haug, A. and Larsen, B. (1971). Biosynthesis of Alginate II. Polymannuronic Acid C-5 epimerase from Azotobacter vinelandii. Carbohydrate Research, 17, 297-308.

Haug, A., Larsen, B. and Smidsrød, O. (1974). Uronic Acid Sequence in Alginate from Different Sources. Carbohydrate Research, 32, 217-225.

Hestrin, S. (1949). The reaction of acetylcholine and other carboxylic acid derivatives with hydroxylamine and its analytical application. Journal of Biological Chemistry, 180, 249-261.

Hirst, E.L., Jones, J.K.N. and Jones, W.O. (1939). The Structure of Alginic Acid. I. Journal of the Chemical Society, 1880-1885.

Hirst, E.L., Percival, E.G.V. and Wold, J.K. (1964). The Structure of Alginic Acid. IV. Partial Hydrolysis of reduced polysaccharide. Journal of the Chemical Society, 1493-1499.

Hoagland, D.A. and Lieb, L.L. (1915). The Complex Carbohydrates and forms of Sulphur in Marine Algae of the Pacific Coast. Journal of Biological Chemistry, 23, 287-297.

Høiby, N. (1974). Epidemiological Investigations of the Respiratory Tract Bacteriology in Patients with Cystic Fibrosis. Acta Pathologica et Microbiologica Scandinavica Section B, 82, 541-550.

Hoffman, P., Linker, A., Lippman, V. and Meyer, K. (1960). The Structure of Chondroitin Sulphate B from Studies with Flavobacterium Enzymes. Journal of Biological Chemistry, 235, 3066-3069.

Hook, M., Lindahl, V., Backstrom, G., Malmstrom, A. and Fransson, L.A. (1974). Biosynthesis of Heparin, III. Formation of Iduronic acid Residues. Journal of Biological Chemistry, 249, 3908-3915.

Hovingh, P. and Linker, A. (1970). The Enzymatic Degradation of Heparin by a Heparitinase and a Heparinase from Flavobacterium. Journal of Biological Chemistry, 245 6170-6175.

Iacocca, V.F., Sibinga, M.S., Barbero, G.J. (1963). Respiratory Tract Bacteriology in Cystic Fibrosis. American Journal of Disease of Children, 106, 315-324.

International Union of Biochemistry (1961). Report of the Commission on Enzymes. Pergamon Press, Oxford.

Jacobson, B. and Davidson, E.A. (1962). Biosynthesis of Uronic Acids by Skin Enzymes. The Journal of Biological Chemistry, 237, 638-642.

Jannasch, H.W. (1965). Continuous Culture in Microbial Ecology. Laboratory Practice, 14,1162-1167.

Johnstone, D.B. (1974) in Bergey's Manual of Determinative Bacteriology (Buchanan, R.E. and Gibbons, N.E., eds.) Williams and Wilkins, Baltimore.

Johnston, C. (1968). Industrial Uses of Algae. Process Biochemistry, 11-14.

Kashiwabara, Y., Suzuki, H. and Nisizawa, K. (1969). Alginate Lyases of Pseudomonads. The Journal of Biochemistry, 66, 503-512.

Kaufman, B. and Repaske, R. (1958). Polysaccharide synthesis by Azotobacter vinelandii. Bacteriological Proceedings, 126.

Keijbets, M.J.H. and Pilnik, W. (1974). β-Elimination of Pectin in the Presence of Anions and Cations. Carbohydrate Research, 33, 359-362.

Kilbourn, J.P. Campbell, R.A., Grach, J.L. and Wills, M.D. (1968). Quantitative Bacteriology of Sputum American Review of Respiratory Diseases, 98, 810-818.

Kilbourn, J.P. (1970). Infection in Cystic Fibrosis. Lancet II, 878-879.

Kiss, J. (1974). β - Eliminative Degradation of Carbohydrates Containing Uronic Acid Residues. Advances in Carbohydrate Chemistry and Biochemistry, 29, 229-303.

Kirkwood, S. (1974). Unusual Polysaccharides. Annual Review of Biochemistry, 43, 401-417.

Knutson, C.A., and Jeanes, A. (1968). A New Modification of the Carbazole Analysis: Application to heteropolysaccharides. Analytical Biochemistry, 24, 470-481.

Kooiman, P. (1954). Enzymic hydrolysis of Alginic Acid. Biochemical et Biophysical Acta, 13, 338-340.

Larsen, B. and Haug, A. (1971a). Composition and Structure of Alginate Produced by Azotobacter vinelandii. Carbohydrate Research, 17, 287-296.

Larsen, B. and Haug, A. (1971b). Biosynthesis of Alginate III. Polymannuronic acid C-5-epimerase from Azotobacter vinelandii. Carbohydrate Research, 20, 225-232.

Lawson, G.J. and Stacey, M. (1954). Immunopolysaccharides. I Preliminary Studies of a Polysaccharide from Azotobacter chroococcum containing a Uronic acid. Journal of the Chemical Society, 1925-1931.

Lesley, S.M. (1961). Degradation of the Polysaccharide of Xanthomonas phaseoli by an Extracellular Bacterial Enzyme. Canadian Journal of Microbiology, I, 815-825.

Lewin, R.A.and Lounsbery, D.M. (1969). Isolation, Cultivation and Characterization of Flexibacteria. Journal of General Microbiology, 58, 145-170.

Lin, L.P. and Sadoff, H.L. (1969). Chemical composition of Azotobacter vinelandii cysts. Journal of Bacteriology, 100, 480-486.

Lin, T.Y. and Hassid, W.Z. (1966a). Isolation of guanosine diphosphate uronic acids from a Marine brown alga, Fucus gardneri Silva. Journal of Biological Chemistry, 241, 3282-3293.

Lin, T.Y. and Hassid, W.Z. (1966b). Pathway of alginic acid synthesis in the marine brown alga, Fucus gardeneri Silva. Journal of Biological Chemistry, 241, 5284-5297.

Linker, A., Meyer, K. and Hoffman, P. (1956). The Production of Unsaturated Uronides by Bacterial Hyaluronidases. Journal of Biological Chemistry, 219, 13-25.

Linker, A. and Jones, R.S. (1964). A. Polysaccharide Resembling Alginic Acid from a Pseudomonas Micro-organism. Nature, 204, 187-188.

Linker, A. and Jones, R.S. (1966). A New Polysaccharide Resembling Alginic Acid Isolated from Pseudomonads. Journal of Biological Chemistry, 241, 3845-3851.

Linker, A. (1966). in Methods in Enzymology (Neufield, E.F. and Ginsburg, V. eds.). Vol. VIII 650-654. Academic Press, New York.

Linker, A. and Hovingh, P. (1973). The Heparitin sulphates. Carbohydrate Research, 29, 41-62.

Lowry, O.H., Rosebrough, N.J. Farr, A.L. and Randall , R.J. (1951). Protein measurement with the Folin-Phenol reagent. Journal of Biological Chemistry., 193, 265-275.

Lucas, H.J. and Stewart, W.T., (1940). Oxidation of alginic acid by periodic acid. Journal American Chemical Society, 62, 1792-1796.

McDowell, R.H. (1958). A Method for the Fractionation of Alginates. Chemistry and Industry, 1401-1402.

McDowell, R.H. (1960). Applications of Alginates. Review Pure and Applied Chemistry, 10, 1-19.

McNeely, W.H. and Pettitt, D.J. (1973) in Industrial Gums: Polysaccharides and their Derivatives Ed. R.L. Whistler. Academic Press, New York and London.

McNicol, L.A. and Baker, E.E. (1970). Pectate Lyase and Polygalacturonate Lyase Activity in a Vi Antigen Degrading Enzyme Preparation. Biochemistry, 9, 1017-1023.

Madgwick, J., Haug, A. and Larsen, B. (1973 a). Alginate Lyase in the Brown Alga. Laminaria digitata (Huds) Lamour. Acta Chemica Scandinavica, 27, 711-712.

Madgwick, J., Haug, A. and Larsen, B. (1973 b). Polymannuronic acid 5-epimerase from the marine alga, Pelvetia canaliculata . Acta Chemica Scandinavica, 27, 3592-3594.

Markley, K. and Smallman, E. (1968). Protection by Vaccination against Pseudomonas infection after thermal injury. Journal of Bacteriology, 96, 867-874.

Marshall, J.J. (1974). Application of Enzymic Methods to the Structural Analysis of Polysaccharides: Part I. Advances in Carbohydrate Chemistry and Biochemistry, 30, 257-370.

Martin, J.P. (1945). Some Observations on the Synthesis of Polysaccharides by Soil Bacteria. Journal of Bacteriology, 50, 349-360.

Mathews, C.K. and Sutherland, K.E. (1965). Comparative Biochemistry of bacterial and phage-induced dihydrofolate reductases. Journal of Biological Chemistry, 240, 2142-2147.

Meland, S.M. (1962). Marine Alginate-decomposing bacteria from North Norway. Nytt Magasin fur Botanik, 10, 53-80.

Misustin, E.N. and Shil'Nikova, V.K. (1971). Biological Fixation of Atmospheric Nitrogen, MacMillan Press Ltd., London.

Nakada, H.I. and Sweeny, P.C. (1967). Alginic Acid Degradation by Eliminases from Abalone Hepatopancreas. Journal of Biological Chemistry, 242, 845-851.

Nelson, W.L. and Cretcher, L.H. (1929). The Alginic Acid from Macrocystis pyrifera. Journal American Society, 51, 1914-1922

Nelson, W.L. and Cretcher, L.H. (1930). The Isolation and Identification of D-Mannuronic acid lactone from Macrocystis pyrifera. Journal American Chemical Society, 52, 2130-2132.

Nelson, N. (1944). A Photometric adaptation of the Somogyi Method for the Determination of Glucose. Journal of Biological Chemistry, 153, 379-380.

Nisizawa, K., Fujibayashi, S. and Kashiwabara, Y. (1968) Alginate Lyases in the Hepatopancreas of a Marine Mollusc, Dolabella auricula Solander. The Journal of Biochemistry, 64, 25-37.

Osborn, M.J., Gander, J.E., Parisi, E. and Carson, J. (1972). Mechanism of Assembly of the outer membrane of Salmonella typhimurium. Journal of Biological Chemistry, 247, 3962-3972.

Oshima, K. (1932). Discovery of an alginic acid cleaving enzyme (alginase) and its properties. Biological Abstracts, 9386.

Parikh, V.M. and Jones, J.K.N. (1963). The Structure of the Extracellular Polysaccharide of Azotobacter indicum. Canadian Journal of Chemistry 41, 2826-2835.

Passmore, R. and Robson, J.S. (1968). A Companion to Medical Studies. Volume I. Blackwell Scientific Publications, Oxford.

Penman, A. and Sanderson, G.R. (1972). A Method for the Determination of Uronic Acid Sequence in Alginates. Carbohydrate Research, 25, 273-282.

Percival, E. and McDowell, R.H. (1967). Chemistry and Enzymology of Marine Algal Polysaccharides. Academic Press, London.

Pike, L. and Wyss, O. (1975). Isolation and Characterization of Phage-resistant Strains of Azotobacter vinelandii. Journal of General Microbiology, 89, 182-186.

Preiss, J. and Ashwell, G. (1962a). Alginic Acid Metabolism in Bacteria. I. Enzymatic formation of unsaturated oligosaccharides and 4-deoxy-L-erythro-5-hexo-seulose uronic acid. Journal of Biological Chemistry, 237, 309-316.

Preiss, J. and Ashwell, G. (1962b). Alginic Acid Metabolism in Bacteria. II. The Enzymatic reduction of 4-deoxy-L-erythro-5-hexoseulose uronic acid to 2-keto-3-deoxy-D-gluconic acid. Journal of Biological Chemistry, 237, 317-321.

Preiss, J. and Ashwell, G. (1963). Polygalacturonic Acid metabolism in Bacteria. Journal of Biological Chemistry, 238, 1571-1576.

Preston, R. D. (1974). The Physical Biology of Plant Cell Walls. Chapman and Hall, Ltd., London.

Rees, W.R. and Reynolds, T. (1958). A solvent for the paper chromatographic Separation of Glucose and sorbitol. Nature, 181, 767-768.

Rees, D.A. (1967). The Shapes of Molecules. Oliver and Boyd, Edinburgh.

Rees, D.A. and Samuel, J.W.B. (1967). The Structure of Alginic Acid IV. Journal of the Chemical Society, 2295-2298.

Rees, D.A. (1972). Shapely Polysaccharides. Biochemical Journal, 126, 257-273.

Reid, D.S., Bryce, T.A., Clark, A.H. and Rees, D.A. (1974). Helix-coil transition in Gelling Polysaccharides, Faraday Discussions of the Chemical Society, No. 57 230-238.

Rogers, K.B. (1960). Pseudomonas infections in a childrens' hospital. Journal of Applied Bacteriology, 23, 533-537.

Rombouts, F.M., (1972). Occurrence and Properties of bacterial pectute lyases. Agricultural Research Reports, 779.

Rubenchik, L.I. (1963). Azotobacter and its Use in Agriculture, National Science Foundation,Washington D.C.

Saito, H., Tatsuya, Y. and Suzuki, S. (1968). Enzymatic Methods for the Determination of Small Quantities of Isomeric Chondroitin Sulphates. Journal of Biological Chemistry, 243, 1536-1542.

Schlegel, H.G. and Jannasch, H.W. (1967). Enrichment Cultures. Annual Reviews of Microbiology, 21, 49-70.

Segeren, A.J.M., Boskamp, J.V. and van der Tempel, M. (1974). Rheological and Swelling Properties of Alginate Gels Faraday Discussions of the Chemical Society, No. 57, 255-274.

Smidsrød, O. (1974). Molecular Basis for Some Physical Properties of Alginates in the Gel State. Faraday Discussions of the Chemical Society No. 57, 263-274.

Stacey, M. and Barker, S.C. (1960). Polysaccharides of Micro-organisms, Clarendon Press, London.

Stanford, E.C. (1883). A New Substance Obtained from Some of the Commoner Species of Marine Algae. Chemical News, 47, 254-257, 267-269.

Stanier, R.Y. Palleroni, N.J. and Doudoroff, M. (1966). The Aerobic Pseudomonads: a Taxonomic Study. The Journal of General Microbiology, 43, 159-271.

Sutherland, I.W. and Wilkinson, J.F. (1965). Depolymerases for bacterial exopolysaccharides obtained from Phage-infected bacteria. Journal of General Microbiology, 37, 373-383.

Sutherland, I.W. (1967). Phage Induced Fucosidases hydrolysing the exopolysaccharide of klebsiella aerogenes type 54 (A3(S1)). Biochemical Journal, 104, 278-285.

Sutherland, I.W. (1971). The Exopolysaccharides of klebsiella Serotype 2 strains as substrates for Phage-induced Polysaccharide Depolymerases. Journal of General Microbiology, 70, 331-338.

Sutherland, I.W. (1972). Bacterial Exopolysaccharides. Advances in Microbial Physiology, 8, 143-213.

Suzuki, S., Saito, H., Yamagata, T., Anno, K. Seno, N., Kawai, Y., and Furulashi, T. (1968). Formation of three Types of Disulphated Disaccharides from Chrondroitin Sulphates by Chondroitinase Digestion. Journal of Biological Chemistry, 243, 1543-1550.

Thurow, H., Niemann, H., and Strim, S. (1975). Bacteriophage-borne Enzymes in Carbohydrate Chemistry. Carbohydrate Research, 41, 257-271.

Torriani, A. and Pappenheimer, A.M. (1962). Inducible Polysaccharide Depolymerases of Bacillus palustris. Journal of Biological Chemistry, 237, 3-13.

Trevelyan, W.E., Procter, D.P. and Harrison, J.S. (1950). Detection of Sugars on Paper chromatograms. Nature, 166, 444-445.

Tsujino, I. and Saito, T. (1961). A New Unsaturated Uronide Isolated from Alginase Hydrolysate. Nature, 192, 970-971.

Vincent, D.I. (1960). Oligosaccharides from Alginic Acid. Chemistry and Industry, 1109-1110.

Voragen, A.G.J. (1972). Characterization of Pectin Lyases on Pectins and Methyl Oligogalacturonates. Agricultural Research Reports 780.

Wallenfels, K., Bechtler, G., Kuhn, R., Trischmann, H. and Egge, H. (1963). Premethylation of Oligomeric and polymeric carbohydrates and quantitative analysis of the cleavage products. Angewandte Chemie, International Edition, 2, 515-523.

Warren, L. (1960). Thiobarbituric acid spray reagent for deoxy sugars and sialic acids. Nature, 186, 237.

Waskman, S.A., Carey, C.L. and Allen, M.C. (1934). Bacteria decomposing alginic acid. Journal of Bacteriology, 28, 213-220.

Wasserman, A. (1949). Cation Adsorption by Brown Algae: the Mode of Occurrence of Alginic Acid. Annals of Botany 13, 79-88.

Weber, K. and Osborn, M. (1969). The Reliability of Molecular Weight Determinations by Dodecyl-sulphate-Polyacrylamide Gel Electrophoresis. Journal of Biological Chemistry, 244, 4406-4412.

Weissbach, A. and Hurwitz, J. (1958). The formation of 2-keto-3-deoxyheptonic Acid in Extracts of Escherichia coli B. Journal of Biological Chemistry, 234, 705-709.

Weissman, B. and Meyer, K. (1954). The structure of hyalobiuronic acid and hyaluronic acid from umbilical cord. Journal of American Chemical Society, 76, 1753-1757.

Wilkinson, J.F. (1958). The Extracellular Polysaccharides of Bacteria. Bacteriological Reviews, 22, 46-73.

Williams, R.J. and Govan, J.R.W. (1973). Pyocine Typing of Mucoid Strains of Pseudomonas aeruginosa isolated from Children with Cystic Fibrosis. Journal of Medical Microbiology, 6, 409-412.

Yamagata, T., Saito, H., Habuchi, O. and Suzuki, S. (1968). Purification and Properties of Bacterial Chondroitinases and Chondrosulphatases. Journal of Biological Chemistry, 243, 1523-1535.