MICROBIAL DEGRADATION OF AGAR:

THE ENZYMIC HYDROLYSIS OF NEOAGAROTETRAOSE

The purified extracellular agarases produced by marine microorganisms hydrolyze the β-1,4 galactosidic linkages of agarose to yield a homologous series of neoagarosaccharides. A predominant end product after complete enzymic hydrolysis of agarose is the tetrasaccharide, neoagarotetraose, which is resistant to further degradation by agarase. This project involved the isolation of a β-tetrasaccharidase from Pseudomonas atlantica, which hydrolyzes the β-1,4 linkage of neoagarotetraose.

The cell-bound β-tetrasaccharidase, selectively released by EDTA-Tris-NaCl treatment of P.atlantica cells, was free of α-disaccharidase activity. β-Tetrasaccharidase and agarase were separated by isoelectric focusing and were shown to possess distinct pI values. Determination of the isoelectric points facilitated separation of the enzymes on DEAE-Sephadex. Specificity studies were carried out on the crude and DEAE-purified β-tetrasaccharidase preparations.

The possible location of the β-tetrasaccharidase in the cell and some of the enzyme characteristics are discussed.

Linda J. Ross

Department of Microbiology

and Immunology

M.Sc. June, 1970

Spine Label:

Enzymic Hydrolysis of Neoagarotetraose

MICROBIAL DEGRADATION OF AGAR:

THE ENZYMIC HYDROLYSIS OF NEOAGAROTETRAOSE

by

Linda J. Ross

A thesis submitted to the
Faculty of Graduate Studies and Research
in partial fulfillment of the requirements
for the degree of Masters of Science

Department of Microbiology and Immunology
McGill University
Montreal

June, 1970

ACKNOWLEDGMENTS

The candidate wishes to extend sincere thanks to her research director, Dr. W. Yaphe, for his supervision and assistance throughout this investigation.

The candidate also wishes to thank Dr. R. W. Reed, Chairman of the Department of Microbiology and Immunology, for permission to carry out the work in this department.

Special thanks are extended to Dr. Michael Duckworth and to Mr. Kwan Young for their interest and help, and for providing the neoagarosaccharides used in this study. I am particularly grateful to Mr. Robert Murgita for his help with isoelectric focusing.

The research project was supported by a grant from the National Research Council of Canada. The candidate also acknowledges the tenure of a National Research Council Bursary, 1968-1969.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

The degradation of agar by microorganisms was first reported by Gran in 1902, and since that time many new species possessing this ability have been isolated and identified. Humm (1946) characterized several new species of Atlantic coast agar-digesters, including Pseudomonas atlantica. A strain of this organism was later isolated by Yaphe (1957) who investigated the enzyme activity. An extracellular enzyme produced by this bacterium caused hydrolysis of internal β-1,4 galactosidic linkages of the agarose molecule to yield a homologous series of oligosaccharides. A predominant end product, after complete enzymolysis of agarose, is the tetrasaccharide, neoagarotetraose. Specificity studies have shown that the purified agarase from P.atlantica has no activity on neoagarotetraose (Yaphe, 1966).

Preliminary evidence has demonstrated an enzyme system associated with the cells of P.atlantica, capable of degrading neoagarotetraose. Yaphe (1966) reported the hydrolysis of neoagarotetraose by an intracellular enzyme preparation from P.atlantica, and suggested the presence of an enzyme which can hydrolyse the β-1,4 linkage of the tetrasaccharide.

The purpose of this investigation was to isolate the β-tetrasaccharidase from cells of P.atlantica and deduce a scheme for the purification of this enzyme. Studies to establish the specificity requirements of the β-tetrasaccharidase have been carried out in order to determine how the enzyme can be used to elucidate the fine structure of agar.

II. LITERATURE REVIEW

Algae are the predominant form of vegetation found in the sea. They encompass a great diversity of form, ranging from unicellular organisms to the giant seaweeds, each species characteristic to a particular habitat. By far the greater weight of marine algae consists of the unicellular forms which are free-floating and are found in the upper layers of all seas. The larger species are generally attached to rocks and are found only in the continental shelf areas. It is this type of algae which is of economic importance and has been used for centuries either as human food, as food for animals, as manure, or as a source of chemicals.

The extensive use of algae and algal products has led to investigation of their structure and composition. The general nature of the main polysaccharide constituents of common marine algae has been known for many years, but it is only recently that the fine structure of algal components has been elucidated.

Classification of algae has been based primarily on their morphology; however, subdivision has been aided by the type of pigmentation present. All marine algae contain chlorophyll a, but its colour is masked by the strong pigments typical of the various classes. The Phaeophyceae (brown seaweeds), Chlorophyceae (green seaweeds), and the Rhodophyceae (red seaweeds) are three of the major classes of algae. Each class is also characterized by particular types of polysaccharide (Percival and McDowell, 1967), although the fine structure varies from species to species. This thesis is concerned only with the polysaccharides of the Rhodophyceae, and the mechanism of their degradation by microorganisms.

A. POLYSACCHARIDES OF THE RHODOPHYCEAE

Polysaccharides of the Rhodophyceae are typified by galactan polymers of alternating α-1,3 and β-1,4 glycosidic bonds. These polysaccharides contain varying proportions of D- and L-galactose, 3,6-anhydro-D- and L-galactose, methyl ethers, and ester sulfates. Various combinations of these residues in the alternating galactan chain have been characterized, demonstrating the wide spectrum of polysaccharides produced by these algae. It has been suggested that modifications of the basic structure are appropriate for a particular algal species growing in a particular environment (Anderson, Dolan and Rees, 1965).

Subdivision of the polysaccharides of the Rhodophyceae is in fairly good agreement with their structural modifications. Agar, porphyran, and carrageenan are the three main groups and each is characterized by an alternating structure based on certain variations of a basic repeating unit.

1. Agar

The source of agar is a group of red algae, classed together as agarophytes (Tseng, 1945), of which Gracilaria and Gelidium are common genera. Agar is extracted from these seaweeds by boiling and is separated by filtration. The filtrate sets to a gel on cooling and can be purified to some extent from soluble impurities by freezing and thawing.

Araki's characterization of agar established two major components, agarose and agaropectin. The original separation was achieved by acetylation of agar, followed by fractionation in chloroform into soluble agarose acetate and insoluble agaropectin acetate (Araki, 1937). The agarose component, as prepared by Araki, was essentially neutral and had the greater

gelling capability. This polysaccharide was shown to be a galactan polymer, composed of alternating residues of 3-linked β-D-galactopyranose and 4-linked 3,6-anhydro-α-L-galactopyranose (Araki, 1956). The disaccharide, agarobiose, is considered to be the basic repeating unit, Figure 1.

Agaropectin, the charged polysaccharide complex of agar, has the same basic structure as agarose, with considerable masking of the repeating sequence by charged groups. The 3,6-anhydro-L-galactose residues can be replaced by sulfated galactoses, Figure 2, (Araki, 1966), and D-galactose residues can be replaced by the pyruvic acid ketal, 4,6-O-(1-carboxyethylidene)-D-galactose, Figure 3, (Hirase, 1957). In both agarose and agaropectin, some of the D-galactose residues can also be replaced with 6-O-methyl-D-galactose, Figure 4, (Hirase and Araki, 1961). The extent of masking varies with the season (Rees and Conway, 1962), and from species to species, and the relative proportions of agarose and agaropectin fluctuate accordingly (Araki, 1966).

Modification or masking of the basic repeating structure is known to alter the physical properties of the agar gel. The physical characteristics of agarose and agaropectin fractions have recently been investigated by Hickson and Polson (1968) who have reported evidence for helical coiling of agarose molecules. Their observation is in agreement with Rees' hypothesis that the gelling property of agar and other polysaccharides extracted from algae is the result of a double helix conformation. Helix formation is thought to be due to the 3,6-anhydrogalactose residue, which is a unique sugar, restricted to gel-forming polysaccharides, Figure 5, (Rees, 1970). The shape of this molecule is such that three hydrogen atoms are arranged equatorially. These cause the formation of a

Figure 1.

Agarobiose

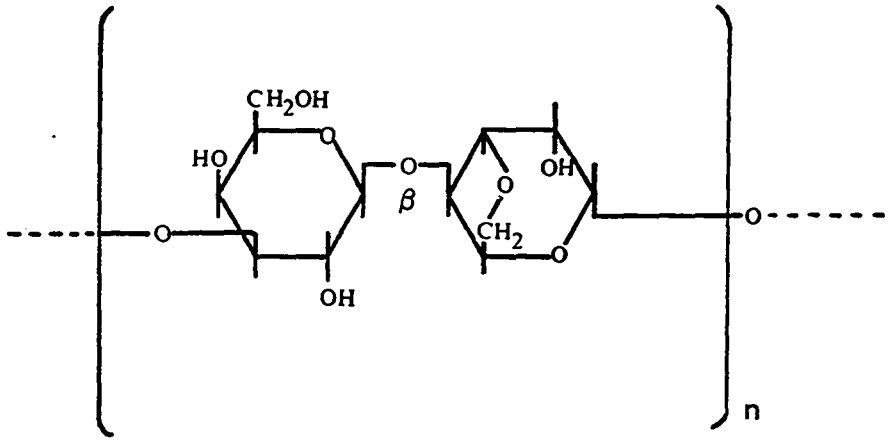


Figure 2.

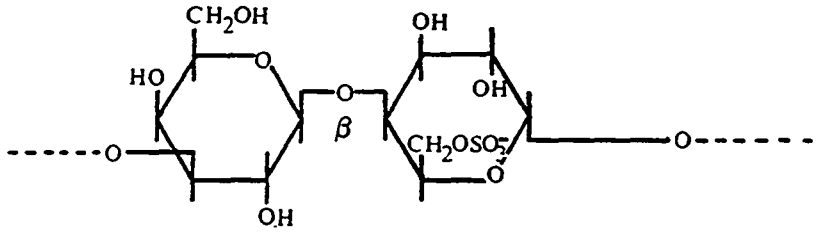


Figure 3.

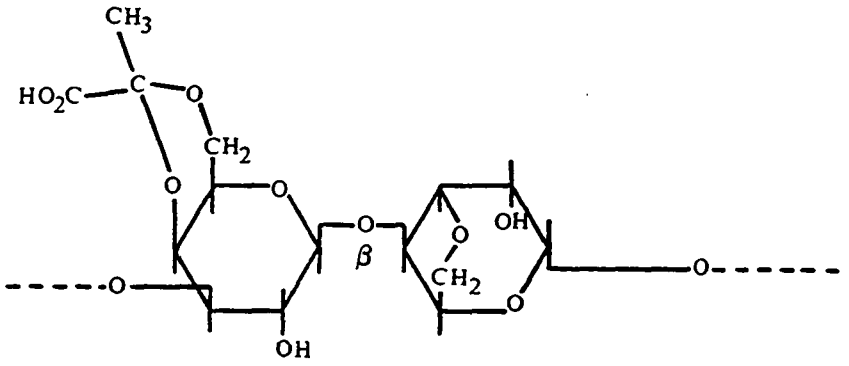


Figure 4.

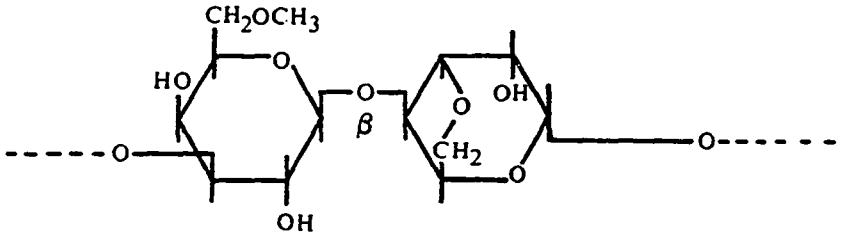


Figure 2 - 4. The structure of the sulfated, pyruvated, and 6-0-methylated derivatives of agarobiose.

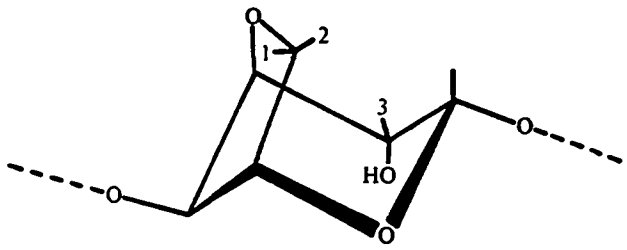


Figure 5. 3,6-anhydro-L-galactose

tight intramolecular helix held by hydrogen bonding; the interaction of many helical molecules results in gel formation. It is apparent that helix formation, and therefore gel strength, are progressively inhibited by an increasing number of charged groups and their interactions with cations.

In the agar family of polysaccharides, the ideal agarose structure, 3-linked β-D-galactopyranose and 4-linked 3,6-anhydro-α-L-galactopyranose, is masked to a variable degree with charged groups. Charged groups, namely ester sulfates and the pyruvic acid ketal, are characteristic to the agaropectin fraction obtained from agar. With increasing concentration of these masking groups, the gelling capability of the polymer is decreased. This explains a current concept that agar consists of a wide spectrum of polysaccharides, which encompasses three extremes: completely neutral agarose, pyruvated agarose, and a sulfated polymer with no 3,6-anhydro-L-galactose (Duckworth and Yaphe, 1970[a]).

2. Porphyran

Porphyran-type polysaccharides, found in Porphyra species, have been shown to be structurally related to agarose. Porphyran consists of a masked repeating structure in which D-galactose can be replaced by 6-O-

methyl-D-galactose, and L-galactose 6-sulfate partially replaces the 3,6-anhydride residue (Turvey and Rees, 1961; Turvey and Williams, 1964). The methyl ethers are thought to be distributed randomly on half the D-galactose units, but the sulfate groups on L-galactose units tend to occur in blocks (Duckworth and Turvey, 1969b).

The essential similarity of porphyran to agarose was confirmed by Anderson and Rees (1965), and recent studies have indicated that occasional sequences identical to agarose can be found in the porphyran polymer (Turvey and Christison, 1967b). The structure of porphyran is represented in Figure 6, where some of the α-1,3 linked D-galactose residues contain a methyl ether group at C_6, and certain of the 3,6-anhydro-L-galactose units are present as L-galactose 6-sulfate.

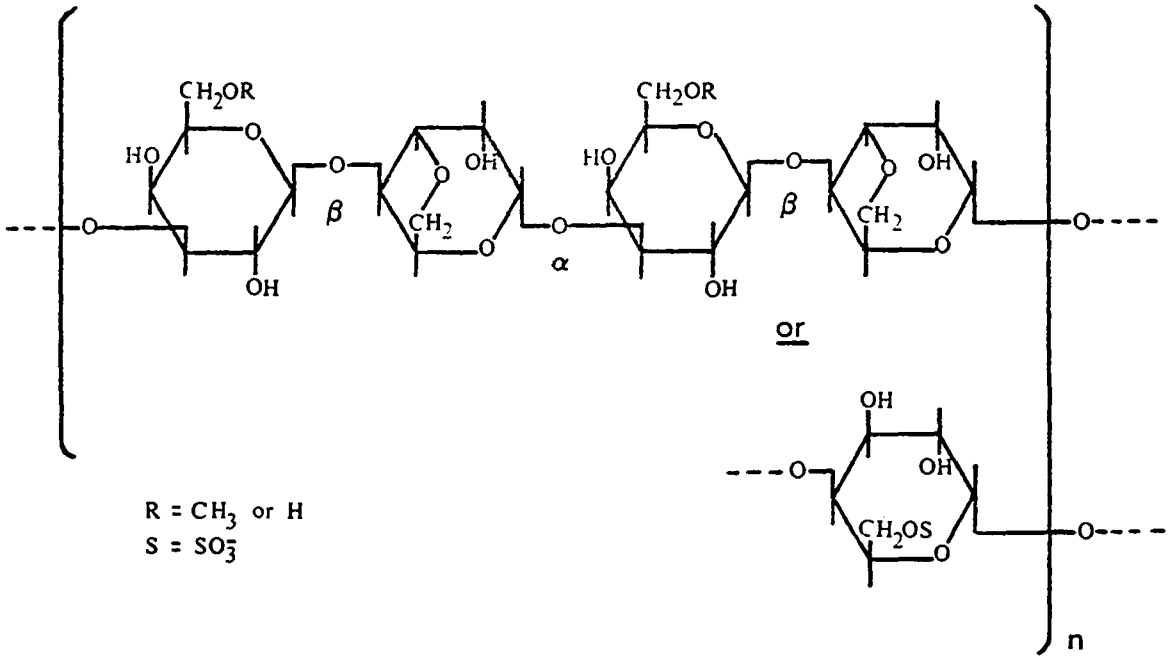


Figure 6. Structure of Porphyran

3. Carrageenan

Carrageenan-type polysaccharides, of which Chondrus crispus is a major source, are also similar to the agar family, being comprised of alternating α-1,3 and β-1,4 linked galactose residues. However, they are distinct from agar in that 3,6-anhydro-α-D-galactose takes the place of the 3,6-anhydro-α-L-sugar (Percival, 1954), and that they have a higher content of ester sulfate (Buchanan et al., 1943).

Like agar, carrageenan constitutes a family of polysaccharides which vary with the season and habitat of a particular species. Carrageenan from Chondrus crispus was originally fractionated into two major polysaccharides, Kappa- and Lambda-carrageenan, by precipitation of the gel-forming κ-carrageenan fraction with dilute potassium chloride (Smith and Cook, 1953). κ-carrageenan was found to be based on the disaccharide, carrabiose, which has the structure 4-O-β-D-galactopyranosyl (1→4) 3,6-anhydro-α-D-galactose, Figure 7, (O'Neill, 1955). Studies by Dolan (1965)

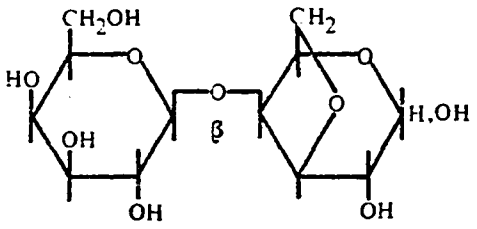


Figure 7. Carrabiose

have established that practically all the 3-linked units occur as D-galactose 4-sulfate. D-galactose 6-sulfate residues replace some 3,6-anhydro-D-galactose, and the 4-linked residues of both types are partly 2-sulfated.

Carrageenan is now believed to comprise four idealized polysaccharide components: Kappa, Lambda, Mu, and Iota carrageenan. Like agar and porphyran, all fractions are based on an alternating sequence of α-1,3 and β-1,4 linked galactose units, and differ from one another only in their proportions of 3,6-anhydro-sugar and ester sulfate, and in the various sites of the sulfate esters.

B. AGAR-DEGRADING MICROORGANISMS

The first isolation of a microorganism capable of degrading agar was made by Gran in 1902, twenty years after the introduction of agar into bacteriological techniques. He reported that a marine bacterium, which he named Bacillus gelaticus, caused depressions in the agar medium, and altered the polymer so that it no longer gave a blue-black colour when flooded with iodine (Gran, 1902).

Since then, many new species of agar-digesters have been isolated from a variety of sources (Waksman and Bavendamm, 1931; Goresline, 1933; Araki and Arai, 1954; Swartz and Gordon, 1959). Angst (1929) reported the isolation of thirteen species of agar-degrading bacteria and placed them in a new genus, Agarbacterium. Stanier (1941), in a study of marine agar-digesting bacteria, found that the ability to hydrolyze this polysaccharide is widespread and encompasses several genera of microorganisms. For this reason, he felt that the physiological genus, Agarbacterium, was taxonomically unsound.

Humm (1946) conducted a survey of Atlantic coast agar-decomposers, isolating and identifying twenty species. He outlined the general

characteristics of the agar-digesters and proposed a key for their classification, concluding that they were not considered a natural group but were physiologically related. This point was reviewed by Yaphe (1963), and has been reinvestigated by Colwell and Gochnauer (1963) who carried out an extensive study on sixty bacterial cultures isolated from marine sources. A computer analysis of the results indicated that these strains should be classified as Pseudomonas and Vibrio types. They concluded that separate genera should not be formed to describe marine species on the basis of a single feature, such as the ability to utilize a specific polysaccharide.

The nutritional requirements of some agarolytic bacteria have recently been investigated (Girard et al., 1968). This work indicates that the nutrition of these organisms is "simple", in that many of them utilize single amino acids with galactose and that a variety of carbon compounds may replace galactose. Mitchell and Nevo (1965) have found that some marine agarolytic bacteria are able to degrade the capsular polysaccharides and cell walls of certain microorganisms. However, further studies are needed to evaluate the role of agar-decomposing bacteria in the marine environment.

C. THE LOCATION AND RELEASE OF ENZYMES PRODUCED BY MICROORGANISMS

It is important to know the cellular location of the enzymes involved in agar decomposition as well as their modes of action, in order to understand how agar is degraded and metabolized by bacteria. The degradation of agar to its monosaccharide components by Pseudomonas atlantica involves

three enzymes: agarase, and the two enzymes which hydrolyse the tetrasaccharide, neoagarotetraose, and the disaccharide, neoagarobiose (see Figure 8). The agarase is believed to be primarily an extracellular enzyme, although the activity is also associated with the cell. The enzymes responsible for tetrasaccharide and disaccharide degradation are known to be present in an intracellular extract of P.atlantica (Yaphe, 1966); however, their location in the cell is not known. It was of interest in this project to determine whether the β-tetrasaccharidase is a truly intracellular enzyme or whether it is located at the cell surface.

The localization of enzymes produced by microorganisms has been a matter of long-standing interest. Pollock (1962) has classified bacterial enzymes into two distinct groups: those that are fixed to the cell and those that are free in the medium around the cells. The first group can be subdivided into those enzymes that are confined within the permeability barrier, usually considered to be the cytoplasmic membrane, and those that are fixed to surface structures, located outside the barrier. Thus, a useful classification of enzymes, according to their location in, on, or around the cell would be: (1) cell-bound: a) truly intracellular, b) surface-bound; and (2) extracellular (Pollock, 1962).

It must be realized, however, that classification of enzymes in this manner is by no means absolute. Functionally similar enzymes are often cell-bound in one species and extracellular in another. For example, the cellulases of Cytophaga species are cell-bound (Stanier, 1942), while those of Cellulomonas are extracellular (Hammerstrom et al., 1955). Similarly, the agarases produced by Cytophaga species are thought to be cell-bound, while those of Pseudomonas and Vibrio species are probably extracellular

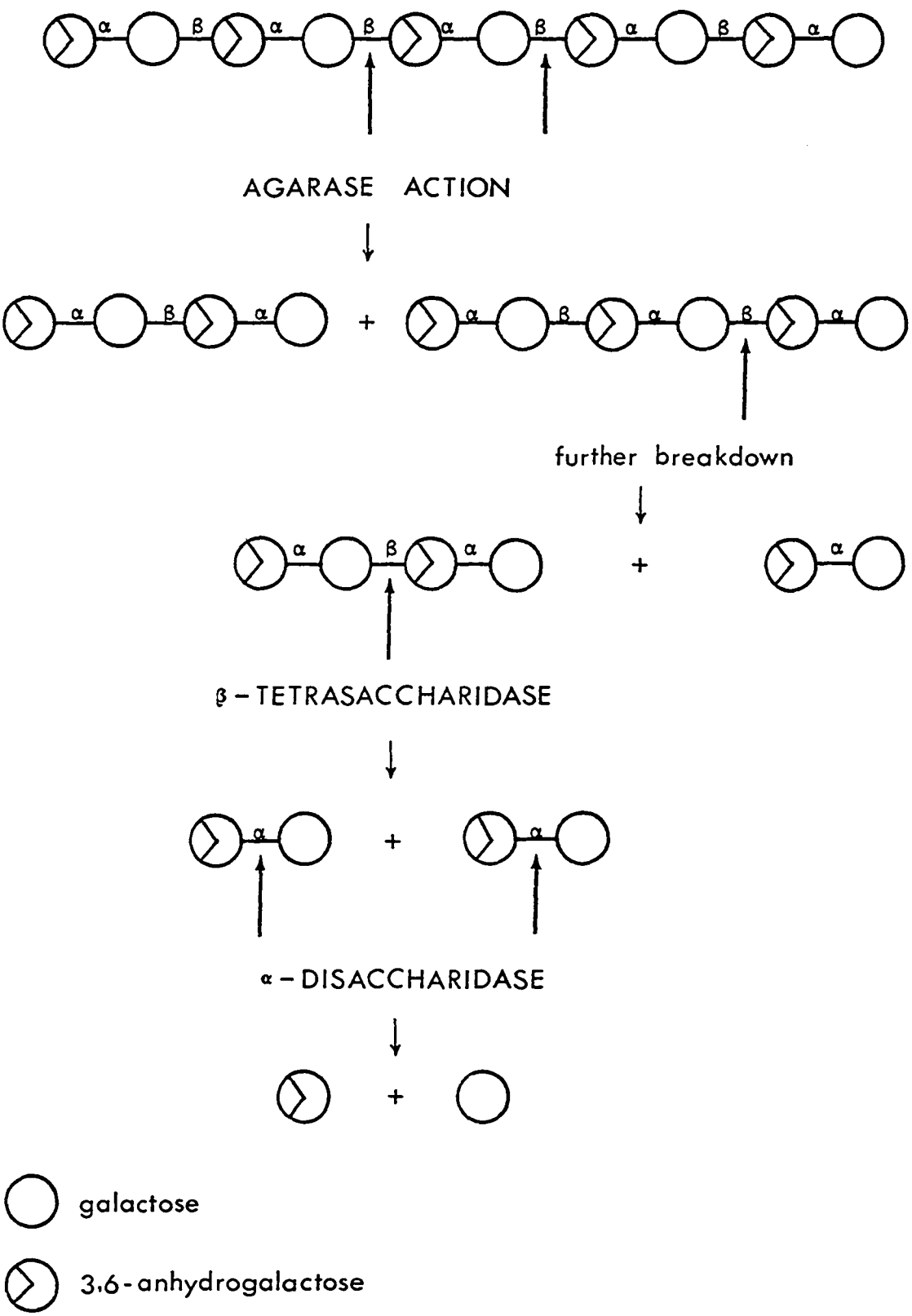


Figure 8. The Degradation of Agarose

(Pollock, 1962); however, the location of agarase enzymes has not been definitively established.

Even in the same bacterium, an enzyme may be partly cell-bound and partly extracellular. This is often a function of the age of the culture or the environmental conditions. For example, penicillinase is an enzyme which can be cell-bound or extracellular or a mixture of the two, depending on the species and the circumstances involved. The production of penicillinase by Staphylococcus aureus and the degree of extracellularity of the formed enzyme has been postulated to be under the control of a plasmid-associated gene (Novick, 1963). However, an alteration of the growth medium caused 98% of the penicillinase produced to be cell-bound (Coles and Gross, 1967a), whereas normally 40% is liberated as extracellular enzyme. These workers found that incubation of the cells with multivalent anions, both inorganic and organic, gave release of the enzyme. Generally, the more ionic groups per molecule, the greater was their effectiveness in stimulating release of penicillinase(Coles and Gross, 1967a, 1967b). Their results indicated that the enzyme is ionically bound to the cell wall and is liberated as an exoenzyme according to the environmental conditions.

The variation in enzyme location is most apparent on comparison of gram-negative and gram-positive bacteria. There is increasing evidence that a group of enzymes that are extracellular in gram-positive organisms are surface enzymes in gram-negative bacteria (Heppel, 1967; Cashel and Freese, 1964). This difference has been attributed to the structural differences in the cell walls of these two groups of cells. The release of exoenzymes from gram-positive cells has been examined in depth by

Lampen (1965). He has shown that penicillinase, alkaline phosphatase, and nuclease are excreted at the cytoplasmic membrane and are released into the medium without interference by the cell wall, in Bacillus licheniformis (Lampen, 1967). In some species of gram-positive organisms there is covalent binding of these enzymes to the cytoplasmic membrane (Chesbro and Lampen, 1968); however, there is no barrier to the passage of large molecules through the cell wall of gram-positive organisms.

On the other hand, Mitchell (1961) has conceived the cell wall of gram-negative bacteria as a "molecular sieve", preventing the loss of enzymes which are released through the cytoplasmic membrane. He coined the term "periplasm" to describe the area of the cell envelope between the plasma membrane and the molecular sieve layer in which certain enzymes can be retained. A number of hydrolytic enzymes involved in phosphate, nucleotide, and sugar degradation are believed to be confined in the periplasm (Heppel, 1967; Costerton, 1970). These enzymes can be released from Escherichia coli by sphaeroplast formation (Neu and Heppel, 1964, 1965), and by osmotic shock treatment (Neu and Heppel, 1965; Nossal and Heppel, 1966; Neu, 1967). The latter procedure involves exposure of the cells to EDTA in 0.5M sucrose, followed by a sudden osmotic transition to cold, dilute $MgCl_2$. Enzyme release by osmotic shock treatment is not peculiar to E.coli, as Neu and Chou (1967) have shown release of similar surface enzymes from a number of species of enteric bacteria.

Recent work has provided definite evidence of the periplasmic location of certain enzymes of gram-negative bacteria. Alkaline phosphatase, an exoenzyme in most gram-positive bacteria (Chesbro and Lampen, 1968), is believed to be localized in the periplasmic space of gram-negative cells

(Kushnarov and Smirnova, 1966; Brockman and Heppel, 1968). Recent evidence by Torriani (1968) and Schlesinger (1968) suggests that the inactive subunits of alkaline phosphatase are synthesized in the cell cytoplasm and diffuse through the cell membrane to the periplasmic space where subsequent dimerization to active enzyme occurs.

Other enzymes thought to be located in the periplasmic space include glucose-6-phosphatase (Mitchell, 1961), acid hexose phosphatase, cyclic phosphodiesterase (Brockman and Heppel, 1968), RNase, DNase (Neu and Heppel, 1965), and 5'-nucleotidase (Neu, 1967a, 1967b). These enzymes are released from the cell by osmotic shock treatment. However, recent work by Nisonson et al. (1969) has shown that 5'-nucleotidase and 3'-nucleotidase are located at the cell surface and enter the periplasmic space when exposed to 0.5M sucrose containing 0.12M Tris buffer and 10^{-3}M EDTA. Resuspension of the cells in distilled water or dilute $MgCl_2$ releases the enzymes. Treatment with EDTA and Tris, in the absence of sucrose, did not allow penetration of the enzymes into the periplasm and did not cause their release (Nisonson et al., 1969). Similarly, treatment with EDTA in Tris buffer does not result in release of alkaline phosphatase or other surface enzymes from the gram-negative organisms studied (Neu and Chou, 1967; Leive, 1965a).

EDTA is thought to serve a double purpose in the osmotic shock procedure. Anraku and Heppel (1967) have shown that the presence of EDTA enables bacteria to withstand osmotic shock more successfully. They have reported reduced leakage of intracellular β-galactosidase, protein, and 260-absorbing material into the shock fluid when EDTA is present. However, the primary function of EDTA in this system is as a chelating agent.

Repaske (1958) reported that EDTA causes removal of metal ions from the cell wall of gram-negative organisms, thus potentiating the action of lysozyme on these cells. Experiments have indicated that EDTA treatment of E.coli causes a non-specific increase in permeability (Leive, 1965a), accompanied by loss of 30% to 50% of the lipopolysaccharide content of the cell wall (Leive, 1965b). Brief treatment with EDTA does not affect cell viability (Leive, 1965a; Gray and Wilkinson, 1965a). Neu, Ashman, and Price (1967) have reported a two-fold effect of EDTA on E.coli: initially, an increase in plasma membrane permeability with loss of lipopolysaccharide and release of the acid-soluble nucleotide pool; this was followed by actual breakdown of ribosomal RNA.

The presence of Tris in the experimental system has been shown to increase the chelating effect. Goldschmidt and Wyss (1967) have stated that Tris acts as a donor group to form a homologue with EDTA, resulting in a more powerful chelating agent than EDTA alone. This effect has been confirmed by Asbell and Eagon (1966) and by Neu and coworkers (1967). Voss (1967) has postulated that chelating agents and organic cations, such as Tris, act together to break the salt bridges which hold together the lipoprotein and lipopolysaccharide polymers of the cell wall. Neu (1969) has found that Tris could not be replaced by other amine buffers in osmotic shock treatment of E.coli. The hypothesis has been made that EDTA, in conjunction with Tris buffer, causes release of lipopolysaccharide material (Anraku and Heppel, 1967), and exposes the area just beneath the cell surface to osmotic changes. The sudden reduction of external osmotic pressure is accompanied by extrusion of molecules previously associated with the cell surface (Leive, 1968).

The effect of osmotic shock treatment on species of Pseudomonas has not as yet been reported. It was noted, however, that P.aeruginosa was lysed to an appreciable extent by EDTA alone, in comparison to E.coli which required both EDTA and lysozyme for lysis (Repaske, 1958). Gray and Wilkinson (1965a) and Wilkinson (1967) have shown that most pseudomonads are highly sensitive to EDTA treatment, with up to 100% loss in viability. There is conclusive evidence that EDTA removes essential multivalent cations from the cell wall of EDTA-sensitive gram negative bacteria (Eagon and Carson, 1965), and solubilizes the lipopolysaccharide component (Gray and Wilkinson, 1965b; Cox and Eagon, 1968), which is thought to be cross-linked via divalent cations. This treatment causes the formation of osmotically fragile rods, "osmoplasts", which can be restored to osmotic stability by the addition of multivalent cations (Asbell and Eagon, 1966a). Monovalent cations, such as sodium, are not able to restore osmoplasts. However, if EDTA treatment is carried out in the presence of 0.5M NaCl instead of sucrose, the sodium ions replace the divalent cations of the cell wall (Asbell and Eagon, 1966b). Since sodium does not form cross-linkages, a weakened cell wall, with increased permeability is formed (Costerton et al.,1967). One would expect that enzymes free in the cell wall space would be released by this treatment.

Costerton and coworkers (1967) have reported that the outer layers of the cell envelope of a marine pseudomonad are removed by washing the cells in 0.5M NaCl followed by suspension in 0.5M sucrose. These forms, termed mureinoplasts, retained a rigid mucopeptide structure, but had lost the outer double tract (De Voe et al., 1970). The alkaline phosphatase is released from the cells on formation of mureinoplasts (R.A. MacLeod,

personal communication). Alkaline phosphatase has been localized in the periplasmic space of Pseudomonas aeruginosa by electron microscopy, and can be completely removed by plasmolysis of the cells in 0.2M Mg^{++} (J. Ingram, personal communication).

D. THE USE OF ENZYMES IN THE DETERMINATION OF STRUCTURE

Hydrolytic enzymes have played a valuable part in the elucidation of the structure of a number of algal polysaccharides. Their use has been of particular value in studies of the galactan sulfates of the Rhodophyceae, because the high incidence of ester sulfate groups interferes with methylation and acetylation techniques, and polysaccharides containing uronic acid residues must be converted to a neutral polymer before they can be subjected to chemical analysis.

Enzyme hydrolysis is often more specific than chemical analysis. Where several types of linkages are present, for example, α-1,3 and β-1,4 linked galactan residues in the Rhodophyceae, certain bacterial enzymes will preferentially cleave the β-1,4 bonds and leave the α-1,3 linkages intact; whereas acid hydrolysis tends to cleave the α-1,3 links. However, suitable degradative enzymes for many of the algal polysaccharides have yet to be found.

The extent of enzyme hydrolysis can be measured by (a) the decrease in viscosity, (b) the change in optical rotation, (c) the increase in reducing power, and (d) chromatographic analysis of the products. The mode of action of the enzyme may be either random cleavage of the polymer or stepwise hydrolysis along the polysaccharide chain. A rapid fall in

viscosity followed by a small increase in reducing power is indicative of random cleavage, while a slow decrease in viscosity with a rapid increase in reducing power and the early appearance of mono- and di-saccharides is proof of endwise fission. Random cleavage of the polymer leads to the formation of a series of oligosaccharides.

Although agar-digestion by bacteria was first observed in 1902, only recently have the enzymes responsible for the degradation of agar and other algal polysaccharides been investigated. Ishimatsu et al. (1954) were the first workers to isolate such an enzyme and use it in studies on the structure of agar. Araki and Arai (1956), using an extracellular agarase from Pseudomonas kyotoensis, isolated a homologous series of four oligosaccharides from the enzyme hydrolysate of agar. These were separated by ethanol elution from a charcoal column and were found to differ from the oligosaccharides released by chemical hydrolysis of agar.

The starting sugar was a disaccharide, which Araki named neoagarobiose (Araki and Arai, 1956), since it was an isomer of agarobiose which had been isolated previously by acid hydrolysis (Araki, 1944). The structure of neoagarobiose was found to be O-3,6-anhydro-α-L-galacto-pyranosyl (1→3)-D-galactose, Figure 9. The disaccharide made up 28% of

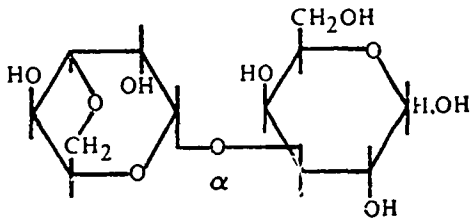


Figure 9. Neoagarobiose

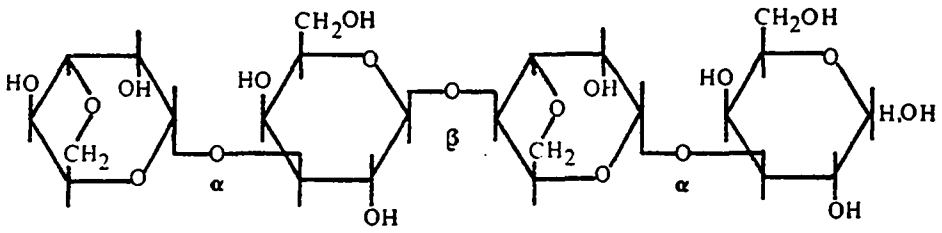


Figure 10. Neoagarotetraose

the total hydrolysate, with the predominant quantity, 40%, being the tetrasaccharide. The structure of this sugar was shown to be two neoagarobiose units linked through a β-1,4 bond; that is, 0-3,6-anhydro-α-L-galactopyranosyl (1→3)-0-β-D-galactopyranosyl (1→4)-0-3,6-anhydro-α-L-galactopyranosyl (1→3)-D-galactose, Figure 10.

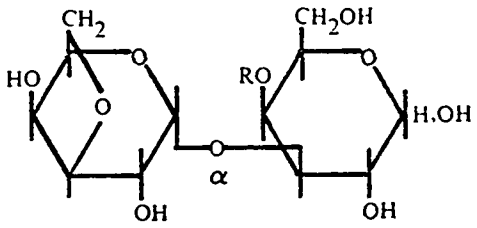
Araki established a scheme for the nomenclature of these oligosaccharides; the prefix "agaro" was given to those sugars with D-galactopyranose at the non-reducing end, and "neoagaro" to those with 3,6-anhydro-L-galactopyranose at the non-reducing end. Therfore, the tetrasaccharide was named neoagarotetraose (Araki and Arai, 1957), and higher homologues in the hydrolysate were the neoagarohexa- and octa-saccharides. Neoagarotetraose and neoagarobiose will be referred to in the text as tetrasaccharide and disaccharide, and the enzymes which hydrolyze these sugars as β-tetrasaccharidase and α-disaccharidase.

Araki proposed that the agarase enzyme was specific for the β-1,4 glycosidic linkage, causing random cleavage of agarose to yield high molecular weight homologues of neoagaro-oligosaccharides, which, in turn, are similarly hydrolyzed to form neoagarobiose as the lowest fragment (Araki and Arai, 1957).

Yaphe, in 1957, reported the isolation of an agarase from the marine

microorganism, Pseudomonas atlantica. The agarase was shown to hydrolyze the β-1,4 linkages of agarose to yield the same series of oligosaccharides as found by Araki and coworkers. It was suggested that agarase activity is specific for the neoagarobiose unit, that is, 3,6-anhydro-α-L-galacto-pyranosyl (1→3)-D-galactose (Yaphe, 1966). Degradation of agaropectin by this enzyme releases a series of both neutral and charged oligosaccharides (Duckworth and Yaphe, 1970a).

A κ-carrageenase was isolated from another marine bacterium, P.carrageenovora (Yaphe and Baxter, 1955). Enzyme activity of the agarase and κ-carrageenase were specific for the agar-type polysaccharides and κ-carrageenan respectively. Recent studies with the κ-carrageenase have led to the separation of a similar series of oligosaccharides based on the disaccharide, neocarrabiose sulfate (Figure 11, R=SO_3), from the enzyme hydrolysate of κ-carrageenan (Weigl et al., 1966; Weigl and Yaphe, 1966a). As with the agarase, enzyme hydrolysis occurs at the β-1,4 linkage. Neocarrabiose sulfate could then be converted into neocarrabiose (Figure 11, R=H), by the action of an enzyme from the cells of P.carrageenovora (Weigl and Yaphe, 1966b).



R = SO_3 or H

Figure 11. Neocarrabiose sulfate (R=SO_3); Neocarrabiose (R=H)

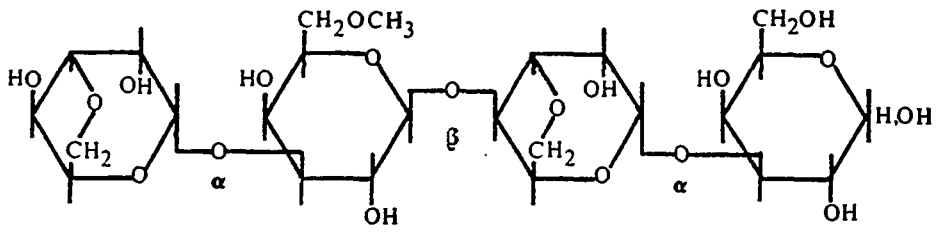


Figure 12. 6^3-O-methyl Neoagarotetraose

Yaphe has made use of the differing specificities of the agarase and κ-carrageenase in order to identify agar and κ-carrageenan type polysaccharides in the mucilages of various members of the Rhodophyceae (Yaphe, 1959; Hong et al., 1970).

An enzyme extract from a Cytophaga species, which was grown on porphyran as the sole carbon source, has been shown to be active against porphyran and agarose, but had little or no effect on carrageenan and structurally similar galactans (Christison and Turvey, 1967a, 1967b). Prolonged action of the enzyme preparation on porphyran released D-galactose, 6-O-methyl-D-galactose, neoagarobiose, and higher homologues including sulfated oligosaccharides and a methylated tetrasaccharide, 6^3-O-methyl neoagarotetraose, represented in Figure 12. The high production of reducing end groups compared to the viscosity change led Christison and Turvey to believe that several enzymes were present in the cell sonicate, with the major activity being agarase.

The agarase from the Cytophaga species has been isolated and purified from the extracellular culture filtrate (Duckworth and Turvey, 1969a). This enzyme is similar to those reported previously (Araki and Arai, 1956; Yaphe, 1957), in that it is specific for the β-D-galactosidic linkage in

agarose and the products of its action on agarose are the series of neoagarosaccharides. Masking of the agarose structure resulted in a decrease in enzyme activity: the enzyme could not hydrolyze β-1,4 linkages near an L-galactose 6-sulfate unit, and the presence of 6-O-methyl-D-galactose residues decreased the rate of hydrolysis by one-fifth (Duckworth and Turvey, 1969b).

Enzymic hydrolysis of agarose resulted in the accumulation of neoagarotetraose in the hydrolysate, and tetrasaccharides were again found to predominate among the small neutral oligosaccharides released on degradation of porphyran (Duckworth and Turvey, 1969b). Araki and Arai (1956) also reported that the tetrasaccharide was the major product of agarose degradation. Yaphe (1966) reported that preliminary hydrolysis of agarose released high molecular weight neoagarosaccharides which were completely degraded to neoagarotetraose and neoagarobiose on addition of fresh enzyme. Specificity studies have shown that purified agarase enzymes have no activity on either neoagarotetraose or neoagarobiose (Yaphe, 1966; Duckworth and Turvey, 1969c). Agarases have been shown to preferentially attack internal β-D-galactosidic linkages (Duckworth and Turvey, 1969c; Young and Yaphe, unpublished results). This explains the predominance of tetrasaccharides in hydrolysates of agar and porphyran, and the inability of the agarase to degrade tetrasaccharides accounts for their accumulation.

It should be emphasized that the presence of disaccharide in the hydrolysate is the result of degradation of neoagarosaccharides larger than the tetrasaccharide. A scheme for the breakdown of agarose by the purified agarase has been outlined in Figure 8, page 12. The pattern

does not agree completely with Araki's original proposal for the mechanism of degradation of agar. He suggested that all neoagarosaccharides were hydrolyzed to yield neoagarobiose as the lowest fragment (Araki and Arai, 1957). This hypothesis was based on his observation that neoagarotetraose was hydrolyzed by the extracellular enzyme to yield the disaccharide. However, it is quite possible that the unpurified agarase, prepared from the extracellular fluid of a one week old culture, contained more than one enzyme, and may well have been contaminated with small amounts of β-tetrasaccharidase.

A mixture of neoagarotetraose and neoagarobiose was degraded to galactose, 3,6-anhydrogalactose, and neoagarobiose on incubation with intracellular enzymes prepared by sonication of Pseudomonas atlantica (Yaphe, 1966). This suggests the existence of two enzymes, one which can hydrolyze the β-1,4 linkage of the tetrasaccharide, and another to cleave the α-1,3 linkage of the disaccharide, Figure 13. A cell extract prepared from a Cytophaga species was also reported to cause release of mono- and di-saccharides from agar and porphyran (Turvey and Christison, 1967a). Although these observations clearly suggest the presence of an α- and β-enzyme, evidence for a specific activity has not yet been reported. No attempt has been made to isolate or purify these enzymes.

This project was initiated to isolate and purify the enzyme from Pseudomonas atlantica capable of hydrolyzing the β-1,4 linkage of neoagarotetraose to produce two molecules of neoagarobiose. The specificity requirements for the enzyme can be established by studying the degradation of model oligosaccharides. The importance of a β-tetrasaccharidase of known specificity is emphasized when we consider that

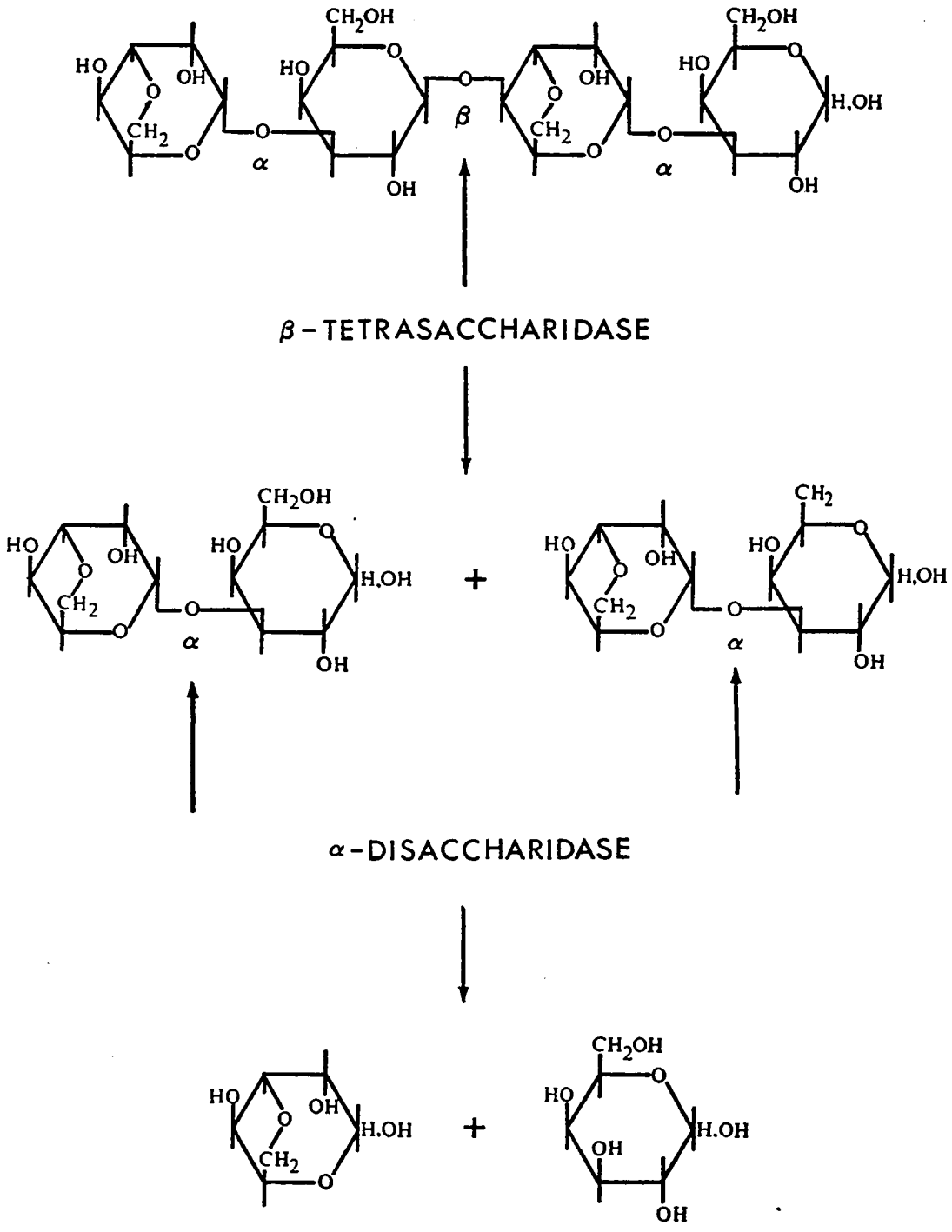


Figure 13. The enzymic degradation of neoagarotetraose and neoagarobiose.

degradation of algal polysaccharides by extracellular agarases usually results in the predominance of tetrasaccharides in the hydrolysate. Once the mode of action of the enzyme has been established, the β-tetrasaccharidase could be used in the determination of the fine structure of unknown oligosaccharides obtained by the enzymic hydrolysis of agars with varying degrees of masking.

III. MATERIALS AND METHODS

A. THE MICROORGANISM

The bacterium used in these studies was a marine microorganism, Pseudomonas atlantica 549, which was originally isolated from a species of Rhodymenia palmata, collected at Point Pleasant Park, Halifax, Nova Scotia. The cultural and morphological characteristics of the bacterium have been described (Yaphe, 1957).

The culture was started from a lyophilized preparation by serial transfer in H-1 mineral salts medium (see appendix) containing 0.1% Bacto-agar. Medium 2216E (Oppenheimer and Zobell, 1953), with 1.5% Bacto-agar (see appendix), was used for maintenance of P.atlantica.

B. PRODUCTION OF EXTRACELLULAR AGARASE

1. Enzyme Preparation

Flasks containing 200 ml of H-1 medium (0.1% Bacto-agar) were inoculated with 1 ml of a 24 hour culture of P.atlantica and incubated on a rotary shaker at 25°. After 16 hours incubation, the residual polysaccharide which had not been degraded by the bacteria, was removed by centrifugation at 500 g for 10 minutes at room temperature. The supernatant solution, containing extracellular enzyme and bacterial cells, was subjected to a second centrifugation at 10,000 g for 20 minutes at 4° to remove the cells.

All further experiments were carried out at temperatures between

0 and 4°. Analar ammonium sulfate was added to the cell-free preparation to a final concentration of 70% saturation to precipitate the crude agarase enzyme. After the slow addition and dissolution of the salt, the solution was stirred for 30 minutes to complete the precipitation. The precipitated enzyme was removed by centrifugation at 10,000 g for 20 minutes. The precipitate was dissolved in a small volume of 0.1M phosphate buffer (pH 7) and dialyzed against dilute buffer, 0.01M. The enzyme solution was stored at -20°.

2. Measurement of Agarase Activity - Iodine Method

An equal volume of the enzyme preparation was added to 1% agarose solution in 0.01M phosphate buffer and the mixture was incubated at 42°. At various time intervals, one drop of the enzymic hydrolysate was mixed with one drop of a solution of I-KI (see appendix) in a spot plate. The absence of a blue-black colour indicated partial hydrolysis of agarose, and the time at which the colour change occurred marked the achroic point.

C. PREPARATION OF OLIGOSACCHARIDES BY HYDROLYSIS OF AGAR

1. Preparation of an Agarose Hydrolysate

Agarose was prepared from Bacto-agar by a modification of the polyethylene glycol method (Russel et al., 1964), and was obtained from Dr. W. Yaphe, this department.

Crude agarase was added to a 1% agarose solution in 0.01M phosphate buffer and the mixture was incubated at 42°. The reaction was followed by the iodine test and by cellulose thin layer chromatography. When hydrolysis had reached the desired level, the enzyme action was stopped

by heating the mixture in a boiling water bath for 15 minutes. The agarose hydrolysate was added to 6 volumes of ethanol to precipitate any residual polysaccharide material, and the precipitate was removed by filtration. The filtrate was evaporated to dryness using a Büchi rotary evaporator (Fisher Scientific Co., Montreal, Quebec). The sugars were redissolved in distilled water and analyzed by cellulose thin layer chromatography.

2. Neoagarobiose Oligosaccharides

Neoagarobiose and neoagarotetraose were obtained from Dr. W. Yaphe, and higher molecular weight oligosaccharides, neoagarohexa-, octa-, and deca-ose, were obtained from Mr. K. Young, this department. These sugars were prepared by gel-filtration of an agarose hydrolysate on Sephadex G-25 and by preparative cellulose thin layer chromatography.

3. 6^3-O-methyl Neoagarotetraose

6^3-O-methyl neoagarotetraose was obtained from Dr. M. Duckworth, this department. This sugar was isolated from an enzymic hydrolysate of porphyran by the method of Duckworth and Turvey (1969b), and was separated in a pure form from neoagarotetraose by preparative cellulose thin layer chromatography.

D. CELLULOSE THIN LAYER CHROMATOGRAPHY

Cellulose thin layer chromatography was used throughout this investigation to determine the purity of oligosaccharide samples and to follow the enzymic hydrolysis of these oligosaccharides.

1. Chromatographic Procedure

Thin layers were prepared as follows: Microcrystalline cellulose

(Camag D.S.O.,15 g) was mixed with 85 ml of distilled water and blended in a Virtis "45" disintegrator (Virtis Research Equipment, Gardiner, New York) for one minute at medium speed. The slurry was spread as a layer 0.25 mm thick over the surface of glass plates 5 x 20 and 20 x 20 cm, using a Desaga spreader.

For the formation of even layers, the plates must be extremely clean, and it was found to be advantageous to soak them in acid or saturated sodium carbonate before washing and spreading. After spreading the layer, the plates were left to dry at room temperature.

The chromatographic procedure was essentially a partition chromatographic separation by an ascending technique, using the solvent systems of Duckworth and Yaphe (1970b). All solvents used were reagent grade from Fisher Scientific Co., Limited, Montreal, Quebec.

Solvent (a) Butan-1-ol - Ethanol - Water, 3:2:2 (v/v)
This solvent gives excellent separation of neutral oligosaccharides obtained from the enzymic hydrolysis of agarose. Only one development is necessary.

Solvent (b) Butan-1-ol - Pyridine - Water, 2:1:1 (v/v)
This solvent has been used for the separation of partially 6-O-methylated oligosaccharides from the agarose series of neoagarosaccharides. Two developments in this solvent are needed for complete separation of sugars.

Solvent (c) Butan-1-ol - Trichloroethylene - Ethanol, 3:1:1, (v/v)
This solvent system has been used by Berger and Agate (1969) to separate large quantities of sucrose from other sugars, and was used in this investigation for assays of fractions obtained from isoelectric focusing of the enzyme. Five developments in this solvent were needed to separate

neoagarobiose, sucrose, and neoagarotetraose.

2. Indicator Reagents

After solvent development was completed, the plates were dried and then sprayed. Two indicator reagents were used in cellulose thin layer chromatography.

(a) Naphthoresorcinol

This was the most commonly used indicator reagent, being very sensitive for the detection of oligosaccharides containing 3,6-anhydrogalactose. Compounds containing this sugar residue showed a pale blue colour after 15 minutes at room temperature. Galactose also gave a blue colour, but did not appear until 30 minutes after spraying. Sucrose gave a red to purple colour, also after 30 minutes. The reaction with this spray reagent was detected more rapidly by examination under ultraviolet light. Naphthoresorcinol reagent was prepared and used as described by Yaphe, 1957 (see appendix).

(b) Aniline hydrogen phthalate

Aniline hydrogen phthalate is specific for sugars with reducing end groups, and is best used for the detection of monomers. After spraying, the plate was air-dried and then heated for 5 minutes at 100°. Galactose appeared as a brown spot, while oligosaccharides gave a poor colour with this reagent. The preparation of this reagent is described in the appendix.

E. PREPARATION OF β-TETRASACCHARIDASE BY SONICATION OF CELLS

1. Growth of Cells

Cells were prepared by growth in H-1 mineral salts medium, with an agarose hydrolysate (approximately 0.05%) as added substrate. Flasks containing 200 ml of medium were inoculated with 1 ml of a 24 hour culture of P.atlantica in agar-free medium, and were incubated on a rotary shaker at 25°. After 20 hours incubation, the cells were recovered by centrifugation at 10,000 g for 20 minutes at 4° and were washed three times in H-1 salts solution (see appendix).

2. Sonication Treatment

All ultrasonic treatments were carried out in an ice-bath, using an M.S.E. ultrasonic disintegrator (Wilson Science Equipment Limited). Washed cells were suspended in a small volume of 0.1M phosphate buffer (pH 7) and were sonicated for 1 minute intervals with 1 to 2 minutes cooling between treatments. A total sonication time of 5 minutes was achieved. The cell debris was removed by centrifugation at 30,000 g for 30 minutes at 4°. This intracellular enzyme preparation was tested for activity against agarose, neoagarotetraose and neoagarobiose.

3. Partial Purification of the Intracellular Preparation

All purification experiments with the intracellular preparation were carried out at 0 to 4°. Enzyme activity was tested after each step against agarose and neoagarotetraose.

a) Streptomycin sulfate precipitation

Nucleic acids and proteins associated with nucleic acid synthesis can be removed by precipitation with streptomycin sulfate. Streptomycin sulfate

(0.5 ml of a 10% solution) was added with stirring to 5 ml of crude enzyme which had been dialyzed against 0.01M phosphate buffer (pH 7). The precipitate was removed by centrifugation at 30,000 g for 25 to 30 minutes. Protein and nucleic acid levels were estimated spectrophotometrically, using the method of Warburg and Christian (1941).

b) Ammonium sulfate precipitation

The amount of ammonium sulfate to be added in order to raise the saturation level, was calculated from the nomograph of Dixon (1953). Saturated ammonium sulfate was added slowly to the supernatant fluid from step (a) to give 50% saturation. The precipitate was removed by centrifugation at 30,000 g for 15 minutes, and the supernatant fluid was adjusted to 75% ammonium sulfate saturation, and the protein precipitate was again collected by centrifugation. The precipitates were redissolved in small volumes of phosphate buffer and dialyzed against 0.01M phosphate at pH 7. Protein levels were estimated spectrophotometrically.

c) Gel-filtration

Sephadex G-50 (Pharmacia Fine Chemicals, Uppsala, Sweden) was allowed to swell in 0.01M phosphate buffer (pH 7) and the fines were removed. The gel was poured as a thin slurry into a glass column, 2.5 x 25 cm, and buffer was passed through the column for several hours to ensure uniform packing. Blue dextran 2000 (Pharmacia Fine Chemicals, Uppsala, Sweden) was passed through the column to determine the void volume and to indicate any flaws in the packing of the gel. A 0.5 ml sample of the enzyme, 50% ammonium sulfate precipitate, was layered onto the column and allowed to drain into the gel before eluting with buffer. Fractions of 1 ml were collected automatically, using an LKB Fraction Collector (LKB-Produkter,

AB S-161 25 Bromma 1,Sweden), and were analyzed for protein concentration and for enzyme activity against agarose and neoagarotetraose.

F. PREPARATION OF β-TETRASACCHARIDASE BY SELECTIVE RELEASE FROM CELLS

All experiments were carried out at temperatures between 0 and 4°, unless otherwise indicated.

1. Cell Suspensions

In all experiments, cells were harvested from solid H-1 media (2% Bacto-agar) after 14 to 20 hours growth at 25° in Roux bottles. The cells were washed three times in MacLeod's complete salts (see appendix), and were suspended in salt solution to a concentration which, when diluted 1 to 100, gave an O.D. reading between 0.50 and 0.55 at 440 nm. After centrifugation, the volume of the supernatant solution was measured and the cell pellet was resuspended in an equal volume of the appropriate solution.

2. Osmotic Shock Treatment

The method of Heppel (1967) was used, with some modifications. Washed cells were resuspended in 0.03M tris (hydroxymethyl) aminomethane (Tris) buffer containing 0.5M sucrose and 10^{-4} M ethylenediaminetetra-acetic acid, dipotassium salt (EDTA), adjusted to pH 7.3. After 10 minutes incubation, the cells were collected by centrifugation and the cell pellet was resuspended in an equal volume of cold $MgCl_2$ (5×10^{-4}M) for 10 minutes, with occasional shaking. The cells were removed by centrifugation. The supernatant solution obtained was the shock fluid, which was tested for β-tetrasaccharidase activity.

3. EDTA-Tris Extraction

The procedure is essentially that reported by Leive (1965b), with some modification. Washed cells were resuspended in 0.12M Tris buffer containing 10^{-3} M EDTA, at pH 7.4. The suspension was mixed for 10 minutes using a magnetic stirrer, and the cells were removed by centrifugation. The supernatant solution was dialyzed against 0.01M phosphate buffer (pH 7) and tested for agarase and β-tetrasaccharidase activity.

Various modifications of this technique were investigated in order to find the best extraction method for release of β-tetrasaccharidase activity. These experiments are discussed in the Results Section. The method used for the subsequent preparation of the β-tetrasaccharidase enzyme involved the same procedure as described above, with addition of 1.5M NaCl to the 0.12M Tris, 10^{-3} M EDTA buffer (pH 7.4).

4. Sucrose Suspension of NaCl-Washed Cells

The method used was that reported by Costerton et al. (1967), who found that the outer double tract layer of the cell envelope of a marine pseudomonad could be removed by washing the cells in 0.5M NaCl followed by suspension in 0.5M sucrose. Cells were harvested and washed three times in 0.5M NaCl. A standard concentration of cells was resuspended in 0.5M sucrose for 10 minutes, with occasional shaking. The cells were removed by centrifugation and the supernatant solution was dialyzed extensively to remove sucrose before testing for enzyme activity.

G. MEASUREMENT OF ENZYME ACTIVITY

1. β-Tetrasaccharidase Activity

Enzymatic hydrolysis of neoagarotetraose was followed by cellulose thin layer chromatography. Enzyme activity was determined by measuring the increase in the reducing power of the hydrolysate. The reducing power was determined by the colorimetric method of Somogyi (1952), using Nelson's arsenomolybdate solution as chromogenic reagent (Nelson, 1944), and galactose as standard. Somogyi's alkaline copper reagent was prepared as two reagents, Solution I and II (see appendix), which were stored at room temperature and combined directly before use. Nelson's chromogenic reagent (see appendix) was stored in a brown bottle at 4^{o}. A typical galactose standard curve is shown in Figure 14.

The assay was carried out as follows:
Duplicate samples (0.05 ml) of enzyme hydrolysate were removed and diluted to 2 ml with distilled water. The samples were boiled for 5 minutes to inactivate enzyme activity. Alkaline copper reagent (2 ml) was added and the mixtures were covered with glass marbles and heated for 20 minutes in a boiling water bath. After cooling for 5 minutes, the chromogenic reagent (2 ml) was added and mixed well to dissolve the cuprous oxide. The time allowed for colour development was 15 minutes. The absorbance of the solutions was measured against a reagent blank using a Coleman Double Beam Spectrophotometer (Model 124, Hitachi Ltd., Tokyo, Japan), in 3 ml glass cuvettes, at 660 nm. The reducing sugars present were expressed as nmoles of galactose.

A unit of enzyme activity is defined as that amount of enzyme which

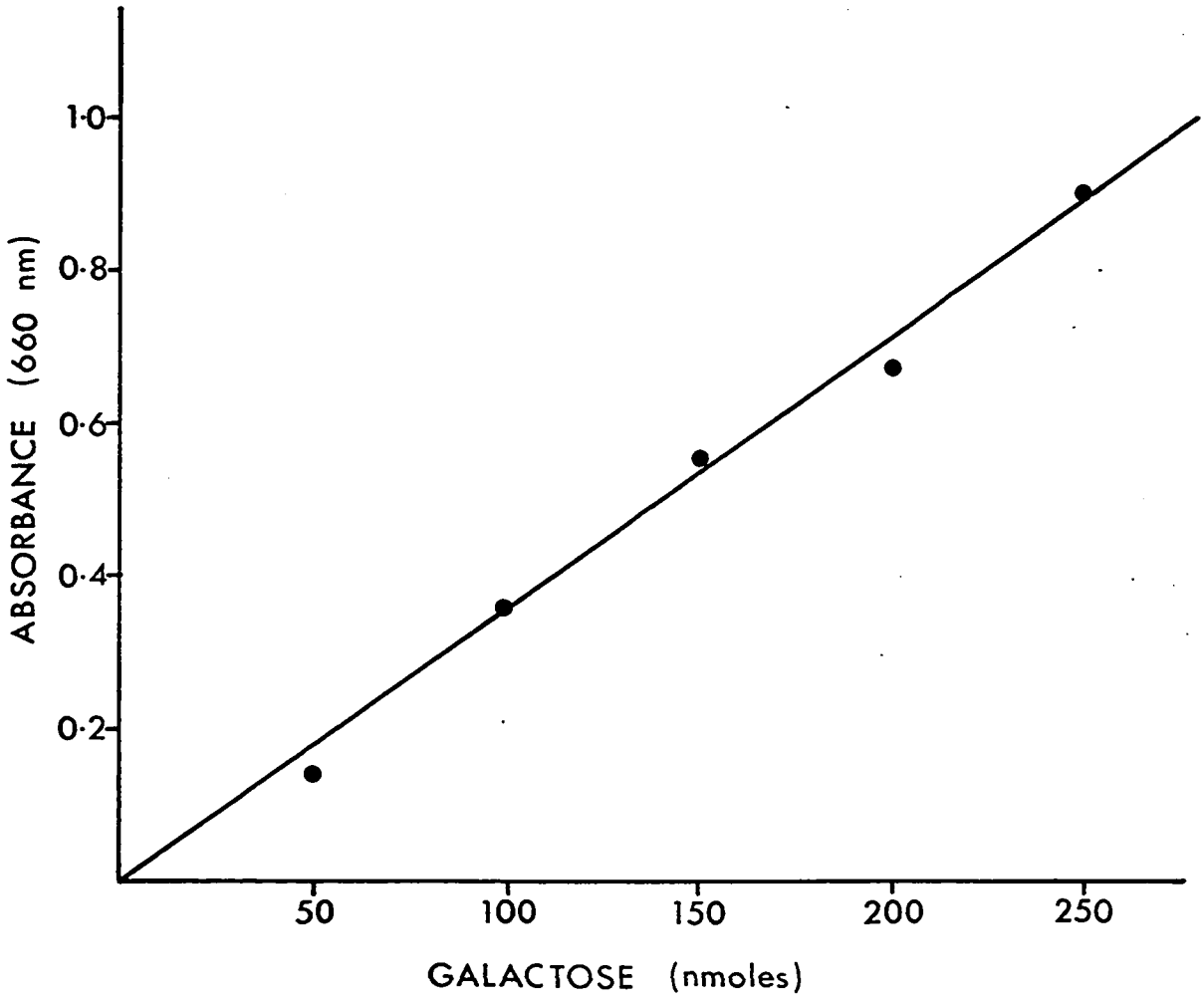


Figure 14. Standardization of the Somogyi colorimetric reagent.

will liberate reducing sugars equivalent to 0.1 nmole of galactose per minute at 25°.

2. Protein Determination

Protein was determined by the spectrophotometric method of Warburg and Christian (1941). The absorbance of enzyme solutions at 260 and 280 nm was measured, and the protein content was calculated from the nomograph of Adams (California Corporation for Biochemical Research).

Protein was also determined by the Lowry method, using the modifications of Oyama and Eagle (1956). Solutions I and II (see appendix) were combined before use and 5 ml of the solution was added to the sample to be analyzed (diluted to 1 ml in 0.1N NaOH). After 10 minutes incubation at room temperature, 0.5 ml of Folin-Ciocalteau reagent (see appendix) was added. The samples were mixed and left at room temperature for 30 minutes. The absorbance was measured at 690 nm using a Spectronic 20 (Bausch and Lomb, Inc., New York), and the values were converted to mg of protein per ml from a standard curve determined for bovine serum albumin.

H. PURIFICATION OF β-TETRASACCHARIDASE

All purification experiments were carried out at temperatures between 0 and 4°.

1. Agarose Adsorption

The specific adsorption of an enzyme onto its substrate is a common technique used in enzyme purification schemes (Pogell, 1966). Preliminary evidence by other workers in this laboratory had indicated that the agarase enzyme could be selectively adsorbed onto a column of agarose particles

(Hong and Yaphe, this department, unpublished results). This technique was attempted in the hope that the β-tetrasaccharidase could be separated from agarase activity. Adsorption of the agarase enzyme was tried several times, using a number of agarose preparations.

Two grams of agarose were stirred into a small volume of 0.01M phosphate buffer (pH 7) and allowed to swell overnight. The slurry was poured into a small column (Pharmacia Fine Chemicals, Uppsala, Sweden), 2.5 x 25 cm, and stabilized by passing buffer through overnight. Enzyme solution (5 ml) was layered carefully onto the packed agarose and was washed through with buffer. The eluant was collected in three fractions, 0 to 15 ml, 16 to 30 ml, and 31 to 50 ml. Each pool was concentrated by ultrafiltration using a Diaflo UM-10 membrane (Amicon Corporation, Lexington, Mass.) to a volume of 3 to 4 ml. The concentrated preparations were tested for enzyme activity on agarose and neoagarotetraose.

2. Isoelectric Focusing

The principle of isoelectric focusing has been known for quite some time, however, it is only recently that the technique has been perfected for practical applications (Svensson, 1961, 1962a, 1962b; Vesterberg and Svensson, 1966). The method is based on the presence of a pH gradient which is obtained by applying a direct current to a system of electrolytes such that the pH increases steadily from anode to cathode. Provided that the pH gradient is sufficiently stable for the duration of an experiment, ampholytes such as proteins and peptides will be repelled by both electrodes and each ampholyte species will collect at the place in the gradient where the pH is equal to the isoelectric point of that species. This collecting, or focusing, is caused by the electric field, thus the name

isoelectric focusing.

A stable pH gradient can be formed by electrolysis of low-molecular "carrier ampholytes" (Svensson, 1962a), which have a considerable buffering action in the isoelectric state and contribute to give very shallow pH gradients, which are essential for a good resolution of considerable quantities of proteins. A necessary prerequisite for isoelectric fractionation is that the electrolyte system is stabilized against uncontrolled convection and against remixing of focused ampholytes. This is achieved by using a density gradient with gradually decreasing concentration from the bottom to the top of the column. In such a stabilized pH gradient, proteins migrate to, and are focused at, their respective isoelectric points. The primary value of this method is that it allows direct measurement of the isoelectric points of fractionated proteins.

The LKB 8100 electrofocusing column of 110 ml capacity, with platinum electrodes and cooling mantle was used (Model 8101, LKB-Produkter AB S-161 25 Bromma 1, Sweden). The instructions of the manufacturer were essentially followed with a few modifications. The cathode was at the bottom of the column, and 2% LKB carrier ampholyte concentration was used. The experiments were performed at room temperature with the internal temperature of the column maintained at 4^{o} using a Neslab pumping and cooling system (Neslab Instruments Inc., Portsmouth, New Hampshire).

The following solutions were prepared:

Bottom Electrode Solution

(mono) ethanolamine	0.8 ml
sucrose	24.0 g
distilled water	28.0 ml

Top Electrode Solution

concentrated H_2SO_4	0.2 ml
distilled water	20.0 ml

Heavy Solution

40% Ampholine	3.75 ml
sucrose	28.0 g
distilled water to	40.0 ml

Light Solution

40% Ampholine	1.25 ml
distilled water to	60.0 ml

The enzyme sample, containing less than 0.5 mmole total salt content, was added in equal amounts to the heavy and light solutions, displacing a corresponding volume of distilled water. Enzyme preparations were concentrated before application by ultrafiltration and the sample volume added was from 8 to 10 ml.

The column was cooled to 4°, one hour before beginning the loading operation. The bottom electrode solution was pumped into the central compartment using a peristaltic pump (Model 600-1200, Harvard Apparatus Co., Dover, Mass.). The sucrose gradient was formed and pumped in using a Model 190 Dialagrad programmed gradient pump (Instrumentation Specialties Co., Lincoln, Nebraska), set to deliver a linear gradient of decreasing sucrose concentration from bottom to top with a flow rate of 4 ml per minute. Any excess sucrose was siphoned off to a level 1 cm below the top electrode. Top solution was layered on slowly with a peristaltic pump to cover the top electrode. A constant voltage of 600 volts and approximately 15 mamp was supplied. The electrofocusing process was usually complete

after 48 hours and was terminated when the current through the column reached a constant value, about 2 mamp. The column was emptied from the bottom using a peristaltic pump, flow rate 4 ml per minute, and the eluant was continuously monitored by an LKB "Uvicord" unit measuring at 254 nm (Model 4701A, LKB-Produkter AB-Stockholm). Two ml fractions were collected in an ice bath. The pH of each fraction was measured to determine the isoelectric points of the focused components. Samples were analyzed spectrophotometrically at 260 and 280 nm and were tested for agarase and β-tetrasaccharidase activity.

Two experiments were carried out; the first was a preliminary run using Ampholine range 3 to 10 to determine the most suitable pH range for the enzymes concerned. The optimum pH range of 5 to 8 was selected and was used in the second experiment. Fractions containing enzyme activity against neoagarotetraose were pooled and dialyzed extensively against 0.01M phosphate buffer (pH 7) to remove sucrose and ampholytes.

3. DEAE-Sephadex Treatment

DEAE-Sephadex is an anion exchanger with high capacity and low non-specific adsorption. It is made in two forms which differ in their capacity. DEAE-Sephadex A-25 is the more highly cross-linked and has a high capacity for molecules smaller than approximately 10,000 molecular weight. The ionic groups of DEAE-Sephadex A-50 are accessible to much larger molecules and this is the most suitable type for protein separation. Complete desorption is generally accomplished by changing the ionic strength of the buffer system or by pH adjustment.

DEAE-Sephadex A-50 (Pharmacia Fine Chemicals, Uppsala, Sweden) was used in all experiments. The gel particles were allowed to swell in

distilled water overnight, and the "fines" were removed. The washed gel was cycled through 1N HCl, distilled water, 0.5N NaOH + 0.5N NaCl, distilled water, and finally 1N HCl. The gel was washed extensively with distilled water until the pH of the washings was equal to the pH of distilled water. The gel was then equilibrated with the appropriate buffer.

a) Column chromatography

DEAE-Sephadex, in 0.1M phosphate (pH 5.3), was poured as a thin slurry into a small column, 2.5 x 25 cm, and was equilibrated overnight with buffer. Crude enzyme preparations were dialyzed against the same buffer for several hours and a 5 to 10 ml sample was applied to the column. Elution was achieved in a stepwise fashion using 0.1M phosphate, 0.1M phosphate containing 0.5M NaCl, and 0.1M phosphate containing 1.0M NaCl, all at pH 5.3. The eluant was monitored spectrophotometrically and by checking for agarase activity. Fractions were collected and concentrated by ultrafiltration using a Diaflo UM-10 membrane. Fractions with high NaCl concentrations were dialyzed against 0.01M phosphate (pH 7) before testing for β-tetrasaccharidase activity.

In subsequent experiments, elution with 0.1M phosphate was omitted, and fractions with agarase activity were eluted from the DEAE-Sephadex with the 0.1M phosphate containing 0.5M NaCl immediately following sample application.

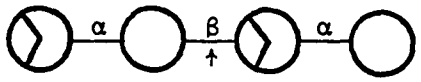
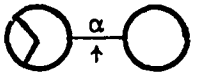
b) Batch-wise separation

DEAE-Sephadex was equilibrated overnight in 0.1M phosphate containing 0.5M NaCl (pH 5.3) in a total volume of 300 ml. Dialyzed enzyme, containing 200 units of β-tetrasaccharidase activity was mixed with the Sephadex slurry for 10 minutes in an ice-bath, using a magnetic stirrer. The

supernatant solution was discarded after centrifugation of the gel. Washing with 0.1M phosphate containing 0.5M NaCl was repeated until the agarase activity could no longer be detected in the washings. The gel was resuspended in 50 ml of 0.1M phosphate containing 1.0M NaCl and the wash solution was collected by filtration on a Buchner funnel. The filtrate was concentrated 8-fold by ultrafiltration and was dialyzed against 0.01M phosphate buffer, pH 7. The units of β-tetrasaccharidase activity recovered were determined.

IV. RESULTS

A. PREPARATION OF β-TETRASACCHARIDASE BY SONICATION OF CELLS

The crude intracellular enzyme, prepared by sonication of Pseudomonas atlantica cells, was incubated with agarose and neoagarotetraose to demonstrate the presence of the agarase, β-tetrasaccharidase, and α-disaccharidase enzymes outlined in Figure 8, page 12. The products of enzymic hydrolysis were examined by cellulose thin layer chromatography, using solvent (a), see Materials and Methods. The pattern of degradation of agarose and neoagarotetraose, with time, is illustrated in Figure 15. After 5 minutes incubation with enzyme, the tetrasaccharide has been partially hydrolyzed  to yield neoagarobiose, which on further incubation is hydrolyzed at the α-1,3 linkage to yield galactose and 3,6-anhydrogalactose,  . These results show that the cell extract contains a mixture of three enzymes: an agarase which attacks the agarose polymer at its occasional β-1,4 linkages to yield a homologous series of oligosaccharides, a β-tetrasaccharidase which hydrolyzes the β-1,4 linkage of neoagarotetraose, and an α-disaccharidase which breaks the α-1,3 linkage of neoagarobiose. The results confirm the original observation that neoagarotetraose can be degraded by an intracellular enzyme preparation of P.atlantica to yield neoagarobiose, galactose, and 3,6-anhydrogalactose (Yaphe, 1966).

β-tetrasaccharidase activity in the crude and partially purified enzyme preparations could only be detected by qualitative methods. The presence of the α-disaccharidase made it impossible to determine the

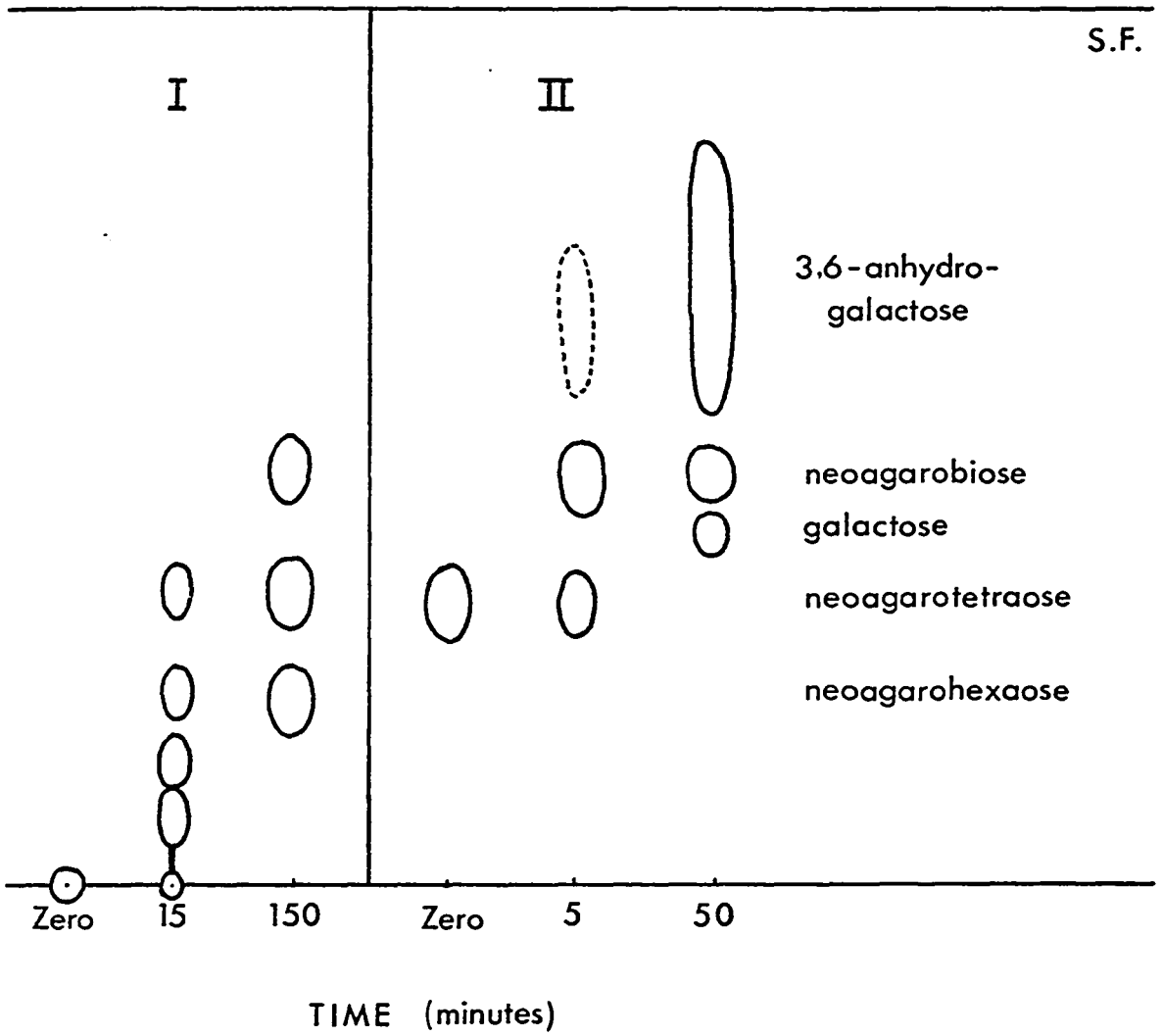


Figure 15. Cellulose thin layer chromatogram of the products of enzymic hydrolysis of agarose (I) and neoagarotetraose (II) after incubation with a cell extract from Pseudomonas atlantica. Solvent (a), Butan-1-ol-Ethanol-Water, 3:2:2 (v/v), was used.

reducing sugar equivalents produced specifically by the degradation of neoagarotetraose to neoagarobiose.

1. Purification of the Cell Extract

The results obtained from streptomycin sulfate and ammonium sulfate precipitations are outlined in Table 1. The increase in the ratio of absorbancy at 280 nm to that at 260 nm indicates a partial removal of nucleic acid material. It is interesting to note that separation of α-disaccharidase activity from the β-tetrasaccharidase enzyme has occurred in the 50-75% ammonium sulfate precipitate.

Sephadex (G-50) gel filtration was carried out to achieve further purification of the β-tetrasaccharidase enzyme. Fractions (1 ml) were collected at 4^{o} and protein was determined spectrophotometrically. Enzyme activity against agarose and neoagarotetraose was assayed by the iodine method and by thin layer chromatography. The elution pattern of the partially purified extract (50% ammonium sulfate precipitate) is shown in Figure 16. Virtually all of the protein added to the column was accounted for by the time Fraction 25 was collected. Most of the protein, which included the β-tetrasaccharidase and α-disaccharidase enzymes, was partially excluded from the gel, while the lower molecular weight agarase was eluted later.

Due to the major losses of β-tetrasaccharidase activity during the purification scheme, and the interference by the α-disaccharidase enzyme in assaying for the β-tetrasaccharidase activity, further experiments using the intracellular extract were not pursued. Other methods of enzyme release were investigated in order to obtain an initial separation of β-tetrasaccharidase from α-disaccharidase activity.

TABLE 1. Partial Purification of a Crude Enzyme Prepared by Sonication of *Pseudomonas atlantica* Cells.

Step and Procedure	Volume (ml)	Ratio $\frac{A280}{A260}$	Total Protein (mg)	Yield of Protein (%)	Enzyme Activity†		
					Agar	Tetra	Di
Crude Extract	6	0.55	36.0	100	***	***	***
Streptomycin sulfate	6	0.84	27.0	75	***	***	***
Ammonium sulfate (0 - 50%)	3	0.78	14.4	40	**	**	**
Ammonium sulfate (50 - 75%)	3	0.89	3.6	10	*	*	-

†Enzyme Activity:

Agar = Agarase, Tetra = β-tetrasaccharidase, Di = α-disaccharidase

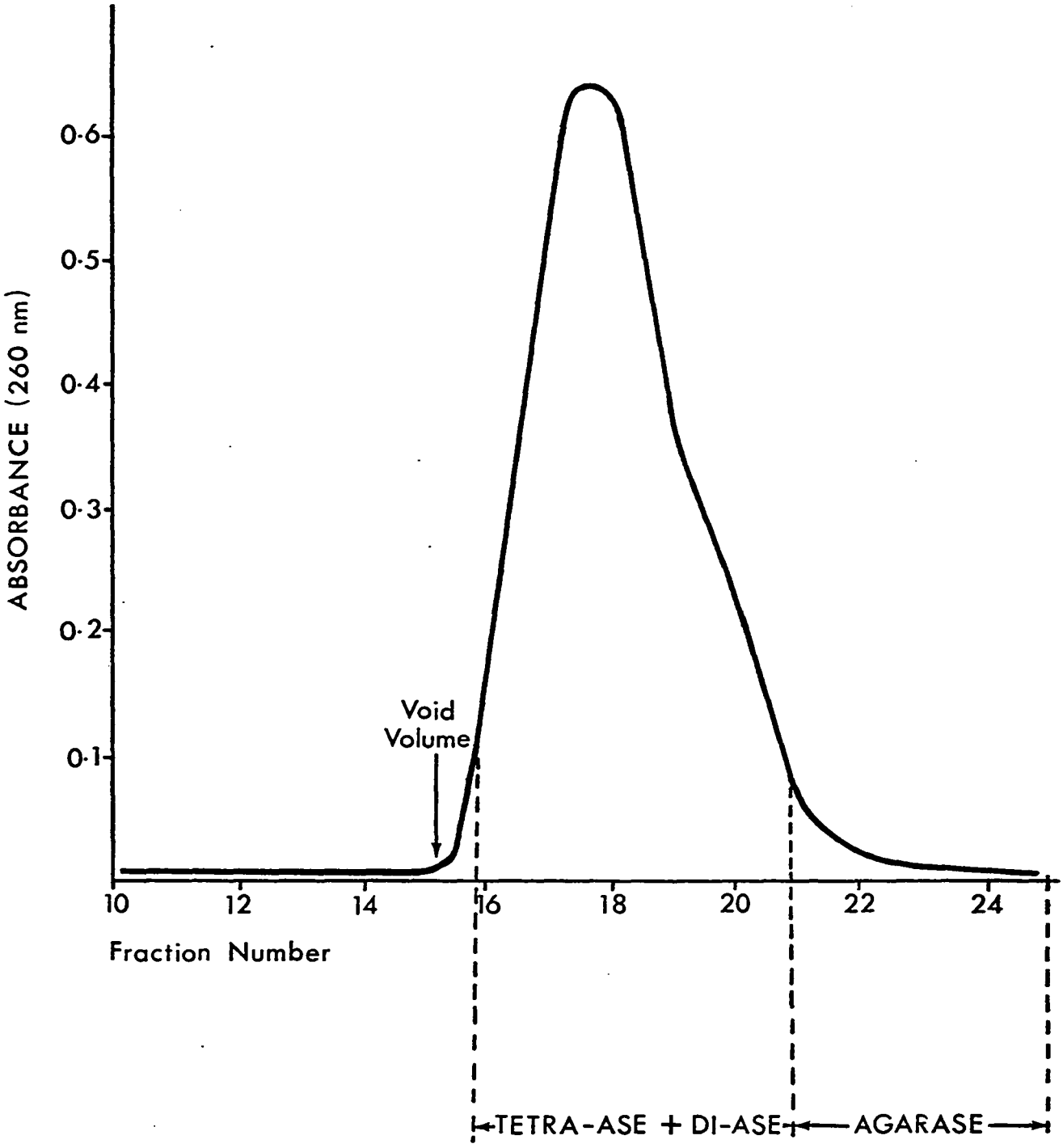


Figure 16. Gel-filtration of a partially purified cell extract from Pseudomonas atlantica on Sephadex G-50 (medium). A 0.5 ml sample of the enzyme (3.7 mg protein) was applied to the column (2.5 x 25 cm) and eluted with sodium phosphate buffer, pH 7. Fractions of 1 ml were collected.

B. PREPARATION OF β-TETRASACCHARIDASE BY SELECTIVE RELEASE FROM CELLS

These experiments were carried out in an attempt to develop a relatively mild treatment which might allow selective release of a surface-located β-tetrasaccharidase from P.atlantica cells, without contamination by a truly intracellular α-disaccharidase enzyme.

1. Osmotic Shock Treatment

Osmotic shock has been used extensively in E.coli and other enteric bacteria to release surface-located and periplasmic enzymes. Lysis of P.atlantica cells was found to occur in the first stage of osmotic shock; that is, suspension in 0.5M sucrose containing 0.03M Tris and 10^{-4} M EDTA. Subsequently, when these cells were resuspended in dilute $MgCl_2$, there was no selective release of β-tetrasaccharidase into the shock fluid. This method is not suitable for Pseudomonas species which are highly sensitive to the chelating effect of EDTA.

2. EDTA-Tris Extraction

Treatment of cells with EDTA is known to cause changes in the permeability characteristics of the cell wall (Leive, 1965a), accompanied by the release of the lipopolysaccharide component of the cell wall and enzymes involved in lipopolysaccharide synthesis (Levy and Leive, 1970). It was anticipated that this treatment would liberate cell wall or surface-located enzymes from P.atlantica, without the release of intracellular components.

The results of preliminary experiments indicated that the β-tetrasaccharidase enzyme could be released from the cells by incubation with EDTA-Tris buffer at 0 to 4°; however, a significant amount of

α-disaccharidase activity was released concurrently into the extracting fluid. The presence of the α-disaccharidase is indicated by the production of 3,6-anhydrogalactose which forms a streaked spot on thin layer chromatography, along with a spot having the same mobility as galactose.

It was found that the addition of sodium chloride to the extracting solution was able to reduce the amount of α-disaccharidase released; the results of this experiment are expressed in Figure 17. At a salt concentration of 1.5M NaCl, the enzyme preparation contained a negligible amount of α-disaccharidase and enzymolysis of neoagarotetraose yields only the disaccharide, neoagarobiose. The presence of EDTA in the extracting fluid decreased the amount of α-disaccharidase released from the cells, but had no apparent effect on the yield of β-tetrasaccharidase. Approximately equal amounts of β-tetrasaccharidase were obtained with and without the addition of EDTA. A comparison of the ability of equimolar concentrations of Tris and phosphate buffers to cause selective release of the β-tetrasaccharidase indicated that Tris buffer was more effective than phosphate in promoting release of the enzyme from P.atlantica cells. The amount of α-disaccharidase enzyme released was similar with either buffer system. The optimal exposure time, which allowed the release of β-tetrasaccharidase with minimal leakage of α-disaccharidase, was found to be 30 minutes.

Based on these observations, the extraction treatment used to release β-tetrasaccharidase activity from P.atlantica was standardized at 30 minutes incubation of washed cells in 0.12M Tris buffer containing 10^{-3} M EDTA and 1.5M NaCl (pH 7.2) at 0 to 4°. Phase microscopy studies indicated

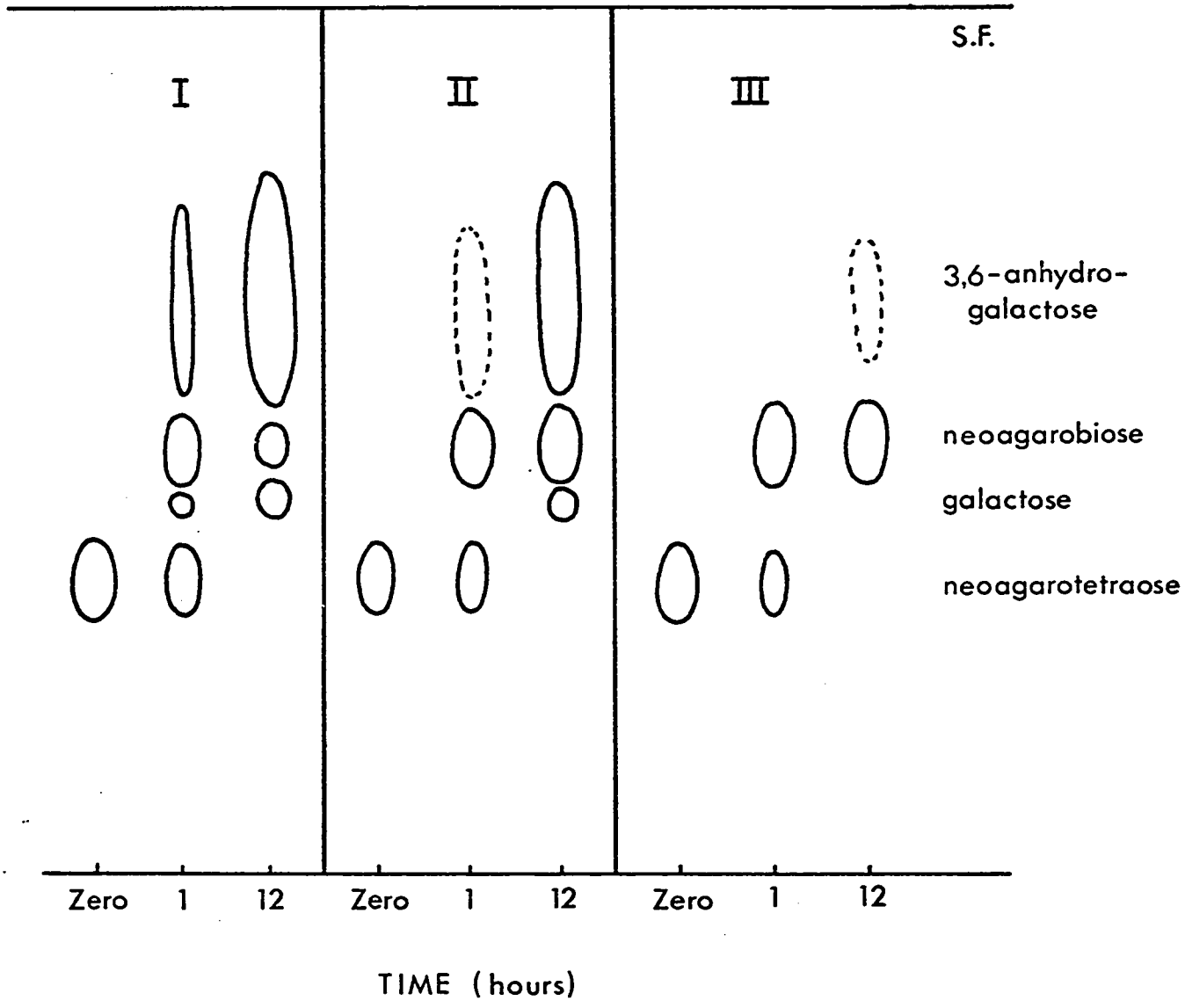


Figure 17. Cellulose thin layer chromatogram of the products of enzymolysis of neoagarotetraose after incubation with three enzyme preparations. The enzyme solutions were prepared by extraction of Pseudomonas atlantica cells with EDTA-Tris buffer (I), EDTA-Tris buffer containing 0.5M NaCl (II), and EDTA-Tris buffer containing 1.0M NaCl (III). Solvent (a), Butan-1-ol-Ethanol-Water, 3:2:2 (v/v), was used.

that cells retained their normal rod shape during this treatment; however, prolonged exposure caused enlargement and rounding of the cells, with some lysis. A similar observation was made by Asbell and Eagon (1966b) who reported the production of osmotically fragile rods, termed osmoplasts, when P.aeruginosa cells were incubated in a Tris-EDTA-sucrose or Tris-EDTA-NaCl solution. The osmoplasts were indistinguishable from normal rods when observed in the light microscope.

3. Sucrose Suspension of NaCl-Washed Cells

This treatment has been shown to cause the removal of the outer layers of the cell envelope of a marine pseudomonad, and allow the escape of enzymes located in the periplasmic space (Costerton et al., 1967; R.A. MacLeod, personal communication). The procedure of Costerton et al. (1967) was followed, and the results showed that β-tetrasaccharidase activity was released into the medium when cells washed with 0.5M NaCl were resuspended in 0.5M sucrose. A considerable amount of material absorbing at 220 nm appeared in solution but there was no evidence of material being released from the cells which absorbed at 260 nm, nor was there any release of α-disaccharidase activity. Phase microscopy showed that the cells retained their normal rod shape when suspended in sucrose, but were lysed when re-suspended in phosphate buffer, indicating that the cells were osmotically fragile. De Voe et al. (1970) have reported that similar forms, termed mureinoplasts, are stable if suspended in a balanced salt solution, (0.5M NaCl, 0.05M $MgCl_2$, 0.01M KCl), but lose U.V. absorbing material if suspended in 0.5M NaCl or 0.05M $MgCl_2$.

C. PURIFICATION OF β-TETRASACCHARIDASE

Extraction of the β-tetrasaccharidase enzyme by EDTA-Tris-NaCl treatment of P.atlantica cells represented an initial step in purification as it resulted in an enzyme solution which was free of α-disaccharidase activity. Further treatment was necessary to remove the agarase activity in order to obtain a pure β-tetrasaccharidase preparation.

1. Agarose Adsorption

The use of an agarose column to selectively remove the agarase enzyme from the crude β-tetrasaccharidase preparation was attempted several times, and representative results are outlined in Table 2. Although a large amount of the agarase could be selectively adsorbed by this treatment, it was found to be impossible to completely remove all agarase activity, even on recycling of the enzyme preparation through a second column.

TABLE 2. Agarose Adsorption of a Crude Enzyme Preparation

Fraction†	Eluant (ml)	Conc. Volume (ml)	Protein (mg/ml)	Enzyme Activity	
				Agarase	β-ase††
A	15	3	0.21	*	***
B	15	3	0.19	*	**
C	20	3	0.06	*	*

†A 5 ml sample of enzyme was applied to an agarose column (2.5 x 25 cm) and eluted with 0.01M sodium phosphate buffer, pH 7.0

††β-ase = β-tetrasaccharidase

2. Isoelectric Focusing

The separation pattern obtained from isoelectric fractionation of a crude enzyme, prepared by EDTA-Tris-NaCl extraction of P.atlantica cells, is represented in Figure 18. The first and last peaks in the diagram are not proteins, but are substances that strongly absorb U.V., which are formed at the electrodes during electrolysis. Separation of the agarase and β-tetrasaccharidase enzymes was not accomplished using the broad pH range 3 to 10, however the results from this preliminary run indicated that separation might be achieved by using a gradient with high resolving power in the pH interval 5 to 8.

The isoelectric focusing profile (Figure 19) obtained by electrolysis of the crude enzyme over the narrow pH range 5 to 8 indicates the definite separation of agarase and β-tetrasaccharidase activities. It is apparent from the focusing pattern that the β-tetrasaccharidase has a distinct isoelectric point, which was estimated at pH 5.6 to 6.1, while the agarase is electrophoretically heterogeneous, focusing between pH 6.6 and 8.1. It was now possible to use these values for the separation of the enzymes by ion-exchange chromatography.

3. DEAE-Sephadex

It was known from the isoelectric focusing data, that the β-tetrasaccharidase enzyme was more acidic than the agarase and could therefore be separated by charge differences on DEAE-Sephadex, which adsorbs acidic molecules.

After adsorption of the enzyme solution onto the gel, the column was eluted in a stepwise fashion with 0.1M phosphate, 0.1M phosphate containing 0.5M NaCl, and 0.1M phosphate containing 1.0M NaCl, all at pH 5.6. The

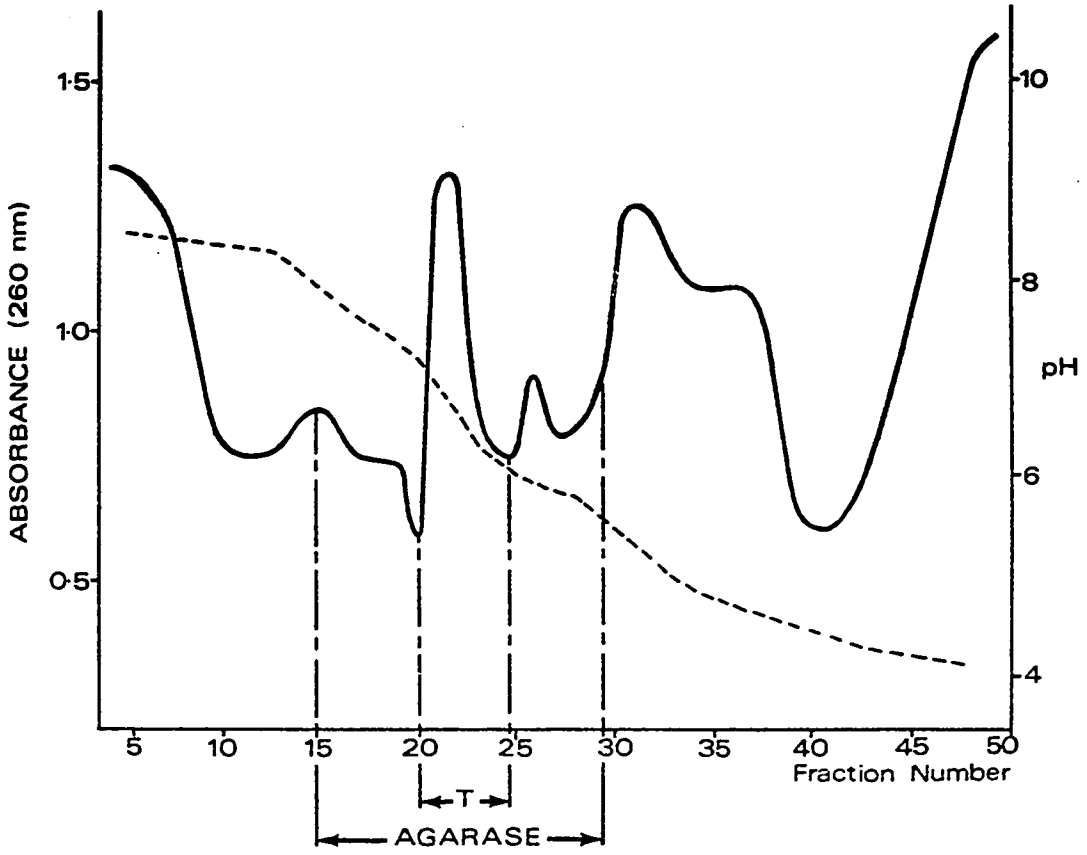


Figure 18. Distribution of protein and enzymic activities from isoelectric separation of a crude enzyme preparation. Carrier ampholytes of pH range 3 to 10 were used and the gradient obtained is indicated by the dotted line. Fractions of 2 ml were collected. T is β-tetrasaccharidase.

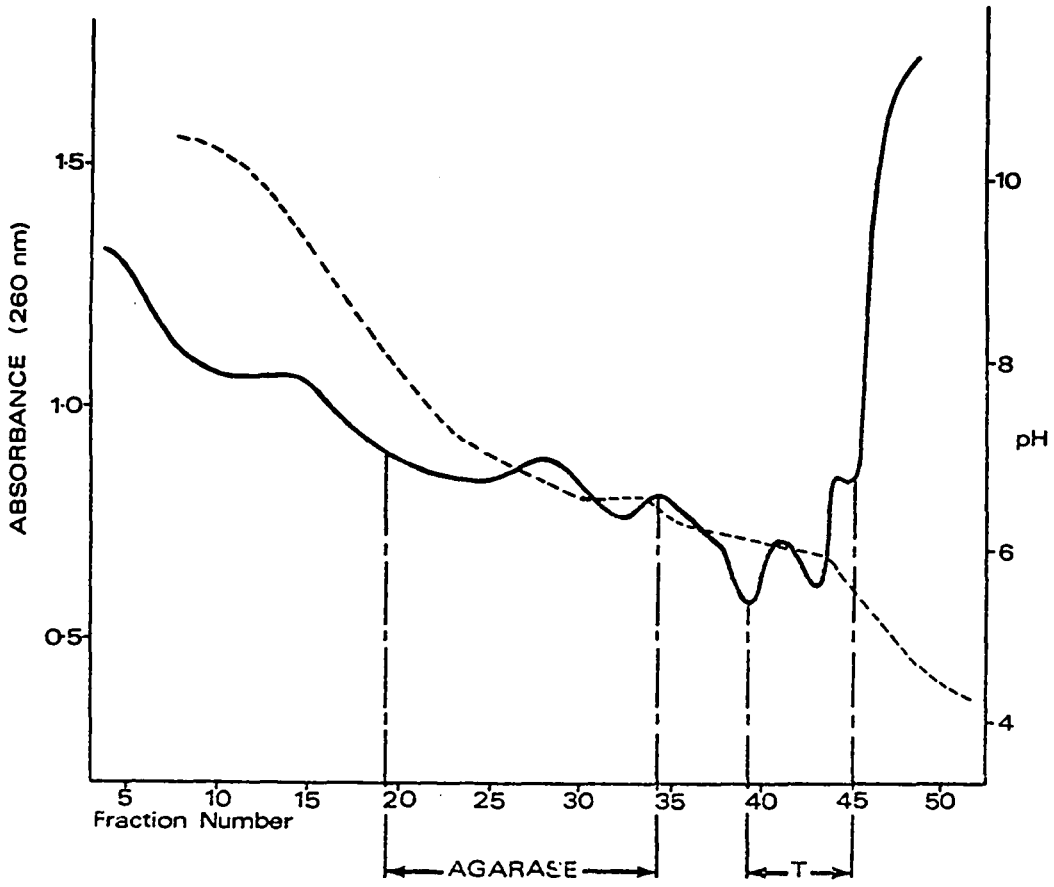


Figure 19. Separation of agarase and β-tetrasaccharidase (T) enzymes by isoelectric focusing of a crude enzyme preparation over a pH range 5 to 8. Protein distribution (solid line) and the pH gradient obtained (dotted line) are illustrated. The fraction volume was 2 ml.

TABLE 3. Purification of a Crude Enzyme Preparation on DEAE-Sephadex A-50 (medium).

Fraction†	Stepwise Elution with:	Enzyme Activity	
		Agarase	β-ase††
A	0.1M phosphate	+	-
B	0.1M phosphate 0.5M NaCl	+	-+
C	0.1M phosphate 0.5M NaCl	+-	-
D	0.1M phosphate 1.0M NaCl	-	+
E	0.1M phosphate 1.0M NaCl	-	+

†Concentrated fractions from gel-filtration on DEAE-Sephadex A-50, see Figure 20.

†† β-ase = β-tetrasaccharidase

elution curve is shown in Figure 20. The five fractions (A,B,C,D and E) collected, were concentrated and examined for agarase and β-tetrasaccharidase activity. The results are outlined in Table 3. This column was successful in separating the agarase and β-tetrasaccharidase enzymes, however the elution volume required to displace all the agarase activity was very large and a considerable amount of inactivation of the β-tetrasaccharidase enzyme occurred during the procedure. It was found to be advantageous to use a batch method for separation of the two enzymes.

The crude enzyme preparation was mixed with a slurry of DEAE-Sephadex A-50 which had been equilibrated with 0.1M sodium phosphate containing 0.5M

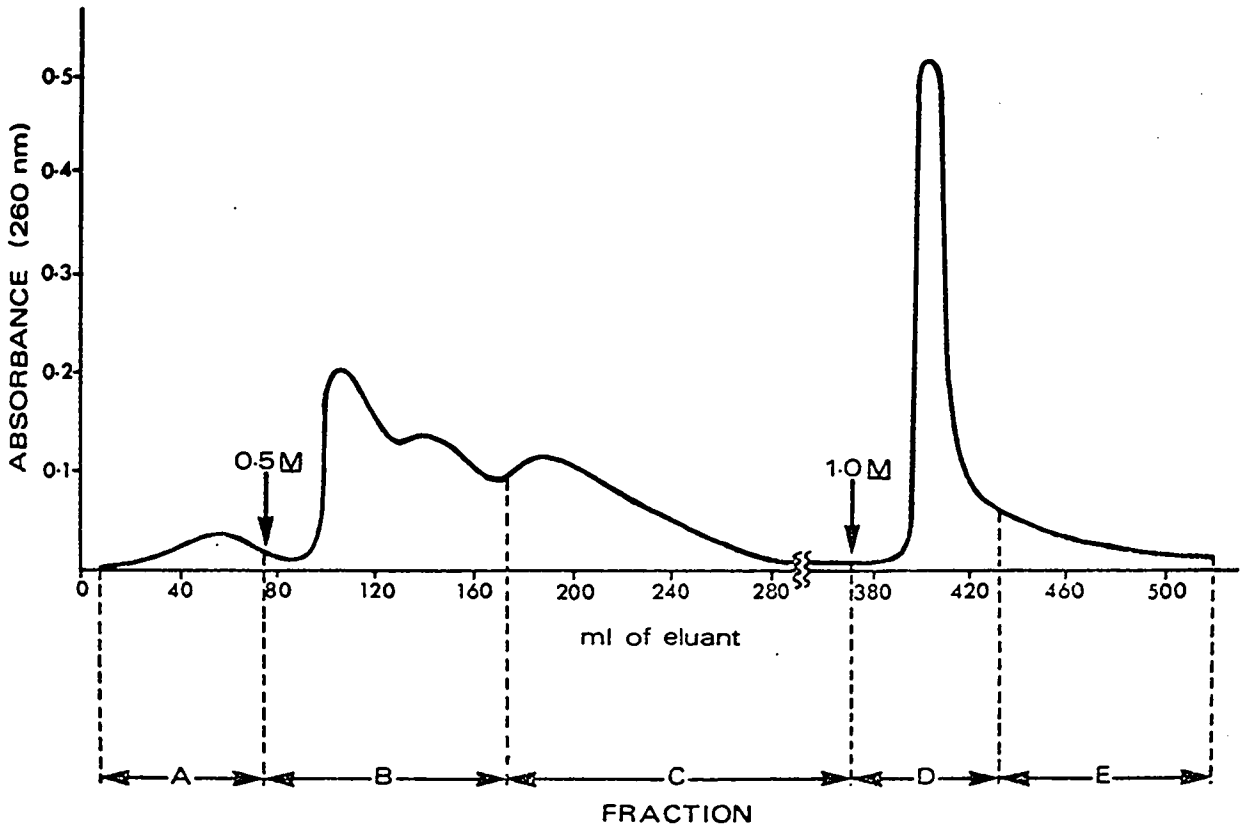


Figure 20. Gel-filtration of a crude enzyme preparation on DEAE-Sephadex. A 6-ml solution of the enzyme was adsorbed onto a column (2.5 x 25 cm) of DEAE-Sephadex A-50 (medium) and elution was carried out by stepwise salt gradient using 0.1M sodium phosphate, pH 5.3, as the starting buffer, followed by buffers of increasing NaCl concentration. Fractions A,B,C,D and E were collected and concentrated by Diaflo ultrafiltration.

NaCl. The β-tetrasaccharidase was adsorbed onto the gel, and the agarase was eluted with three to four 300-ml washes with 0.1M phosphate containing 0.5M NaCl. β-tetrasaccharidase was then eluted with 1.0M NaCl in sodium phosphate, pH 5.6. There was greater recovery of β-tetrasaccharidase activity using this method because the elution time was reduced by one-tenth, and hence the enzyme was in contact with the gel for a much shorter period of time. A batch method of ion-exchange was also capable of handling a much larger volume of enzyme solution. The final recovery of purified β-tetrasaccharidase varied between 7.5% and 10% of the total activity in the crude preparation. The results of purification are summarized in the following table.

TABLE 4. Purification of β-Tetrasaccharidase from Pseudomonas atlantica.

Step and Procedure	Volume (ml)	Total Units	Yield of Units (%)	Total Protein (mg)	Specific Activity (units/mg)
Crude Enzyme (prepared by EDTA-Tris-NaCl extraction of cells)	25	200	100	15.6	12.9
DEAE-Sephadex A-50 (Fraction D, Figure 20)	8	15	7.5	0.8	18.8

D. CHARACTERIZATION OF THE β-TETRASACCHARIDASE

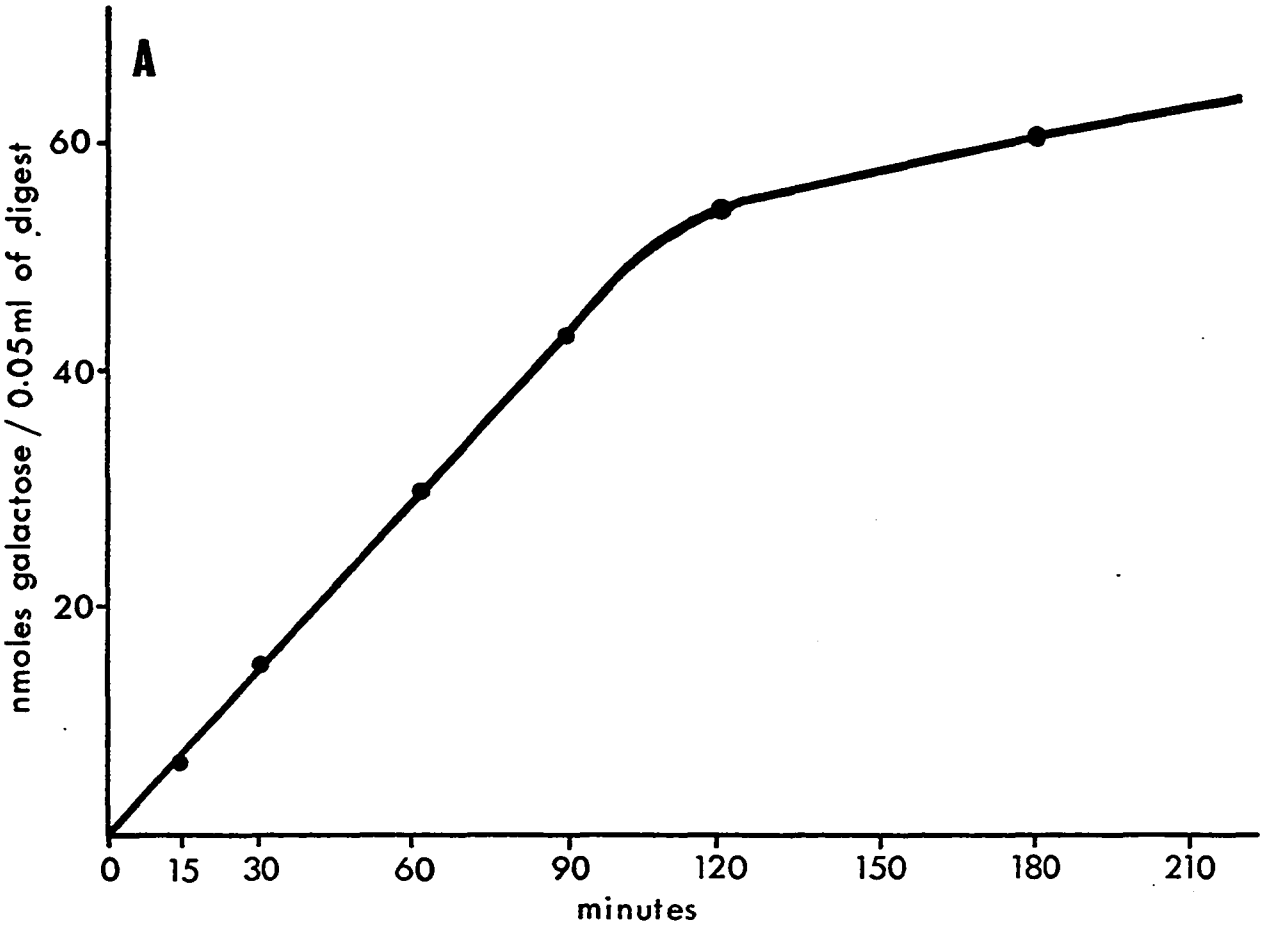
Experiments to characterize the enzyme activity were carried out with the DEAE-purified enzyme and with the crude β-tetrasaccharidase released by EDTA-Tris-NaCl extraction of washed cells. The crude preparation contained agarase but no α-disaccharidase activity, and could be used to examine the breakdown of neoagarotetraose since previous workers had shown that the purified agarase from P.atlantica has no activity on neoagarotetraose. Purified β-tetrasaccharidase was not used in all experiments because of the difficulty in obtaining an active preparation in high yield.

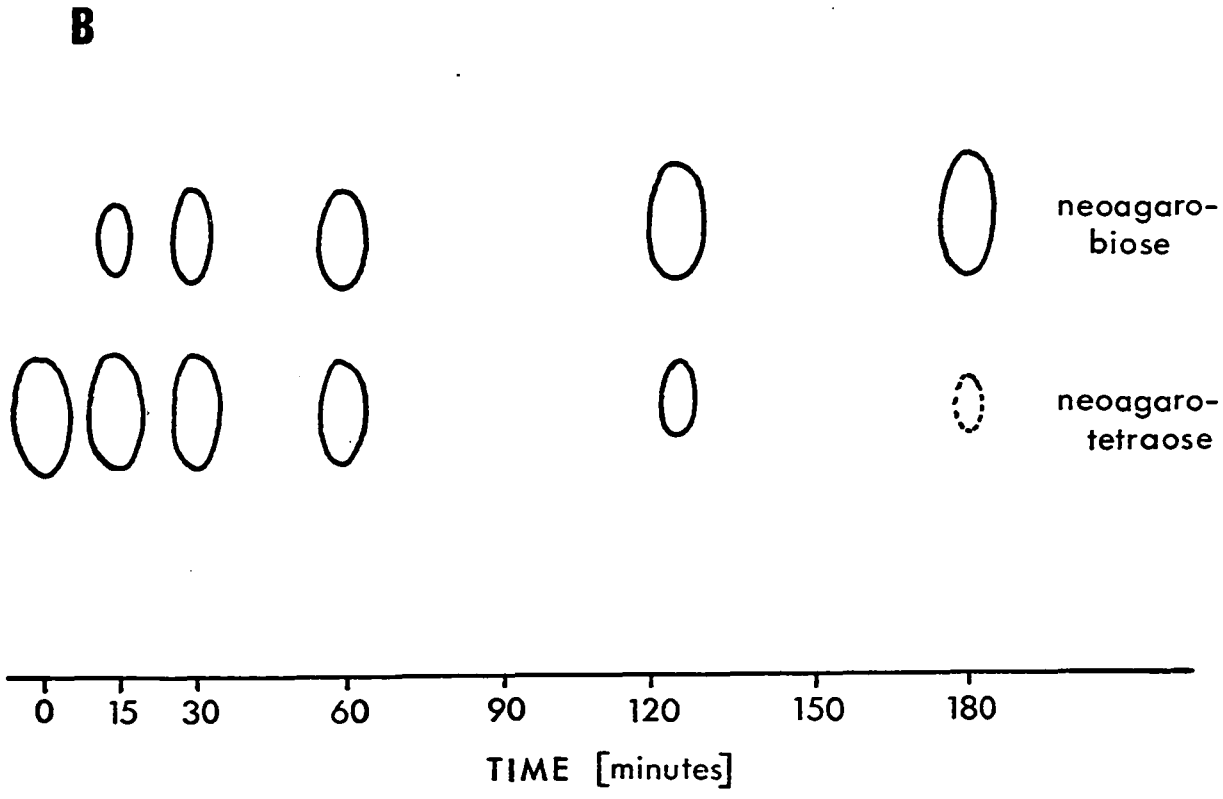
1. Degradation of Neoagarotetraose

Crude enzyme solution (0.6 ml) was incubated at 25° with 0.4 ml of sugar solution, giving a final concentration of 4 mmoles of neoagarotetraose per ml of hydrolysate. Duplicate 0.05 ml samples were withdrawn at selected time intervals and the amount of reducing sugar was determined.

The rate of hydrolysis of neoagarotetraose by enzyme was followed by the release of reducing sugar with time, and the products of enzymolysis were examined by cellulose thin layer chromatography, using solvent (a), see Materials and Methods. The amount of reducing sugar released was calculated by subtracting the zero-time value from the total reducing power of the sample. The enzyme preparation was found to contain 8.05 units of enzyme activity per ml, where 1 unit is defined as the amount of enzyme which liberates reducing sugars equivalent to 0.1 nmole of galactose per minute at 25°. The results are represented in Figure 21, in which the rate of release of reducing sugar by enzymic hydrolysis is correlated with the production of neoagarobiose from neoagarotetraose.

Figure 21. Degradation of neoagarotetraose. A, analysis of the reducing sugar released, expressed as nmoles of galactose per 0.05 ml of digest, with time. B, cellulose thin layer chromatogram of the products of enzymolysis of neoagarotetraose with time. The solvent was Butan-1-ol-Ethanol-Water, 3:2:2 (v/v).

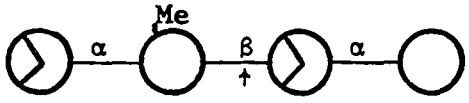

A
nmoles galactose / 0.05ml of digest
60
40
20
0
15
30
60
90
120
150
180
210
minutes


B
neoagaro-
biose
neoagaro-
tetraose
0
15
30
60
90
120
150
180
TIME [minutes]

2. Substrate Specificity

DEAE-purified β-tetrasaccharidase was tested for activity against neoagarohexaose, -octaose, and -decaose, since the crude enzyme preparation contained agarase which would degrade these higher molecular weight oligosaccharides. Aliquots were withdrawn from the hydrolysates at various time intervals and analyzed by cellulose thin layer chromatography, using solvent (a), see Materials and Methods. The products of hydrolysis after 5 hours incubation with enzyme are illustrated in Figure 22. The rate of hydrolysis of these oligosaccharides seems to decrease with increasing molecular weight, with no degradation of neoagarodecaose and higher neoagarosaccharides.

The action of the β-tetrasaccharidase on 6^3-O-methyl neoagarotetraose (see Figure 12, page 22) was also examined. Crude enzyme was used in this study since the purified agarase from P.atlantica has no activity on this sugar. The enzyme was incubated with excess substrate and the products of hydrolysis were examined chromatographically using double development in solvent (b), see Materials and Methods. Figure 23 illustrates the breakdown of 6^3-O-methyl neoagarotetraose



after incubation with enzyme, to yield neoagarobiose and 6-O-methyl neoagarobiose.

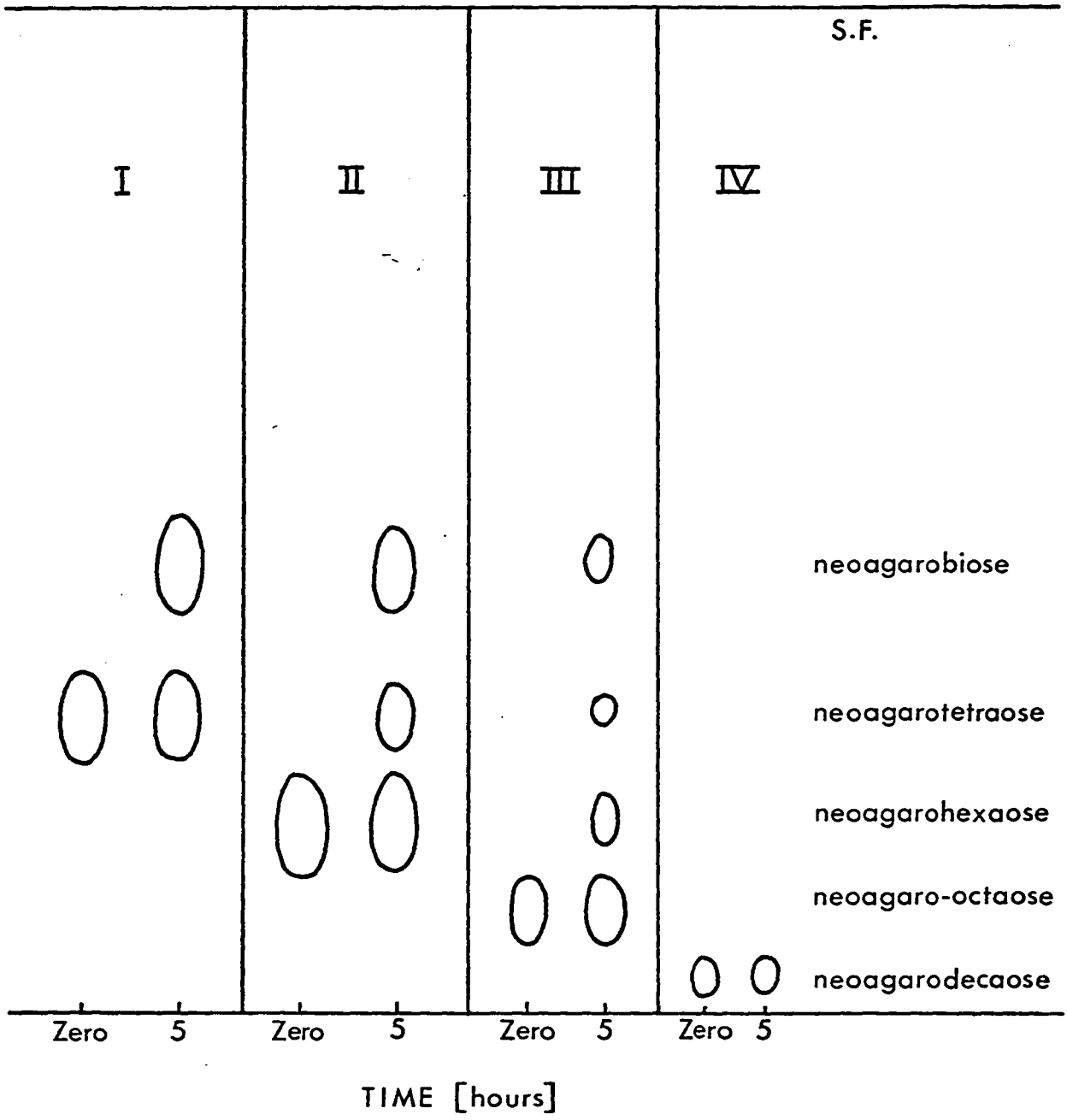


Figure 22. Thin layer chromatogram of the enzymic hydrolysates after 5 hours incubation with DEAE-purified enzyme. The substrates examined were neoagarotetraose (I), -hexaose (II), -octaose (III), and -decaose (IV). Solvent (a), Butan-1-ol-Ethanol-Water, 3:2:2 (v/v), was used.

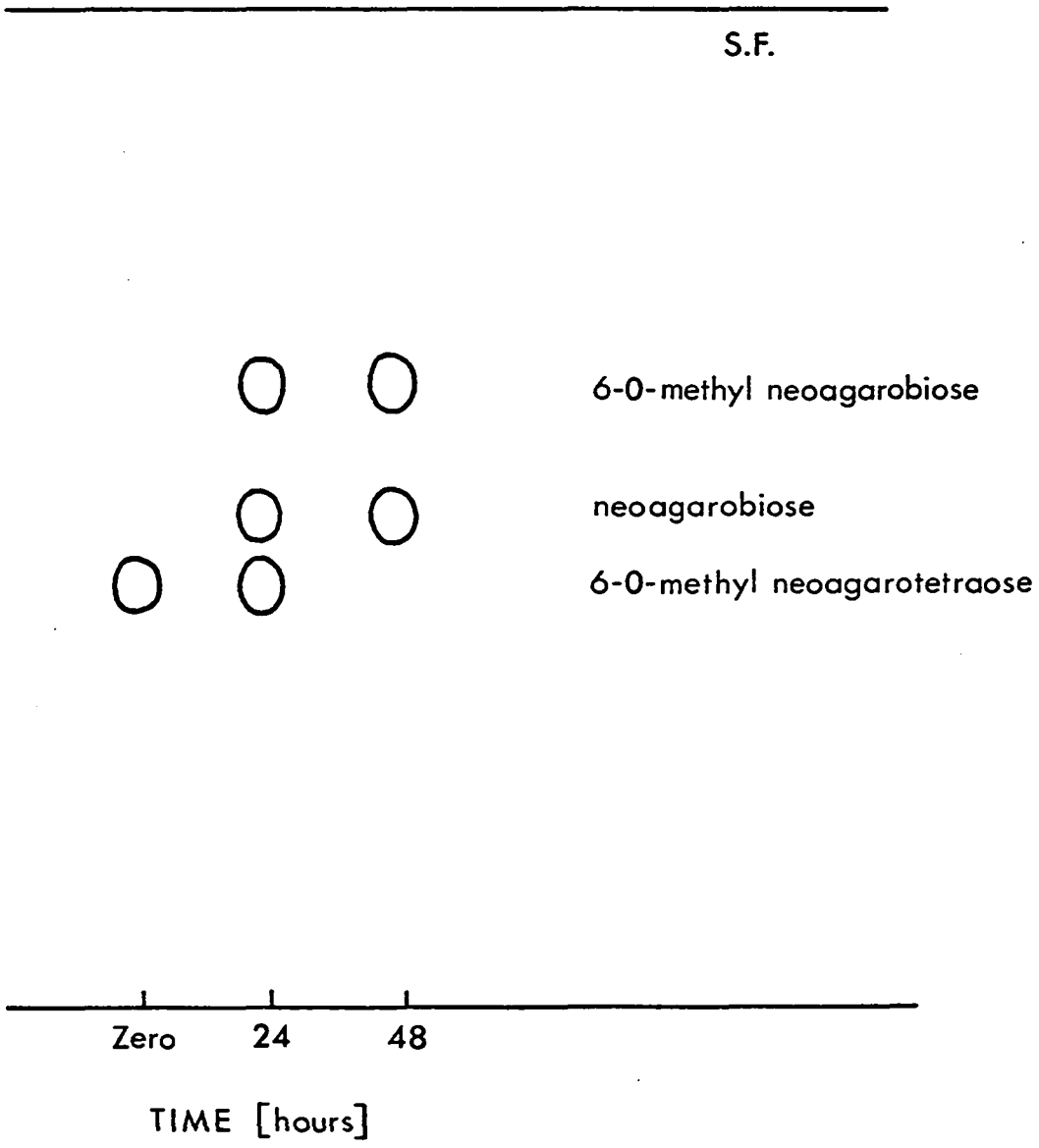


Figure 23. Examination of the products of enzymolysis of 6^3-O-methyl neoagarotetraose by cellulose thin layer chromatography, using double development in solvent (b), Butan-1-ol-Pyridine-Water, 2:1:1 (v/v).

V. DISCUSSION

Marine agar-digesting bacteria have been isolated in large numbers from three major habitats: sea water, bottom sediments, and on marine algae. Their importance as a group has been indicated in that they are not limited to the use of agar as nutrient, but have the potential to utilize a wide variety of other substances (Humm,1946). They can degrade the major cell wall materials of marine plants and animals, including cellulose, starch, and chitin, and such polysaccharide extracts of algae as agar, carrageenan, porphyran, and alginic acid (Yaphe, 1963; Colwell and Gochnauer, 1963; Girard et al., 1968). Mitchell and Nevo (1965) have isolated several strains of agarolytic microorganisms which degrade bacterial capsular and cell wall polysaccharides. Shilo (1967) reported the isolation of several agar-decomposers which have a lytic action against living blue-green algae. The agar-digesting bacteria can degrade complex polysaccharides natural to the marine environment, and therefore, have an important role in the transformation of organic matter in the sea.

A large and varied population of agar-digesting bacteria has been demonstrated in sea water and shore regions, representing approximately 10% of the total marine population. Many marine microbiologists have commented on the ecological significance of these agar-decomposing microorganisms in the cycle of matter in the ocean. Waksman and Bavendamm (1931) put forward a theory, based in part on the earlier work of H. and E. Pringsheim (1910), that a close relation exists in the ocean between algae, agar-digesting microorganisms, and the nitrogen-fixing bacteria. They suggested that the nitrogen-fixing bacteria utilize the

monosaccharides released by the decomposition of agar as a carbon and energy source, and thus supply both the agarolytic organisms and possibly the higher plants with available nitrogen. However, as pointed out by Humm (1946), this theory was based on observations of these bacteria in laboratory culture. All studies to date, with the exception of Shilo's work, have dealt with the degradation of algal extracts, which may not represent the natural state of these polysaccharide materials in the plant. The hydrolysis of agar is one aspect of bacterial metabolism which can be readily observed in the laboratory, but it may be remote from the actual functions of these organisms in nature. It is apparent that the role of the agar-digesting bacteria in the marine environment can only be evaluated by studying the actual degradation of natural algal material.

Extracellular agarases, which have been isolated from agar-decomposing bacteria, have been shown to hydrolyze the internal β-D-galactosidic linkages of agarose, releasing a homologous series of neoagarosaccharides. The degradation of agar by purified agarases results in the accumulation of neoagarotetraose in the enzyme hydrolysate. This study reports the isolation of a β-tetrasaccharidase which is capable of hydrolyzing neoagarotetraose and 6^3-O-methyl neoagarotetraose to the disaccharide components.

In nature, it is a common phenomenon that enzymes which degrade polymers are limited to a small number of microorganisms, while the ability to utilize the products of enzymolysis of the polymer is distributed amongst a wide range of bacteria. For example, Reese and Mandels (1964) have reported a limited distribution of mycodextranase activity in soil

fungi, while the presence of enzymes capable of hydrolyzing degraded mycodextran or mycodextran oligosaccharides was found to be more widespread. A similar situation may exist in the marine environment where microorganisms which do not possess agarase activity may have enzymes similar to the β-tetrasaccharidase reported here, and may be able to utilize the oligosaccharides released by the enzymolysis of various algal polysaccharides.

It was of importance in this study to determine the cellular location of the β-tetrasaccharidase from Pseudomonas atlantica. Osmotic shock treatment, which has been used to liberate periplasmic enzymes, is not suitable for Pseudomonas species, which are highly sensitive to the antibacterial action of EDTA. Asbell and Eagon (1966b) have proposed that the subunits of the cell wall lipopolysaccharide of P.aeruginosa are held together by divalent cation linkages, and that treatment with EDTA removes these bridging cations, resulting in cell lysis. The presence of sucrose does not stabilize the cell against this effect.

Evidence has been presented that Mg^{++} is the essential cation involved in cross-linkage of the lipopolysaccharide complex (Costerton et al., 1967). Sodium would not form cross-linkages but could serve to screen the negative charges involved in Mg^{++} binding sufficiently to prevent repulsion of the subunits and consequent envelope disintegration. De Voe and Oginsky (1969a) have presented evidence of competition between Na^{+} and Mg^{++} for anionic groups in the cell envelope which are exposed when the bridging cations are chelated by EDTA; and with increased NaCl concentration, the amount of cell lysis is decreased. The cell envelope remains intact during this treatment, however as reported by Leive (1965b),

and Wilkinson (1968), the action of EDTA may cause solubilization of lipopolysaccharide. Release of this material by EDTA-Tris-NaCl extraction of P.atlantica cells would account for the heavy precipitate which formed at the anode during isoelectric treatment of the crude enzyme solution. Recently, Winshell and Neu (1970) have correlated the release of surface enzymes from E.coli with the release of additional surface lipopolysaccharide after osmotic shock, following the initial solubilization of lipopolysaccharide during EDTA-Tris-sucrose exposure. Their results suggest that the release of large amounts of lipopolysaccharide (approximately 40% of the cell total) is necessary to free the enzymes from their surface binding sites.

The replacement of Mg^{++} by Na^{+} in the cell envelope results in a weakened structure which would be more permeable to the exit of proteins and other components located in the cell wall. The β-tetrasaccharidase is released by EDTA-Tris treatment, and in the presence of 1.5M NaCl, there is no leakage of intracellular α-disaccharidase, indicating that the cytoplasmic membrane is not damaged. Based on this evidence, it is postulated that the β-tetrasaccharidase enzyme from P.atlantica is, at least in part, a surface or cell wall located enzyme. It is possible that neoagarotetraose and similar tetrasaccharides diffuse into the cell wall of this organism where they are hydrolyzed by the β-tetrasaccharidase. The disaccharide molecules released would then be available at the cytoplasmic membrane for transport into the cell and degradation by the intracellular α-disaccharidase.

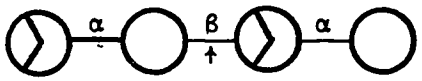
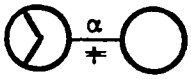
Further evidence for the surface location of the β-tetrasaccharidase was obtained by suspension of P.atlantica cells, which had been washed in

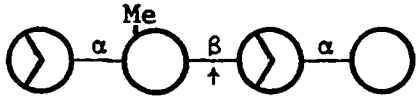
0.5M NaCl, in 0.5M sucrose. This treatment has been reported to cause the removal of the outer double tract layer of the cell wall of a marine pseudomonad (Costerton et al.,1967), and allow the escape of periplasmic enzymes (R.A.MacLeod, personal communication). This treatment resulted in the release of β-tetrasaccharidase from P.atlantica with no leakage of α-disaccharidase activity, and gave a further indication that the β-tetrasaccharidase has a surface location and the α-disaccharidase is an intracellular enzyme.

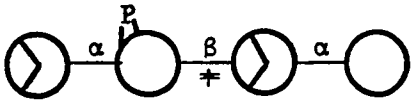
The data reported suggests a cell wall location for the β-tetrasaccharidase, however this theory is based primarily on indirect evidence. The α-disaccharidase has been used as a marker for leakage of intracellular material, but there is no absolute proof of the intracellular location of this enzyme. It is possible that the α-disaccharidase is also located in the cell wall or perhaps bound to the cytoplasmic membrane of P.atlantica. In further studies, an enzyme which is known to be located inside the cell should be used as a marker for cell lysis.

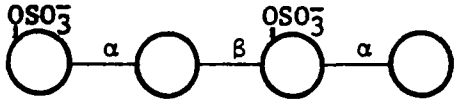
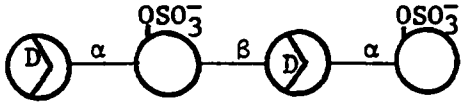
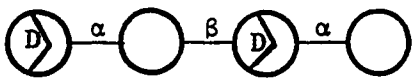
The cellular location of the enzymes involved in agar degradation by P.atlantica is not as yet positively established. The agarase enzyme is believed to be extracellular, however, the activity could not be completely removed from the cells even after six washings, indicating that the enzyme may be partially cell-bound. The β-tetrasaccharidase is found in a cell extract prepared by sonication of cells, and can also be obtained by a treatment which would allow release of cell wall located enzymes. Further studies are needed to determine whether all the β-tetrasaccharidase can be released from the cell by stripping off the outer double tract layer of the cell wall, or by protoplast formation. These experiments would also

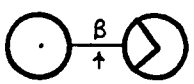
serve to verify the intracellular location of the α-disaccharidase.

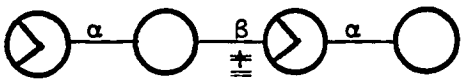
Preliminary experiments to characterize the β-tetrasaccharidase have been carried out on the crude and DEAE-purified enzyme preparations. The activity was found to be specific for the β-1,4 linkage of neoagarotetraose  , forming two molecules of disaccharide. The purified enzyme did not degrade the α-1,3 linkage of neoagarobiose  . The rate of hydrolysis of substrate by the β-tetrasaccharidase was found to decrease with increasing chain length. Neoagarohexaose was susceptible to hydrolysis, but the activity against higher members of the series of neoagarosaccharides was markedly decreased. This characteristic is common to most glycosidases, which do not act on the polymers, but hydrolyze the dimers, trimers, and tetramers produced by the action of polysaccharases (Reese, 1968). With the agarase, on the other hand, the rate of hydrolysis increases with chain length, with no activity against neoagarotetraose.

The effect of substitution on the rate of hydrolysis of neoagarotetraose by the β-tetrasaccharidase has been examined. Modification of the oligosaccharide substrate is known to reduce both the rate and extent of its hydrolysis by enzymes (Reese, 1968). Substitution of the sugar moiety at C_6 is generally less inhibitory than at other carbon atoms, and usually, the smaller the substituent the faster the hydrolysis. The degradation of 6^3-O-methyl neoagarotetraose, obtained from an enzyme hydrolysate of porphyran, was examined. Substitution by the methyl group had little effect on enzyme action, and this sugar was completely hydrolyzed to the disaccharides, 6-O-methyl neoagarobiose and neoagarobiose,  . However, substitution of the internal

galactose residue by a pyruvic acid ketal between carbon atoms 4 and 6 (see Figure 3, page 5) prevented enzymolysis of the β-1,4 linkage  (Duckworth and Yaphe, unpublished results).

The effect of other substituents on the rate of enzyme hydrolysis has not been determined. The enzymolysis products of agar include high molecular weight sulfated oligosaccharides (see Figure 2, page 5) which are resistant to degradation by the agarase from P.atlantica. Another enzyme is needed to degrade these highly charged sugars before breakdown of sulfated neoagarotetraose  by the β-tetrasaccharidase can be studied. Further studies are also required to determine whether the β-tetrasaccharidase is active against the products of enzymolysis of carrageenan, such as neocarratetraose sulfate  (Weigl and Yaphe, 1966a). It is conceivable that this tetrasaccharide could be desulfated by the glycosulfatase of P.carrageenovora (Weigl and Yaphe, 1966b), to yield neocarratetraose  . It would be of interest to determine whether the β-tetrasaccharidase can hydrolyze neocarratetraose, which differs from neoagarotetraose only by the replacement of 3,6-anhydro-L-galactose by the D-isomer.

The requirements for the active site of the β-tetrasaccharidase could be estimated by studying the enzymolysis of agarobiose. The β-1,4 linkage of this disaccharide  can be hydrolyzed by emulsin (Araki, 1958), however, a similar enzyme preparation does not cause

degradation of neoagarotetraose  (Yaphe, unpublished results. Reese (1968) has proposed that the enzyme site is a spacial image of the dominant product of hydrolysis; thus, the β-tetrasaccharidase may be specific for the neoagarobiose moiety and, if so, would not cause hydrolysis of agarobiose.

In conclusion, the isolation of the β-tetrasaccharidase from Pseudomonas atlantica has provided further information into the mechanism by which this organism can degrade and utilize agar. A purification scheme has been deduced to separate the β-tetrasaccharidase from agarase and α-disaccharidase activities, and some specificity characteristics of the purified enzyme have been established.

VI. SUMMARY

A method has been devised for the preparation of a β-tetrasaccharidase, which hydrolyzes the β-1,4 linkage of neoagarotetraose, from cells of Pseudomonas atlantica. The enzyme can be obtained by a simple isolation procedure involving EDTA-Tris-NaCl extraction of washed cells. The evidence presented suggests a surface or cell wall location for the β-tetrasaccharidase.

The crude enzyme preparation is free from α-disaccharidase but is contaminated with agarase activity. β-Tetrasaccharidase and agarase were separated by isoelectric fractionation, and the pI values of the enzymes were found to be 5.6-6.1 and 6.6-8.1 respectively. The difference in the isoelectric points has been used to deduce a method for the separation of these two enzymes on DEAE-Sephadex A-50.

Specificity studies have shown that the β-tetrasaccharidase hydrolyzes the β-1,4 linkage of neoagarotetraose, with decreased activity on neoagarohexaose and neoagaro-octaose. Higher molecular weight neoagarosaccharides are not degraded. 6^3-O-methyl neoagarotetraose is hydrolyzed by the β-tetrasaccharidase, but substitution of the internal galactose residue by the pyruvic acid ketal, 4,6-O-(1-carboxyethylidene)-D-galactose, prevents enzyme action.

APPENDIX

A. MEDIA

1. Zobell Medium 2216E

Proteose peptone	5.0 g
Yeast extract	2.5 g
Na_2HPO_4	0.1 g
$FeSO_4 \cdot 7H_2O$	10 ml of a 0.2% solution
Sea water to	1000 ml

Solid media is prepared by dissolving 15 g of Bacto-agar in 1000 ml of media. The pH is adjusted to between 6.8 and 7.2 with 0.1N NaOH. Sterilize at 15 pounds pressure (121°) for 20 minutes.

2. H-1 Mineral Salts Medium

NaCl	75 g
K_2HPO_4	0.3 g
$MgSO_4 \cdot 7H_2O$	15 g
KCl	3 g
$CaCl_2$	0.6 g
Casamino acids	7.5 g
$FeSO_4 \cdot 7H_2O$	20 ml of a 0.3% solution
Tris buffer (pH 7)	150 ml of a 0.2M solution
Distilled water to	3000 ml

Solid media is prepared by adding 20 g of Bacto-agar per liter of media. Semi-solid media is prepared by adding 1 g of Bacto-agar per liter of media. The medium is adjusted to pH 7.2 with 0.1N NaOH, and is dispensed

in 500 ml Erlenmeyer flasks or Roux bottles, 200 ml per flask. Sterilize at 15 pounds pressure (121°) for 20 minutes.

B. INDICATOR REAGENTS

1. Naphthoresorcinol Reagent

Solution I.

Ethanol	375 ml
Conc. H_2SO_4	100 ml

Solution II.

Naphthoresorcinol	0.1 g
Ethanol	50 ml

Store Solution I at room temperature, Solution II at 4°. Before use, two parts of Solution I are mixed with one part of Solution II.

2. Aniline Hydrogen Phthalate Reagent

Aniline hydrogen phthalate	1 g
Butan-1-ol	5 ml
Distilled water	95 ml

C. SOLUTIONS

1. MacLeod's Complete Salts (De Voe et al., 1970)

NaCl (0.3M)	17.6 g
$MgSO_4 \cdot 7H_2O$ (0.05M)	12.3 g
KCl (0.01M)	0.75 g
Distilled water to	1000 ml

2. H-1 Mineral Salts Solution

NaCl	25 g
K_2HPO_4	0.1 g
$MgSO_4 \cdot 7H_2O$	5 g
KCl	1 g
$CaCl_2$	0.2 g
$FeSO_4 \cdot 7H_2O$	10 ml of a 0.2% solution
Tris buffer (pH 7)	50 ml of a 0.2M solution
Distilled water to	1000 ml

Adjust the pH to 7.2 with 0.1N NaOH and store at 4°.

3. I-KI Solution

Iodine	50 g
KI	100 g
Distilled water	950 ml

This solution is diluted 1:5 with distilled water and stored in the dark.

4. Somogyi Reagents for Analysis of Reducing Sugars

Solution I.

Na_2CO_3	72 g
$NaHCO_3$	48 g
Sodium potassium tartrate	36 g
Na_2SO_4	432 g
Distilled water (CO_2-free)	1800 ml

Dilute to 2400 ml with CO_2-free (boiled), distilled water. After cooling, the solution is stoppered and left to stand overnight at room temperature. The reagent is filtered into, and stored in, an aspirator bottle with an Ascarite vent tube.

Solution II.

$CuSO_4 \cdot 5H_2O$	12 g
Na_2SO_4	108 g
Distilled water (CO_2-free)	400 ml

Dilute to 600 ml with CO_2-free (boiled), distilled water. After cooling, the solution is allowed to stand overnight and then filtered into a glass-stoppered bottle. Store in the dark.

Working Solution.

Four volumes of Solution I plus one volume of Solution II are mixed immediately prior to use.

5. Nelson's Arsenomolybdate Reagent

Ammonium molybdate	25 g
Distilled water	450 ml
Conc. H_2SO_4	21 ml
Sodium arsenate	3 g dissolved in 25 ml of distilled water

The components are dissolved in the above order, and the solution is incubated for 24-48 hours at 37°. Store in a brown bottle at 4° in the dark.

6. Folin-Ciocalteau Phenol Reagent for Protein Determination

Solution I.

Na_2CO_3	20 g
Sodium potassium tartrate	0.2 g
Distilled water	1000 ml

Solution II.

$CuSO_4 \cdot 5H_2O$	0.5 g
Distilled water	100 ml

Store solutions at room temperature, Solution II in the dark.

Solutions I and II are mixed (50 parts I plus one part II) before using, to form the copper carbonate reagent.

Folin-Ciocalteau's phenol reagent is diluted 5 in 12 with distilled water, immediately prior to use.

BIBLIOGRAPHY

ANDERSON, N.S., Dolan, T.C.S., and Rees, D.A.: 1965. Evidence for a common structural pattern in the polysaccharide sulfates of the Rhodophyceae. Nature 205: 1060-1062.

ANDERSON, N.S. and Rees, D.A.: 1965. Porphyran: A polysaccharide with a masked repeating structure. J. Chem. Soc.(1965) 5880-5887.

ANGST, E.C.: 1929. Some new agar-digesting bacteria. Publ. Puget Sound Biol. Sta. 7: 49-63.

ANRAKU, Y. and Heppel, L.A.: 1967. On the nature of the changes induced in Escherichia coli by osmotic shock. J. Biol. Chem. 242: 2561-2569.

ARAKI, C.: 1937. Acetylation of the agar-like substance of Gelidium amansii. J. Chem. Soc. Japan 58: 1338-1350.

ARAKI, C.: 1944. Studies on the chemical constitution of agar-agar. XIII. Chemical constitution of agarobiose. J. Chem. Soc. Japan 65: 627-632.

ARAKI, C.: 1956. Structure of the agarose constituent of agar-agar. Bull. Chem. Soc. Japan 29: 543-544.

ARAKI, C.: 1958. Seaweed polysaccharides. In Proc. Fourth Intern. Congress of Biochemistry, Vienna, Volume I, pp. 15-30, (M.L. Walfrom, Ed.). Pergamon Press, London.

ARAKI, C.: 1966. Some recent studies on the polysaccharides of agarophytes. In Proc. Fifth Intern. Seaweed Symp., pp. 3-17, (E.G. Young and J.L. McLachlan, Ed.). Pergamon Press, London.

ARAKI, C. and Arai, K.: 1954. Studies on the agar-digesting bacteria. The isolation of agar-digesting bacteria and their enzymatic activities. Memoirs of the Faculty of Industrial Arts, Kyoto Technical Univ. 3B: 7-23.

ARAKI, C. and Arai, K.: 1956. Studies on the chemical constitution of agar-agar. XVIII. Isolation of a new crystalline disaccharide by enzymatic hydrolysis of agar-agar. Bull. Chem. Soc. Japan 29: 339-345.

ARAKI, C. and Arai, K.: 1957. Studies on the chemical constitution of agar-agar. XX. Isolation of a tetrasaccharide by enzymatic hydrolysis of agar-agar. Bull. Chem. Soc. Japan 30: 287-293.

ASBELL, M.A. and Eagon, R.G.: 1966a. The role of multivalent cations in the organization and structure of bacterial cell walls. Biochem. Biophys. Res. Commun. 22: 664-671.

ASBELL, M.A. and Eagon, R.G.: 1966b. Role of multivalent cations in the organization, structure, and assembly of the cell wall of Pseudomonas aeruginosa. J. Bact. 92: 380-387.

BERGER, P.D. and Agate, A.S.: 1969. Thin-layer chromatography of simple sugars in the presence of large quantities of sucrose. J. Chromatography 39: 232-234.

BROCKMAN, R.W. and Heppel, L.A.: 1968. On the localization of alkaline phosphatase and cyclic phosphodiesterase in Escherichia coli. Biochem. 7: 2554-2562.

BUCHANAN, J., Percival, E.E., and Percival, E.G.V.: 1943. Polysaccharide of carrageen moss. J. Chem. Soc. (1943) 51-54.

CASHEL, M. and Freese, E.: 1964. Excretion of alkaline phosphatase by Bacillus subtilis. Biochem. Biophys. Res. Commun. 16: 541-544.

CHESBRO, W.R. and Lampen, J.O.: 1968. Characteristics of secretion of penicillinase, alkaline phosphatase, and nuclease by Bacillus species. J. Bact. 96: 428-437.

COLES, N.W. and Gross, R.: 1967a. Liberation of surface-located penicillinase from Staphylococcus aureus. Biochem. J. 102: 742-747.

COLES, N.W. and Gross, R.: 1967b. Influence of organic anions on the liberation of penicillinase from Staphylococcus aureus. Biochem. J. 102: 748-752.

COLWELL, R.R. and Gochnauer, M.B.: 1963. The taxonomy of marine bacteria. Bacteriol. Proc.(1963) p.40.

COSTERTON, J.W.: 1970. The structure and function of the cell envelope of gram-negative bacteria. Revue Canadienne de Biologie (in the press).

COSTERTON, J.W., Forsberg, C., Matula, T.I., Buchmire, F.L.A., and MacLeod, R.A.: 1967. Nutrition and metabolism of marine bacteria: XVI. Formation of protoplasts, spheroplasts, and related forms from a gram-negative marine bacterium. J. Bact. 94: 1764-1777.

COX, S.T. and Eagon, R.G.: 1968. Action of ethylenediaminetetraacetic acid, tris(hydroxymethyl)-aminomethane, and lysozyme on cell walls of Pseudomonas aeruginosa. Can. J. Microbiol. 14: 913-922.

DE VOE, I.W. and Oginsky, E.L.: 1969a. Antagonistic effect of monovalent cations in maintenance of cellular integrity of a marine bacterium. J. Bact. 98: 1355-1367.

DE VOE, I.W. and Oginsky, E.L.: 1969b. Cation interactions and biochemical composition of the cell envelope of a marine bacterium. J. Bact. 98: 1368-1377.

DE VOE, I.W., Thompson, J., Costerton, J.W., and MacLeod, R.A.: 1970. Stability and comparative transport capacity of cells, mureinoplasts, and true protoplasts of a gram-negative bacterium. J. Bact. 101: 1014-1026.

DIXON, M.: 1953. A nomogram for ammonium sulfate solutions. Biochem. J. 54: 457-458.

DOLAN, T.C.S.: 1965. Ph.D. Thesis, Edinburgh.

DUCKWORTH, M. and Turvey, J.R.: 1969a. An extracellular agarase from Cytophaga species. Biochem. J. 113: 139-142.

DUCKWORTH, M. and Turvey, J.R.: 1969b. The action of a bacterial agarase on agarose, porphyran, and alkali-treated porphyran. Biochem.J. 113: 687-692.

DUCKWORTH, M. and Turvey, J.R.: 1969c. The specificity of an agarase from a Cytophaga species. Biochem. J. 113: 693-696.

DUCKWORTH, M. and Yaphe, W.: 1970a. Structural analysis of agaropectin with an agarase from Pseudomonas atlantica. Bacteriol. Proc. (1970) p. 24.

DUCKWORTH, M. and Yaphe, W.: 1970b. Thin layer chromatographic analysis of enzymic hydrolysates of agar. J. Chromatography (in the press).

EAGON, R.G. and Carson, K.J.: 1965. Lysis of cell walls and intact cells of Pseudomonas aeruginosa by ethylenediamine tetraacetic acid and by lysozyme. Can. J. Microbiol. 11: 193-201.

GIRARD, A.E., Buck, J.D., and Cosenza, B.J.: 1968. A nutritional study of some agarolytic marine bacteria. Can. J. Microbiol. 14: 1193-1198.

GOLDSCHMIDT, M.C. and Wyss, O.: 1967. The role of Tris in EDTA toxicity and lysozyme lysis. J. gen. Microbiol. 47: 421-431.

GORESLINE, H.E.: 1933. Studies on agar-digesting bacteria. J. Bacteriol. 26: 435-457.

GRAN, H.H.: 1902. Studien über Meeresbakterien. II. Über die Hydrolyse des Agar-Agars durch ein neves Enzym, die Gelase. Bergens Museums Aarbog 2: 1-16.

GRAY, G.W. and Wilkinson, S.G.: 1965a. The action of ethylenediaminetetra-acetic acid on Pseudomonas aeruginosa. J. Appl. Bacteriol. 28: 153-164.

GRAY, G.W. and Wilkinson, S.G.: 1965b. The effect of ethylenediaminetetra-acetic acid on the cell walls of some gram-negative bacteria. J. gen. Microbiol. 39: 385-399.

HAMMERSTROM, R.A., Claus, K.D., Coghlan, J.W., and McBee, R.H.: 1955. The constitutive nature of bacterial cellulases. Arch. Biochem. Biophys. 56: 123-136.

HEPPEL, L.A.: 1967. Selective release of enzymes from bacteria. Science 156: 1451-1455.

HICKSON, T.G.L. and Polson, A.: 1968. Some physical characteristics of the agarose molecule. Biochim. Biophys. Acta. 165: 43-58.

HIRASE, S.: 1957. Studies on the chemical constitution of agar-agar. XIX. Pyruvic acid as a constituent of agar-agar. Bull. Chem. Soc. Japan 30: 68-70.

HIRASE, S. and Araki, C.: 1961. Isolation of 6-O-methyl-D-galactose from the agar of Ceramium boydenii. Bull. Chem. Soc. Japan 34: 1048.

HONG, K.C., Goldstein, M.E., and Yaphe, W.: 1970. A chemical and enzymic analysis of the polysaccharides from Gracilaria. In Proc. Sixth Intern. Seaweed Symp., pp. 473-482, (R. Margalef, Ed.). Subsecretaria de la Marina Mercante, Madrid.

HUMM, H.J.: 1946. Marine agar-digesting bacteria of the south atlantic coast. Bull. Duke Univ. Marine Sta. 3: 43-75.

ISHIMATSU, K., Kibesaki, Y., and Maitani, S.: 1954. Agar-liquefying bacteria. XIV. Properties of reducing sugars formed from agar by agarase. Science and Industry (Japan) 28: 100-103.

KUSHNAREV, V.M. and Smirnova, T.A.: 1966. Electron microscopy of alkaline phosphatase of Escherichia coli. Can. J. Microbiol. 12: 605-608.

LAMPEN, J.O.: 1965. Secretion of enzymes by microorganisms. In Function and Structure in Microorganisms. 15th Symposium of the Society for General Microbiology, pp. 115-133, (M.R. Pollock and M.H. Richmond, Ed.). Cambridge University Press, London.

LAMPEN, J.O.: 1967. Release of penicillinase by Bacillus licheniformis. J. gen. Microbiol. 48: 261-268.

LEIVE, L.: 1965a. A nonspecific increase in permeability in Escherichia coli produced by EDTA. Proc. Natl. Acad. Sci. 53: 745-750.

LEIVE, L.: 1965b. Release of lipopolysaccharide by EDTA treatment of Escherichia coli. Biochem. Biophys. Res. Commun. 21: 290-296.

LEIVE, L.: 1968. Studies on the permeability change produced in coliform bacteria by ethylenediaminetetraacetate. J. Biol. Chem. 243: 2373-2380.

LEVY, S.B. and Leive, L.: 1970. Release from Escherichia coli of a galactosyltransferase complex active in lipopolysaccharide synthesis. J. Biol. Chem. 245: 585-594.

MITCHELL, P.: 1961. Approaches to the analysis of specific membrane transport. In Biological Structure and Function, Volume II, pp. 581-603, (I.W. Goodwin and O. Lindberg, Ed.). Academic Press, New York.

MITCHELL, R. and Nevo, Z.: 1965. Decomposition of structural polysaccharides of bacteria by marine microorganisms. Nature 205: 1007-1008.

NELSON, N.: 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153: 375-380.

NEU, H.C.: 1967a. The 5'-nucleotidase of Escherichia coli. I. Purification and properties. J. Biol. Chem. 242: 3896-3904.

NEU, H.C.: 1967b. The 5'-nucleotidase of Escherichia coli. II. Surface localization and purification of the Escherichia coli 5'-nucleotidase inhibitor. J. Biol. Chem. 242: 3905-3911.

NEU, H.C.: 1969. The role of amine buffers in EDTA toxicity and their effect on osmotic shock. J. gen. Microbiol. 57: 215-220.

NEU, H.C., Ashman, D.F., and Price, T.D.: 1967. Effect of ethylenediaminetetra-acetic acid-tris(hydroxy)aminomethane on release of the acid-soluble nucleotide pool and on breakdown of ribosomal RNA in Escherichia coli. J. Bact. 93: 1360-1368.

NEU, H.C. and Chou, J.: 1967. Release of surface enzymes in Enterobacteriaceae by osmotic shock. J. Bact. 94: 1934-1945.

NEU, H.C. and Heppel, L.A.: 1964. The release of ribonuclease into the medium when Escherichia coli cells are converted to sphaeroplasts. Biochem. Biophys. Res. Commun. 14: 109-112.

NEU, H.C. and Heppel, L.A.: 1965. The release of enzymes from Escherichia coli by osmotic shock and during the formation of sphaeroplasts. J. Biol. Chem. 240: 3685-3692.

NISONSON, I., Tannenbaum, M., and Neu, H.C.: 1969. Surface localization of Escherichia coli 5'-nucleotidase by electron microscopy. J. Bact. 100: 1083-1090.

NOSSAL, N.G. and Heppel, L.A.: 1966. The release of enzymes by osmotic shock from Escherichia coli in exponential phase. J. Biol. Chem. 241: 3055-3062.

NOVICK, R.P.: 1963. Analysis by transduction of mutations affecting penicillinase formation in Staphylococcus aureus. J. gen. Microbiol. 33: 121-136.

O'NEILL, A.N.: 1955. Derivatives of 4-O-β-D-galactopyranosyl-3,6-anhydro-D-galactose from κ-carrageenin. J. Am. chem. Soc. 77: 6324-6326.

OPPENHEIMER, C.H. and Zobell, C.E.: 1952. The growth and viability of sixty-three species of marine bacteria as influenced by hydrostatic pressure. J. Marine Res. 11: 10-18.

OYAMA, V.I. and Eagle, H.: 1956. Measurement of cell growth in tissue culture with a phenol reagent (Folin-Ciocalteau). Proc. Soc. Exptl. Biol. Med. 91: 305-307.

PERCIVAL, E.E.: 1954. The mercaptolysis of the polysaccharide from Chondrus crispus. Chemy. Ind.(1954) p. 1487.

PERCIVAL, E. and McDowell, R.H.: 1967. Chemistry and Enzymology of Marine Algal Polysaccharides, pp. 1-4. Academic Press, London.

POGELL, B.M.: 1966. Enzyme purification by specific elution procedures with substrate. In Methods in Enzymology, Volume IX. pp. 9-15, (W.A. Wood, Ed.). Academic Press, New York.

POLLOCK, M.R.: 1962. Exoenzymes. In The Bacteria, Volume IV, pp. 121-178, (I.C. Gunsalus and R.Y. Stanier, Ed). Academic Press, New York.

PRINGSHEIM, E. and Pringsheim, H.: 1910. Über die Verwendung von Agar-agar als Energiequelle zur Assimilation des Luftstickstoffs. Centrbl. Bakt. II, 26: 227-231.

REES, D.A.: 1970. Structure, conformation, and mechanism in the formation of polysaccharide gels and networks. Adv. in Carbohydrate Chemistry (in the press).

REES, D.A. and Conway, Elsie: 1962. The structure and biosynthesis of porphyran: A comparison of some samples. Biochem. J. 84: 411-416.

REESE, E.T.: 1968. Microbial transformation of soil polysaccharides. Pontificiae Academiae Scientiarum Scripta Varia 32: 535-582.

REESE, E.T. and Mandels, Mary: 1964. A new α-glucanase: Mycodextranase. Can. J. Microbiol. 10: 103-114.

REPASKE, R.: 1958. Lysis of gram-negative organisms and the role of versene. Biochim. Biophys. Acta. 30: 225-232.

RUSSELL, B., Mead, T.H., and Polson, A.L.: 1964. A method of preparing agarose. Biochim. Biophys. Acta. 86: 169-174.

SCHLESINGER, M.J.: 1968. Secretion of alkaline phosphatase subunits by sphaeroplasts of Escherichia coli. J. Bact. 96: 727-733.

SHILO, M.: 1967. Formation and mode of action of algal toxins. Bacteriol. Rev. 31: 180-193.

SMITH, D.B. and Cook, W.H.: 1953. Fractionation of carrageenin. Arch. Biochem. and Biophys. 45: 232-233.

SOMOGYI, M.: 1952. Notes on sugar determination. J. Biol. Chem. 195: 19-23.

STANIER, R.Y.: 1941. Studies on marine agar-digesting bacteria. J. Bact. 42: 527-559.

STANIER, R.Y.: 1942. The Cytophaga group: A contribution to the biology of Myxobacteria. Bacteriol. Rev. 6: 143-196.

SVENSSON, H.: 1961. Isoelectric fractionation, analysis and characterization of ampholytes in natural pH gradients. I. The differential equation of solute concentrations at a steady state and its solution for simple cases. Acta. Chem. Scand. 15: 325-341.

SVENSSON, H.: 1962a. Isoelectric fractionation, analysis and characterization of ampholytes in natural pH gradients. II. Buffering capacity and conductance of isoionic ampholytes. Acta. Chem. Scand. 16: 456-466.

SVENSSON, H.: 1962b. Isoelectric fractionation, analysis and characterization of ampholytes in natural pH gradients. III. Description of apparatus for electrolysis in columns stabilized by density gradients and direct determination of isoelectric points. Arch. Biochem. Biophys., Suppl.1, 132-138.

SWARTZ, M.N. and Gordon, N.: 1959. Agarase from an agar-digesting bacterium. J. Bact. 77: 403-409.

TORRIANI, A.: 1968. Alkaline phosphatase subunits and their dimerization in vivo. J. Bact. 96: 1200-1207.

TSENG, C.K.: 1945. The terminology of seaweed colloids. Science 101: 597-602.

TURVEY, J.R. and Christison, J.: 1967a. The hydrolysis of algal galactans by enzymes from a Cytophaga species. Biochem. J. 105: 311-316.

TURVEY, J.R. and Christison, J.: 1967b. The enzymic degradation of porphyran. Biochem. J. 105: 317-321.

TURVEY, J.R. and Rees, D.A.: 1961. Isolation of L-galactose-6-sulfate from a seaweed polysaccharide. Nature, Lond. 189: 831-832.

TURVEY, J.R. and Williams, T.P.: 1964. Sugar sulfates from the mucilage of Porphyra umbilicalis. In Proc. Fourth Intern. Seaweed Symp. Biarritz, pp. 370-373. Pergamon Press, London.

VESTERBERG, O. and Svensson, H.: 1966. Isoelectric fractionation, analysis and characterization of ampholytes in natural pH gradients. IV. Further studies on the resolving power in connection with separation of myoglobins. Acta. Chem. Scand. 20: 820-834.

VOSS, J.G.: 1967. Effects of organic cations on the gram-negative cell wall and their bactericidal activity with ethylenediaminetetra-acetate and surface active agents. J. gen. Microbiol. 48: 391-400.

WAKSMAN, S.A. and Bavendamm, W.: 1931. On the decomposition of agar-agar by an aerobic bacterium. J. Bact. 22: 91-102.

WARBURG, O. and Christian, W.: 1941. Isolierung und Kristallisation des Gärungsferments Enolase. Biochem. Z. 310: 384-421.

WEIGL, J., Turvey, J.R., and Yaphe, W.: 1966. The enzymic hydrolysis of κ-carrageenan with κ-carrageenase from Pseudomonas carrageenovora. In Proc. Fifth Intern. Seaweed Symp., pp. 329-332, (E.G.Young and J.L. McLachlan, Ed.). Pergamon Press, London.

WEIGL, J. and Yaphe, W.: 1966a. The enzymic hydrolysis of carrageenan by Pseudomonas carrageenovora: Purification of a κ-carrageenase. Can. J. Microbiol. 12: 939-947.

WEIGL, J. and Yaphe, W.: 1966b. Glycosulfatase of Pseudomonas carrageenovora: Desulfation of disaccharide from κ-carrageenan. Can. J. Microbiol. 12: 874-876.

WILKINSON, S.G.: 1967. The sensitivity of pseudomonads to ethylene-diaminetetra-acetic acid. J. gen. Microbiol. 47: 67-76.

WILKINSON, S.G.: 1968. Studies on the cell walls of Pseudomonas species resistant to ethylenediaminetetra-acetic acid. J. gen. Microbiol. 54: 195-213.

WINSHELL, E.B. and Neu, H.C.: 1970. Relation of lipopolysaccharide and fatty ester release to the ethylenediaminetetraacetic acid alteration of permeability in Enterobacteriaceae. J. Bact. 102: 537-539.

YAPHE, W.: 1957. The use of agarase from Pseudomonas atlantica in the identification of agar in marine algae (Rhodophyceae). Can. J. Microbiol. 3: 987-993.

YAPHE, W.: 1959. The determination of κ-carrageenin as a factor in the classification of the Rhodophyceae. Can. J. Botany 37: 751-757.

YAPHE, W.: 1963. Proposals on the classification of microorganisms which utilize the polysaccharides of marine algae and a definition for agar. In Symp. on Marine Microbiology, pp. 588-593, (C.H. Oppenheimer, Ed.). C.C.Thomas Publishers, Illinois.

YAPHE, W.: 1966. The purification and properties of an agarase from a marine bacterium, Pseudomonas atlantica. In Proc. Fifth Intern. Seaweed Symp., pp. 333-335, (E.G. Young and J.L. McLachlan, Ed.). Pergamon Press, London.

YAPHE, W. and Baxter, Beverly: 1955. The enzymic hydrolysis of carrageenin. Appl. Microbiol. 3: 380-383.