AN ENZYMATIC AND CHEMICAL STUDY OF AGAR

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by

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ABSTRACT

Agars from five major groups of agarophytes (Rhodophyceae) have been fractionated on DEAE Sephadex A-50 (Cl^-). The results indicate that agar is a complex mixture of polysaccharides all having the same backbone structure but masked to a variable degree with charged groups, ranging from the essentially neutral agarose to highly sulfated galactans. The agarases which degrade agarose have been isolated from marine and soil bacteria. The mode of action of these agarases and the end products of the hydrolysis have been investigated. Purified bacterial agarases can be classified into two major groups, being specific for either the β linkages, P. atlantica and strain 3A agarases; or α linkages, strain GJ1B agarase, in agarose. A proposal has been made for a practical definition of agar and agarose.

RESUME

L'agar de cinq importants groupes d'agarophytes (Rhodophyceae) a été fractionné sur DEAE Sephadex A-50 (Cl^-). Les résultats obtenus indiquent que l'agar est un mélange complexe de polysaccharides qui possèdent tous la même structure de base masquée à des degrés différents par des groupements chargés; lesquels peuvent varier de degrés essentiellement neutres, comme dans le cas de l'agarose, à des degrés hautement chargés, comme dans le cas des galactanes sulfatés. Des agarases qui dégradent l'agarose ont été isolées des bactéries du sol et de la mer. Les modes d'action de ces agarases et les produits de leur hydrolyse ont été recherchés. Les agarases bactériennes purifiées peuvent être classifiées en deux groupes selon leur activité. Un premier groupe d'agarases est spécifique pour le lien β de l'agarose et provient des bactéries P. atlantica et 3A. Dans un deuxième groupe l'on retrouve l'agarase isolée de la bactérie GJ1B qui attaque les liens α de l'agarose. Finalement l'on suggère une définition pratique de l'agar et de l'agarose.

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I. INTRODUCTION

Agar, a complex mixture of polysaccharides, is extracted from certain species of red algae (Rhodophyceae) known as the agarophytes. It was originally separated into two components, agarose and agaropectin (Araki, 1937a). Agarose, a neutral polysaccharide, "is composed of 1,3-linked β-D-galactopyranose and 1,4-linked 3,6-anhydro-α-L-galactopyranose, which are repeated in an alternate way" (Araki, 1956) and agaropectin "is a more complicated polysaccharide containing uronic acid and sulfuric acid as well as D-galactose and 3,6-anhydro-L-galactose as constituents" (Araki, 1956). Recent studies on the structure of agar (Duckworth and Yaphe, 1971a) have revealed that agar is a spectrum of polysaccharides ranging from an essentially neutral agarose to a highly sulfated galactan; the basic structure being modified and substituted to varying degrees with sulfate and pyruvic acid.

The degradation of agar by microorganisms was first observed by Gran (1902) with an extracellular fluid of a Bacillus strain. The enzyme responsible for the degradation of agar was not purified. Araki and Arai (1956; 1957) isolated an unpurified bacterial agarase (β-agarase) capable of hydrolyzing the β-1,4-glycosidic linkages of agarose, yielding a homologous neoagarobiose series of oligosaccharides, thus confirming his earlier results on the structure of agarose as obtained by chemical analysis. Later similar bacterial β-agarases

were reported by Yaphe (1957), and Duckworth and Turvey (1969a) from various microorganisms.

Agar has been used widely in the food processing industry, as well as in biochemical research. Due to the lack of standards for agar and the incomplete knowledge of its chemical and physical properties, many non-reproducible results have appeared in the literature. In order to establish standards for agar, the fine structure of agar must be investigated. Studies on the fine structure of agar by means of chemical analysis is inadequate and laborious. Enzymolysis has proved to be a useful tool in the structural characterization of agar.

In this study, an α-agarase has been isolated, and the mode of action of the agarases has been studied. A combination of the α- and β-agarases has been used in the structural characterization of agar, in order to establish an example upon which standards for agar for biochemical use may be based. Agars obtained from several species of agarophytes have also been investigated.

II. LITERATURE REVIEW

Algae are the predominant vegetation in the sea, having great diversity, ranging from the free floating type, most of which are unicellular algae, to giant seaweeds. The economic importance of the unicellular algae is mainly indirect, as they form the first stage in the food cycle of life in the sea. The larger species, however, which are found on the shores in the continental shelf area of many countries, have been used for centuries as food for humans and animals, as manure, or as a source of chemicals. The extensive use of the algae and their products has led to investigation of their habitat, morphology and composition.

The predominant algal species in any area depends on the shores, which have a considerable tidal range. The intertidal areas are occupied mainly by green seaweeds and members of the order Fucales of the brown seaweeds. Other species of brown seaweeds are predominant in low tide areas to a depth of ten to twenty meters. With increasing depth, few plants are present, mainly the red species. An important factor in the zoning of algae with depth is the change in spectral distribution of light penetrating the water; those algae with suitable pigments for making use of light are most abundant.

The botanical classification of the seaweeds is based primarily on their morphology, particularly in respect of the reproductive systems. Division

into classes has been aided by the type of pigmentation present. All the seaweeds contain chlorophyll a, but the color of the chlorophyll in some classes is masked strongly by other typical pigments. The Phaeophyceae (brown seaweeds), Chlorophyceae (green seaweeds) and the Rhodophyceae (red seaweeds) are the three major classes. Like the characteristic pigmentation, each class may have a particular type of polysaccharide, although the fine structure may vary from species to species (Percival and McDowell, 1967).

This thesis is concerned with the degradation by microorganisms of agar, one of the major families of polysaccharides found in the Rhodophyceae.

A. GALACTANS OF THE RHODOPHYCEAE

The majority of the water-soluble polysaccharides of the Rhodophyceae contain galactose as the D- or as the D- and L-enantiomorphs, with the occurrence of the same basic structure throughout the polysaccharides, i.e., a chain of galactose units linked alternately α-1,3 and β-1,4 (Anderson, Dolan and Rees, 1965). Thus ..... A-B-A-B-A-B-A-B-A-B-A-B ..... where B is D-galactose and A can be either the L-isomer as for example in the agar-type polysaccharides, or the D-isomer as in the case of the carrageenan-type polysaccharides. However, the AB structure in different algae can be modified and substituted by various groups such as 3,6-anhydride, half-ester sulfate, pyruvic acid, and in some instances methoxyl groups, to produce a striking variety of polysaccharides. This

modified and substituted basic repeating structure has been called a "masked repeating structure" (Rees, 1969). Galactans based on the basic repeating structure have been isolated and characterized from different genera of the Rhodophyceae. Hence a whole spectrum of polysaccharides constitutes a family of polysaccharides which appears to fall into two groups, agar and carrageenan, in fairly good agreement with their structural modifications and substitutions.

These galactans are easily isolated by hot-water extraction followed by precipitation from the aqueous extract by addition of alcohol. Alternatively, agar which forms a gel on cooling is purified by freezing and thawing. Soluble impurities are drained off when the gel thaws.

The precise function of these polysaccharides in the plants is not known. They may act as the structural material of cells, giving the plant flexibility against the wave motion (Rees and Conway, 1962). Their mucilaginous and hygroscopic nature probably hinders the dessication of the plant when it is exposed to air. Such polysaccharides probably act as ion-exchangers, whereby cations such as potassium and calcium are selectively absorbed from seawater (Eppley, 1958).

(1) Agar

The word "agar-agar", according to Tseng (1944) is of Malayan origin. It was originally applied to several species of red algae, primarily Eucheuma

musicatum f. *depauperata* (E. *speciosum*), which the Malayans use for food. The word "agar", or "Kanten" as the Japanese call it, has come since to apply to the jelly-like extractive from certain red algae. What is agar? According to the U.S. Pharmacopoeia (1970), agar is "the dried, hydrophilic, colloidal substance extracted from Gelidium cartilagineum (Linne) Gaillon (Fam. Gelidiaceae), Gracilaria confervoides (Linne) Greville (Fam. Sphaerococcaceae), and related red algae (Class Rhodophyceae)." A similar definition is given in the U.S. Dispensatory and Physician's Pharmacology (1968). The Society of American Bacteriologists (1956) suggested that agar be defined as "any phycocolloid derived from the Rhodophyceae, which will melt at a minimum temperature of 70°C and gel between 33° and 39°C". The Extra Pharmacopoeia; Martindale (1967) described agar as "the solid residue obtained by concentrating a decoction from various species of Gelidium and other algae belonging to Rhodophyceae." These definitions and descriptions of agar, mainly emphasize the physical properties of the preparations. Little or no data is provided on the chemical properties of the polysaccharides.

Agar is largely used in the food-processing industry, since it forms stable viscous solutions at low concentration and is very resistant to bacterial attack. The chief producers are Japan and to a lesser extent, the United States, New Zealand, Russia, Spain and many other countries. It is known that most commercial agar is a blended mixture of agar extracted from different species of red seaweeds (Araki, 1958; Rees, 1969; Duckworth and Yaphe, 1971a). The

seaweeds commonly used as source of agar by industry are summarized as follows:

a. Japanese agar: Gelidium amansii, G. subcostatum, G. japonicum, Pterocladia tenuis, Acanthopeltis japonica, Gracilaria confervoides, Ceramium rubrum, C. boydenii, Campylaephora hypnaeoides (Araki, 1958).

b. American agar: Gelidium cartilagineum, Gracilaria confervoides (Humm and Wolf, 1946).

c. New Zealand agar: Pterocladia lucida, P. capillacea (Forsdike, 1950).

d. Russian agar: Ahnfeltia plicata, Phyllophora spp., Iridaea sp. (Forsdike, 1950).

e. Australian agar: Gracilaria confervoides (Forsdike, 1950), Pterocladia-Gelidium group, Hypnea musciformis, Eucheuma spp. (Ferguson Wood, 1945).

f. South African agar: Gelidium cartilagineum, Gracilaria confervoides, Suhria vittata, Hypnea specifera, Gelidium pristoides (Ferguson Wood, 1945; Forsdike, 1950).

g. British agar: Gigartina stellata, Chondrus crispus (Forsdike, 1950).

h. Malayan agar: Eucheuma speciosum (Ferguson Wood, 1945).

i. Danish agar: Furcellaria fastigiata (Yaphe, 1957).

j. Spanish agar: Gelidium corneum (Yaphe, personal communication).

Yaphe (1959) showed that extracts from Iridaea, Hypnea, Eucheuma, Gigartina, Chondrus and Furcellaria are preparations which contain carrageenan and therefore should not be called agar.

The agars from the different species of seaweed differ markedly, however, in their physical properties (Humm and Wolf, 1946; Forsdike, 1950), and therefore, lack uniformity (Humm and Wolf, 1946).

The introduction of agar as a solidifying agent for culture media was an important step in the advancement of the science of bacteriology. Credit for the use of agar for this purpose should be given to Fannie Hesse (Hitchens and Leikind, 1939). It was she who suggested to her husband that agar, which she had been using for the preparation of fruit and vegetable jellies, could be used as solidifying agent in his studies on the atmospheric bacteria. The discovery was communicated without delay to Robert Koch who made the first printed reference to the use of agar in bacterial culture techniques (Koch, 1882). The use of agar as solidifying agent has made possible many of the advances in bacteriological and biochemical research.

The raw product, in the form in which it is supplied to the food-processing industry, contains impurities and should be purified before being employed for biochemical systems. The first purification which appeared in the

literature, was described by Noble (1928) in his preparation of a cyanide citrate pour plate medium in which he washed agar repeatedly for four days with distilled water. The same procedure was suggested by Kendall (1928), and the product obtained by washing agar powder showed its purity by its markedly lower electro-osmotic flow. This washing method was modified by Grabar and Williams (1955) who washed the agar gel, instead of the powder. A two-stage purification procedure was described by Karjala in 1958. In the first step, the water soluble material was removed by repeated washing of the powder with distilled water. The wet agar was then dissolved, heated with activated coal, centrifuged and filtered. In the second step, the agar solution was frozen, allowed to thaw and the fluid portion decanted. The residue was refrozen and lyophilized. Reuter's (1959) method for purification was somewhat similar to Karjala's, however, the extraction with activated coal was improved by boiling the agar solution with ammonium oxalate, sodium bicarbonate and activated coal, and the freeze-thawing procedure was repeated four times before lyophilization.

Bockemüller and Oerter (1956) proposed a procedure based on electrodialysis which yielded an agar of great transparency. The agar powder was washed with distilled water and dissolved. The solution was poured into a column and allowed to gel. Electrodialysis was then carried out for 24 hr. at 220 v with tap water, which was renewed continuously, as an electrolyte. The agar gel was then taken out and dissolved in water. The procedure was repeated before the agar was dialyzed against distilled water and dehydrated.

The above procedures to remove impurities from agar have been reviewed by Wieme (1965).

Many different brands of commercial agar for bacteriological work have been marketed: Danagar (Denmark), Merck Agar (Germany), Davies Agar (England), Difco Bacto-agar (U.S.A.), and a number of others. For special biological work, these agars have to be purified further. However, some brands have been purified by the company for a particular purpose and further treatment is therefore not necessary. For example, Difco Special Agar-Noble was purified for cyanide citrate pour plate medium.

Araki's characterization of agar into agarose and agaropectin was achieved by acetylation of agar with acetic acid and pyridine, and fractionation with chloroform into soluble agarose acetate and insoluble agaropectin acetate. Subsequent deacetylation of the polysaccharide acetates in alcoholic alkali yielded agarose and agaropectin (Araki, 1937a). The agarose fraction was virtually free from uronic acid and ester sulfate. The proportion of agarose and agaropectin varies from species to species (Araki, 1966).

Methylation followed by acid hydrolysis of agarose yields equal amounts of 2,4,6-tri-O-methyl-D-galactose and 2-O-methyl-3,6-anhydro-L-galactose (Percival and Somerville, 1937; Araki, 1937b; 1956; Araki and Hirase, 1960) providing evidence that agarose is composed of 1,3-linked D-galactose and 1,4-linked 3,6-anhydro-L-galactose. Further evidence to substantiate the structure of agarose was obtained by isolating agarobiose (Fig. 1), 4-O-β-D-galactopyranosyl-

Fig. 1. Agarobiose

Fig. 2. Agarobiose diethyldithioacetal

Fig. 3. Agarobiose dimethyl acetal

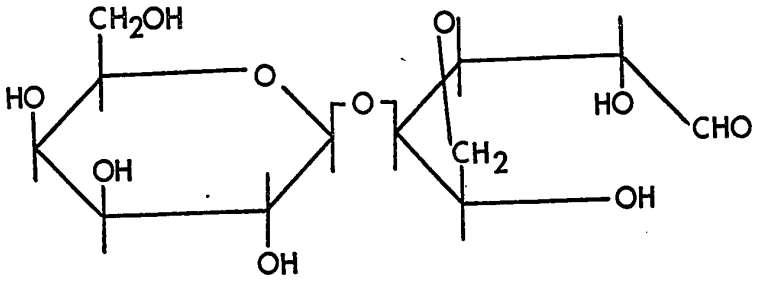


FIG. 1

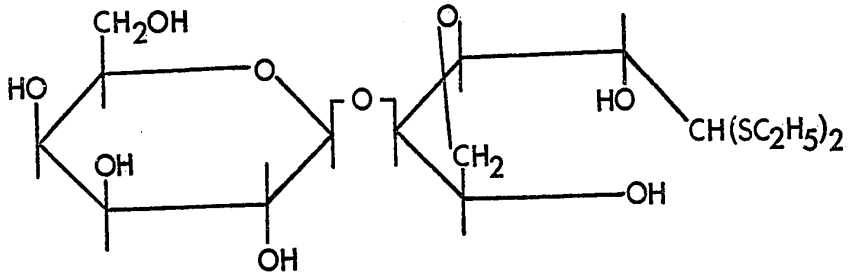


FIG. 2

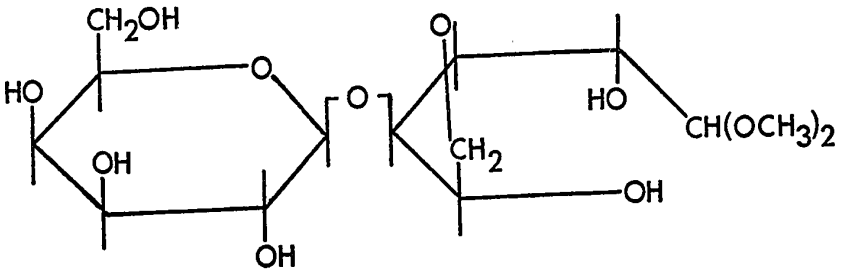


FIG. 3

3,6-anhydro-L-galactose was obtained, as the free sugar, as its diethyldithioacetal (Fig. 2), and as its dimethyl acetal (Fig. 3), by partial acid hydrolysis, mercaptolysis and methanolysis, respectively, of commercial agar and agar from Gelidium amansii (Araki and Hirase, 1954) and from Gracilaria confervoides (Clingman et al.,1957). Enzymic hydrolysis of agarose by the enzyme from Pseudomonas kyotoensis (Araki and Arai, 1956; 1957) led to the isolation of a second disaccharide, neoagarobiose, O-3,6-anhydro-α-L-galactopyranosyl (1 → 3)-D-galactose, and a tetrasaccharide, 4-O-β-neoagarobiosyl-neoagarobiose. This evidence proved that the major structural feature of agarose is a linear chain of alternating 1,3-linked β-D-galactopyranose and 1,4-linked 3,6-α-L-galactopyranose. 6-O-methyl-D-galactose, together with small quantities of L-galactose, D-xylose and 4-O-methyl-L-galactose have also been found in agarose from different species of agarophytes (Hirase and Araki, 1961; Araki, 1966; Araki, Arai and Hirase, 1967).

The word, agaropectin, was first used by Samec and Isajevic (1922) to describe the sulfated galactans in agar. Agaropectin, the charged fraction of agar, has the same basic repeating residues as agarose, with considerable masking or modification by charged groups. The D-galactose residue may be substituted with pyruvic acid acetal, 4,6-O-(1'-carboxyethylidene)-D-galactose (Hirase, 1957), and some of the 3,6-anhydro-L-galactose residues may be replaced with sulfated L-galactose residues (Araki, 1966). D-glucuronic acid has also been reported to be present (Araki, 1937c).

Thus, Araki stated (1956) that "agar is not a single substance, but is a mixture of two polysaccharides, agarose and agaropectin, resembling starch in the respect that starch is a mixture of amylose and amylopectin. Agarose, a main polysaccharide, is composed of 1,3-linked β-D-galactopyranose and 1,4-linked 3,6-anhydro-α-L-galactopyranose, which are repeated in an alternate way .....; whereas, agaropectin is a more complicated polysaccharide containing uronic acid and sulfuric acid as well as D-galactose and 3,6-anhydro-L-galactose as constituents."

Hjertén (1962) reported a simpler method to separate agarose and agaropectin, by precipitation of the agaropectin with quaternary ammonium compounds such as cetyl pyridinium chloride. Blethen (1966) modified this method by adding carrageenan, which is more highly sulfated than the agaropectin component of agar to co-precipitate agaropectin in the presence of a quaternary ammonium salt, benzyldimethyl (2-(2-(p-1,1,3,3-tetramethylbutylphenoxy)-ethoxy)-ethyl) ammonium chloride. The inventor claimed that the product was free of sulfate.

Agarose has been prepared by fractional precipitation of agar with polyethylene glycol (PEG) (6000) (Russell, Mead and Polson, 1964). PEG was added to an agar solution at 80°C. The precipitate obtained by filtration, was washed at 40°C with distilled water. The residue was dissolved in water, and PEG fractionation was repeated. The product obtained by this method is claimed by the investigator to contain no detectable sulfate, and to exhibit no electro-endosmosis. A modified method has been described by Hegenauer and Nace (1965)

using lower molecular weight PEG (4000).

Zabin (1969) described a method for separating agarose from agaropectin by passing an agar solution through a hydrophilic loose lattice anion exchanger, such as diethylaminoethyl (DEAE) cellulose, by a batch process. Microanalysis of the product obtained indicated a sulfur content of 0.1% by weight (0.3% sulfate).

Separation of agarose by adsorption of agaropectin onto an insoluble adsorbent, such as charcoal, bentonite gel, magnesium pyrophosphate gel and aluminium hydroxide gel has been reported by Barteling (1969). The best results were obtained with aluminium hydroxide gel, especially when the agar was prewashed with an EDTA solution. The product obtained had a low sulfur content (0.08%) as compared to "Araki agarose" (0.04%), "Hjertén agarose" (0.14%) and Difco Special Agar-Noble (0.35%). The agarose also exhibited very good electroendosmotic properties.

Araki's concept of agar being composed of two types of polysaccharides, neutral agarose and charged agaropectin, has been re-evaluated, by Duckworth and Yaphe (1971a), and found to be an oversimplification. They fractionated agar on DEAE Sephadex A-50 (Cl^- form) and showed that agar is a mixture of polysaccharides, all having the same backbone structure, but substituted to a variable degree with charged groups. There are three extremes in structure in this spectrum of polysaccharides (Fig. 4): (a) The essentially neutral agarose. This polysaccharide is essentially the same as the idealized "agarose" defined by Araki, but in nature the

Fig. 4. Structural extremes in agar.

1: Theoretical structure of neutral agarose

2: Agarose containing galactose units substituted with pyruvate

3: Sulfate galactan

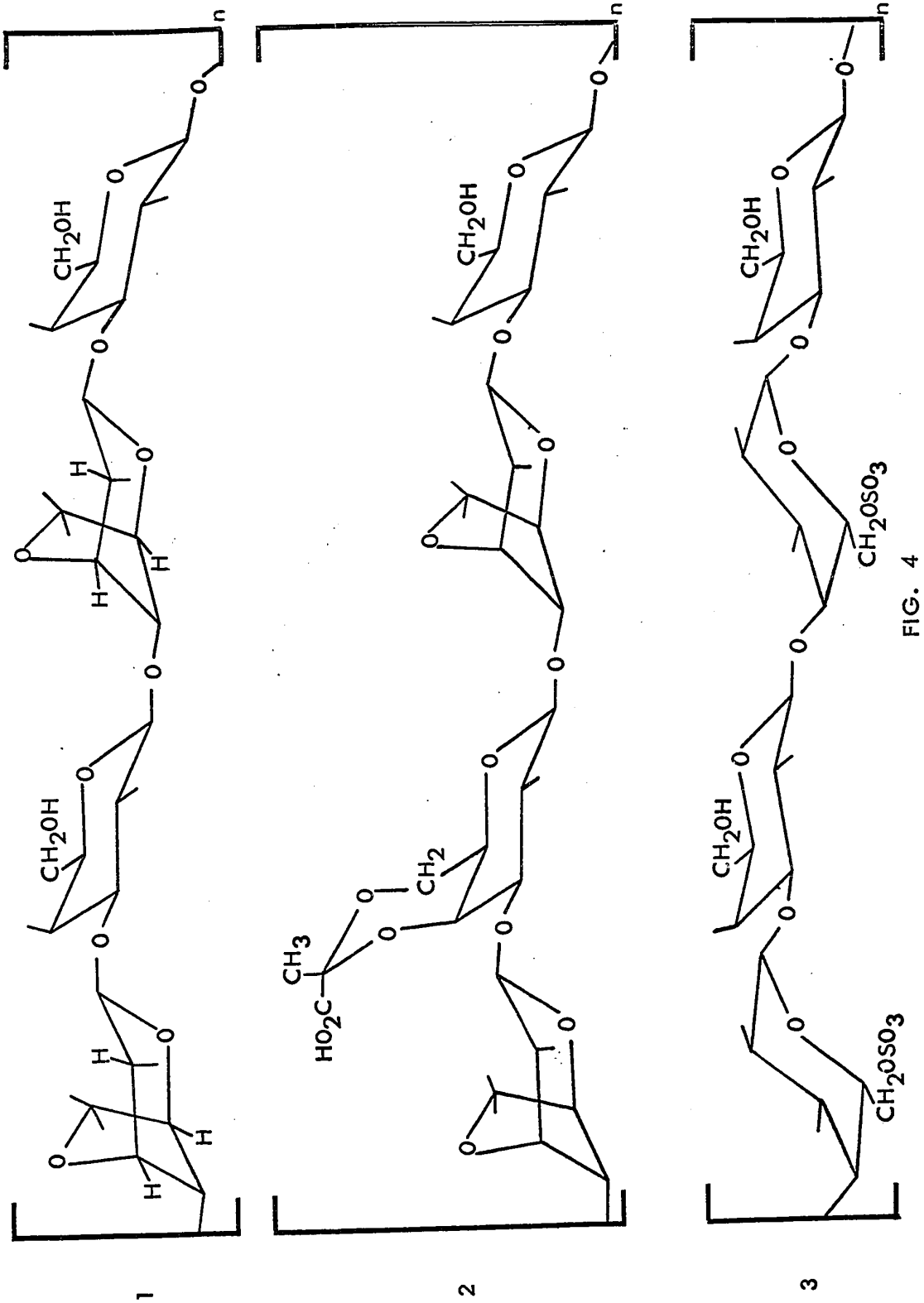


FIG. 4

completely neutral agarose is present in very small amounts, if at all. A small amount of charged groups is always present in the water eluant from ion-exchange chromatography. (b) Pyruvated polysaccharides with little sulfation. From the first extreme of neutral agarose, the masking of the basic structure with charged groups gradually increases. The D-galactose residues are substituted with 4,6-O-(1'-carboxyethylidene)-D-galactose residues. At the same time, sulfated L-galactose residues also gradually replace the 3,6-anhydro-L-galactose. (c) Sulfated galactans. This extreme contains little or no 3,6-anhydro-L-galactose or pyruvated D-galactose. Therefore, the practical definition of agarose will have to indicate that agarose is not a neutral polysaccharide obtained by separation from the charged agaropectin but is that fraction of agar having the lowest charged content and hence the greatest gelling ability, fractionated from a whole complex of molecules called agar, all differing in their extent of substitution with charged groups. Also the word "agaropectin" is redundant and should no longer be used to describe the charged component in agar. Similar results to those of Duckworth and Yaphe have also been reported by Izumi (1971).

Porphyran-type polysaccharides which were described as a separate group of polysaccharides (Percival, 1970) can be extracted from Porphyra, Laurentia species and Bangia fuscopurpurea. However, it has been shown that porphyran has the same arrangement of alternating glycosidic linkages as found in agarose, in which a greater portion of the D-galactose and 3,6-anhydro-L-galactose residues are partially replaced by 6-O-methyl-D-galactose, and L-

galactose 6-sulfate, respectively. Treatment of porphyran from Porphyra umbilicalis with alkali (Rees, 1961a) or an enzymic extract of the weed (Rees, 1961b), quantitatively converts the L-galactose 6-sulfate into 3,6-anhydro-L-galactose. Complete methylation of this polysaccharide yields a product which is apparently identical to methylated agarose (Anderson and Rees, 1965). This indicates that porphyran should be considered a member of the agar family, rather than an independent type of polysaccharide.

Analysis of the major groups of agarophytes by anion-exchange chromatography is important in their evaluation as source of agar, as shown by Duckworth, Hong and Yaphe (1971).

(2) Carrageenan

The carrageenans, like the agars, constitute a family of polysaccharides. They are extracted from Chondrus, Gigartina, Eucheuma, Hypnea, Fucellaria, and Irideae species (Percival, 1970) and are composed of galactose residues which are linked alternately α-(1 → 3) and β-(1 → 4), with a masked repeating structure (Rees, 1969).

The carrageenans can be isolated into two basic fractions with an aqueous solution of KCl (Smith et al.,1954). The KCl-insoluble fraction is called, in classical nomenclature, kappa-carrageenan, and the KCl-soluble, lambda-carrageenan. However, recent studies have shown carrageenan extracts to contain

a variety of extremes of structures, namely kappa, iota, mu and lambda-carrageenan (Anderson, Dolan, Penman, Rees, Mueller, Stancioff and Stanley, 1968; Mueller and Rees, 1968). Also, all these structures may not be present in the same species of carrageenan-producing seaweed.

a. Kappa-carrageenan

This polysaccharide forms a specific precipitate with potassium ions, and consists largely of alternating 1,3-linked β-D-galactose 4-sulfate and 1,4-linked 3,6-anhydro-α-D-galactose (Fig. 5a) residues (O'Neill, 1955; Bayley, 1955). D-galactose 6-sulfate residues replace some of the 3,6-anhydro-D-galactose residues, and there is evidence for the presence of minor amounts of 3,6-anhydro-D-galactose 2-sulfate and galactose 2,6-disulfate units (Anderson, Dolan and Rees, 1968). That the polymer is made up entirely of carrabiose 4-sulfate units, (Fig. 5b), and modified carrabiose 4-sulfate units are demonstrated by the fact that after treatment with alkaline borohydride and methanolysis, the yield of dimethyl acetal obtained, when corrected for side reaction, is close to the theoretical value for total carrabiose composition (Rees, 1961a).

In comparison with agarose, kappa-carrageenan forms a less rigid gel, and requires a higher concentration of polysaccharide for gel formation. Gelation occurs in the presence of K^+, Rb^+, Cs^+ or NH_4^+ but not with Li^+ or Na^+.

Fig. 5. a. Repeating structure of the major portion of kappa-carrageenan.

b. Carrabiose 4-sulfate.

n: 1

Fig. 6. Repeating structure of the major portion of iota-carrageenan.

Fig. 7. Repeating structure of the major portion of mu-carrageenan.

Fig. 8. Proposed structure of lambda-carrageenan (Rees, 1969).

R: H or SO_3^-

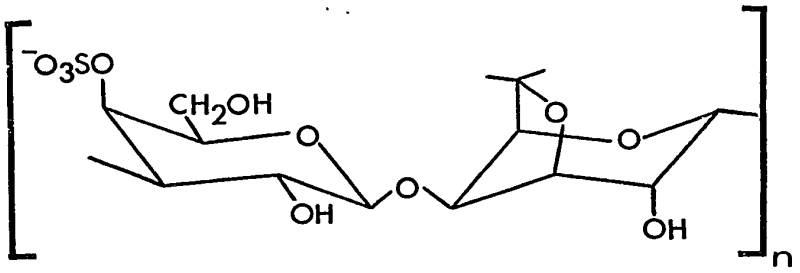


FIG. 5

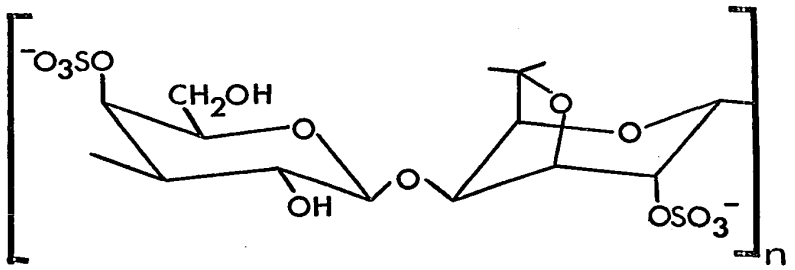


FIG. 6

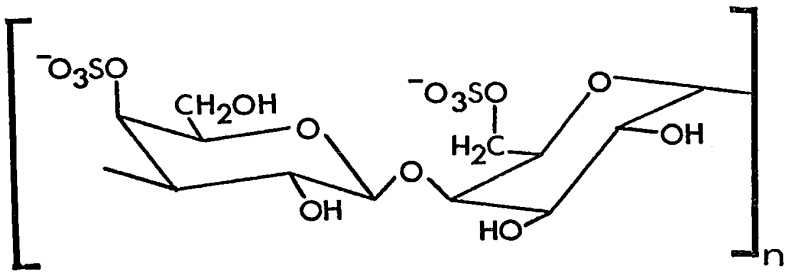


FIG. 7

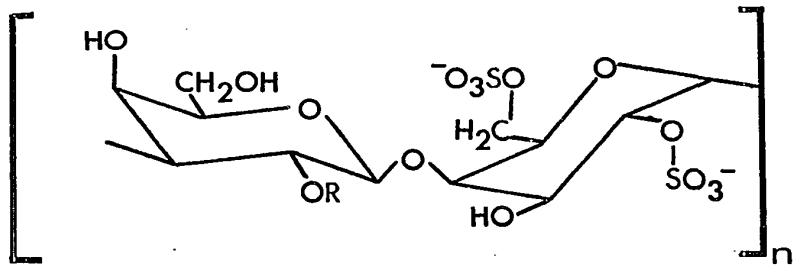


FIG. 8

b. Iota-carrageenan

The structure of this polysaccharide, as extracted from Eucheuma spinosum and Agardhiella tenera, is similar to that of kappa-carrageenan, but the 4-linked 3,6-anhydro-D-galactose residues are 2-sulfated (Fig. 6), with about 10% of the 3,6-anhydro-D-galactose 2-sulfate replaced by D-galactose 2,6-disulfate (Mueller and Rees, 1968; Anderson, Dolan, Lawson and Rees, 1968). Iota-carrageenan forms a more elastic gel than that of kappa-carrageenan in the presence of K^+.

c. Mu-carrageenan

This polysaccharide, like lambda-carrageenan, is KCl-soluble (Dolan and Rees, 1965; Anderson, Dolan, Lawson, Penman and Rees, 1968). No method has yet been developed for separating these polysaccharides without modifying the structure of mu-carrageenan. After alkaline borohydride treatment, the modified mu-carrageenan can be then precipitated as the potassium salt. The results from methylation and infra-red spectra (Anderson and Rees, 1966) indicate that mu-carrageenan consists of repeating units of 3-linked D-galactose 4-sulfate and 4-linked D-galactose 6-sulfate (Fig. 7). To some extent, the 4-linked moieties are replaced by 3,6-anhydro-D-galactose (Anderson, Dolan, Lawson, Penman and Rees, 1968), and a small proportion of the 4-linked residues are 2-sulfated. Mu-carrageenan has little tendency to gel.

d. Lambda-carrageenan

In the older literature, lambda-carrageenan was the name given to the material remaining in the supernatant after KCl fractionation of carrageenan. However, this material is heterogenous, more often than not contaminated with mu-carrageenan. It has been proposed (Rees, 1969) that the name "lambda-carrageenan" be reserved for the components having the structure shown in Fig. 8, i.e. an alternating sequence of 4-linked D-galactose 2,6-disulfate and 3-linked D-galactose residues. The latter residue may be present as the 2-sulfate derivative.

Aqueous solutions of lambda-carrageenan, and of its simple salts, are viscous, but do not gel.

The structure of the major gelling and non-gelling components of carrageenan have been determined. However, in agar, emphasis has been placed mainly on the structure of the gelling component. Only limited information on the structure of the sulfated, non-gelling components of agar is known. In this thesis, the distribution of the components in agar from a few species of agarophytes were examined, but major emphasis was placed on the fine structure of the gelling components.

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 micro-

biological techniques. The bacterium, which he named Bacillus gelaticus, causes depressions in agar medium and alters the polymer structure. When the agar plate is flooded with iodine, a blue-black color no longer appears. Since then, many agar-degrading microorganisms have been isolated from different sources (Angst, 1929; Waksman and Bavendamm, 1931; Goresline, 1933; Araki and Arai, 1954; Swartz and Gordon, 1959). Among them Angst isolated thirteen species and placed them into a new genus, Agarobacterium. Stanier (1941) found that the ability to degrade agar was very widespread among several genera of microorganisms. For this reason, he felt the genus Agarobacterium was taxonomically unsound.

Humm (1946), while conducting a survey of agar-decomposers along the Atlantic coast, isolated and identified twenty species. He outlined the general characteristics of these bacteria and proposed a key for their classification, concluding that they were not considered as a natural group, but only physiologically related. This point was reviewed by Yaphe (1963), and re-investigated by Cowell and Gochnauer (1963) on sixty species of agar-degrading bacteria. Their results from computer analysis indicated that these bacteria would be classified as Pseudomonas and Vibrio species. They concluded that a separate genus should not be introduced merely on the basis of a single feature, such as the ability to utilize a specific polysaccharide. A Cytophaga species has also been reported to degrade agar (Duckworth and Turvey, 1969a).

The nutritional requirements of agarolytic bacteria has recently been

studied (Girard et al., 1968). This work indicated that the nutrition of these bacteria is "simple", in that many of them utilize single amino acids with galactose, and that a variety of carbon compounds may replace galactose. Some agar-decomposers are able to degrade the capsular polysaccharides and cell walls of certain microorganisms (Mitchell and Nevo, 1965). However, little is known with regard to the role of the bacteria in their marine and soil environments.

C. ENZYMOLOGY OF THE RHODOPHYCEAN GALACTANS

The use of enzymes in elucidating the structure of algal polysaccharides is more specific and the rate of reaction is more easily controlled, than with chemical methods. Enzymic hydrolysis is particularly useful in studying the galactan sulfates of the Rhodophyceae, in which the high incidence of sulfate esters interferes with methylation and acetylation techniques. However, suitable degradative enzymes for many of these polymers have yet to be found. The enzymes use the polysaccharides as a substrate cleaving specific glycosidic linkages in the molecule, and therefore provide information concerning the structure of the polysaccharide.

(1) Agar-degrading enzymes

The enzymic hydrolysis of agar was first demonstrated by Gran (1902) from a extracellular culture filtrate of Bacillus gelaticus. He assigned the name "gelase" to the enzyme which was responsible for the degradation of agar. Kadota

(1951) also prepared an enzyme solution from Vibrio purpureus capable of hydrolyzing agar. Fukumoto and Ishimatsu (1951, cited in Araki and Arai, 1956) described the agar-degrading enzyme as an agarase. Little information can be found in the literature concerning the isolation and characterization of the hydrolyzed products of agar, until Ishimatsu and his co-workers (1954) reported the characterization of a dissaccharide produced by the action of agarase on agar. Araki and Arai (1956; 1957), using an unpurified agarase preparation from Pseudomonas kyotoensis, isolated a homologous series of four oligosaccharides from the enzymic hydrolysate of agarose, two of which were identified as a di- and tetrasaccharide. The disaccharide was shown to be O-3,6-anhydro-α-L-galactopyranosyl (1 → 3)-D-galactose (Fig. 9). Since it was an isomer of agarobiose, which had previously been isolated by acid hydrolysis of agar (Araki, 1944), it was named neoagarobiose. The tetrasaccharide was demonstrated to consist of two neoagarobiose units linked through a β-1,4-linkage (Fig. 10). Araki and Arai (1957) proposed a scheme of nomenclature for the oligosaccharides of agar: the prefix "agaro" was given to those oligosaccharides whose non-reducing ends are presented by D-galactopyranose, and the prefix "neoagaro" to those oligosaccharides whose non-reducing ends are presented by 3,6-anhydro-α-galactopyranose. Using this scheme the tetrasaccharide was called neoagarotetraose, while the other two higher molecular weight oligosaccharides were called neoagarohexaose and neoagarooctaose, respectively. Araki's agarase catalyses the random hydrolysis of the β-1,4-linkages of agar.

Fig. 9. Neoagarobiose.

Fig. 10. Neoagarotetraose.

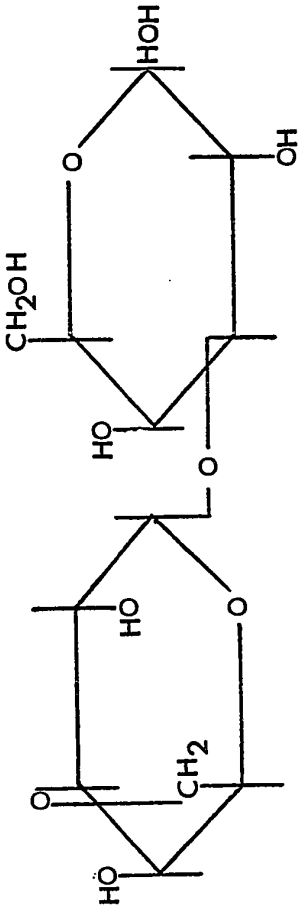


FIG. 9

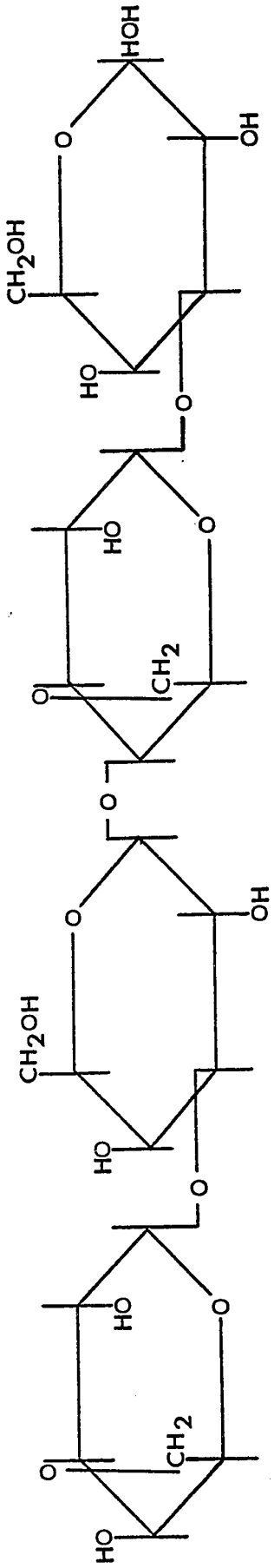


FIG. 10

Yaphe (1957) isolated an extracellular agarase, from a marine bacterium, Pseudomonas atlantica, which cleaved the β-1,4-linkages of agarose, yielding the same series of oligosaccharides as found by Araki and Arai (1956; 1957). Preliminary hydrolysis of agarose by this enzyme released high molecular weight neoagaro-oligosaccharides which were completely degraded to neoagarotetraose and neoagarobiose on addition of fresh enzyme (Yaphe, 1966). The enzyme was not capable of hydrolyzing either neoagarotetraose or neoagarobiose. Enzymolysis of charged agarose also yielded both neutral and charged neoagaro-oligosaccharides (Duckworth and Yaphe, 1971b).

A mixture of neoagarotetraose and neoagarobiose were degraded to 3,6-anhydro-L-galactose, galactose and neoagarobiose on incubation with the sonicates of Pseudomonas atlantica (Yaphe, 1966). This led to the isolation of a β-oligosaccharidase (Ross, 1970), which hydrolyzes neoagarotetraose to neoagarobiose by breaking the β-1,4-linkage, and an α-oligosaccharidase which cleaves the α-1,3-linkage of neoagarobiose to 3,6-anhydro-L-galactose and galactose. (Papenburg and Yaphe, unpublished results).

An extracellular agarase has also been isolated and purified from a Cytophaga species (Duckworth and Turvey, 1969a). This agarase resembles those reported previously (Araki and Arai, 1956; Yaphe, 1957), in that it is specific for the β-1,4-linkages of agarose. However, masking of the agarose molecule resulted in a decrease in enzyme activity: the enzyme could not hydrolyze β-1,4-linkages near a D-galactose 6-sulfate residue, and the rate of hydrolysis was decreased

Fig. 11. Neocarrabiose 4-sulfate.

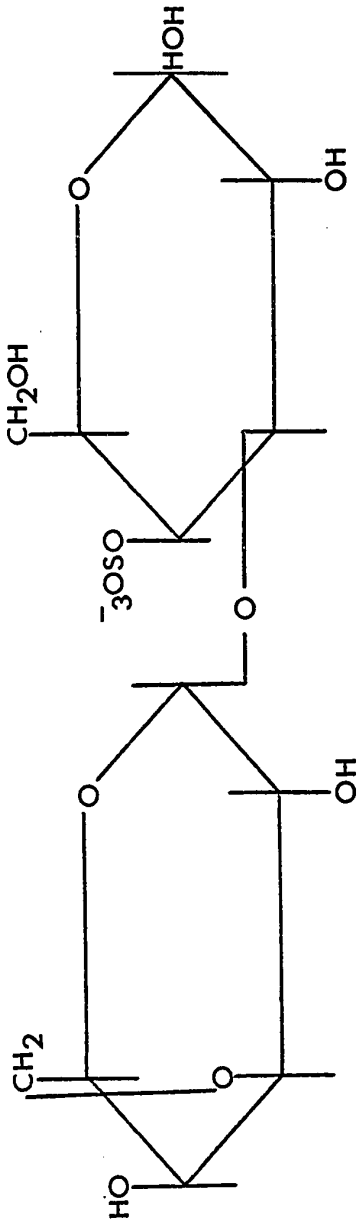


FIG. 11

by one fifth in the presence of 6-O-methyl-D-galactose residues (Duckworth and Turvey, 1969b).

(2) Carrageenan-degrading enzymes

A kappa-carrageenase, which specifically hydrolyzes kappa-carrageenan but not agar-type polysaccharides, has been isolated from another marine bacterium, Pseudomonas carrageenovora (Yaphe and Baxter, 1955). The enzymic hydrolysates of kappa-carrageenan yielded a homologous series of neocarrabiose 4-sulfate oligosaccharides (Fig. 11) (80%), and an enzyme resistant fraction (20%) (Weigl, Turvey and Yaphe, 1966; Weigl and Yaphe, 1966). Alkali treatment of the enzyme resistant fraction removed 19% of the sulfate esters with formation of an additional 14% of 3,6-anhydro-D-galactose residues. The alkali-treated fraction was then found to be susceptible to further enzymolysis by kappa-carrageenase. Weigl and Yaphe (1966) therefore concluded that the enzyme resistant region was a biogenetically "unfinished" region of kappa-carrageenan which still contained D-galactose 6-sulfate residues.

As indicated previously, lambda-carrageenan, as classically defined by Smith et al.(1954), is often contaminated with mu-carrageenan. Therefore, the specificity of the"lambda-carrageenase activity" described by Weigl and Yaphe (1966) is not known.

The specificities of the agarases and carrageenases, not only prove to be a very useful method in determination of the structures of agar and carrageenan-

type polysaccharides, but also serve as an easy tool for identifying these polysaccharides in various members of the Rhodophyceae (Yaphe, 1959; Hong, Goldstein and Yaphe, 1969).

(3) Mode of action of polysaccharidases

Enzymic hydrolysis of polysaccharides occurs by two general mechanisms. The exo-mechanism, as shown by β-amylase, is an endwise enzymic hydrolysis in which the enzyme sequentially cleaves mono- or oligosaccharides from one end of the chain. In the endo-mechanism, on the other hand, the enzyme initiates hydrolysis at random interior positions along the polymer chain (e.g. α-amylase). Several techniques are currently used to determine whether an enzyme is of the endo- or exo-type: 1) Analysis of the enzymic hydrolysate by chromatography. This relies on the accumulation of oligosaccharides as the criterion of endo-attack (French et al., 1965). 2) Modification of both the non-reducing and reducing end of polysaccharide. (Smith and Montgomery, 1959). 3) The rate of formation of end products of enzymolysis either by measurement of the increase in reducing sugar (Nelson, 1944) or by analysis of end products (Huggett and Nixon, 1957). 4) The change in degree of polymerization during the enzyme action by measurement of the iodine value, by viscometry and estimating the molar ratio of carbohydrate to reducing value (Robyt and French, 1967; Tung and Nordin, 1969).

Typical of an endo-mechanism is the initial release of large oligosaccharides, followed by their conversion into end products, whereas, an exo-attack releases only the low molecular weight oligosaccharides. However, this reasoning

has been complicated by the discovery of an endo-multiple attack mechanism (Robyt and French, 1967). For enzymic action on polymeric substrates, three distinctive action patterns have been proposed by Robyt and French (1967). These are single chain, multichain and multiple attack degradations. In single chain degradation, once the enzyme forms an active enzyme-substrate complex, the enzyme completely hydrolyzes one entire polymer chain before it attacks another. The multichain degradation is the classical random action in which, after the formation of enzyme-substrate complex, the enzyme diffuses away from the substrate molecule after splitting a single bond. In the multiple attack degradation, once the enzyme-polymer complex is formed, the enzyme may catalyze the hydrolysis of several bonds before it dissociates and forms a new active complex with another polymer chain. The degree of multiple attack may be defined as the average number of catalytic events, following the first, during the life-time of an individual enzyme-substrate complex. The single chain and multichain mechanisms represent the two extreme cases of polymer degradation. The multiple attack mechanism is an intermediate state between single chain and multichain degradation.

Originally, this concept was proposed for the endwise enzymic attack, i.e. exo-mechanism, on a polymer (Bailey and French, 1957). For example, sweet potato β-amylase was shown to exhibit four attacks per effective encounter with the substrate molecule. Endo-enzymes, such as α-amylases, have also been shown to have a varying degree of multiple attack depending on the experimental conditions

used (Robyt and French, 1967). It is, therefore, clear that this concept originally developed for exo-enzymes, may also be applied to the endo-enzyme action.

The bacterial agarases have been known to hydrolyze agar by random degradation (Araki and Arai, 1957; Yaphe, 1966; Duckworth and Turvey, 1969a). However, the degree of multiple attack has not yet been investigated.

D. MECHANISM OF GELLING

The remarkable gelling properties of agar, kappa-carrageen and other related polysaccharides are well known to biologists. The low concentration at which these gels form and the properties that then result, have few parallels that are quite as striking among natural or synthetic polymers.

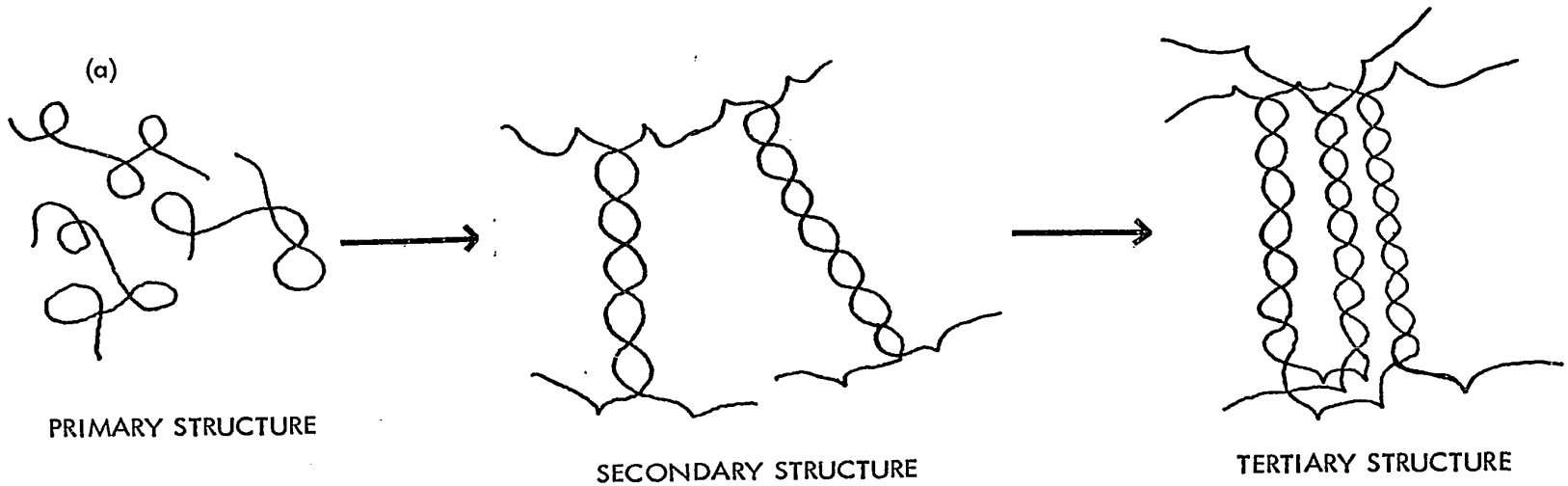
Rees (1972) has proposed a "network theory of gel formation" for agar- and carrageenan-type polysaccharides. The gel formation of the polysaccharides can be described, in the language of protein biochemistry, as showing primary, secondary, tertiary and quaternary structures. The primary structure, is the alternating arrangement of α-(1 → 3)-and β-(1 → 4)-linked galactose residues as described in the previous section. At the temperature above the liquefaction point of the gel, the polysaccharide exists in the random coil or primary structure (Fig. 12). The secondary structures are the formation of a polysaccharide double helix. As the temperature drops to a certain point, the random coils adopt the energetically more favorable double helix conformation (secondary structure). The stabilization of the double strand may be due to the interstrand H-bonding between O-2 and O-6 of the

Fig. 12. Network Theory of Gel Formation

(Rees, 1972; Dea, McKinnon and Rees, 1972).

a: Agar or carrageenan

b: Agar or carrageenan in the presence of a galactomannan


(a)
PRIMARY STRUCTURE
SECONDARY STRUCTURE
TERTIARY STRUCTURE

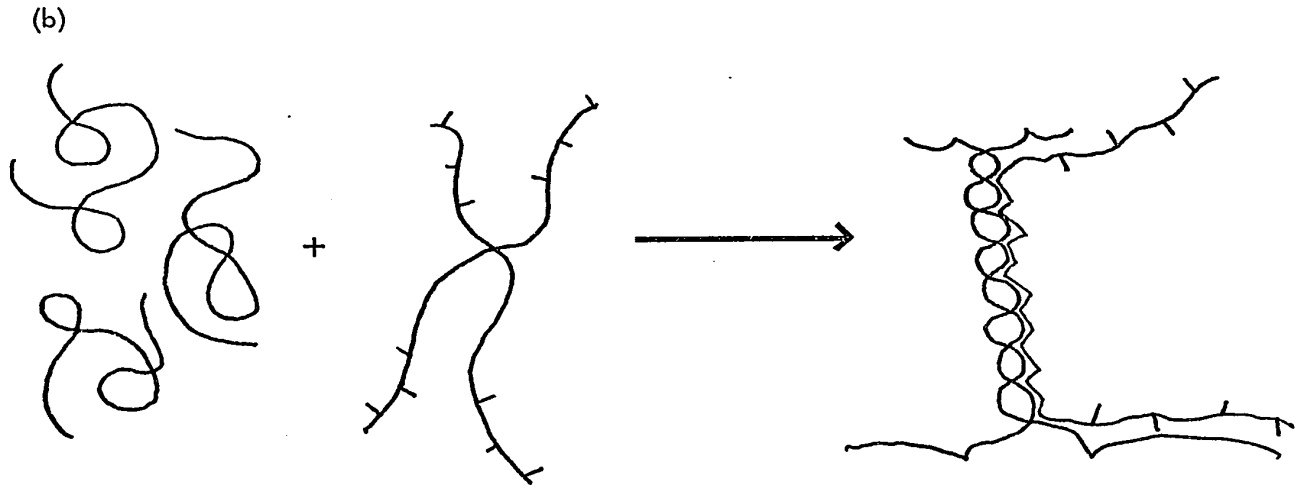

(b)
+
QUATERNARY STRUCTURE

FIG. 12

D-galactose residues of the polysaccharides. Mismatching of the helical strands, or the conformational kinkings due to the presence of masking groups in some of the strands, give rise to a three-dimensional framework of the double helices. Further interactions among the double helices produce aggregates of double helices giving the final structure (tertiary structure). The process of aggregation to form such a network can occur between unlike polysaccharides (quaternary structure). For example, when a carrageenan solution is diluted until it does not itself gel and then is mixed with a suitable non-gelling galactomannan, the mixture may gel even though this is not to be expected from the properties of the individual components. Similar observations have been made for agarose-galactomannan mixtures (Dea, McKinnon and Rees, 1972).

It is a well-known fact that agar dissolves above 90°C and gels between 35 and 40°C. However, Guiseley (1970) investigated a variety of agaroses from different species of agarophytes and found that the gelling temperature of agarose solutions increased with increasing methoxyl content of the agarose. Some of the agaroses he used were found to gel at 53°C. In order to explain Guiseley's observation, Yaphe and Duckworth (1971) used a 6-O-methylated agarose obtained from alkali treated porphyran and found that the polysaccharide had the same gelling temperature as non-methylated agarose. They concluded that the 6-O-methyl (primary methoxyl) group may not affect the gelling temperature. However, the secondary methoxyl groups in the agar molecules may fix the conformation of these molecules more rigidly than would the primary hydroxyl groups, thus allowing the

double helix to form at a higher temperature.

The charged groups present in agar interfere with the gelling properties of agar (Yaphe and Duckworth, 1971). An increase in the sulfate content of agar is associated with a decrease in brittleness of the gel state and an increase in the elasticity of the gel (Rees, 1969). The sulfate groups interfere with gel formation in two ways: (a) Sulfated sugars introduce a "kink" into the helical chain, (Fig. 12), as in the case where 3,6-anhydro-L-galactose is substituted by L-galactose 6-sulfate. (b) The presence of a sulfate group in the C-2 position of (1 → 3)-linked D-galactopyranosyl residues would prevent gel formation, since hydrogen bonding is inhibited between O- 2 of the residues of one strand and O- 6 of the same residue of the second strand in the double helix, as in the enantiomorphic polysaccharide carrageenan (Anderson et al., 1969). Sulfate groups in other positions would not be expected to affect double helix formation (secondary structure formation), but they would probably affect the aggregation of the double helices (tertiary structure formation) due to electrostatic interactions.

The pyruvic acid is present in agar as the 4,6-O-(1'-carboxyethylidene) groups on a portion of the (1 → 3)-linked D-galactose residues, and thus would not be expected to affect secondary structure formation, but would possibly inhibit aggregation (tertiary structure formation) by electrostatic interaction (Yaphe and Duckworth, 1971).

The mechanism of gelling of the polysaccharides as proposed by Rees (1972) is based on background knowledge in which there are many gaps, since

there are, as yet, no standard rigorous experimental methods available to demonstrate the mechanisms. The mechanism of gelling of agarose is based on the more detailed study on the carrageenans, for although the carrageenans are chemically different from agar, they exhibit similar properties.

III. MATERIALS AND METHODS

A. PREPARATION OF POLYSACCHARIDES

(1) Source and extraction of seaweeds

The seaweed was collected, air dried and stored at 4°C. The source of agarophytes is given in Table I. The dried seaweed (10 to 20 g) was soaked in water for at least 3 hr. and extracted with distilled water (150 ml) at 121°C for 3 hr. The hot extract was filtered through a double-layered cheese cloth and a pad of Celite (545). Solid sodium chloride was added to the filtrate to a final concentration of 0.1 M. The filtrate was poured into a tray, allowed to gel at room temperature, cut into strips and then frozen and thawed. The thawed strips of agar were washed twice with 0.1 M NaCl, and dehydrated with ethanol. To those filtrates which did not gel, ethanol (3 vol) was added and the precipitated polysaccharide was collected by centrifugation.

(2) Fractionation of agar

Agar (1 g), suspended in three changes of distilled water for 3 hr. to remove NaCl, was dissolved in distilled water (50 ml) at 100°C. The agar was fractionated by column chromatography on DEAE Sephadex A-50 (Duckworth and Yaphe, 1971a). The agar solution was added to DEAE Sephadex A-50 (Cl^-) in a

jacketted column (4 x 30 cm) at 60°C and eluted stepwise with water, 0.5 M, 1 M and 2.5 M NaCl. Before eluting with the next higher concentration of NaCl, the eluate was checked to ensure it was free of polysaccharide by the phenol-sulfuric acid method (Hay, Lewis and Smith, 1965). Each fraction was dialyzed, precipitated into ethanol (3 vol) and centrifuged. The polysaccharides were air dried and weighed.

(3) Preparation of washed agar and agarose

The washed agar was prepared by washing commercial Difco Bacto-agar (lot No. 616480) successively at 20 and 60°C in a column with 0.1 M NaCl until the eluates were free of detectable carbohydrate (Duckworth and Yaphe, 1971c). The agarose was prepared from the washed agar by fractionation on DEAE Sephadex A-50 (Cl^-) as described (A-2), but only the water eluted fraction was collected.

(4) Sulfate determination

The method used was that of Jones and Letham (1954). To the weighed polysaccharide (approx. 8 to 10 mg, depending on the sulfate concentration), nitric acid (0.175 ml) was added. The tube was sealed and heated at 110°C overnight, cooled to room temperature, opened and heated to complete dryness in a sand bath. The residue was dissolved in distilled water (0.5 ml) and then sulfate

reagent (see Appendix) was added. A small quantity of Cetavlon was added, and the solution was allowed to stand for 3 hr. and centrifuged. 200 µl of the supernatant fluid was taken and diluted with 0.1 M HCl to 10 ml in a volumetric flask, and the absorbance read at 252 nm. Sodium sulfate (0 to 200 µg) was used as a standard.

(5) 3,6-Anhydrogalactose determination

The procedure of Yaphe and Arsenault (1965) was followed. This is a modified procedure originally described by Yaphe (1960). The instructions laid out in the paper were followed completely in order to obtain reproducible results. The 1,1-diethoxy-ethane (Acetal, Eastman) was prepared fresh just before use and the resorcinol stock solution was kept in the dark and prepared weekly.

The concentration of 3,6-anhydrogalactose in an unknown solution was determined by reference to a calibration curve, obtained at the same time, using standard solutions of either fructose or neoagarobiose. The latter reference sugar showed the same molar response as 3,6-anhydrogalactose.

(6) Pyruvic acid determination

Pyruvic acid was determined by the lactate dehydrogenase method (Duckworth and Yaphe, 1970a). The analysis was carried out only on those fractions obtained by ion-exchange chromatography of freeze-thawed agar, which contained more than 0.2% pyruvic acid.

B. CHROMATOGRAPHY

(1) Paper and thin-layer chromatography

a. Paper chromatography

For descending paper chromatography, Whatman No. 1 and 3MM papers were used for qualitative and preparative purposes, respectively. In preparative work, the paper was washed thoroughly with distilled water before use.

b. Thin-layer and wedge chromatography

Microcrystalline cellulose (Camag D.S.O.) or cellulosepulver MN 300 (Macharey, Nagel and Co.) were used as supporting material for thin-layer chromatography (TLC). Plates (20 x 20 cm or 20 x 40 cm) were coated with cellulose to a thickness of 0.25 mm (for qualitative work) or 0.5 mm (for preparative work). Ascending chromatography was used.

The wedge TLC technique was used for sharp separation and identification of the oligosaccharides. The cellulose TLC surface was cut into a wedge-shape with an angle ranging from 45 to 90°; diagonal lines were then drawn to allow the slow movement and curving of the solvent front. This gives the most satisfactory results for separating charged oligosaccharides from neutral ones. It also gives better results in identifying sugars, in that the spurs of identical sugars form a line of identity as in the immunodiffusion technique.

c. Solvents

All the solvents used were reagent grade (Fisher Scientific Co.);

Solvent (A): Butane-1-ol : ethanol : water (3:2:2; V/V)

Solvent (B): Butane-1-ol : acetic acid : water (4:1:2; V/V)

Solvent (C): Butane-1-ol : acetic acid : water (4:1:5; V/V)

Solvent (D): Butane-1-ol : pyridine : water (10:3:3; V/V)

Solvent (E): Butane-1-ol : ethanol : water (1:1:1; V/V)

d. Indicator reagents

After solvent development, the chromatogram was air dried and sprayed with one of the following indicator reagents:

Naphthoresorcinol (Yaphe, 1957)

The naphthoresorcinol reagent is a sensitive indicator reagent for detecting small amounts (10^{-3} µg) of 3,6-anhydrogalactose present in oligosaccharides. Visualization of the sugar is enhanced by the use of shortwave U.V. light (254 nm). The preparation of this reagent is described in the Appendix.

Aniline hydrogen phthalate (Block et al., 1955)

This reagent is specific for sugars with reducing end groups. After spraying, the chromatogram was heated for at least 10 min. at 100°C. The sensitivity of the test is improved if the sugars are observed under long wave U.V. light

(360 nm). The preparation of this reagent is described in the Appendix.

Ammoniacal silver nitrate (Block et al., 1955)

This is a universal spray reagent. After the spraying and air drying, the chromatogram was heated at 100°C for 5 to 10 min. The sugars appear as grayish black spots against a brown background. The background was washed off with 5% sodium thiosulfate to which a few drops of ammonia had been added (see Appendix).

Silver nitrate-sodium hydroxide (Smith, 1960)

A universal spray reagent; reducing sugars appear immediately after spraying, whereas the non-reducing sugars show up after 10 to 20 min. in the dark. This reagent was used for the primary detection of 3,6-anhydrogalactitol as end sugar in the sodium borohydride reduced enzymic hydrolysate of agarose. The preparation of the reagent is described in the Appendix.

(2) Column chromatography

a. Gel filtration

Sephadex G-series (Pharmacia Fine Chemicals) gel and column packings were prepared according to the manufacturer's instructions. Void volumes were measured by passing Dextran Blue (0.2%) through the columns. For chromatography of enzymes, G-100, G-150 and G-200 were used, and for separation of oligo-

saccharides, G-25 superfine was used. The columns were equilibrated with the appropriate buffer and the flow rates were controlled by a peristaltic pump (model 600-1200, Harvard Apparatus Co., Mass.).

b. Ion-exchange chromatography

Mannex DEAE-cellulose (Mann Research Laboratories, Inc., New York) was regenerated in the OH form. The cellulose slurry was packed in a column under a pressure of 10 lbs., and the column was equilibrated with the buffer at 4°C before use.

(3) Gas-liquid chromatography

The use of trimethylsilylation reagents affords a simple, rapid and quantitative method for forming derivatives of monosaccharides suitable for gas-liquid chromatography (GLC) (Sweeley et al., 1963). Separation of a mixture of monosaccharides by GLC of the trimethylsilys (TMS) derivatives is complicated by the fact that each monosaccharide can give rise to up to four peaks due to the various structural and anomeric isomers of the monosaccharides. However, this can be overcome by reduction of the monosaccharides to the corresponding sugar alcohols, followed by trimethylsilation, thus yielding only one peak per monosaccharide alcohol TMS on GLC (Sweeley et al., 1963; Sawardeker et al., 1965). The single peak per monosaccharide also offers an easier, more accurate method for the

quantitative determination of a monosaccharide mixture. This method was therefore adopted in the quantitative determination of monosaccharides in the hydrolysates of oligosaccharides.

Two types of liquid phases were used: 1.5% XE-60 for the TMS derivatives and 10% carbowax for methyl glycoside TMS derivatives. Chromosorb-Q (100 to 200 mesh) was used as solid support. The stationary phase was packed into U-shaped glass columns (8 ft). Each column was "bled" with nitrogen gas overnight at 200°C before use.

A Hewlett-Packard 402 high efficiency gas chromatograph with a dual flame ionization detector attached to an integrator (H/P, type 3373B) was employed. Nitrogen with a flow rate of 12.5 ml/min. was the carrier gas and the oven temperature was 140°C.

The TMS derivates were prepared by the method of Ellis (1969). Commercial monosaccharides (chromatographically-pure grade) or hydrolysates of the oligosaccharides were dried at 40°C in vacuo and dissolved in sufficient dimethylsulfoxide (DMSO) to yield a concentration of 0.2 to 6.0 mg per ml. To the DMSO solution, hexamethyldisilazane (0.3 vol) and trimethylchlorosilane (0.2 vol) were successively added, with intermediate mixing. The reaction mixture, or the upper phase, was chromatographed no sooner than ten min. after trimethylsilylation. For quantitative determination the upper phase was transferred to a second tube, and washed with a few drops of water. The top layer was again transferred to a screw-cap test tube and dried with anhydrous sodium sulfate. The water washed reaction

mixture gave a sharp solvent front and a steady base line when chromatographed.

Identification of a monosaccharide was done by comparison of its retention time with that of a known standard, and by the injection of the standard together with the unknown. Quantitative analysis was determined initially by measuring the peak area; later it was measured with an electronic digital integrator with an automatic base line corrector.

C. PREPARATION OF α- AND β-AGARASES

(1) Microorganisms and enzyme production

The microorganisms used in this study were marine bacteria Pseudomonas atlantica 549 ATCC 19262 and strain GJ1B, and a soil bacterium strain 3A.

The Pseudomonas atlantica was originally isolated at Point Pleasant Park, Halifax, Nova Scotia. The morphology and cultural characteristics have been described (Yaphe, 1957). The culture, started from a lyophilized preparation, was maintained on 1.5% Difco Bacto-agar slants of ZoBell's medium Z2216E (Oppenheimer and ZoBell, 1952, see Appendix). For the preparation of the inoculum, serial transfers were made in H-1 medium (see Appendix) containing 0.2% washed agar. Flasks containing 200 ml H-1 medium were inoculated with 1 ml of a 18 hr. culture and incubated on a rotary shaker for 18 hr. at 25°C for the maximum production of agarase.

Marine bacterium strain GJ1B was received from Dr. G. Jones as a

mixed culture which was collected at Great Bay - Little Bay estuarine complex, New Hampshire, U.S.A. The bacterium was separated from a non-agar decomposer, strain GJ1A, on H-1 medium, and then lyophilized. GJ1B is a Gram negative rod-shaped, unipolar flagellated, non-pigmented bacterium which bores a hole through 5 mm of agar in a petri plate in 24 hr. The inoculum was prepared by subculturing 3 times at 24 hr. intervals in H-2 medium (see Appendix), containing 0.2% washed agar. The culture medium was incubated on a rotary shaker at 25°C. 5 ml of the inoculum was transferred to flasks containing 200 ml H-2 medium, and incubated under the same conditions for the inoculum preparation.

The soil strain 3A was obtained from Dr. F.D. Cooke, isolated in Alberta. It is a Gram negative rod with unipolar flagellation and yellow pigmentation. The culture was started from a lyophilized preparation by serial transfer in Alexander salt medium (see Appendix), containing 0.2% washed agar. After three successive transfers, 5 ml of the culture was transferred to flasks containing 200 ml of Alexander salt medium and incubated on a rotary shaker at 25°C for three days.

(2) Measurement of agarase activity

a. Qualitative iodine method

Qualitative determination of agarase activity was done by the iodine method. An equal volume of enzyme preparation and 0.2% washed agar in 0.01 M Tris-buffer pH 7.2 was mixed and incubated in a 42°C water bath. At various inter-

vals, one drop of I_2/KI solution (see Appendix) was mixed with one drop of enzymic hydrolysate on a spot plate. The disappearance of a blue-black color indicated partial hydrolysis of the agar. The time required for the color to disappear (the achroic point) gave an indication of the amount of enzyme present.

b. Quantitative viscometric method

Two quantitative methods may be used for the assay of agarase activity:

i) The measurement of the rate of decrease in viscosity of the hydrolysate (Tracey, 1955),

ii) the increase in the reducing power present in the hydrolysate (Somogyi, 1952).

Because the determination of the reducing power is laborious and much more time consuming than the viscometric method, the latter was adapted for the quantitative assay of the enzyme activity in this study. The method used was a modification of the method of Tracey (1955).

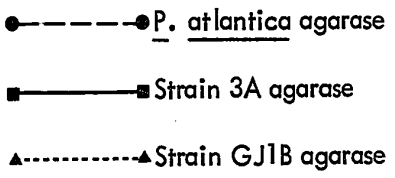
The addition of enzyme to an agar solution decreases the specific viscosity of the hydrolysate. The specific viscosity (η_{sp}) is defined as $\eta_{sp} = \frac{\text{flow time of the hydrolysate}}{\text{flow time of the buffer}} - 1$. An Oswald capillary viscometer with working volume of 5 ml was used. The flow time of 0.01 M Tris-HCl buffer (pH 7.2)

ranged from 50 to 60 sec. at 42°C. 4 ml of 1% washed agar was pipetted into the viscometer and equilibrated at 42°C. 1 ml enzyme solution, equilibrated at the same temperature was then pipetted into the viscometer. Immediately the digest was mixed thoroughly by bubbling air through the viscometer. The flow time of the digest was determined as soon as possible, and at regular intervals thereafter. The exact incubation period was calculated from the time of mixing the solutions. Since hydrolysis was in progress while the flow time was determined, the incubation period was taken as half of the flow time plus the period from the time of mixing to the time when the flow time determination was started. The rate of decrease of specific viscosity was then plotted. The time, in minutes, required to halve the specific viscosity was determined.

c. Definition of the unit of enzyme activity

A unit of the enzyme activity is defined as that amount of enzyme which will halve the specific viscosity of 5 ml of 0.8% washed agar solution within 100 minutes at 42°C. In order to measure the enzyme activity accurately, the concentration of enzyme was such that it halved the specific viscosity within 10 to 30 minutes. For agarases, a linear relationship was found between the enzyme dilutions and the units of activity (Fig. 13).

Fig. 13. Relationship between enzyme dilution and units of enzyme activity as determined by the viscometric method.



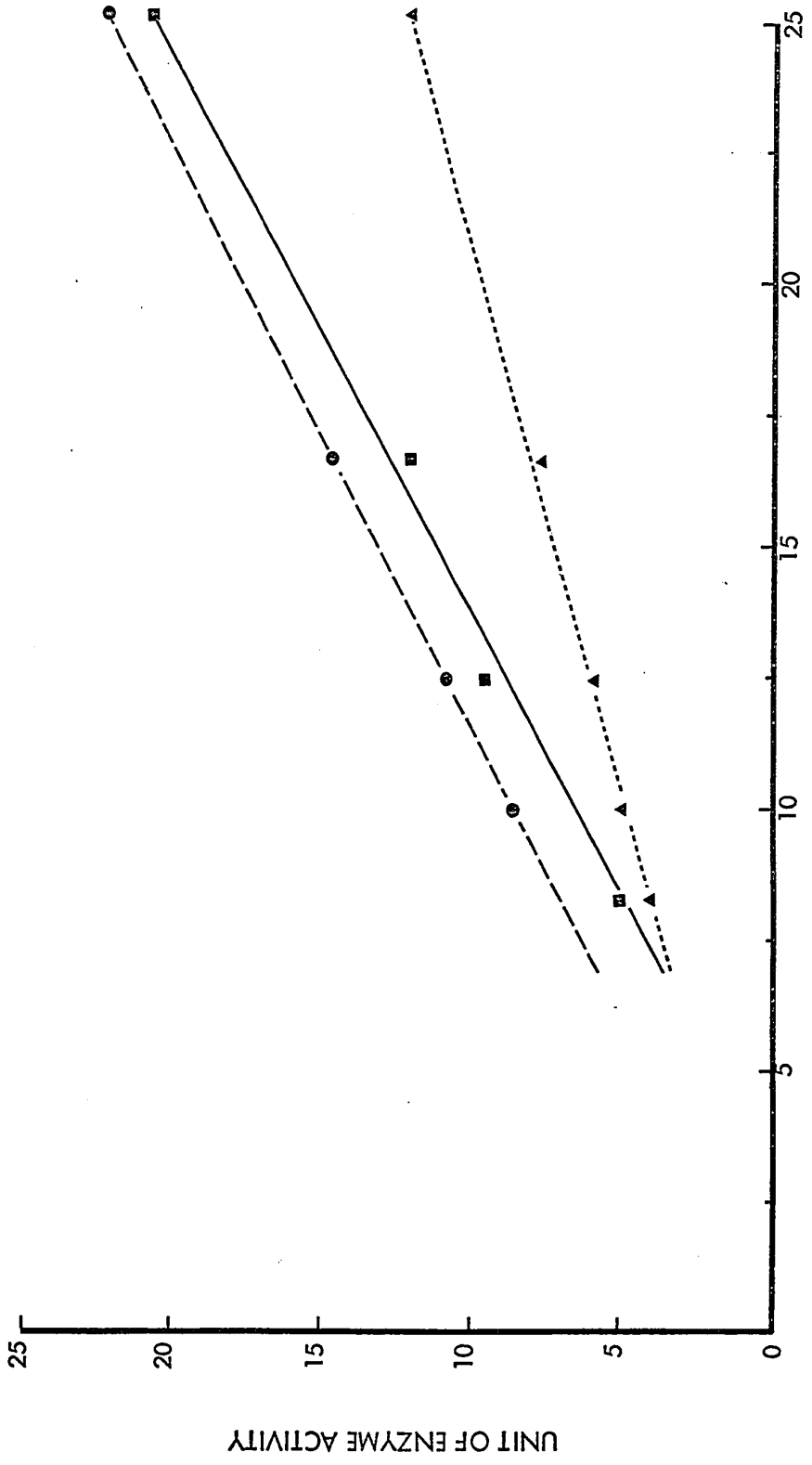

UNIT OF ENZYME ACTIVITY
0
5
10
15
20
25
RELATIVE ENZYME CONCENTRATION
5
10
15
20
25

FIG. 13

(3) Enzyme purification

The crude agarases obtained from the cell-free culture medium were always contaminated with oligosaccharidases. The cultures were harvested and the cell-free culture medium was obtained by centrifugation. To the cell-free supernatant, solid ammonium sulfate was added to a final concentration of 70% saturation. The precipitate was collected by centrifugation and dissolved in the smallest amount possible of Tris-HCl buffer (pH 7.2, 0.01 M). The enzyme solution was dialyzed against the same buffer for 4 hours. For the agarases from strains GJ1B and 3A, the enzymes were first dialyzed against buffer containing 0.1 M $CaCl_2$ for 1 hr. and then against buffer without $CaCl_2$. The dialyzed ammonium sulfate preparation (A/S) was kept frozen when not in use.

The A/S enzyme solution (5 to 10 ml) was applied to a DEAE-cellulose (OH^-)column (2.5 x 30 cm), equilibrated with Tris-buffer (pH 7.2, 0.01 M). Elution of the enzyme was achieved with a salt gradient ranging from 0 to 0.2 M to establish the salt concentration which was required to elute the agarase. In the subsequent experiments, the column was eluted in a stepwise fashion using 0.01 M Tris-buffer pH 7.2 containing different salt concentrations; 0.025 M NaCl for the P. atlantica agarase, 0.01 M $CaCl_2$ for the strain 3A agarase, and 0.1 M $CaCl_2$ for the strain GJ1B agarase. The column was first eluted with 0.01 M Tris-buffer and treated with salt solutions of higher ionic strength in the same buffer. Fractions were collected and dialyzed against 0.01 M Tris-buffer. The absence of oligosaccharidases was

demonstrated by mixing a small portion of the purified enzyme with neoagarotetraose. The purified enzyme was concentrated by filtration through a diaflow membrane UM-10 (Amicon Corp.), and kept frozen.

(4) Isoelectric focusing

The principle of isoelectric focusing is based on the presence of a pH gradient which is obtained by a direct current to an electrolyte system, so 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 species of ampholyte will collect at that place in the gradient where the pH is equal to the isoelectric point of that ampholyte. This collecting, or focusing, is caused by an electric field, thus the name isoelectric focusing.

A stable pH gradient can be obtained by electrolysis of low molecular weight "carrier ampholytes" (Sevensson, 1962) which contribute a narrow pH gradient and a considerable buffering capacity in their isoelectric state. This is essential for good resolution of considerable quantities of protein, especially when the isoelectric points (pI) are close. Another necessity for isoelectric separation is the prevention of convection and remixing of focused ampholytes. This can be achieved by using a density gradient with gradually decreasing concentration from bottom to the top of the column. In such a stabilized condition, the isoelectric focusing technique not only renders good resolution in separation of ampholytes,

but also allows direct measurement of the pIs of the fractionated ampholytes.

A LKB isoelectric focusing column (model 8101, LKB Produkter, Sweden) of 110 ml capacity was used. The manufacturer's instructions were followed completely. 2% Ampholine carrier ampholytes were used for the pH gradient, and sucrose for the density gradient. The column was maintained at 4°C throughout the experiment using a Neslab pumping and cooling system (Neslab Instrument Inc., Portsmouth, New Hampshire).

The following solutions were prepared:

Bottom electrode solution	
Monoethanolamine	0.8 ml
Distilled water	28.0 ml
Sucrose	24.0 g
Top electrode solution	
Conc. sulfuric acid	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 5 mM of salt, was added to the heavy and light solutions, displacing a corresponding volume of distilled water. The bottom electrode solution was pumped into the central compartment of the column using a peristaltic pump (model 600-1200, Harvard Apparatus Co., Dover, Mass.). The linear sucrose gradient was formed using a Dialagrad programmed gradient pump (model 190, Instrumentation Specialties Co., Lincoln, Nebraska) with a flow rate of 4 ml per minute. The top solution was then layered slowly, with the peristaltic pump, to cover the top electrode. A constant 600 volts was supplied for 48 hr. and was terminated when the current through the column reached a constant value (approx. 2 mA). The column was emptied from the bottom using a peristaltic pump with a flow rate of 4 ml per minute, and the eluate was constantly monitored at 254 nm through a LKB "Uvicord" unit (model 4701A, LKB Produkter, Sweden). Two ml fractions were collected, and the pH of each fraction was measured to determine the isoelectric point of the focused protein. Fractions were also analyzed spectrophotometrically at 260 and 280 nm, and were tested for agarase and oligosaccharidase activities.

D. PREPARATION OF OLIGOSACCHARIDES BY HYDROLYSIS OF AGAROSE

(1) Preparation of an agarose hydrolysate

DEAE-fractionated agarose (2 g) (see Materials and Methods, A-3) was dissolved in Tris-buffer (pH 7.2, 0.01 M, 200 ml) at 100°C. Each purified agarase (ca. 100 units) was added to the solution of agarose at 42°C, and incubated until the achroic point was reached. The hydrolysate was boiled at 100°C for 10 min. to stop the reaction. 3 volumes of ethanol were added to the hydrolysate to precipitate the high molecular weight fragments. The precipitate was removed by centrifugation, and the supernatant was evaporated to a small volume. This solution was used for isolating the high molecular weight oligosaccharides. For low molecular weight oligosaccharides, after the achroic point was reached, the hydrolysate was allowed to cool from 42°C to 25°C. Additional enzyme was added and incubated overnight, and then concentrated in vacuo at 35°C to approximately 20 ml.

(2) Isolation of the neoagarobiose series of oligosaccharides

The low molecular weight neoagarobiose series of oligosaccharides with degree of polymerization (DP) of 2 to 8 were separated on 80 cm of a Sephadex G-25 column (2.5 x 100 cm, flow rate 7 ml/hr./cm^2). A 5 ml aliquot of agarose hydrolysate was applied to the column, and eluted with distilled water. Fractions (3 ml) were collected, and separation was monitored by TLC with solvent B. The high

molecular weight oligosaccharides (DP 8 to 16) were partially separated on a G-25 column, and then purified by thin-layer or paper chromatograms. The isolated sugars were concentrated and lyophilized.

(3) Isolation of the agarobiose series of oligosaccharides

The end sugar of the agarobiose series of oligosaccharides, i.e. the sugar at the reducing end, is 3,6-anhydro-L-galactose, a labile sugar. Separation of this series of oligosaccharides is difficult without converting the 3,6-anhydro-L-galactose to a stable derivative. The method of borohydride reduction was used (Wolfrom and Thompson, 1963) to convert the end sugar into the stable sugar alcohol. Sodium borohydride solution (200 mg in 5 ml distilled water) was added dropwise at 4°C to the oligosaccharides (1 g in 3 ml distilled water). The mixture was allowed to react overnight at 4°C and then was acidified with 0.4 M acetic acid, and deionized, first with strong acid resin (Dowex-50W x 8, H^+ form) and then with weak base resin (AGA-316) at 4°C. The solution was evaporated to dryness under reduced pressure and repeated distillation with methanol. The sugar alcohols were dissolved in a small aliquot of distilled water, and chromatographed on G-25 as well as on thin-layer or paper chromatograms as described.

E. IDENTIFICATION OF THE GJ1B ENZYMIC HYDROLYSATE OF AGAROSE

(1) Chromatography of the enzymic hydrolysate

Good resolution of the products from strain GJ1B agarase hydrolysate was obtained on TLC, the cellulose being impregnated with a freshly prepared solution of 0.1 M sodium bisulfite buffer at pH 4.7. This method was adapted from that of Theander (1957) in which aldehydes and ketones were separated in the presence of hydrogen sulfite by paper ionophoresis. The buffer was freshly prepared by dissolving 9.5 g sodium pyrosulfite and 8.8 g sodium acetate. $3H_2O$ in 1 liter distilled water. The pH was adjusted to 4.7 with acetic acid and the TLC plate was sprayed with the buffer and allowed to air dry before the hydrolysate was applied. The plate was developed in solvent B.

(2) Acid hydrolysis of the sugar alcohol

The reduced hydrolysate or the isolated sugar alcohol was hydrolyzed in 1 M sulfuric acid at 100°C for 4 hr. The hydrolysate was cooled and neutralized with barium carbonate. The precipitate was removed by centrifugation, and the supernatant was evaporated to dryness. For paper chromatography, the hydrolysate was redissolved in a small aliquot of distilled water, whereas for GLC, it was redissolved in DMSO for the preparation of TMS-derivatives.

(3) Identification of the agarobiose series of oligosaccharides

The degree of polymerization (DP) of the oligosaccharides was determined by measuring the ratio of 3,6-anhydro-L-galactitol to galactitol by quantitative GLC using ribitol as internal standard. The oligosaccharide was mixed with a known amount of ribitol, and acid hydrolyzed (H_2SO_4, 1 M, 4 hr., 100°C). The hydrolysate was neutralized and reduced with sodium borohydride as described in a previous section. The solution was acidified with acetic acid (0.4 M), deionized with Dowex-50W x 8 (H^+ form), and the borate removed by repeated distillation with methanol. The hydrolysate was then redissolved in DMSO for GLC examination.

The DP of the oligosaccharides was also determined colorimetrically by their 3,6-anhydro-L-galactose content by the resorcinol method (Yaphe and Arsenault, 1965), using a portion of oligosaccharide solution prepared for the 3,6-anhydro-L-galactitol/galactose ratio experiment. The amount of oligosaccharide in the solution was calculated by GLC from the ratio of 3,6-anhydro-L-galactitol to ribitol. The DP was then determined from the ratio of moles of 3,6-anhydro-L-galactose to the moles of oligosaccharide.

F. DETERMINATION OF THE MODE OF ACTION OF THE BACTERIAL AGARASES

(1) Hydrolysis of oligosaccharides

In order to determine which bond of the oligosaccharide (DP 6 and 8) was hydrolyzed by the agarase, each oligosaccharide was labelled by converting the end

sugar into the corresponding alcohol with sodium borohydride, as described on page 54. The mixture of reducing and non-reducing sugars, after the oligosaccharide alcohol had been hydrolyzed by the enzyme, was easily distinguished by separation on TLC and by spraying with a specific indicator reagent such as aniline hydrogen phthalate, which reacts only with a reducing sugar. A sample of the enzyme hydrolysate of the oligosaccharide alcohol was applied to each of two thin-layer plates and developed in solvent B. After development, one plate was sprayed with naphthoresorcinol or silver nitrate reagent, the other with aniline hydrogen phthalate reagent. The failure of a spot to appear with the latter spray reagent, indicated a non-reducing sugar.

(2) Hydrolysis of agarose

The method used for measuring the degree of multiple attack of the agarase on agarose was based on that devised by Robyt and French (1967). To a 1% solution of agarose in Tris-buffer, pH 7.2, was added 2 viscosity units per ml of reaction mixture. The solution was incubated at 42°C and 10 ml aliquots were removed at various time intervals. The enzyme activity was terminated by boiling the hydrolysate for 10 min. The solution was cooled to 42°C in a water bath. 5 ml of the hydrolysate was removed for the viscometric assay, 1 ml for the total reducing sugar determination and 1 ml for determination of the reducing sugar of the 75% ethanol precipitate. The latter sample was mixed with 3 ml of ethanol and the

precipitate removed by centrifugation. The supernatant fluid was kept for analysis. The precipitate was washed twice with 75% ethanol, once with absolute ethanol and then dissolved in 1 ml Tris-buffer pH 7.2 for the reducing sugar determination.

The reducing value was determined by the colorimetric method of Somogyi (1952), using Nelson's arsenomolybdate solution as chromogenic reagent (Nelson, 1944). Galactose was used as standard. Somogyi's alkaline copper reagent was prepared as two reagents, Solution I and II (see Appendix), which were combined before use. Nelson's chromogenic reagent (see Appendix) was stored in a brown bottle in the dark.

(3) Assay for reducing sugars

Duplicate samples of the enzymic hydrolysate were diluted to 1 ml with Tris-buffer. Alkaline copper reagent (1 ml) was added, and the mixture was heated for 20 min. in a boiling water bath. After cooling to room temperature, the chromogenic reagent (1 ml) was added, mixed and set aside for 15 min. to allow for color development. 5 ml DMSO was then added to dissolve the agarose. The precipitated salt was removed by centrifugation, and the absorbance of the supernatant read at 560 nm. The reducing value was calculated from a galactose standard curve and expressed as mμM of galactose.

The degree of multiple attack of the agarase is expressed as the r-value, which is the ratio of the total reducing value of the hydrolysate to the reducing value of the polymer precipitated with 75% ethanol (Robyt and French, 1967).

IV. RESULTS

A. FRACTIONATION AND ANALYSIS OF AGAR

The yield of agar obtained by extraction at 121°C was found to vary according to the agarophyte used. Table I shows the percentage yield on a dry-weight basis, the pyruvic acid and sulfate contents of each agar. The pyruvic acid and sulfate content varied with the source of the agar. A 1% aqueous solution of each agar gelled at room temperature. Acid hydrolysis of the agars, followed by paper chromatography, revealed in each case spots with the same mobility as galactose, methyl galactose and xylose (Fig. 14). Gelidiella acerosa agar was found to contain the largest quantity of methyl galactose.

The fractions obtained by separation of the agars on DEAE Sephadex A-50 (Cl^-) varied with the source of the agar. The yield of polysaccharide in each fraction, and its 3,6-anhydrogalactose and sulfate content, is given in Table II. In each case, with increasing ionic strength of the eluant, the sulfate content of the eluted polysaccharide increased. On the other hand, the 3,6-anhydrogalactose content decreased as the ionic strength of the eluant increased. Since Pterocladia pinnata agar was found to contain the highest amount of pyruvic acid, each fraction obtained by separation of this agar on DEAE Sephadex A-50 (Cl^-) was analyzed for its pyruvic acid content. The fractions eluted from the DEAE Sephadex column with distilled water, 0.5 M, 1 M and

Fig. 14. Paper chromatography of the acid hydrolysate of agars extracted from algae. The chromatogram was developed in solvent C and sprayed with aniline hydrogen phthalate reagent.

1: Gelidium sesquipedale

2: Pterocladia pinnata

3: Digenia simplex

4: Gelidiella acerosa

5: Ahnfeltia plicata

XYL: Xylose

GAL: Galactose

MGAL: 6-O-Methyl galactose

HMF: Hydroxymethylfurfuraldehyde

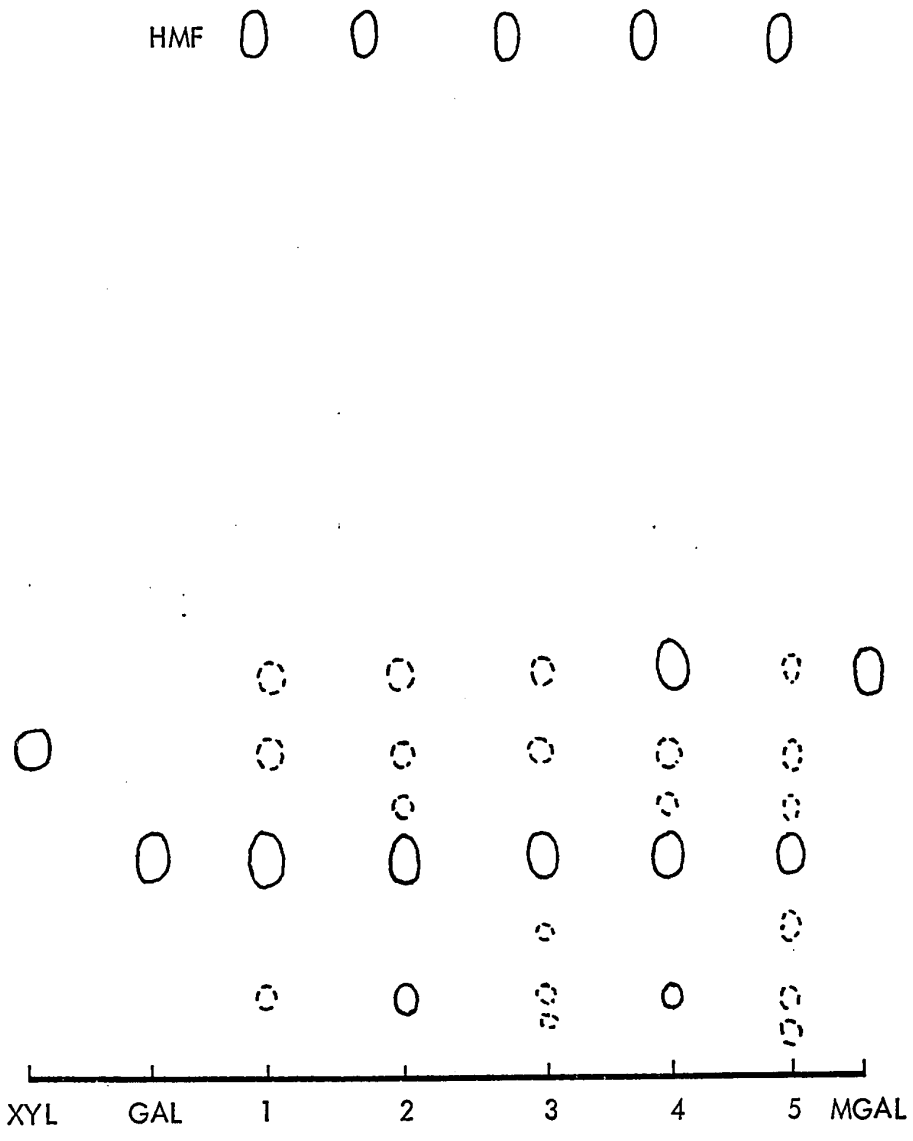


FIG. 14

2.5 M NaCl fractions contained 0.02%, 0.88%, 0.53% and 0.42% of pyruvic acid, respectively. Thus the pyruvic acid content was highest in the 0.5 M NaCl fraction, and gradually decreased as the sulfate increased.

In order to show that the polysaccharides obtained were part of the agar family of polysaccharides, each polysaccharide fraction eluted from the DEAE Sephadex A-50 column was hydrolyzed with the β-agarase obtained from P. atlantica, and the resulting hydrolysate examined by TLC (Fig. 15). The products of enzymic hydrolysis were the neoagarobiose series of oligosaccharides, charged oligosaccharides, and a sugar (A) with a mobility greater than that of neoagarobiose. This sugar (A) which was found in the Gelidiella acerosa fractions may be methylated neoagarobiose.

TABLE I

Sulfate and Pyruvic Acid Content of Agars from Different Agarophytes

Algae	Country of origin	Sulfate (%)	Pyruvic acid (%)	% yield from dry algae
Pterocladia pinnata	Barbados	3.7	0.65	15.3
Digenia simplex	Barbados	7.0	0.11	8.5
Gelidiella acerosa	Barbados	4.6	0.12	6.0
Gelidium sesquipedale	Spain	3.2	0.04	28.5
Ahnfeltia plicata	NFLD, Canada	-	ND *	8.3

* ND: not detectable

TABLE II

3,6-Anhydrogalactose and Sulfate Content of the Agars Fractionated on DEAE Sephadex A-50 (Cl^-)

Algae	Eluant	% yield	3,6-Anhydro-galactose (%)	Sulfate (%)
Pterocladia	Distilled water	12.6	42.4	0.63
pinnata	NaCl 0.5 M	47.9	37.1	1.60
	NaCl 1.0 M	4.1	-	9.50
	NaCl 2.5 M	1.8	-	9.60
	Total	66.4		
Gelidium	Distilled water	28.6	45.7	0.17
sesquipedale	NaCl 0.5 M	28.1	40.8	1.86
	NaCl 1.0 M	8.8	26.1	5.04
	NaCl 2.5 M	8.8	16.3	12.40
	Total	74.3		
Digenia	Distilled water	10.4	16.8	0.57
simplex	NaCl 0.5 M	40.5	30.7	1.68
	NaCl 1.0 M	33.8	25.2	3.90
	NaCl 2.5 M	6.1	9.2	6.40
	Total	90.8		
Gelidiella	Distilled water	39.6	38.4	0.13
acerosa	NaCl 0.5 M	15.4	22.3	1.46
	NaCl 1.0 M	13.0	20.1	4.70
	NaCl 2.5 M	5.7	15.1	6.93
	Total	73.7		
Ahnfeltia	Distilled water	7.2	33.1	0.47
plicata	NaCl 0.5 M	34.4	26.9	1.03
	NaCl 1.0 M	5.0	21.0	2.53
	NaCl 2.5 M	0.6	18.2	5.44
	Total	47.2		

Fig. 15. *P. atlantica* agarase hydrolysates of polysaccharide fractions eluted from a DEAE Sephadex A-50 (Cl^-) column. The chromatogram was developed in solvent A and sprayed with naphthoresorcinol reagent.

1: *Gelidium sesquipedale*

2: *Pterocladia pinnata*

3: *Digenia simplex*

4: *Gelidiella acerosa*

5: *Ahnfeltia plicata*

A: Distilled water fraction

B: 0.5 M NaCl fraction

C: 1.0 M NaCl fraction

D: 2.5 M NaCl fraction

GAL: Galactose

S: Standard contains neoagarobiose, -tetraose and -hexaose

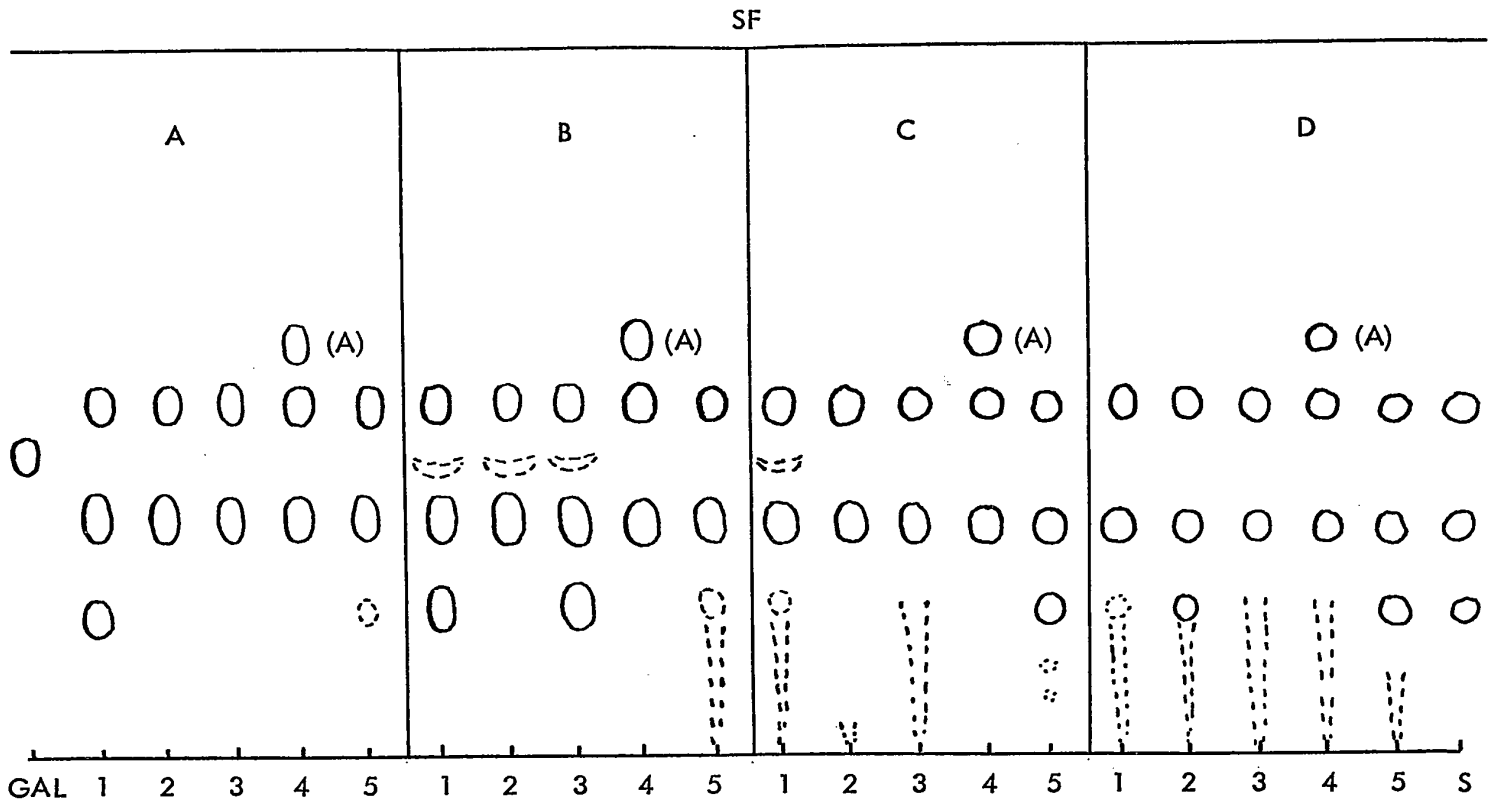


FIG. 15

B. PURIFICATION AND PROPERTIES OF THE BACTERIAL AGARASES

The cell-free culture medium, and the A/S preparations obtained from P. atlantica, strain 3A and strain GJ1B, were incubated with agarose and neoagarotetraose to demonstrate the presence of agarase and oligosaccharidases. The products of enzymic hydrolysis were examined by TLC. The pattern of degradation of agarose and neoagarotetraose is illustrated in Fig. 16. All the preparations contained agarase activity, since each agarose hydrolysate showed the presence of low molecular weight oligosaccharides. However, neoagarotetraose was hydrolyzed by the P. atlantica and 3A preparations to yield mainly neoagarobiose with traces of 3,6-anhydrogalactose and by the GJ1B preparation, to yield sugars which have not been identified. Since neoagarotetraose (Fig. 10) consists of one β- and two α-linkages, the results showed that the agarase preparations were contaminated with β- and/or α-oligosaccharidases. The presence of oligosaccharidases complicated the determination of the mode of action, and the analysis of the end products in the agarase hydrolysate.

(1) Isoelectric focusing

The pI of each agarase was determined before its purification. The advantage of this is that purification of the enzyme on the basis of its pI can be easily accomplished by ion-exchange chromatography.

Originally, an attempt was made to separate the P. atlantica agarase

Fig. 16. Pattern of degradation of agarose and neoagarotetraose by the ammonium sulfate precipitated (A/S) enzyme, on TLC. The chromatogram was developed in solvent A and sprayed with naphthoresorcinol reagent.

A: P. atlantica A/S preparation

B: Strain 3A A/S preparation

C: Strain GJ1B A/S preparation

1: Agarose hydrolysate

2: Neoagarotetraose hydrolysate

S: Standard contains neoagarobiose and -tetraose

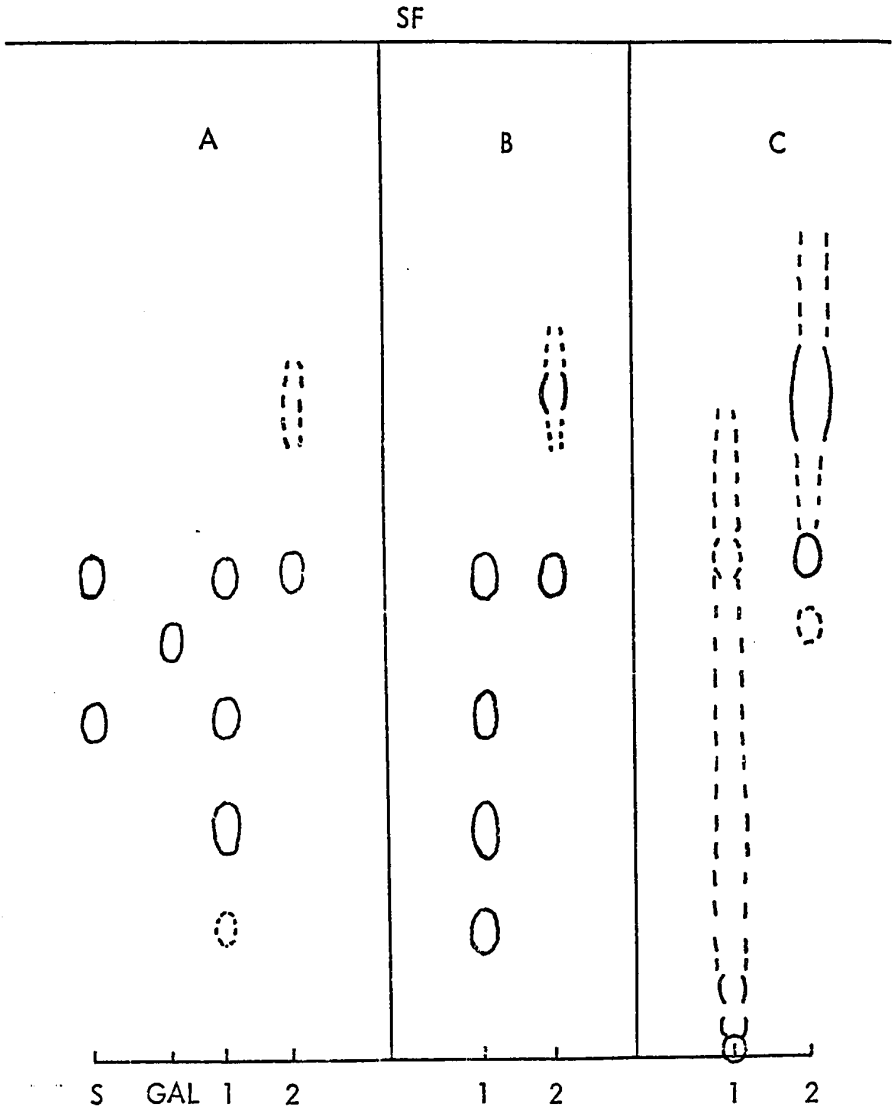
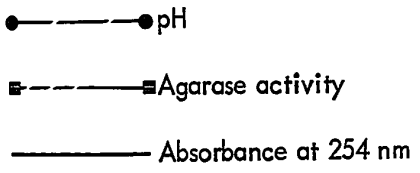


FIG. 16

from the oligosaccharidases by isoelectric focusing, using broad pH Ampholine (pH 3 to 10). However, it was not possible to achieve separation using the broad pH Ampholine. In addition, during the preliminary run, a thick band of polysaccharide-like material appeared at pH 5 which interfered with separation and pI determination. The pH range for the agarase activity was found to be from 5.2 to 8.9, thus the experiment was repeated using narrow pH Ampholine (pH 5 to 8), after removal of the polysaccharide-like material from the A/S preparation by a batchwise process with DEAE Sephadex A-50 (OH^-) in acetate buffer (0.1 M, pH 5.6). The profile of isoelectric focusing (Fig. 17) obtained by electrolysis of the DEAE-treated enzyme preparation indicated that the agarase could be separated from the oligosaccharidases. The oligosaccharidase (which was shown later to be a β-tetrasaccharidase) had a distinct isoelectric point, which was estimated to be at pH 4.8 to 5. The agarase was electrophoretically heterogenous, focusing between pH 5.4 and 8. However, the majority of enzyme activity was found between 5.75 and 6.2, with maximum activity at pH 6. The α-oligosaccharidase was not detected in this experiment. However, it has been shown to have a pI lower than that of the β-tetrasaccharidase (Ross, 1970).

Similar results were obtained with the 3A agarase using the A/S preparation and broad pH Ampholine (pH 3 to 10). However, when the A/S preparation was treated batchwise with DEAE Sephadex A-50 (phosphate buffer 0.01 M, pH 6), the oligosaccharidase activity disappeared, since neoagarotetraose

Fig. 17. Profile of the isoelectric focusing of P. atlantica agarase using Ampholine pH 5 to 8.



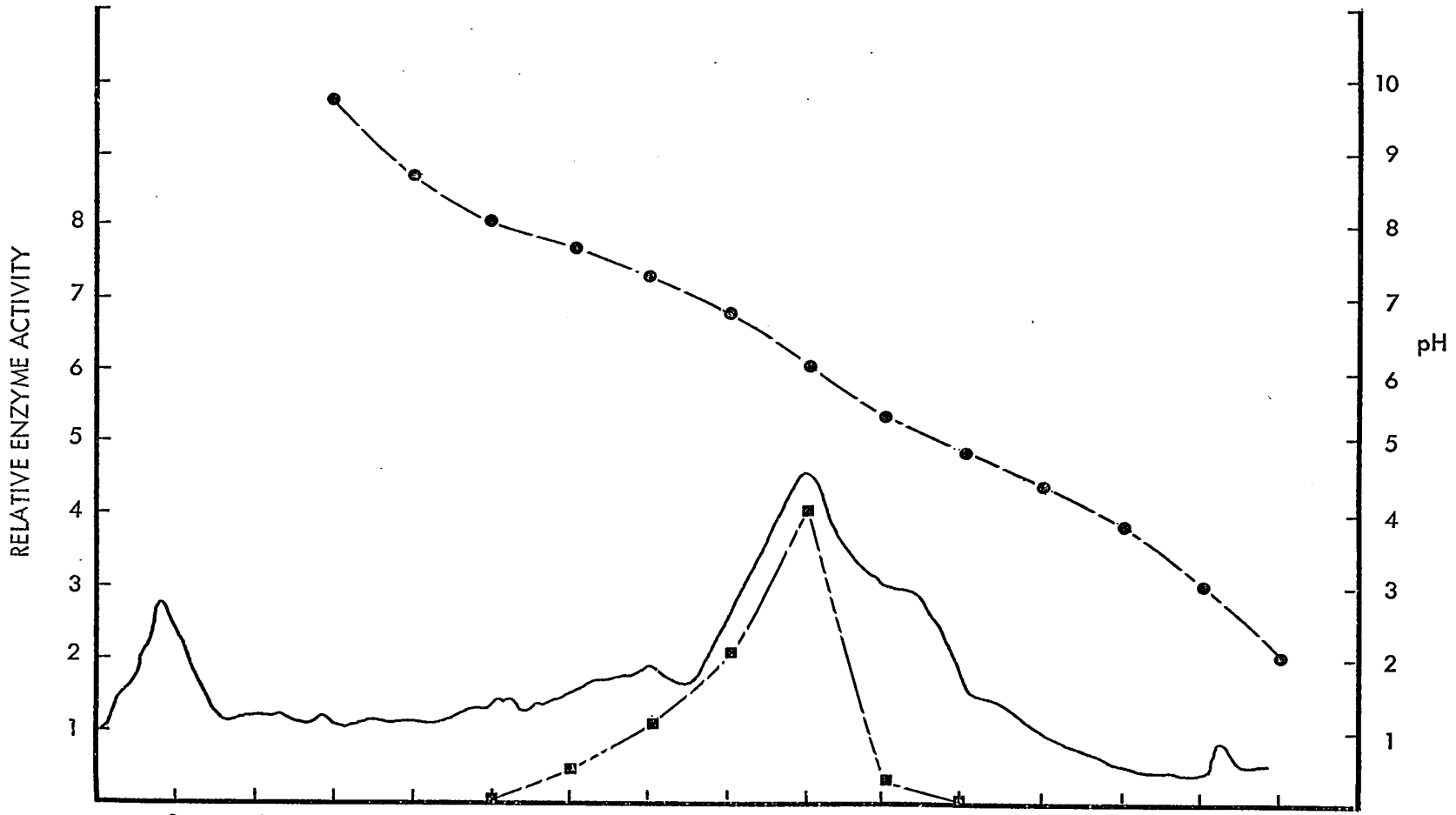

RELATIVE ENZYME ACTIVITY
1
2
3
4
5
6
7
8
pH
1
2
3
4
5
6
7
8
9
10
2
4
8
12
16
20
24
28
FRACTIONS

FIG. 17

was not hydrolyzed after overnight incubation with the enzyme. This indicated that the pIs of these oligosaccharidases are below pH 6. The DEAE-treated preparation was subjected to isoelectric focusing with Ampholine having pH range of 5 to 8. The profile of the isoelectric focusing is represented in Fig. 18. The pH range of the 3A agarase is less electrophoretically heterogenous than the P. atlantica agarase. It ranges from pH 6.0 to 7.2, with maximum enzyme activity at pH 6.5.

Isoelectric focusing of the GJ1B agarase was carried out on the A/S preparation with Ampholine having pH range of 3 to 10. The profile of the experiment is given in Fig. 19. The pH range of this agarase was found to be between 3 and 5.6, with maximum activity at pH 3.8.

(2) Ion-exchange chromatography

Based on the results obtained from the isoelectric focusing experiments, the agarases were purified on DEAE-cellulose (Tris-buffer 0.01 M, pH 7.2). The optimum salt concentration for elution of the agarases from the DEAE-cellulose was determined by gradient elution. The P. atlantica, strain 3A and strain GJ1B agarases were found to be eluted with 0.025 M NaCl, 0.01 M $CaCl_2$ and 0.1 M $CaCl_2$, respectively. In the subsequent experiments, the agarases were recovered from the DEAE-cellulose column by stepwise elution with the respective salt concentration. The agarases thus obtained showed no oligosaccharidase activity.

Fig. 18. Profile of the isoelectric focusing of strain 3A agarase using Ampholine pH 5 to 8.

●———●pH

■– – –■Agarase activity

————Absorbance at 254 nm

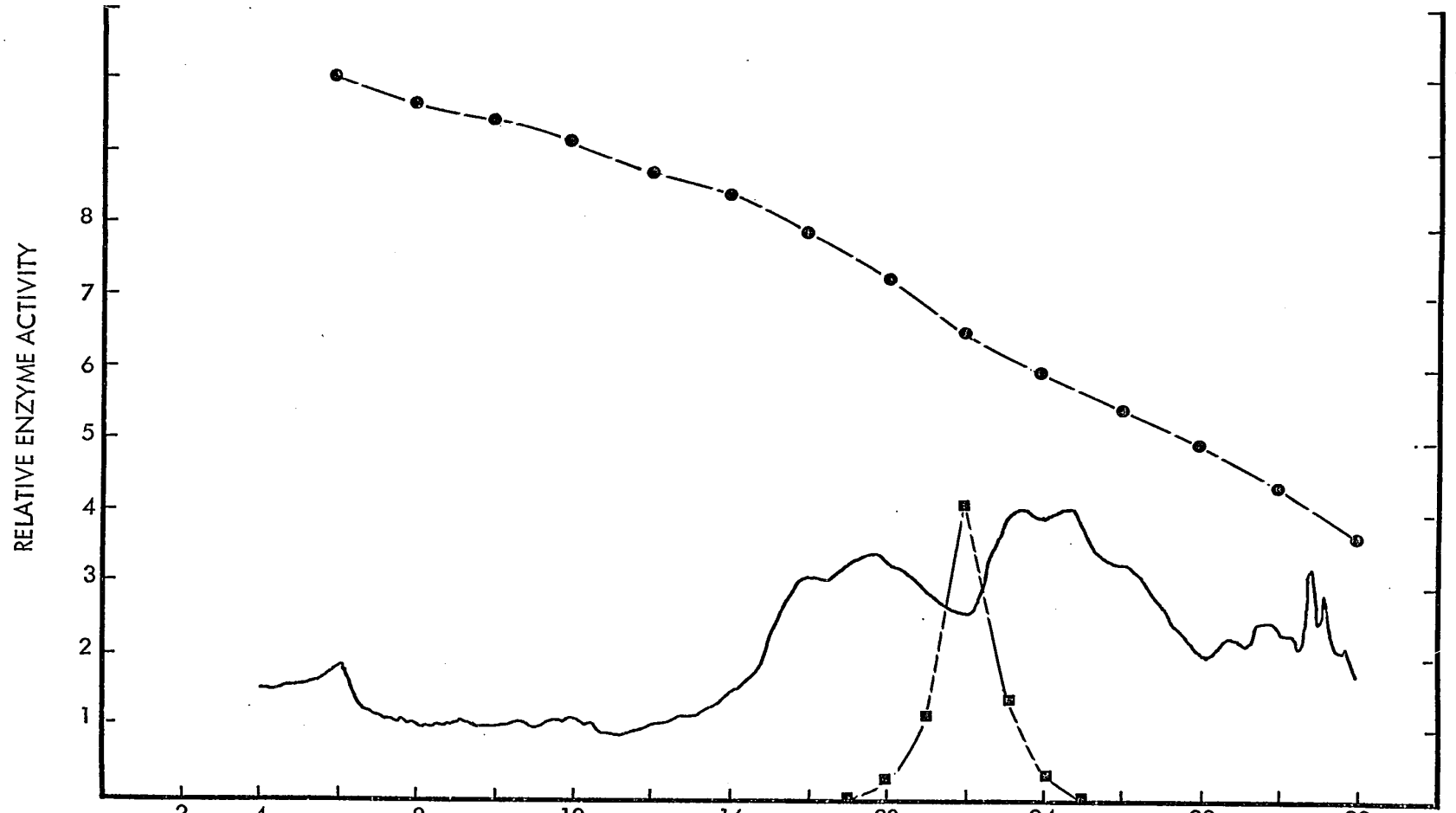

RELATIVE ENZYME ACTIVITY
1
2
3
4
5
6
7
8
pH
1
2
3
4
5
6
7
8
9
10
2
4
8
12
16
20
24
28
32
FRACTIONS

FIG. 18

Fig. 19. Profile of the isoelectric focusing of strain GJ1B agarase using Ampholine pH 3 to 10.

●———●pH

▪– – –▪Agarase activity

———Absorbance at 254 nm

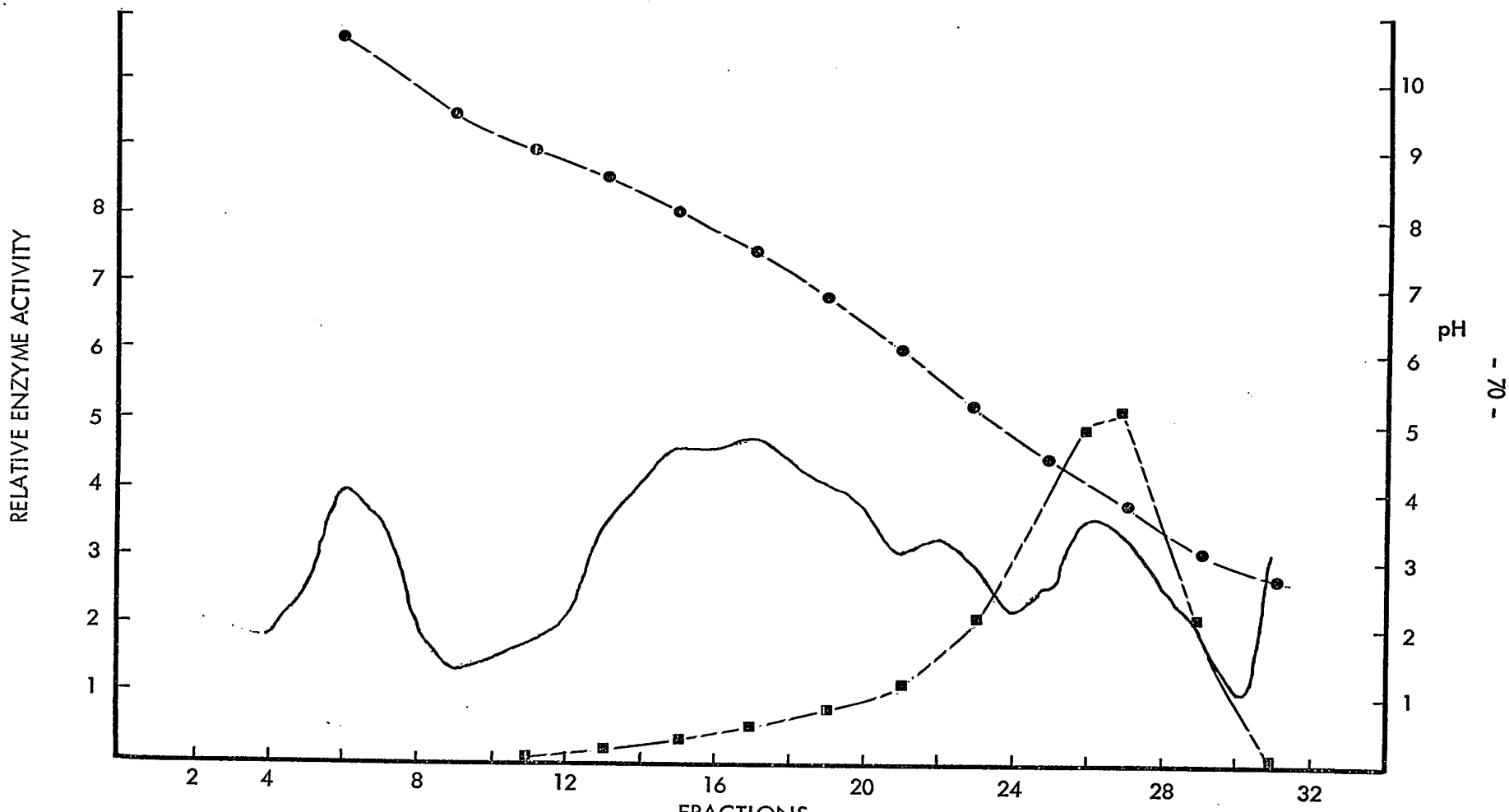

RELATIVE ENZYME ACTIVITY
1
2
3
4
5
6
7
8
pH
1
2
3
4
5
6
7
8
9
10
2
4
8
12
16
20
24
28
32
FRACTIONS

FIG. 19

Quantitative data of the purification process is summarized in Table III. A 38-fold increase in specific activity was obtained with the P. atlantica enzyme, a 45-fold increase with the strain 3A enzyme and only a 3.5-fold increase with the strain GJ1B.

(3) Optimal pH

To determine the optimal pH for GJ1B agarase activity, the viscometric assay procedure was used with Tris-maleate buffer (0.01 M). The A/S preparation showed maximum activity between pH 7.0 and 7.4 (Fig. 20). The optimal pH 7.2 of the GJ1B agarase is similar to that of the P. atlantica and the 3A agarase (pH 7.2).

(4) Stability

The agarase from P. atlantica was stable at 42°C in the presence of substrate, whereas the 3A and GJ1B agarases were labile, specially the latter enzyme. Several methods of stabilization were tried with the GJ1B agarase, but without success. These included the incorporation into the enzyme preparation of bovine serum albumin, glycerol and dithiothreitol. However, the GJ1B agarase was found to be stable in the presence of calcium ions as demonstrated by the following experiments.

TABLE III

Purification of the Agarases

	Step	Volume (ml)	Units	Protein (mg)	Specific * activity	Yield (%)
P. atlantica	Culture medium	116	38,628	232	166.5	100
	Ammonium sulfate	5	22,220	25.6	868.0	57.5
	DEAE (0.025 M NaCl) **	11	29,326	4.6	6348.6	75.9
Strain 3A	Culture medium	660	10,164	528	19.2	100
	Ammonium sulfate	5	9,780	45.6	214.5	96.2
	DEAE (0.01 M $CaCl_2$)	13	2,250	2.6	865.4	22.3
Strain GJ1B	Culture medium	828	124,200	67.7	1835	100
	Ammonium sulfate	10	93,650	17.4	5388	75.4
	DEAE (0.1 M $CaCl_2$)	16	26,720	4.2	6423	21.5

* Specific activity: Units per mg protein

** Ionic strength of NaCl or $CaCl_2$ used to elute the agarase from the column

Fig. 20. Effect of pH on strain GJ1B agarase activity.

Fig. 21. Effect of calcium and EDTA treatment on the strain GJ1B agarase activity.

▲——▲Non-calcium-treated enzyme preparation

■——■Calcium-treated enzyme preparation

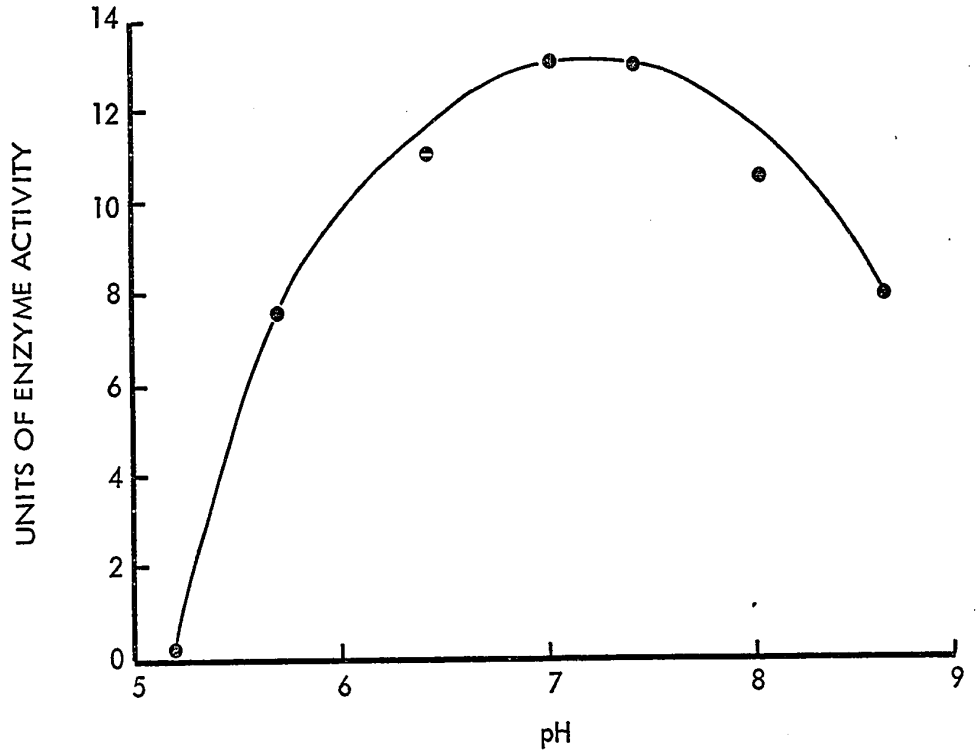

UNITS OF ENZYME ACTIVITY
14
12
10
8
6
4
2
0
5
6
7
8
9
pH

FIG. 20

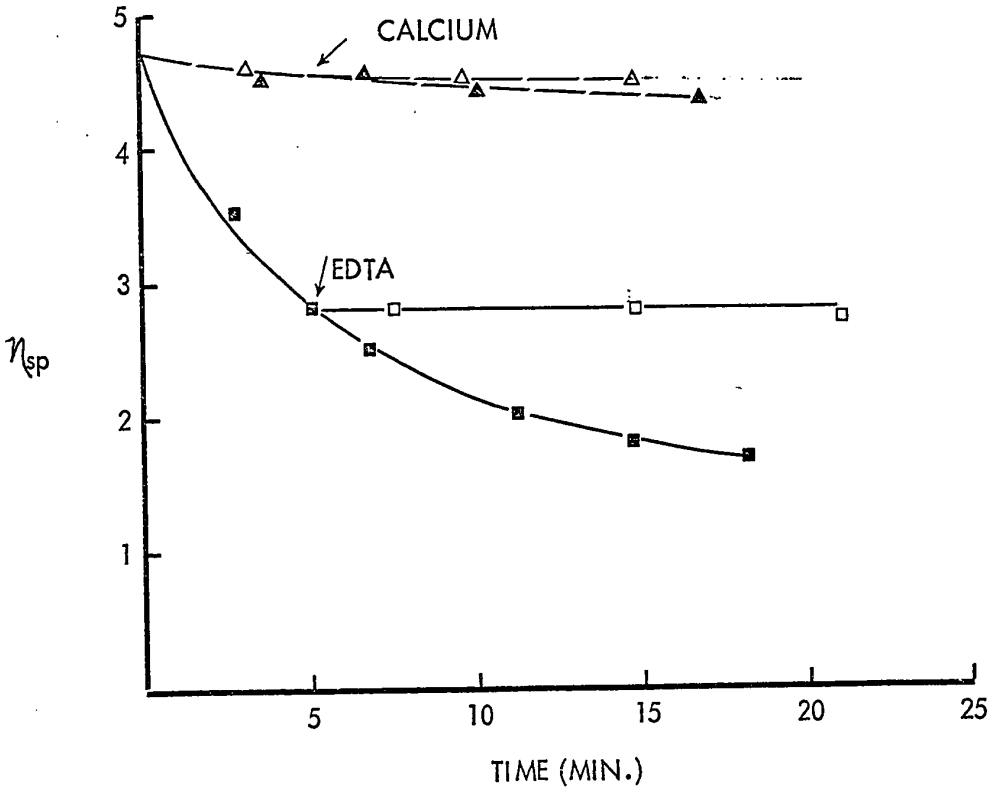

CALCIUM
EDTA
η_{sp}
5
4
3
2
1
5
10
15
20
25
TIME (MIN.)

FIG. 21

(a) The precipitate obtained by ammonium sulfate precipitation from the cell-free culture medium of strain GJ1B was dissolved in Tris-buffer (pH 7.2, 0.01 M), and divided into two equal parts. One sample was dialyzed initially against the buffer containing 0.1 M $CaCl_2$ for 1 hr., and then against the same buffer without $CaCl_2$ for 3 hr. The other sample was dialyzed against the buffer in absence of $CaCl_2$ for 4 hr. An identical dilution was made from each of these preparations, and the enzyme activity was determined viscometrically. The results are given in Fig. 21, from which it can be seen that without calcium treatment, the enzyme exhibits almost no action on the substrate. On the other hand, the calcium-treated preparation reduces the viscosity of the substrate very rapidly. Addition of EDTA (10^{-3} M, 0.1 ml) to the calcium-treated sample during the progress of the enzyme reaction completely destroyed the enzyme activity. However, addition of $CaCl_2$ (0.1 M, 0.1 ml) to the digest of the non-calcium-treated sample during the progress of enzyme reaction, did not restore the enzyme activity.

(b) To eliminate the possibility that EDTA may directly inhibit the enzyme activity, EDTA (10^{-3} M) was added to the calcium-treated A/S preparation, and it was allowed to stand for 30 min. at 4°C. The enzyme was then diluted with and without calcium (0.1 M), and the activity was determined. The results (Fig. 22) showed that the sample without calcium had very little activity, whereas the calcium-treated preparation regained its activity to a large extent.

Fig. 22. Effect of calcium on strain GJ1B agarase activity after EDTA treatment.

⊙——⊖ Preparation without the addition of calcium

●——● Preparation with the addition of calcium

Fig. 23. Effect of calcium concentration on the strain GJ1B agarase activity.

1: 10^{-1} M $CaCl_2$

2: 10^{-2} M $CaCl_2$

3: 10^{-3} M $CaCl_2$

4: 10^{-4} M $CaCl_2$

5: No $CaCl_2$

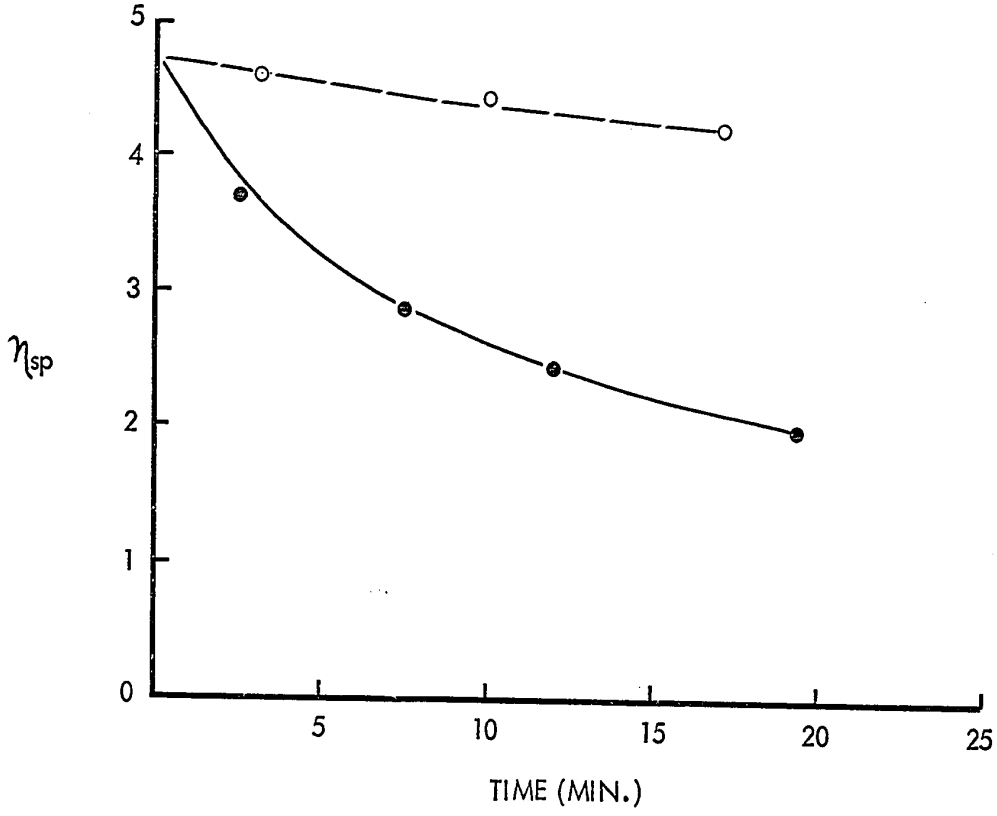

5
4
3
2
1
0
η_{sp}
5
10
15
20
25
TIME (MIN.)

FIG. 22

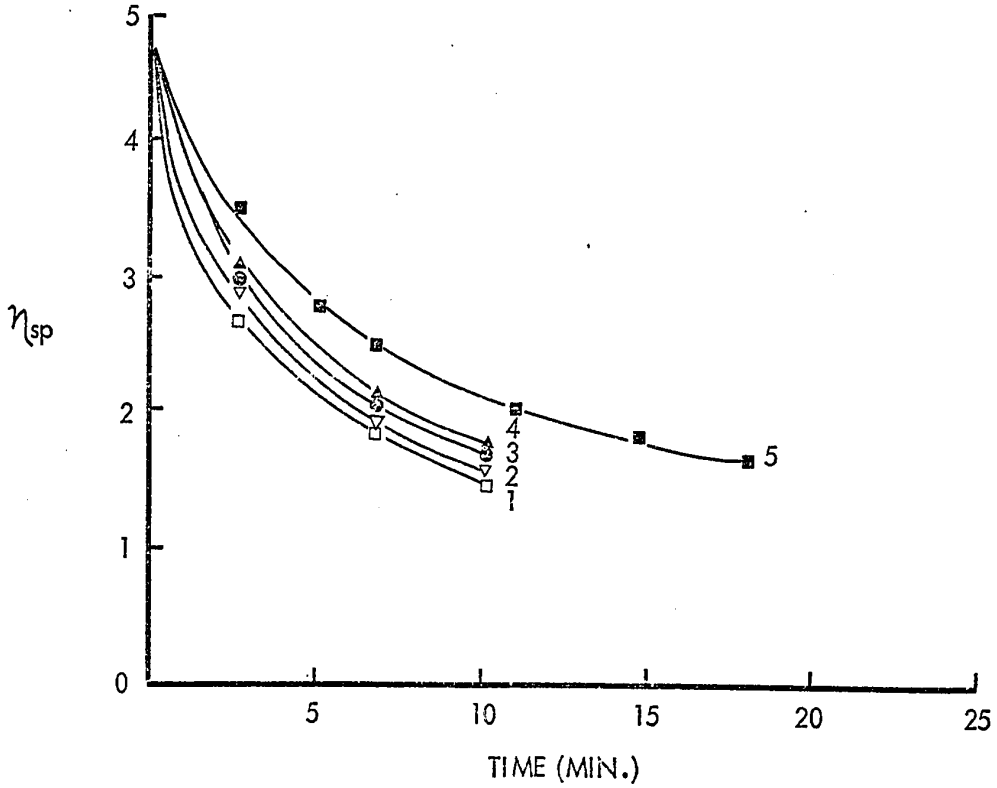

5
4
3
2
1
0
η_{sp}
4
3
2
1
5
5
10
15
20
25
TIME (MIN.)

FIG. 23

(c) The calcium-treated A/S sample prepared in experiment (a) was diluted with various concentrations of $CaCl_2$ ranging from 0 to 10^{-1} M in Tris-buffer and incubated with a 1% washed agar solution made in Tris-buffer containing the corresponding concentration of $CaCl_2$. The enzyme activity was determined viscometrically and the results are shown in Fig. 23. The enzyme activity was found to increase as the concentration of $CaCl_2$ increased.

A similar stabilization of enzyme activity was also observed with the 3A agarase.

C. PREPARATION OF THE OLIGOSACCHARIDES

(1) Neoagarobiose series

a. Isolation

DEAE-fractionated agarose (1%) was hydrolyzed with the purified β-agarase from P. atlantica or 3A at 42°C. At the achroic point, i.e. when the hydrolysate no longer gave a blue-black color with iodine, the hydrolysate was examined by wedge TLC in solvent E, and shown to consist of a homologous series of oligosaccharides with DPs ranging from 4 to 20 (Fig. 24), having Rgal 0.97, 0.87, 0.77, 0.66, 0.55, 0.45, 0.36, 0.27, 0.20, respectively. The oligosaccharides were first partially separated on a Sephadex G-25 column, and then oligosaccharides up to DP 16 were isolated by TLC or paper chromatography (Fig. 25A).

Fig. 24. Wedge cellulose-TLC showing the separation of the neoagarobiose series of oligosaccharides (DP 4 to 20) obtained with the P. atlantica or the strain 3A agarase hydrolysate of agarose at the achroic point. The chromatogram was double-developed in solvent E and sprayed with naphthoresorcinol reagent.

GAL: Galactose

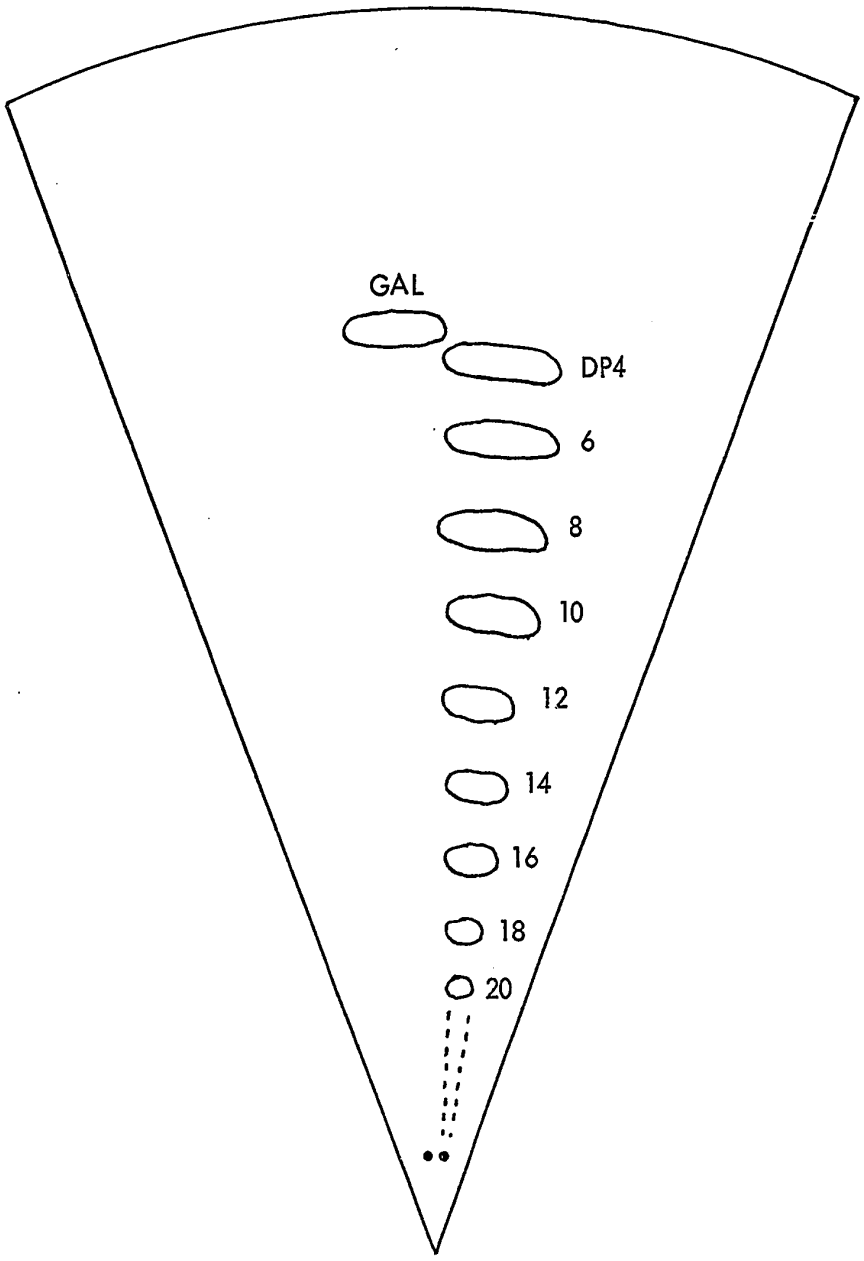


FIG. 24

Fig. 25. TLC of purified neoagarobiose series of oligosaccharides (A) DP 6 to 16, (B) DP 2 and 4. The chromatogram was developed in solvent E and sprayed with naphthoresorcinol reagent.

AH-1: Agarose hydrolysate obtained at achroic point with the β-agarase

AH-2: Final end products of β-agarase on agarose.

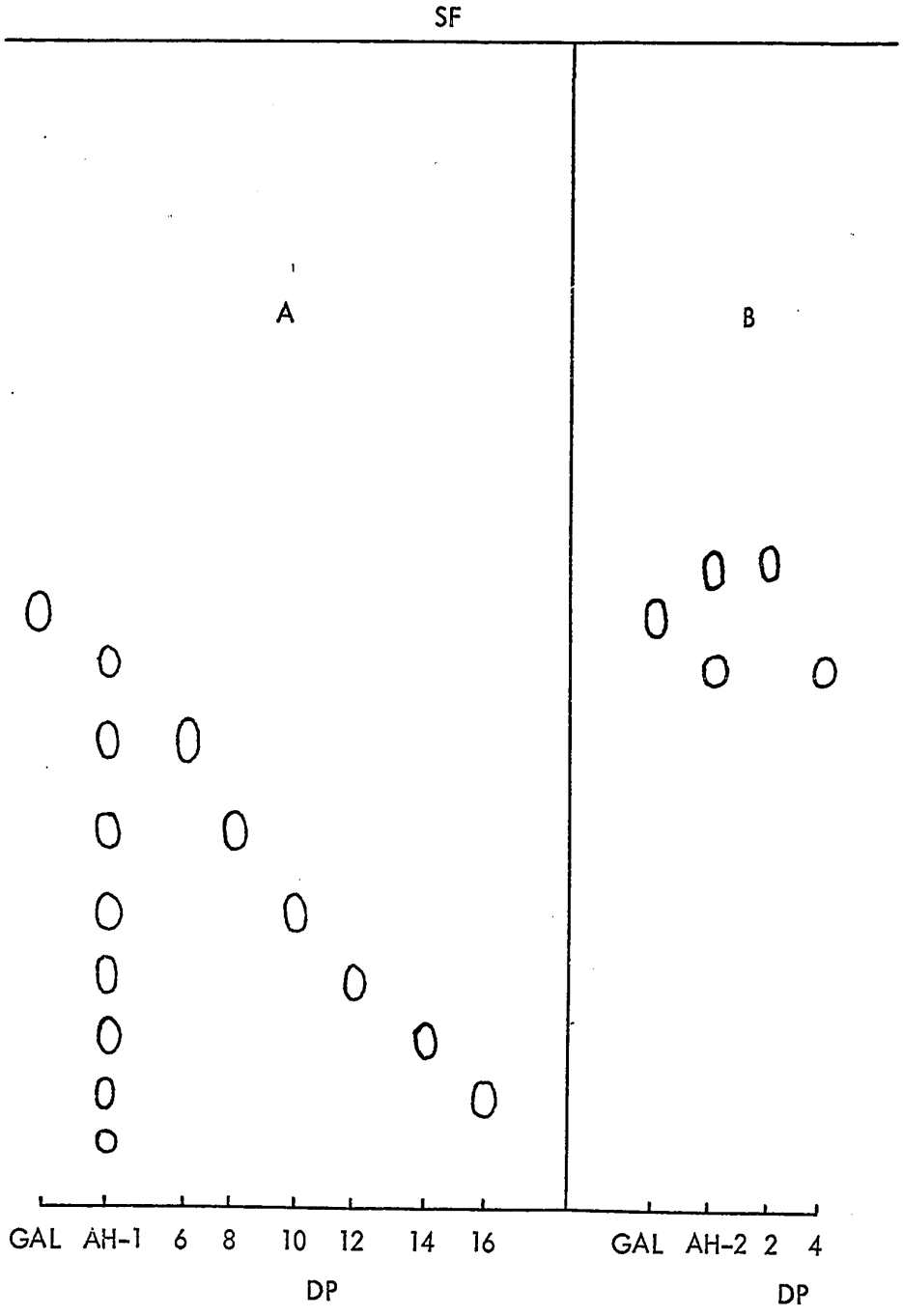


FIG. 25

For complete enzymic hydrolysis additional enzyme was added to the hydrolysate after the achroic point, and the digest was incubated at 25°C overnight, the final end products of the reaction were found to be neoagarobiose and neoagarotetraose (Fig. 25 B). These oligosaccharides were separated on a Sephadex G-25 column.

b. Solubility

The agarose hydrolysate (1%) obtained at the achroic point with the P. atlantica β-agarase when concentrated (5 times), remained in solution at 25°C. However, when kept at 4°C overnight, a powdery precipitate appeared. This precipitate remained undissolved at 25°C, but dissolved completely at 55°C. The precipitate was isolated by centrifugation, redissolved in distilled water at 55°C and chromatographed on wedge TLC. It was found to be enriched in high molecular weight oligosaccharides (DP 12 and up), as compared to the original hydrolysate.

(2) Agarobiose series

The agarose hydrolysate obtained with the purified GJ1B agarase, unlike that obtained with the β agarases, streaked on examination by TLC (Fig. 26). 3,6-Anhydrogalactose is present as end sugar in the agarobiose series of oligosaccharides. This sugar is unstable and the oligosaccharides could only be

Fig. 26. TLC of the agarose hydrolysate obtained with strain GJ1B agarase. The chromatogram was developed in solvent A and sprayed with naphthoresorcinol reagent.

A: Unmodified agarose hydrolysate

B: Agarose hydrolysate modified by bisulfite impregnation of the cellulose-TLC plate

C: Agarose hydrolysate after treatment with borohydride

GAL: Galactose

AH: Agarose hydrolysate

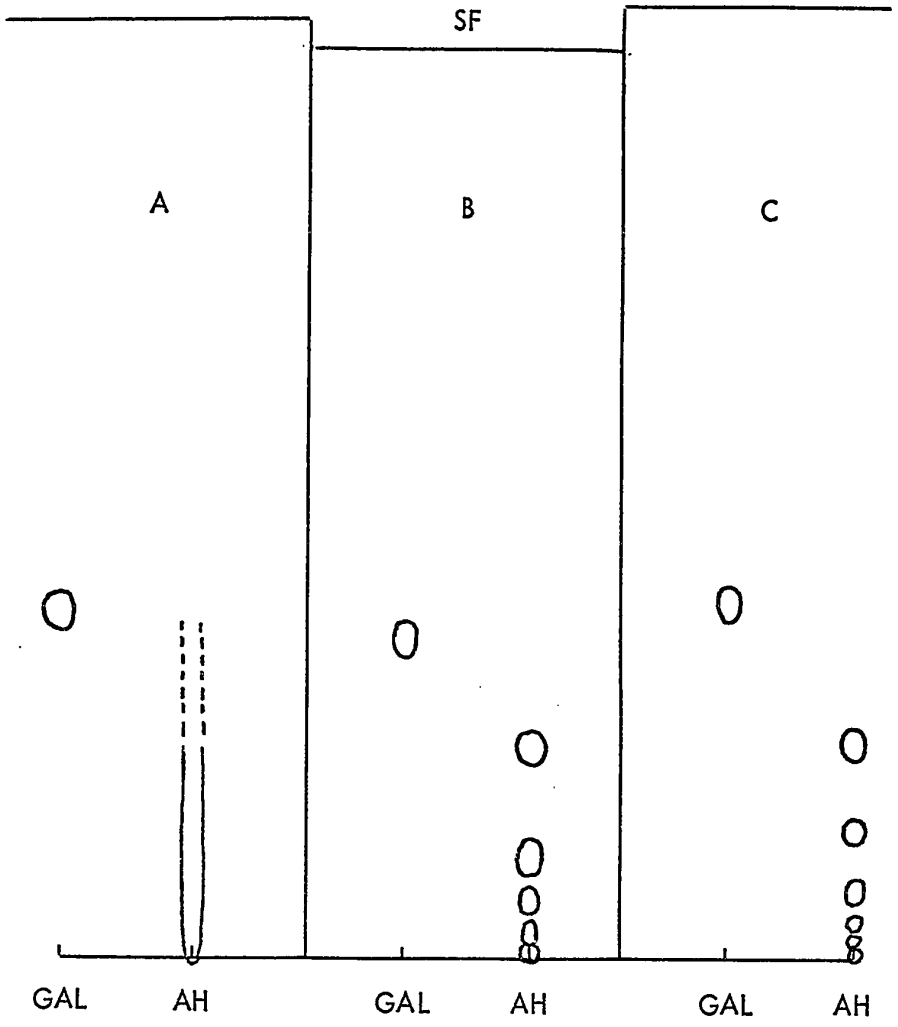


FIG. 26

separated by impregnation of the TLC plate with bisulfite, or by reduction of the 3,6-anhydrogalactose to the stable 3,6-anhydrogalactitol by borohydride reduction (Fig. 26). The reduced oligosaccharides were separated by TLC or on a Sephadex G-25 column. The isolated sugars were pure as demonstrated by TLC in solvent B (Fig. 27).

D. CHARACTERIZATION OF α-AGARASE AND ITS PRODUCTS

The oligosaccharides obtained by hydrolysis of agarose with the purified GJ1B agarase were shown to be different to those obtained with β-agarase (for example P. atlantica), by co-chromatographing the reduced oligosaccharides from each hydrolysate on TLC, using the wedge technique (Fig. 28). Since agarose is essentially a neutral polysaccharide, consisting of an alternating sequence of (1 → 4)-linked 3,6-anhydro-α-L-galactose and (1 → 3)-linked β-D-galactose units (Araki, 1956; Duckworth and Yaphe, 1971a), the β-agarase catalyzes the hydrolysis of agarose at the β-1,4 linkages yielding oligosaccharides with D-galactose as end sugar (Araki and Arai, 1956; 1957; Yaphe, 1957; Duckworth and Turvey, 1969a). On the other hand, an α-agarase hydrolyzes agarose by breaking the α-1,3 linkages and yielding oligosaccharides with 3,6-anhydro-L-galactose as end sugar (Fig. 29).

E. IDENTIFICATION OF THE PRODUCTS OF ENZYMIC HYDROLYSIS OF GJ1B AGARASE

The oligosaccharides present in the enzymic hydrolysate from the GJ1B

Fig. 27. TLC of the isolated oligosaccharide alcohols obtained with the strain GJ1B agarase. The chromatogram was developed in solvent B and sprayed with naphthoresorcinol reagent.

AH: Agarose hydrolysate after treatment with borohydride

GAL: Galactose

(A): Agarohexaitol

(B): Agaro-octaitol

(C): Agarodecaitol

Fig. 28. Co-chromatography using the wedge technique of the oligosaccharide obtained with P. atlantica and strain GJ1B. The chromatogram was developed in solvent B and sprayed with naphthoresorcinol reagent.

A: P. atlantica oligosaccharides

B: GJ1B oligosaccharide alcohols

C: P. atlantica oligosaccharide alcohols

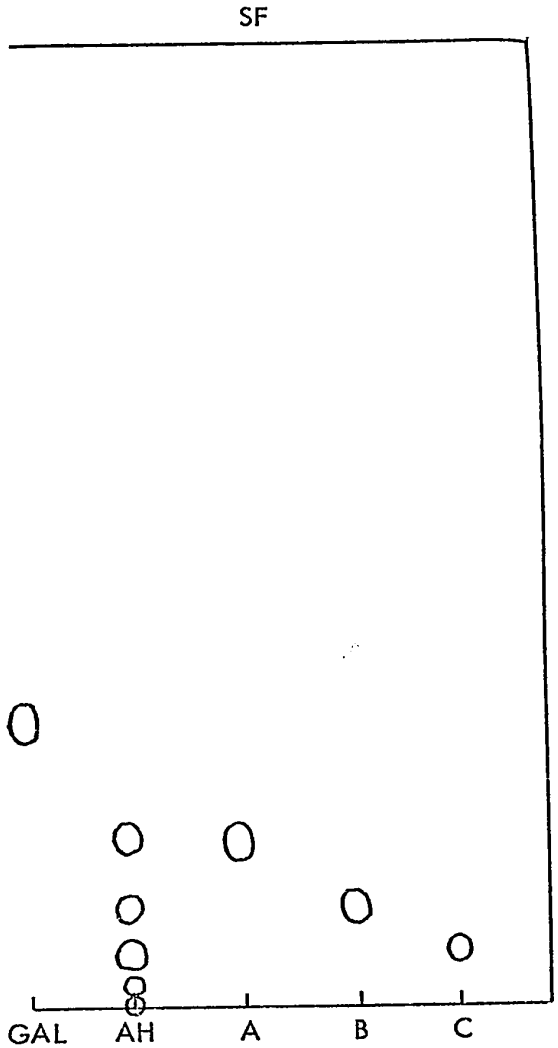


FIG. 27

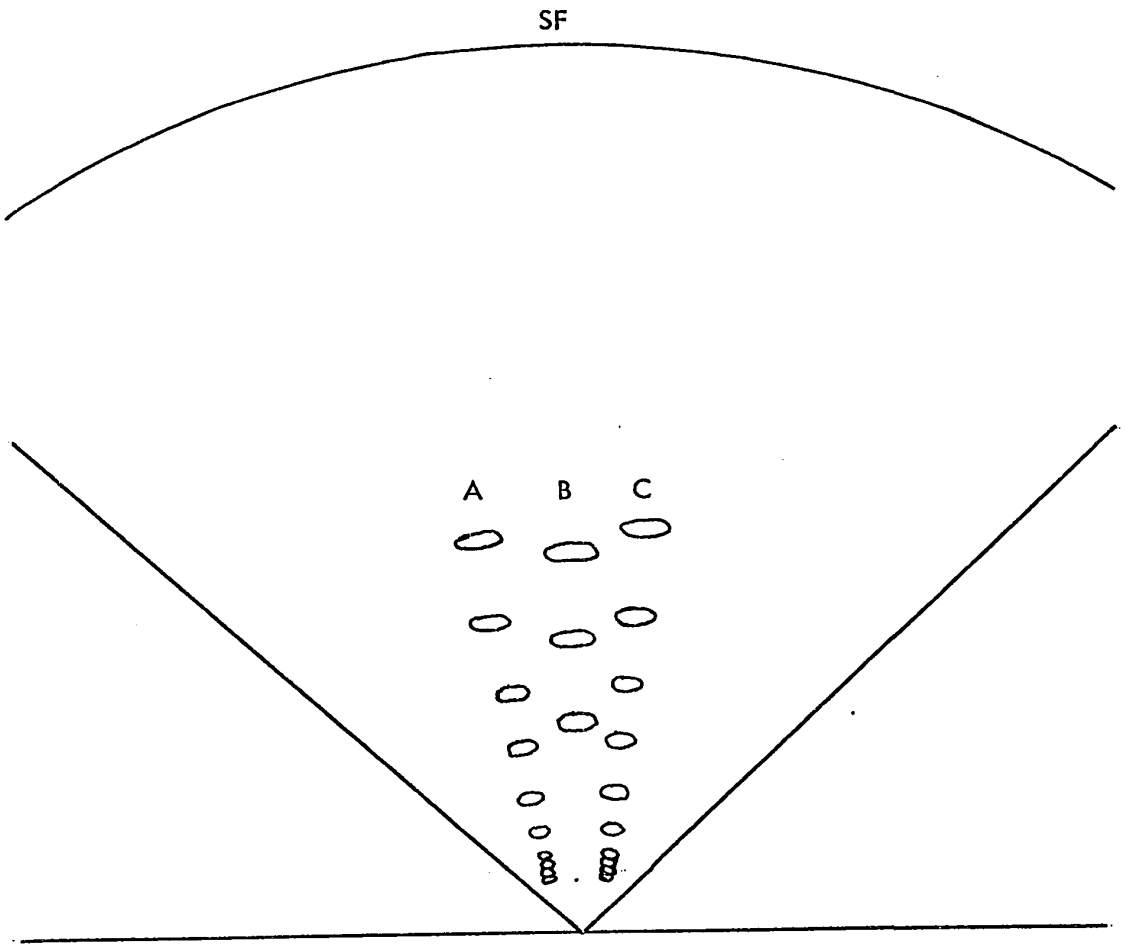


FIG. 28

Fig. 29. Schematic illustration showing the production of agarobiose and neoagarobiose series of oligosaccharides by α- and β-agarase, respectively.

Fig. 30. Modification of 3,6-anhydrogalactose with bisulfite.

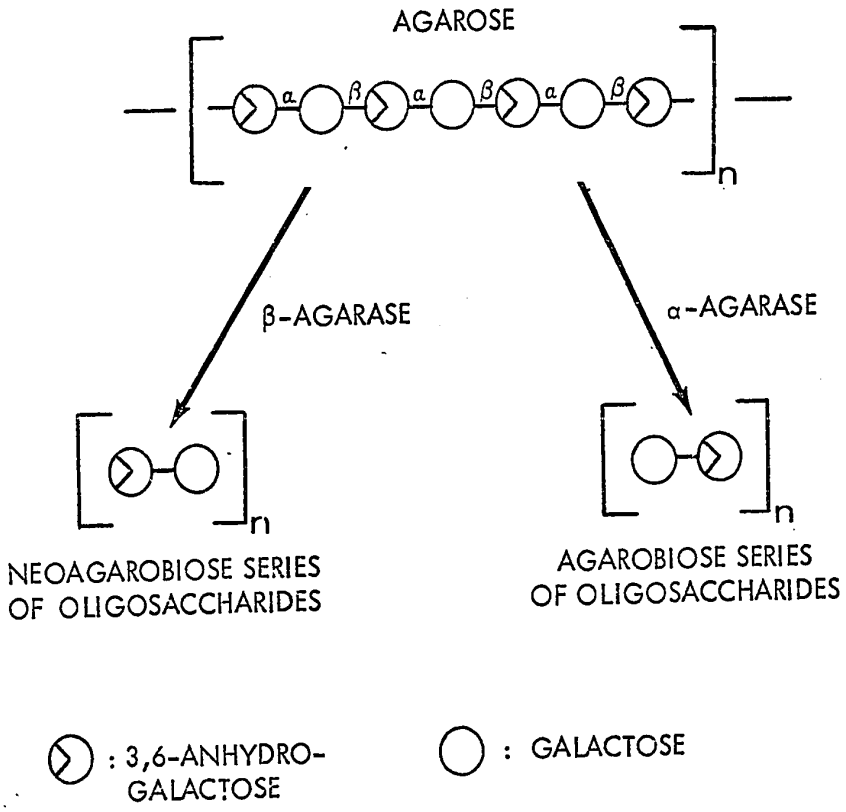

AGAROSE
α
β
α
β
α
β
n
β-AGARASE
α-AGARASE
n
n
NEOAGAROBIOSE SERIES
OF OLIGOSACCHARIDES
AGAROBIOSE SERIES
OF OLIGOSACCHARIDES
: 3,6-ANHYDRO-
GALACTOSE
: GALACTOSE

FIG. 29

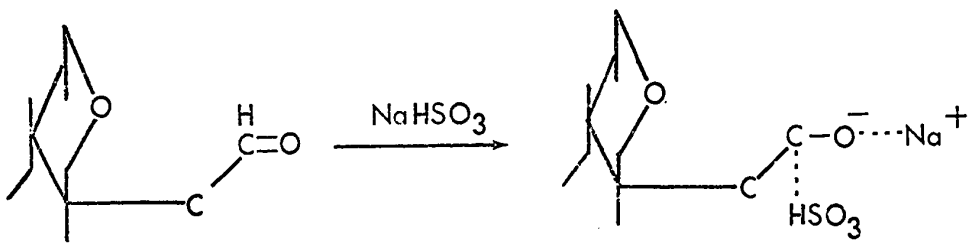

O
H
C=O
C
NaHSO3
O
C–O····Na+
C
HSO3

FIG. 30

agarase streak on cellulose-TLC. These sugars were separated on a TLC impregnated with bisulfite buffer (Fig. 26). 3,6-Anhydrogalactose, in contrast to galactose, is not a hemiacetal, but is a true aldehyde. A true aldehyde sugar would streak on TLC unless modified, for example, by forming an additional compound with bisulfite (Fig. 30). Further proof for 3,6-anhydrogalactose as the end sugar in the agarobiose series of oligosaccharides was obtained by reducing the oligosaccharides with borohydride, followed by acid hydrolysis, and examination of the product by paper chromatography. 3,6-Anhydrogalactitol, but not galactitol, was observed (Fig. 31) indicating that 3,6-anhydrogalactose was the sugar at the reducing end of the oligosaccharides. Examination by GLC of the acid hydrolysates of the pure oligosaccharide alcohols, after separation and isolation from the hydrolysate, showed in each case a peak corresponding to that of 3,6-anhydrogalactitol (Fig. 32).

The chromatographically pure oligosaccharide alcohols isolated from the agarose hydrolysate after treatment with the GJ1B agarase, were identified by several methods. Since the oligosaccharides were isolated as the alcohol derivatives, acid hydrolysis would destroy the internal 3,6-anhydrogalactose units, leaving only 3,6-anhydrogalactitol and galactose as the end products. The ratio of the 3,6-anhydrogalactitol to galactose could thus be used to indicate the DP of the oligosaccharides. Each oligosaccharide was acid hydrolyzed (H_2SO_4, 1 M, 100°C) and reduced with sodium borohydride to convert galactose into galactitol in order to obtain a single peak equivalent to galactose on examination by GLC. The sugar (A) (Fig. 27) with Rgal 0.6 was found to have a ratio of 2.72 (galactitol/3,6-

Fig. 31. Paper chromatography of acid hydrolysate of the oligosaccharide alcohol obtained with the GJ1B agarase. The chromatogram was developed in solvent D and sprayed with silver nitrate-sodium hydroxide reagent.

GAL: Galactose

H: Acid hydrolysate of the oligosaccharide alcohol

A: 3,6-Anhydrogalactitol

B: Galactitol

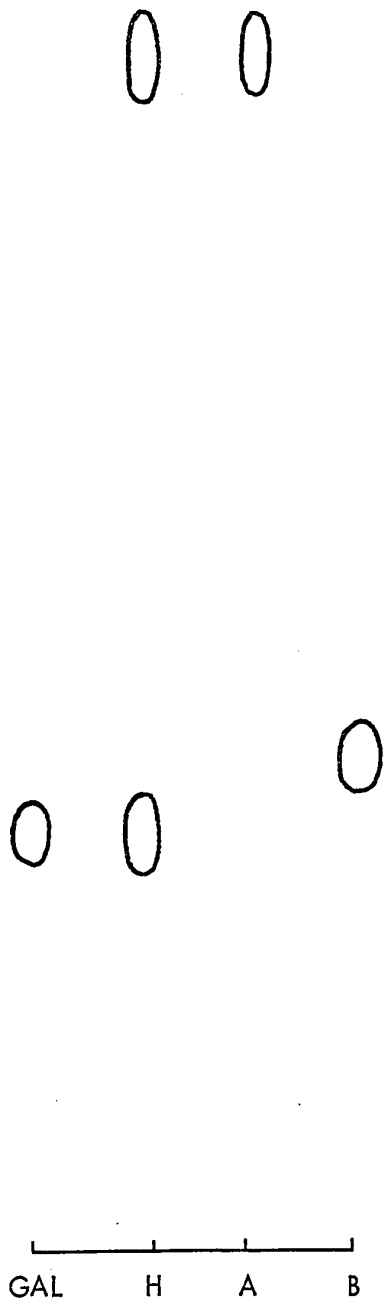


FIG. 31

Fig. 32. Gas-liquid chromatography (GLC) of the acid hydrolysate of the oligosaccharide alcohol obtained with the GJ1B agarase.

A: Acid hydrolysate-TMS

B: Co-chromatography of acid hydrolysate-TMS and 3,6-anhydrogalactitol-TMS

1: 3,6-Anhydrogalactitol-TMS

2 and 3: Galactose-TMS

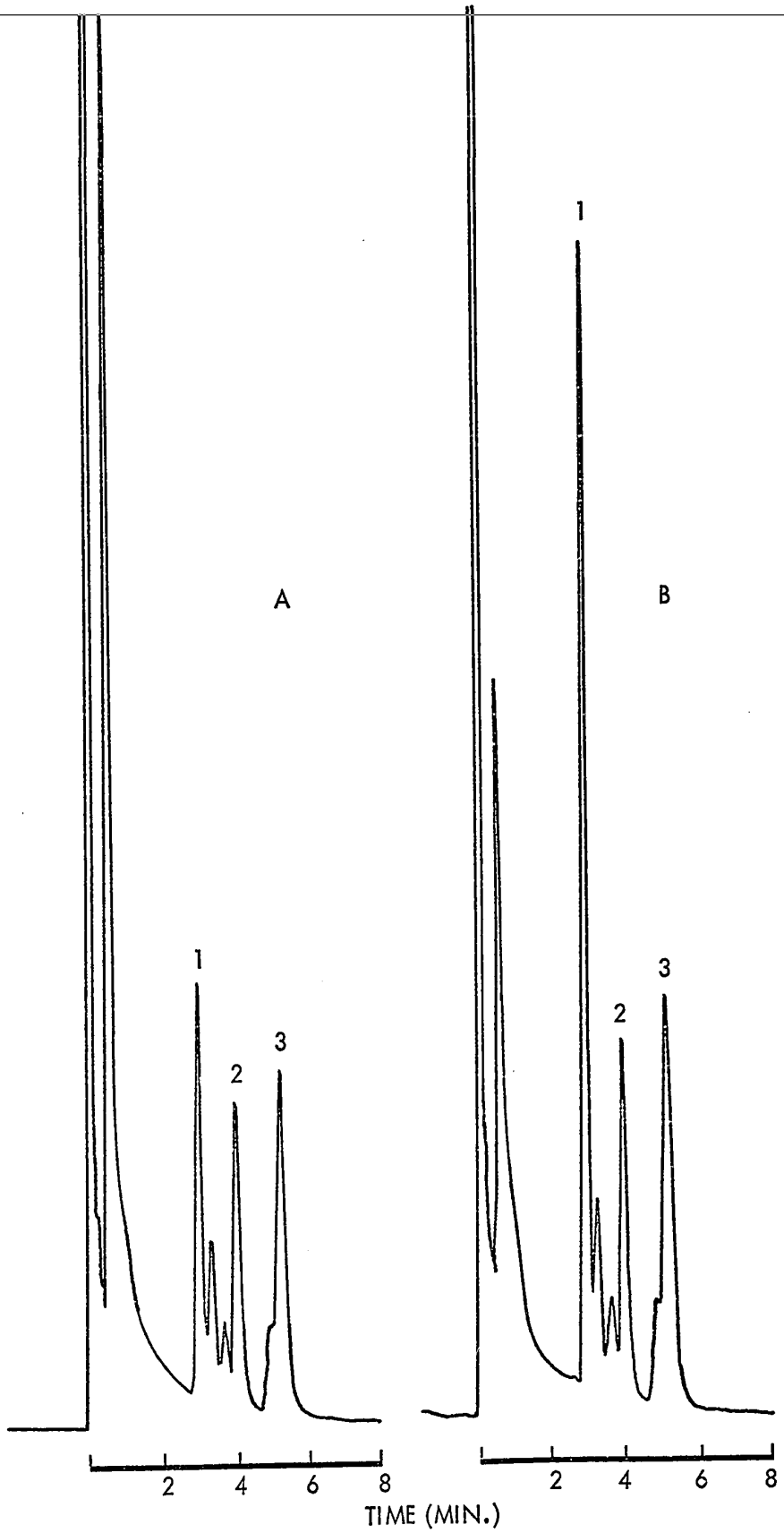


FIG. 32

anhydrogalactitol) indicating that it is a hexasaccharide, i.e. agarohexaitol. The theoretical ratio for this sugar is 3. Similarly, the oligosaccharide (B) with Rgal 0.35 was identified as an octasaccharide having a ratio of 3.81, the theoretical ratio of agaro-octaitol being 4. The results of quantitative GLC are shown in Fig. 33 using ribitol as internal standard.

This method for measuring the ratio of 3,6-anhydrogalactitol to galactose is incomplete, since the amount of internally linked 3,6-anhydrogalactose has not been determined. Although, in theory, methanolysis will fulfill this purpose, in practice a portion of the 3,6-anhydrogalactose is lost during methanolysis (Araki, 1937b). An attempt was therefore made to determine the internal 3,6-anhydrogalactose by the resorcinol method (Yaphe and Arsenault, 1965). The same oligosaccharide solution used in the quantitative GLC, was used in this experiment. The concentration of the oligosaccharide was calculated from the results obtained by GLC, using a known amount of ribitol as internal standard. Oligosaccharides A and B were shown to contain 2 and 3.1 moles, respectively, of 3,6-anhydrogalactose per mole of oligosaccharide, confirming that A is a hexa- and B an octasaccharide. The results of the DP determination of the oligosaccharides are summarized in Table IV.

Fig. 34 shows the Bate-Smith and Westall relationship for oligosaccharides from the agarose hydrolysates obtained with both the α- and β-agarases. The Rm values according to Bate-Smith and Westall (1950) were determined for each sugar, where $Rm = \log (\frac{1}{Rf} - 1)$, and plotted against the number of disaccharide

Fig. 33. Quantitative GLC of the acid hydrolysate of oligosaccharide alcohols (A) and (B) obtained from GJ1B agarase.

1: Ribitol-TMS, internal standard

2: 3,6-Anhydrogalactitol-TMS

3: Galactitol-TMS

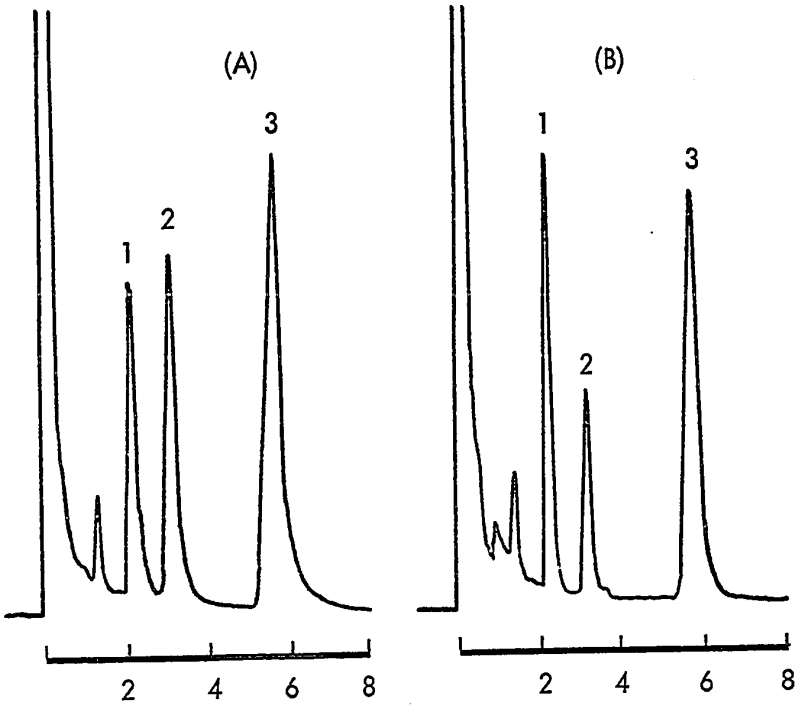


FIG. 33

repeating units. A linear relationship was obtained in both cases as shown in Fig. 34, indicating that both hydrolysates contained a homologous series of oligosaccharides. Thus, the oligosaccharide (C) (Fig. 27) isolated from the GJ1B enzymic hydrolysate, with a Rgal 0.19, should have a DP 10, i.e. agarodecaitol. Further identification of this sugar was not carried out.

TABLE IV

DP Determination for the Oligosaccharides Obtained with the GJ1B Agarase

		Galactitol/3,6-anhydrogalactitol		3,6-Anhydrogalactose		
Sugar	Rgal	Theor.	Exp.	Theor.	Exp.	DP
A	0.6	3	2.72	2	2	6
B	0.35	4	3.81	3	3.1	8

Fig. 34. Bate-Smith and Westall plot for the oligosaccharides obtained with the α- and β-agarases.

A: Oligosaccharide alcohols from strain GJ1B α-agarase

B: Oligosaccharides from P. atlantica β-agarase

Rm: $\log\left(\frac{1}{Rf} - 1\right)$

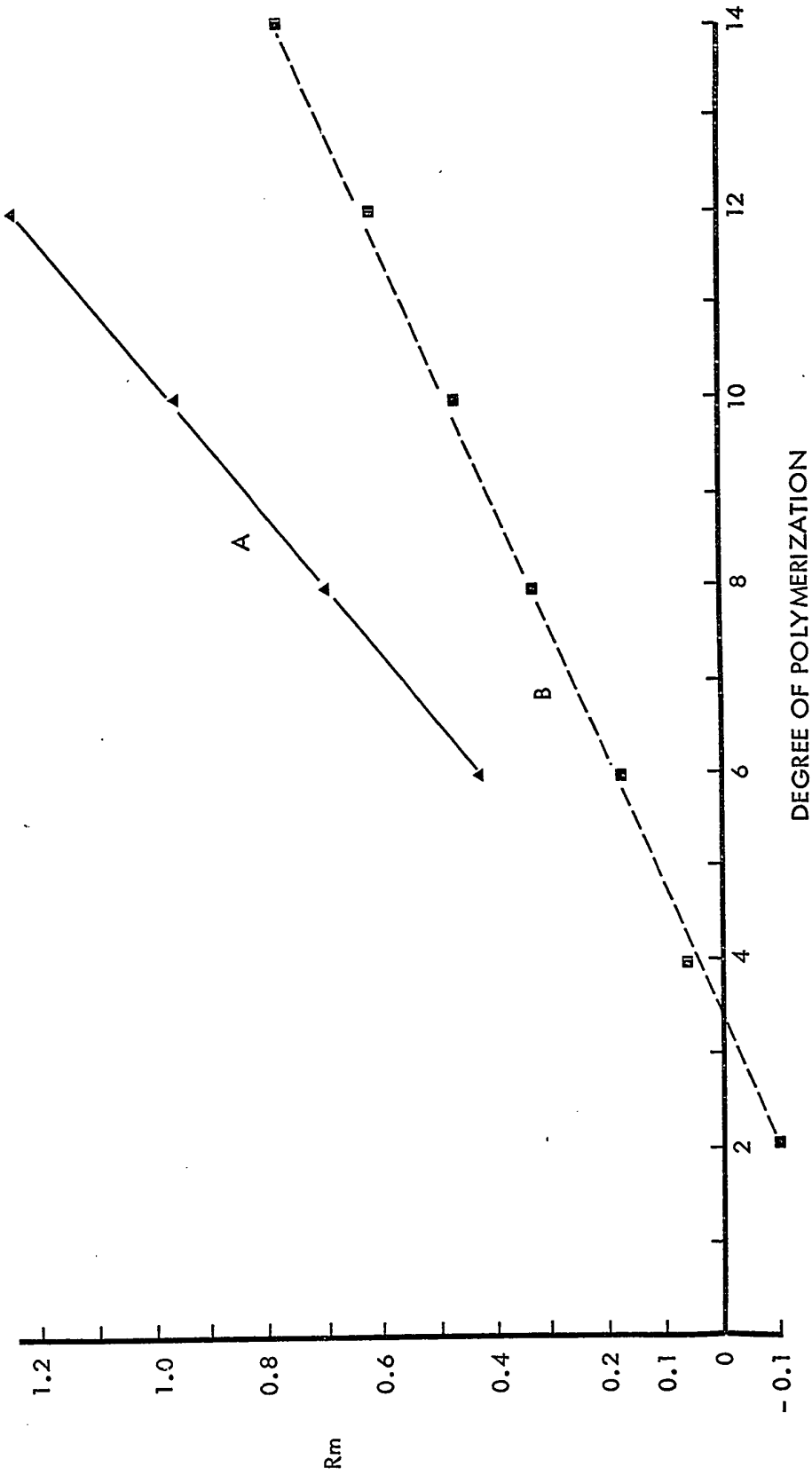

Rm
1.2
1.0
0.8
0.6
0.4
0.2
0.1
0
- 0.1
2
4
6
8
10
12
14
A
B
DEGREE OF POLYMERIZATION

FIG. 34

F. MODE OF ACTION OF THE BACTERIAL AGARASES

(1) Hydrolysis of oligosaccharides

The final end products of the enzymic hydrolysis of agarose by the β-agarases obtained from 3A and P. atlantica, were neoagarotetraose and -biose. The enzymes had no activity on these two sugars. However, the β-agarases hydrolyzed the neoagarobiose series of oligosaccharides having a DP higher than 4.

a. P. atlantica agarase

Fig. 35 illustrates the breakdown of the neoagarobiose series of oligosaccharides by the P. atlantica agarase with time. Neoagarohexaose was hydrolyzed to yield neoagarobiose and -tetraose. Initial hydrolysis of neoagaro-octaose by the enzyme produced mainly neoagarotetraose and traces of neoagarohexaose and -biose. Neoagarohexaose was further hydrolyzed to neoagarotetraose and -biose. Similarly, neoagarodecaose was hydrolyzed to produce mainly neoagarohexaose and -tetraose, together with a small quantity of neoagaro-octaose and -biose. The neoagaro-octaose and -hexaose produced from the decamer were further hydrolyzed to the end products, neoagarobiose and -tetraose.

b. Strain 3A agarase

The pattern of hydrolysis of the neoagarobiose series of oligosaccharides by the 3A agarase is similar to that by the P. atlantica enzyme, except for the

Fig. 35. Hydrolysis of neoagarohexaose, -octaose and -decaose by *P. atlantica* agarase. The chromatogram was developed in solvent A and sprayed with naphthoresorcinol reagent.

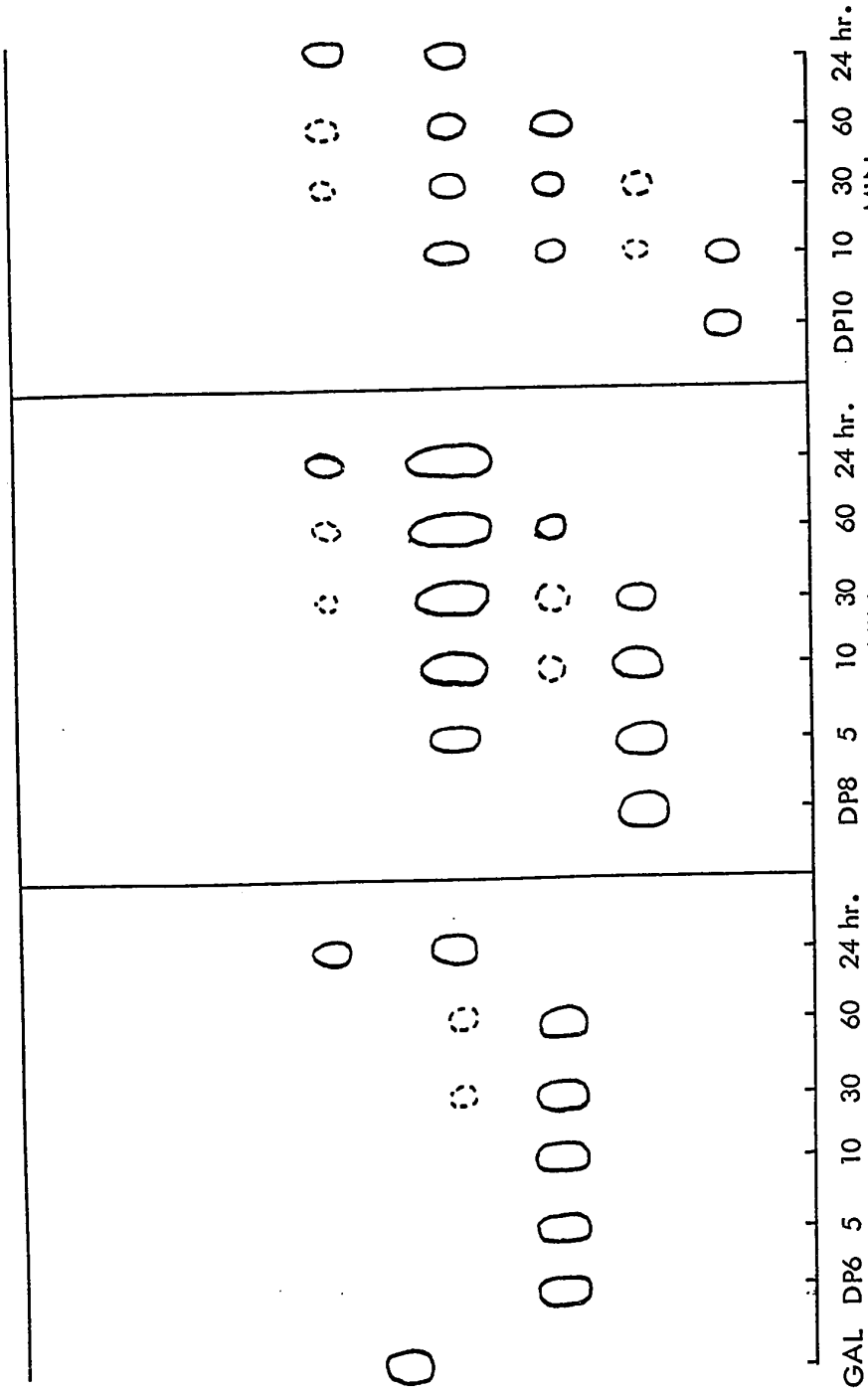

GAL
DP6
5
10
30
60
24 hr.
MIN.
DP8
5
10
30
60
24 hr.
DP10
10
30
60
24 hr.
MIN.

FIG. 35

hydrolysis of neoagaro-octaose. The 3A agarase hydrolyzed neoagaro-octaose mainly to neoagarohexaose and -biose, with a relatively small amount of neoagarotetraose (Fig. 36). The rate of hydrolysis of neoagaro-octaose by P. atlantica and 3A β-agarases was greater than the breakdown of neoagaro-hexaose, since neoagaro-octaose disappeared long before neoagarohexaose was completely hydrolyzed (Fig. 35, 36).

The action of the purified β-agarase on neoagaro-octaose to yield neoagarohexaose, -tetraose and -biose led to the speculation that the agarase preparation may consist of two agarases, one enzyme cleaving the central β-linkage of neoagaro-octaose, the other hydrolyzing the peripheral β-linkages. Since ion-exchange chromatography of the β-agarase did not separate the purified preparation into two agarases, an attempt was made to fractionate the P. atlantica preparation on a molecular weight basis, by gel filtration. The profile of chromatography of the β-agarase from P. atlantica on G-100 is given in Fig. 37. The enzyme obtained from different fractions (Fraction No. 6, 14, 16, 28, 38, 48) was used to hydrolyse neoagaro-octaose. The hydrolysates when examined by TLC, showed identical patterns, i.e. all the fractions tested showed dual action on neoagaro-octaose. Recycling the enzyme (from Fraction No. 26 to 35) through the column gave the same result. Gel filtration on Sephadex G-150 and G-200 was also attempted, but similar results to those with Sephadex G-100 were obtained, indicating that the β-agarase from P. atlantica may be a single enzyme having dual specificity on neoagaro-octaose.

Fig. 36. Hydrolysis of neoagaro-octaose and -hexaose by strain 3A agarase. The chromatogram was developed in solvent A and sprayed with naphthoresorcinol reagent.

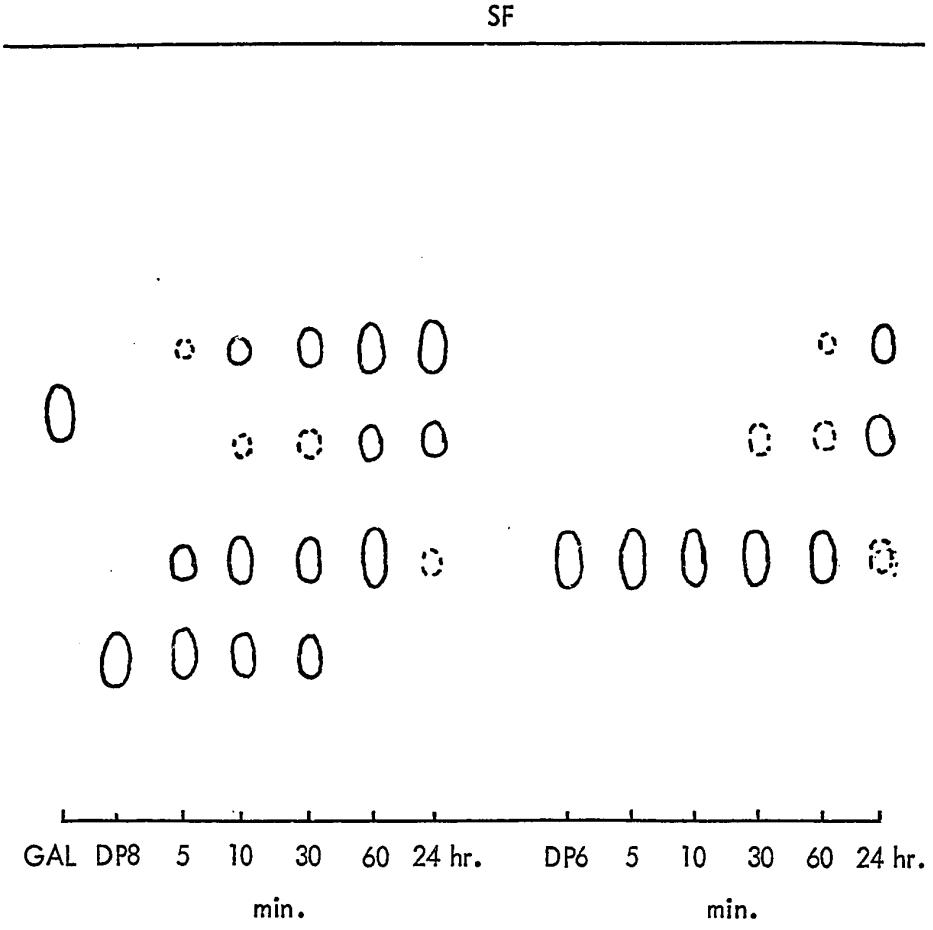
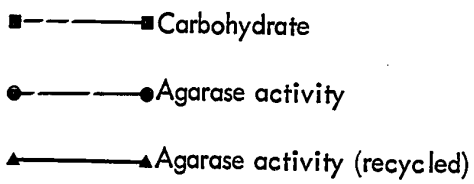


FIG. 36

Fig. 37. Purification of the P. atlantica agarase on Sephadex G-100.



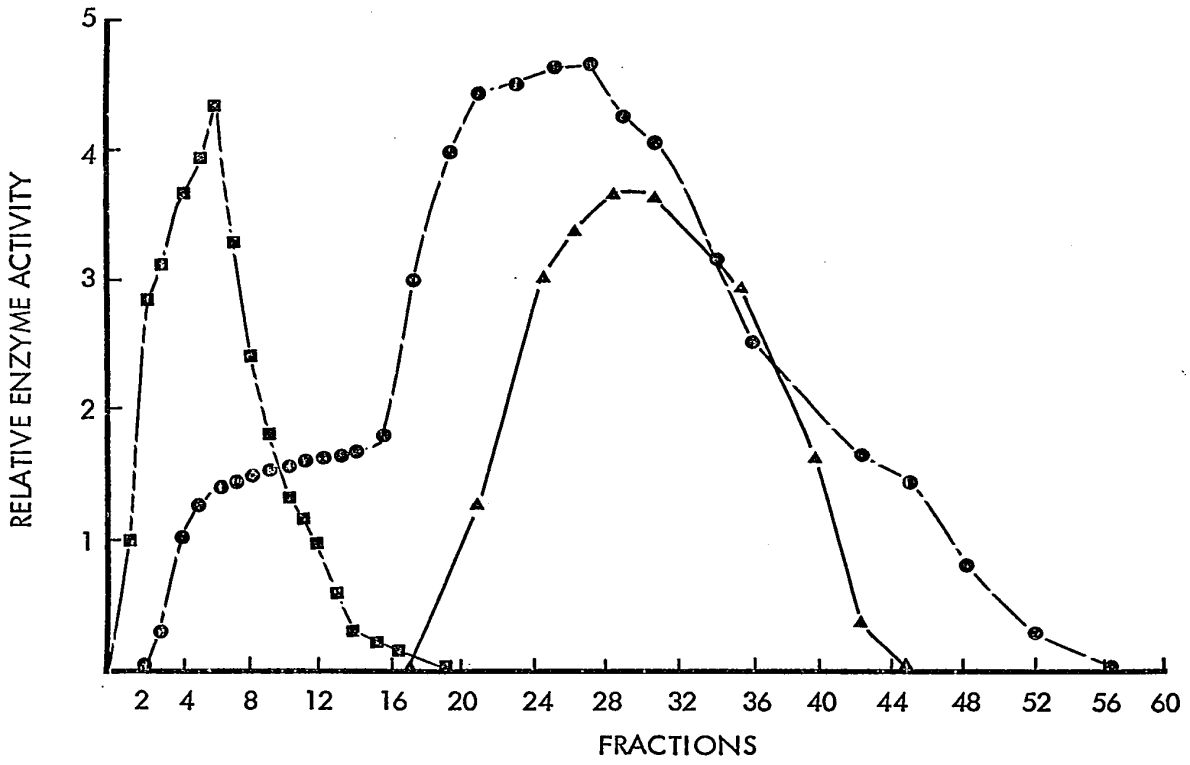

RELATIVE ENZYME ACTIVITY
5
4
3
2
1
2 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60
FRACTIONS

FIG. 37

c. Site of hydrolysis

i) Neoagarobiose series of oligosaccharides

In order to determine which oligosaccharide linkage was hydrolyzed by the P. atlantica agarase, the oligosaccharides were reduced with sodium borohydride which converts the reducing sugar to a sugar alcohol. Fig. 38 and 39 show the products of the enzymolysis of neoagarohexaitol and -octaitol. The TLC plates were sprayed with naphthoresorcinol reagent and aniline hydrogen phthalate. The former reagent is specific for compounds containing 3,6-anhydro-galactose and the latter only reacts with substances which have a free aldehyde group. The breakdown products of enzymic action on neoagarohexaitol were neoagarotetraose and neoagarobiitol; the latter was not stained with aniline hydrogen phthalate reagent, indicating that its end sugar is present as the alcohol (Fig. 38). This suggests that the agarase hydrolyzes the hexasaccharide by breaking the β-1,4-linkage close to the reducing end sugar (Fig. 39 A), and confirms a previous observation made by Hong and Yaphe (unpublished results). The β-agarase of P. atlantica hydrolyzed neoagaro-octaitol yielding, in the early stages of the reaction, neoagarotetraose and its sugar alcohol, as well as small amounts of neoagarohexaose and neoagarobiitol. These products indicated that the enzyme catalyzes the hydrolysis mainly at the central linkage, but also to a lesser extent, at the peripheral linkage near the reducing end (Fig. 39 B).

Fig. 38. Hydrolysis of neoagarohexaitol and -octaitol by P. atlantica agarase. The chromatogram was developed in solvent B. Side A was sprayed with naphthoresorcinol reagent, an indicator reagent for oligosaccharide containing 3,6-anhydrogalactose, and side B with aniline hydrogen phthalate reagent, an indicator reagent for a reducing end sugar in the oligosaccharide.

1: Neoagaro-octaitol + β-agarase

2: Neoagarohexaitol + β-agarase

GAL: Galactose

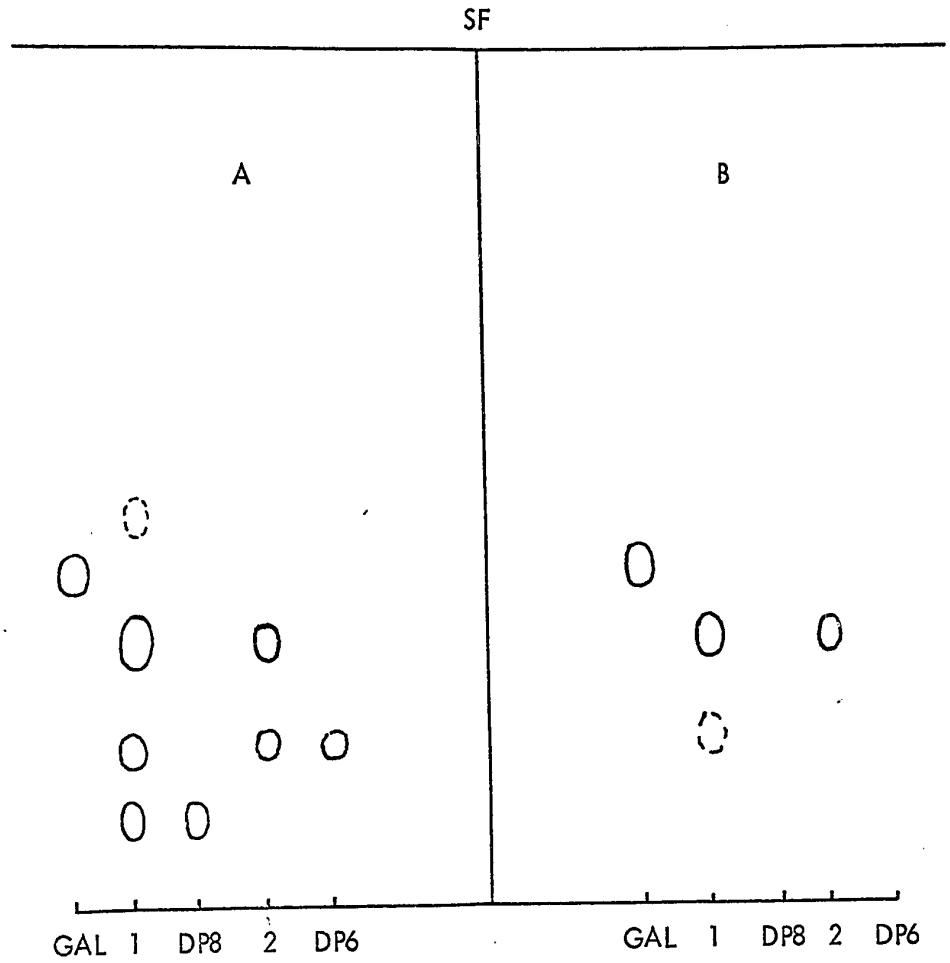


FIG. 38

Fig. 39. Diagramatic illustration of the site of hydrolysis of neoagarohexaitol (A) and -octaitol (B) by the P. atlantica agarase.

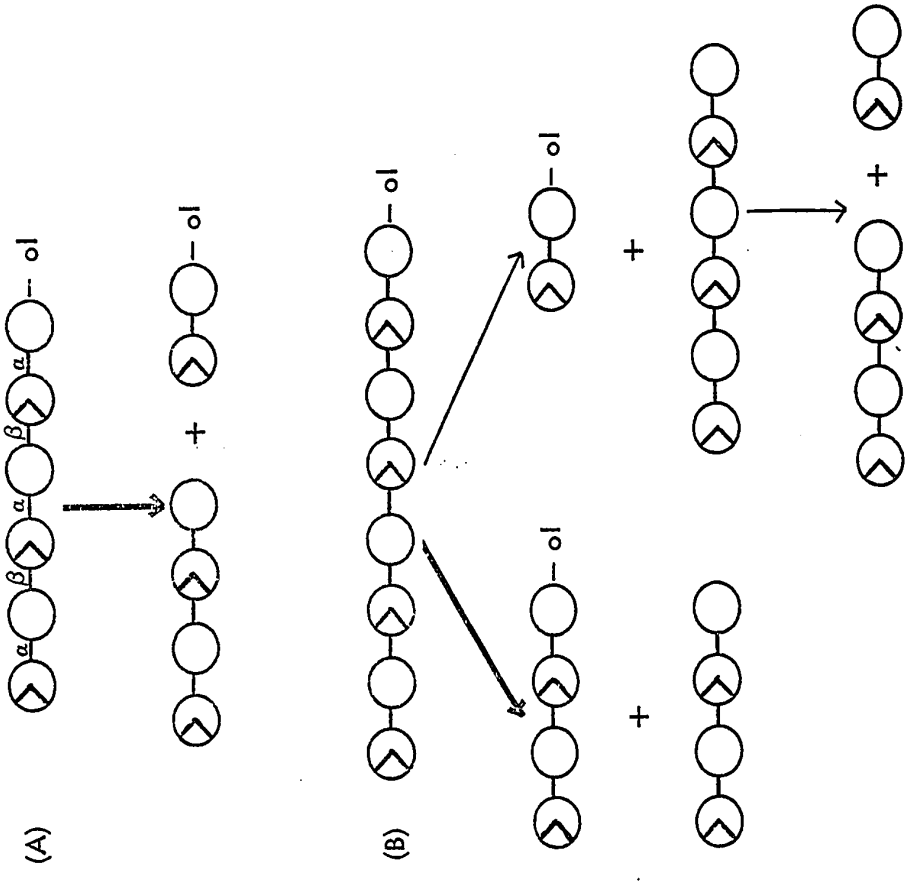


FIG. 39

ii) Agarobiose series of oligosaccharides

The agarobiose series of oligosaccharides obtained with the GJ1B agarase were also used as substrate to study the enzyme specificity of the β-agarase from P. atlantica as well as to produce oligosaccharides having an odd number of monosaccharide units. The β-agarase from P. atlantica showed no action on agarohexaitol (Fig. 40).

Agaro-octaitol was hydrolyzed by the P. atlantica β-agarase yielding two sugars, A1, Rgal 1.10, with the end sugar in the alcohol form and A2, Rgal 0.6, with the end sugar in the aldehyde form as shown in Fig. 40. Previously it was shown that neoagarohexaose was hydrolyzed at the β-1,4 linkage near the reducing end. Therefore, A1 may be a trisaccharide having 3,6-anhydro-L-galactitol at one end and 3,6-anhydro-L-galactose at the other (Fig. 41 B). A2 may be a pentasaccharide having galactose at either ends (Fig. 41 B). The TLC pattern of the β-agarase hydrolysate from agarodecaitol is given in Fig. 40. At least three sugars, Rgal 1.10 (B1), 0.74 (B2) and 0.60 (B3) were produced by the action of P. atlantica agarase on agarodecaitol. B2 was shown to be an oligosaccharide alcohol, since it did not react with the aniline hydrogen phthalate reagent, whereas B3 reacted on spraying with this reagent (Fig. 40 B). B1 was produced in trace amounts and could only be detected by the naphthoresorcinol reagent, and hence the nature of its end sugar could not be determined. Co-chromatography of the breakdown products of the β-agarase on agaro-octaitol

Fig. 40 Hydrolysis of the agarohexaitol, -octaitol and -decaitol by the P. atlantica agarase. The chromatogram was developed in solvent B. Side A was sprayed with naphthoresorcinol reagent, and side B with aniline hydrogen phthalate reagent.

GAL: Galactose

S: Standard containing neoagarobiose

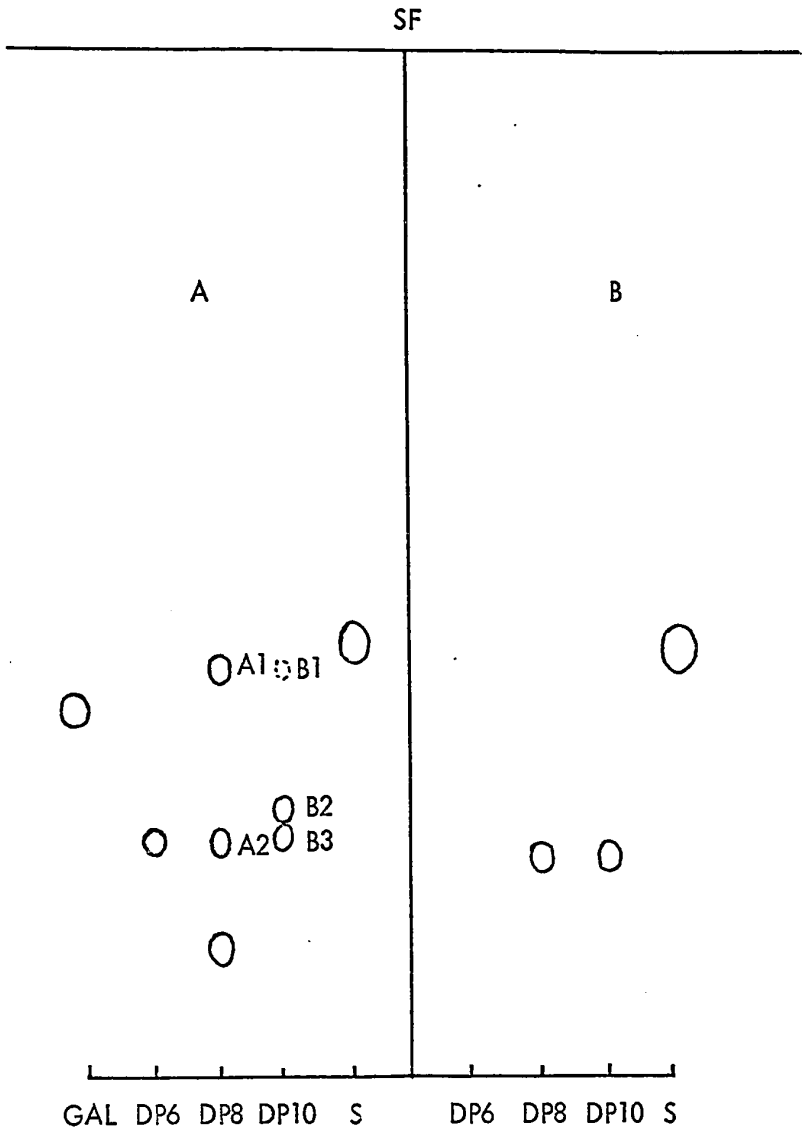


FIG. 40

Fig. 41. Diagramatic illustration of the site of hydrolysis of agarohexaitol (A), -octaitol (B) and -decaitol (C) by the *P. atlantica* agarase.

(1), (3), (7): Agaropentaose

(2), (4): Neoagarotriitol

(5): Neoagaropentaitol

(6): Agaroheptaose

(8): Neoagarobiose

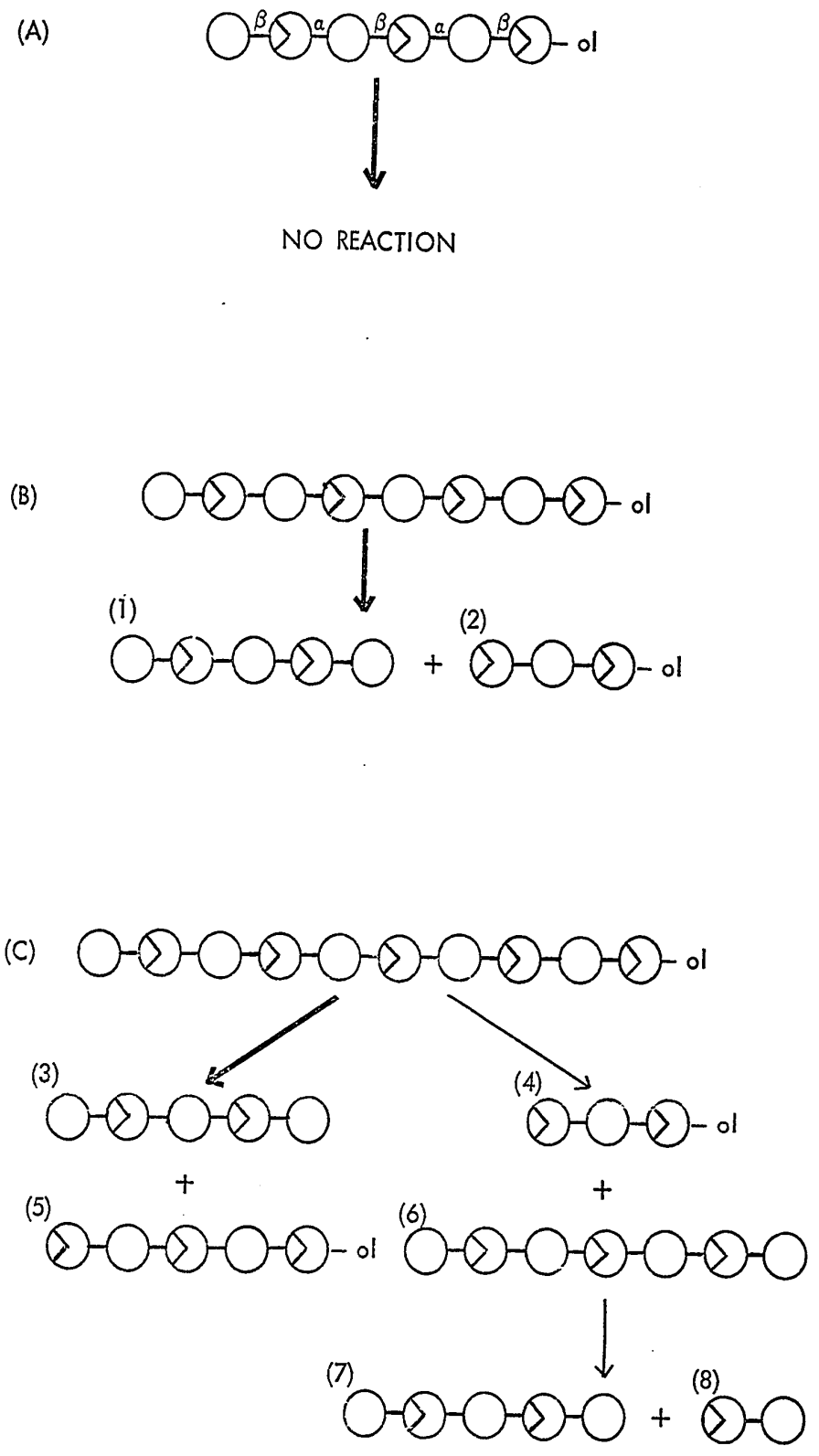


FIG. 41

and agarodecaitol on TLC, using the wedge technique, is illustrated in Fig. 42. Comparison of the Rgal values of the sugars, suggests that A1 and B1, and A2 and B3 are identical sugars. For the same reasons as discussed above for A1 and A2, sugar B2 was believed to be pentasaccharide having 3,6-anhydrogalactose at one end and 3,6-anhydrogalactitol at the other (Fig. 41 C).

Theoretically, the production of neoagarotriose (B1) from agarodecaitol (Fig. 41 C) should be accompanied by the production of agaroheptaose. It is possible that further hydrolysis of the agaroheptaose with the agarase yielded agaropentaose (B3) and neoagarobiose, where the latter sugar was present in trace amounts and was not detected with the indicator reagent.

The end products of the action of the GJ1B agarase on agarose were agarohexaose, -octaose and -decaose, which were obtained as the corresponding sugar alcohol derivatives. The enzyme showed no further activity on these sugars. Since higher oligosaccharides were not isolated, the mechanism of action of the α-agarase on the agarobiose series of oligosaccharides was not determined.

(2) Hydrolysis of agarose

The mechanism of action of the bacterial agarases on agarose were carried out with the DEAE-purified enzymes. Agarose was hydrolyzed at 42°C and samples were removed at various time intervals for the reducing value and viscosity determinations. The specific viscosity was plotted against the total reducing value, and the result is shown in Fig. 43. All the agarases used reduced

Fig. 42. Comparison of P. atlantica agarase hydrolysate of agaro-octaitol and -decaitol by wedge TLC. The chromatogram was developed in solvent B and sprayed with naphthoresorcinol reagent.

1: Agaro-octaitol

2: Agaro-octaitol + β-agarase

3: Agarodecaitol + β-agarase

4: Agarodecaitol

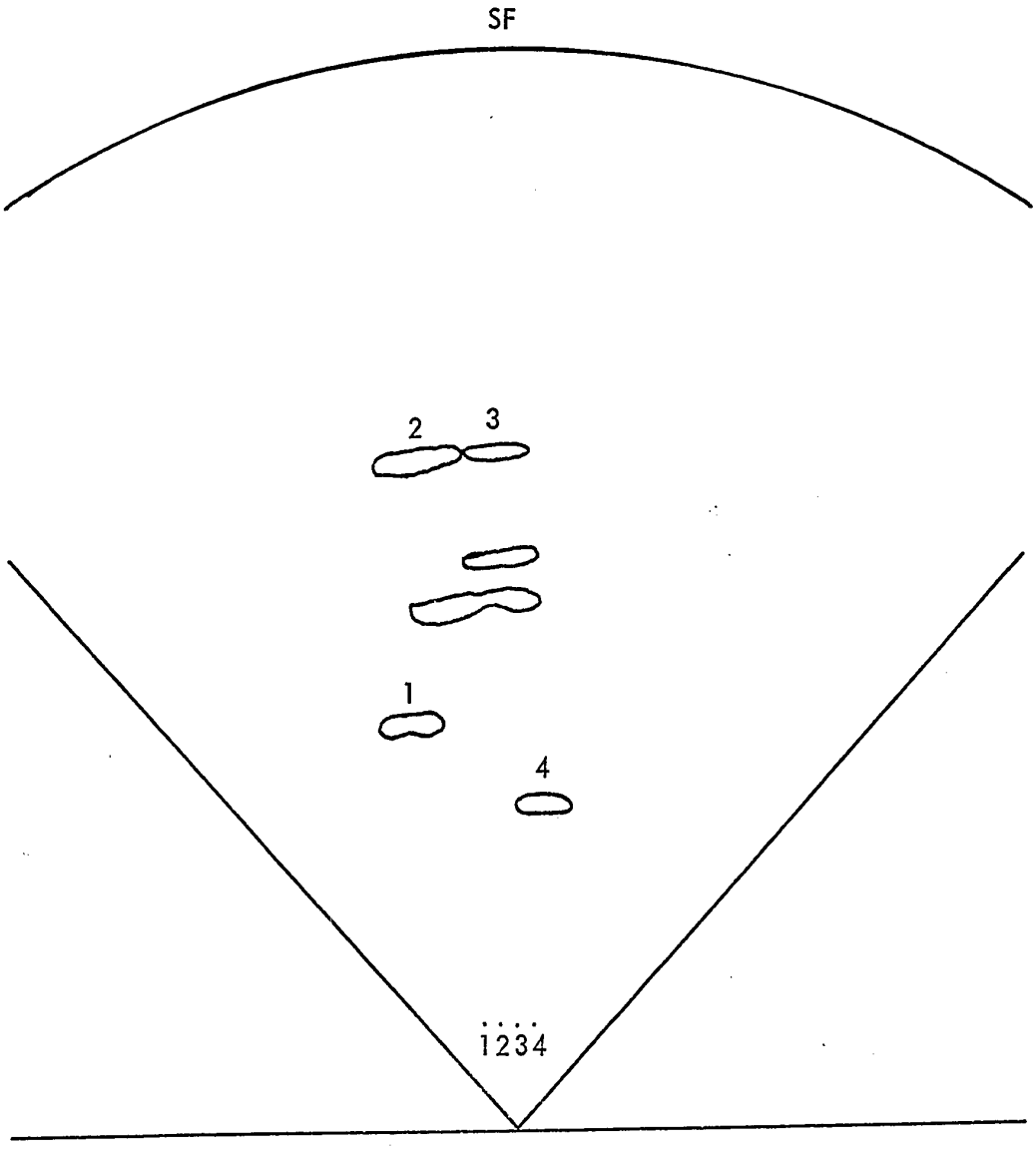


FIG. 42

Fig. 43. Comparison of specific viscosity and reducing values of agarose hydrolysates obtained with P. atlantica, strain 3A and strain GJ1B agarases.

A, B, C: Specific viscosity

A', B', C': Reducing value

A, A': Strain GJ1B agarase

B, B': P. atlantica agarase

C, C': Strain 3A agarase

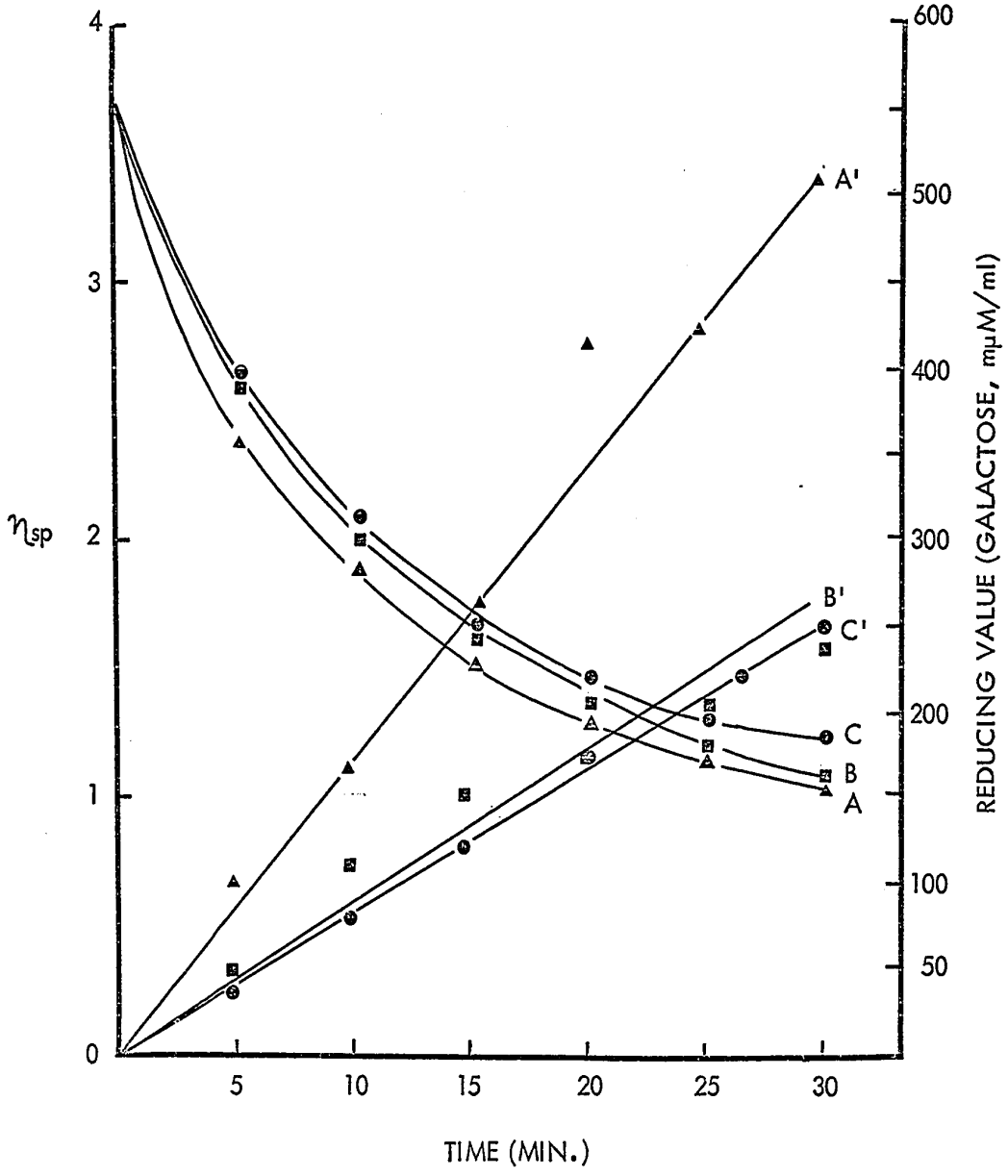

4
3
2
1
0
η_{sp}
600
500
400
300
200
100
50
REDUCING VALUE (GALACTOSE, mμM/ml)
A'
B'
C'
C
B
A
5
10
15
20
25
30
TIME (MIN.)

FIG. 43

the viscosity of agarose rapidly, but released a relatively small amount of reducing sugar, indicating that the enzymes catalyze the hydrolysis of agarose by a random or endo-mechanism. Although two viscosity units of enzyme per ml were used in all cases, the amount of reducing sugar generated by the GJ1B agarase was greater than that produced by the 3A or P. atlantica agarase, suggesting that there is a difference between the α- and β-agarases in their endo-mechanism of action. The degree of multiple attack (r - 1), as defined by Robyt and French (1967), was calculated for agarases from P. atlantica (0.54), 3A (0.08) and GJ1B (1.58). The degree of multiplicity of the β-agarases from P. atlantica and strain 3A was less than 1, indicating that these enzymes hydrolyze agarose in a random fashion, i.e. they have a multichain mechanism of action. On the other hand, the GJ1B agarase, having a degree of multiplicity of 1.58, catalyzes the hydrolysis of agarose via a multiple attack mechanism. The TLC pattern of the supernatant obtained from the 75% ethanol precipitation of the hydrolysate, is illustrated in Fig. 44. Oligosaccharide fragments of a suitable size for detection by TLC were found after incubation of agarose for 20 min. with the enzyme from P. atlantica and 3A, starting with large fragments which were broken down to smaller fragments as the incubation proceeded. However, in the case of the GJ1B agarase, low molecular weight oligosaccharides appeared in the hydrolysate within 5 min. of incubation with the enzyme, and the series of oligosaccharides produced was not the same as that found in the β-agarase hydrolysates. The rapidity with which low molecular weight oligosaccharides were produced by the GJ1B agarase further substantiates the data obtained in the degree of multiplicity experiment.

Fig. 44. Oligosaccharides in supernatants obtained from 75% ethanol precipitation of agarose hydrolyzed with strain GJ1B (A), P. atlantica (B) and strain 3A (C) agarases. The chromatogram was developed in solvent B and sprayed with naphthoresorcinol reagent.

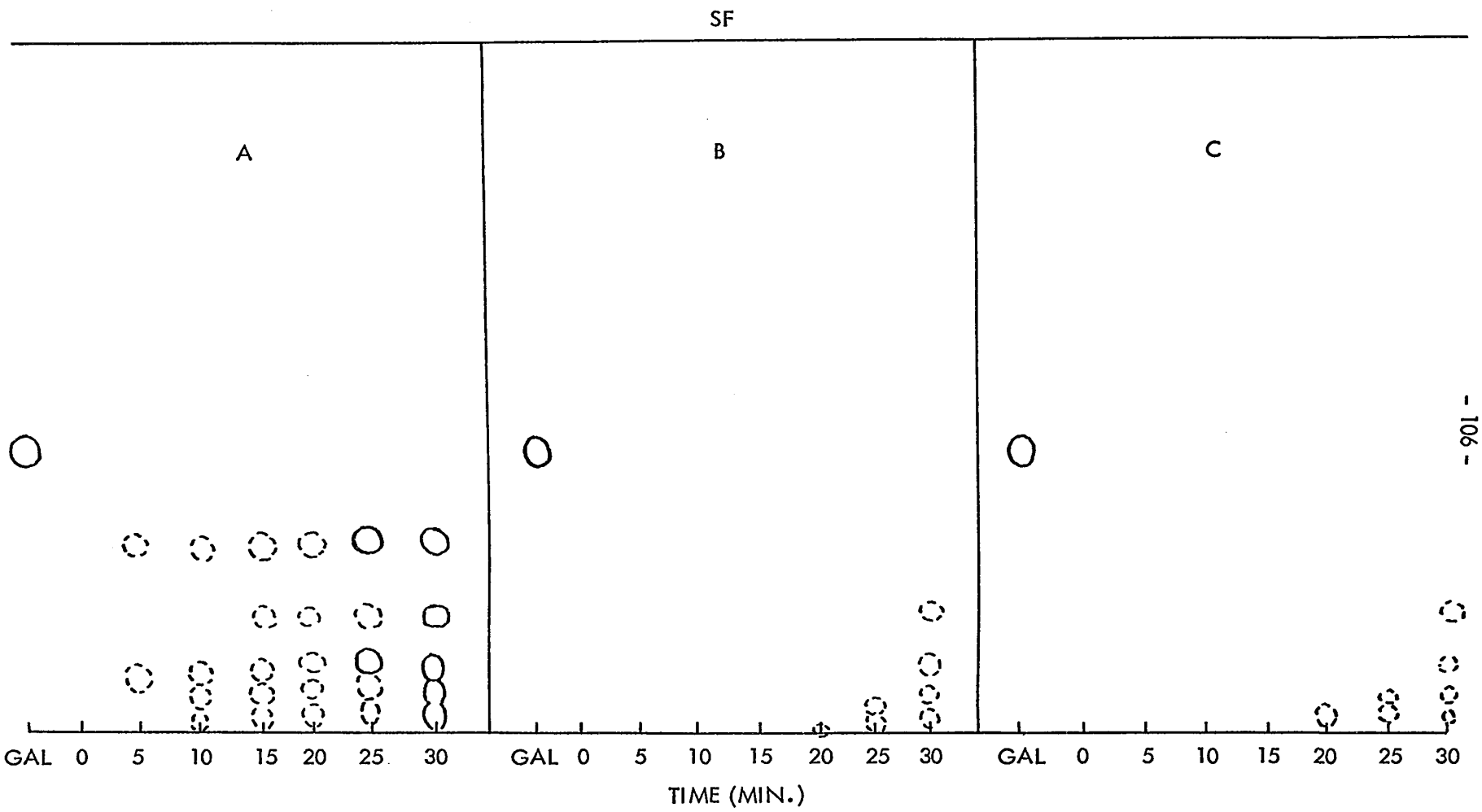

SF
A
B
C
GAL 0 5 10 15 20 25 30
GAL 0 5 10 15 20 25 30
GAL 0 5 10 15 20 25 30
TIME (MIN.)

FIG. 44

V. DISCUSSION

Algae, in particular the marine algae, are important as a source of several valuable chemicals such as agar, carrageenan and alginates. A knowledge of the structures of these algal products, their chemical and physical properties, as well as the distribution of the sources in nature, is thus of great importance.

It is important for the agar industry to find algae which contain good quality agar, i.e., which produce a high yield of agar which gels at a low concentration. The yield of agar from the seaweeds used in this study (Table I) varied from species to species. Gelidium sesquipedale had the highest yield of agar and this agar was shown to contain a low sulfate and pyruvate content. According to the theory of gel formation proposed by Rees (1972) and Yaphe and Duckworth's proposals (1971), the presence of charged groups interfere with the gel-forming capacity of agar. Since Gelidium sesquipedale contains a good yield of agar, and a low content of charged groups, as shown in the sample analyzed, it would appear that this algal species would be a good choice for the production of a high quality agar.

During the fractionation of the agars on DEAE Sephadex A-50 (Cl^-), (Table II), a considerable portion of material in each case was irreversibly bound to the Sephadex. This phenomenon has been observed for other charged polysaccharide complexes (Hirst et al., 1965). The material bound to the column is

thought to be composed of highly charged galactans which were not removed during the freeze-thaw process. This study suggests that Ahnfeltia plicata agar would appear to contain a large amount of highly charged material, since only a small portion of polysaccharide was recovered from the column. In general, the yield of polysaccharide in each fraction eluted from the DEAE Sephadex column with different algae was found to vary with the source of the agar. The properties of each unfractionated agar also varied as to the source of the agar. However, regardless of the different yields obtained (Table I), a general pattern was observed. From the analysis for the polysaccharides eluted in each fraction, the sulfate content of the eluted polysaccharide increased, with increasing ionic strength of the eluant, whereas the 3,6-anhydrogalactose content decreased. An exception to this was found to be the Digenia simplex agar, where the distilled water fraction contained only 10% of 3,6-anhydrogalactose. This may be due to contamination with other neutral polymers. A study of this fraction of the polysaccharide should be carried out in order to find the reason for this phenomenon. With an increase in sulfation and a decrease in 3,6-anhydrogalactose content, a decrease in gel strength of the polysaccharide was observed. This phenomenon can be explained by Rees' gelling mechanism in that a high concentration of 3,6-anhydrogalactose favors the helix formation of the polysaccharide. The replacement of a 3,6-anhydro-L-galactose residue by a sulfated galactose would change the conformation of the L-galactose residue, hence causing a "kink" in the helix, and impairing the gelling capacity.

From the pyruvic acid content in each fraction of eluted polysaccharides from Pterocladia pinnata agar, it can be seen that the percentage of both pyruvic acid and sulfate does not increase concomitantly with increasing ionic strength of the eluant. The masking of the basic unit with 4,6-O-(1'-carboxyethyiidene)-D-galactose reaches a peak in the 0.5 M NaCl fraction. As indicated by Duckworth and Yaphe (1971a), the masking of the basic chain with 4,6-O-(1'-carboxy-ethylidene)-D-galactose residues appears to occur away from the sulfated galactose residues. However, whether the masking occurs with 4,6-O-(1'-carboxy-ethylidene)-D-galactose on some polysaccharide molecules and sulfated galactose residues on others, or whether both are laid down away from each other on the same molecule, is not known.

The presence of pyruvate on the D-galactose residues of a polysaccharide would not be expected to interfere with the helix formation (secondary structure) of the polymer, since it does not alter the conformation of the L-galactose residue. However, it may sterically hinder aggregate formation (tertiary structure), and this may be the reason why the highly pyruvated fraction (0.5 M, NaCl fraction) produces a clearer gel than does the distilled water fraction.

The results obtained from the fractionation of the agars from different species of agarophytes, are in fairly good agreement with those obtained from Difco Bacto-agar (Duckworth and Yaphe, 1971a). Thus agar cannot be regarded as containing two components, neutral agarose and charged agaropectin as proposed by Araki (1937a), but as a complex of polysaccharides varying as to the degree of

masking. Like the carrageenan polysaccharides (Rees, 1969), agar may be regarded in terms of extremes of structure (Duckworth and Yaphe, 1971a). These are (a) neutral agarose, (b) pyruvated agarose with little sulfation and (c) sulfated galactans containing no or little 3,6-anhydrogalactose or 4,6-O-(1'-carboxyethylidene)-D-galactose residues (see Literature Review). The ratio of the amounts of each extreme differs with the source of the agars and, possibly, also with seasonal variations, the stage of growth of the plant and with environmental conditions. It has been recently shown that the ratio of kappa- to lambda-carrageenan in Chondrus crispus and Gigartina stellata is related to the nuclear phase of the plant (Chen et al., 1972). This ratio is low in tetrasporic plants (2n) and relatively high in the gamatophytes (n). It is not known whether a comparable situation exists in agarophytes. Each agarophyte used in this study was collected from a particular locality on the same day, and probably consists of a mixture of both nuclear phases. In further studies on the agarophytes, emphasis should be placed on the cultivation of each of the nuclear phases of the agarophytes, followed by the analysis of their polysaccharides, to determine the ratio of the extremes of structure in each phase.

Araki, Arai and Hirase (1967) have isolated D-xylose from acid hydrolysates of agar. However, they found no evidence to indicate whether the xylose is indeed part of the agar, or whether is associated with a contaminating xylan. Hydrolysis of the Ahnfeltia plicata agar with P. atlantica agarase was followed by acid hydrolysis of the oligosaccharide fragments. The resulting acid

hydrolysate of the enzymic hydrolysis was found to be devoid of xylose whereas acid hydrolysis of the complete agar revealed the presence of xylose. It is thought that the xylose is either present as part of a contaminating polysaccharide which was not removed during the preparation of the agar, or that it is indeed part of the agar polysaccharide, but is laid down in a region of the polysaccharide molecule in such a way that the enzyme failed to hydrolyze the polymer into fragments of oligosaccharides small enough to be isolated from the unhydrolyzed polysaccharide. Accordingly, unless one can isolate small quantities of the sugars which have been claimed to be a part of the agar structure, such as xylose, 4-O-methyl-L-galactose, O-methylpentose (Araki, Arai and Hirase, 1967), arabinose (Percival and McDowell, 1967), uronic acid (Araki, 1937c), from the oligosaccharide fragments produced by the enzymic hydrolysis, it is not convincing to state that these sugars are part of the agar family of polysaccharides.

The method described by Araki (1937a) for separation of agarose from agaropectin, based on the different solubilities of agarose acetate and agaropectin acetate in chloroform, has been extremely useful for the purpose for which it was introduced, namely the elucidation of the structure of agar, but is tedious and impractical for the routine preparation of agarose (Hjertén, 1962). The acetylation procedure may also lead to degradation of the agarose, thus producing a lower gel strength than the original agar.

The cetyl pyridium chloride method (Hjertén, 1962), introduced as an alternative method for preparation of agarose, is based on complex formation between

the positively charged quaternary ammonium salt and the negatively charged agaropectin. This method is claimed to effect an excellent separation of the two components, agarose and agaropectin, but is expensive.

Blethen (U.S. patent, 3281409, 1966), prepared agarose from crude agar by co-precipitating the agaropectin with a quaternary ammonium salt in the presence of carrageenan. The agarose, which was obtained in high yield (53 to 67.5%), was claimed to be sulfate free. This is surprising, since in the present study, as well as in that reported by Duckworth and Yaphe (1971a), a very small fraction of the agar (if any at all) was found to be completely devoid of sulfate.

Agarose prepared by fractional precipitation of agar with polyethylene glycol (Russell, Mead and Polson, 1964; Hegenauer and Nace, 1965) is based on the different solubilities of agarose and agaropectin in polyethylene glycol. The charged agaropectin is more soluble than the neutral agarose. Although this method is relatively simple and inexpensive, there is some doubt as to whether the product is free from sulfate.

The methods discussed above are based on the concept that agar consists of two components, neutral agarose and charged agaropectin. The low concentration of sulfate found in agarose was attributed to incomplete separation of these two groups of polysaccharides. However, fractionation of Difco Bacto-agar on DEAE Sephadex A-50 (Cl^-) (Duckworth and Yaphe, 1971a) and fractionation of Japanese agar by Izumi (1971) have shown that agar is a complex family of polysaccharides which differ from one member to the next in the extent of masking of

the basic repeating units with sulfate and pyruvic acid, and that agarose is probably one of the theoretical extremes in the polysaccharide family. This finding is confirmed in the present study using different agars prepared from different species of agarophytes. Also the methods of preparation of agarose based on the "two components" concept, attempt to isolate a molecule that may not exist in nature. The definition of agarose as a neutral polysaccharide as proposed by Araki, must be modified and a more practical definition of agarose should therefore be considered, since fractionation of agar on anion-exchange chromatography did not show complete removal of the charged components.

The pyruvic acid content of agarose prepared by the methods described above, as well as that of commercial agarose, has not been reported. Lowering the sulfate content alone does not necessarily indicate an increase in the purity of the preparation with reference to the total charged content. Therefore, any purification method should be concerned with the total charge content, rather than the sulfate content alone. Thus purification of agarose using a method based on ion-exchange chromatography would seem to be the method of choice.

Although Zabin (1969) was not aware that agar is a complex mixture of polysaccharides, he proposed the purification of agarose on DEAE-cellulose. However, his batchwise process yielded products which still contained 0.3% sulfate. The pyruvic acid content was not reported. Based on the same principle of purification, Duckworth and Yaphe (1971c) successively removed polysaccharide fractions with a decreasing content of charged groups by continuously washing the

agar granules with distilled water at 20°C and 50°C, and the partially purified washed agar was further purified by polyethylene glycol treatment before applying the purified agar to a DEAE Sephadex A-50 (Cl^-) column. This method has several advantages, (a) the highly charged molecules and other inorganic impurities were removed by washing with distilled water, (b) further removal of the charged polysaccharides by polyethylene glycol precipitation lowered the total charged content to an extent that enabled the anion-exchange chromatography to achieve its maximum performance, (c) DEAE Sephadex A-50 is more porous than DEAE-cellulose and thus the polysaccharide molecules could penetrate into the gel and hence the adsorption of the charged molecules is more effective. The disadvantage of using DEAE Sephadex A-50 is that it cannot be regenerated and reused, and since the purification procedure has to be carried out at a high temperature (60°C), degradation of the DEAE Sephadex A-50 may occur. Washing the agar granules with distilled water is a liquid-solid extraction, whereas washing the agar gel is a liquid-liquid extraction. The latter case appears to be more effective with regard to the removal of impurities and the highly charged polysaccharides (Hjertén, 1971).

Washed agar which is free of the highly sulfated agar molecules is suitable as an overlay medium in the viral plaque assay of some viruses (Fiala and Kenny, 1966), but other viruses are more sensitive and require agarose with a much lower charged content (Borden, Gray and Murphy, 1970). Similarly, for agarose gel electrophoresis, an essentially neutral agarose is required

(Johansson and Stenflo, 1971).

The purity of the agarose used in the bio-medical fields is critical in obtaining reproducible results. There are no recognized standards for agarose, and commercial agaroses are found to vary with brand and batch, and it is thus difficult to use these materials in bio-medical research for reproducible results. Since agar is a complex family of polysaccharides and agarose is only one of the theoretical extremes in the agar family of polysaccharides, the definition of commercial agarose as a neutral polysaccharide is impractical. The writer feels that a more practical definition for commercial agarose would be that portion of the agar family of polysaccharides which contains less than 0.05% of sulfate and 0.01% of pyruvic acid, and which retains a gelling capacity of at least that of the unpurified agar. This proposal for agarose is based on an unsophisticated, inexpensive method of purification (Duckworth and Yaphe, 1971c) but which still meets the original specifications as described by Araki (1937a). Therefore, commercial preparations (Barteling, 1969) which contain more than 0.05% sulfate should not be considered as agarose, but as highly purified agar.

The original material used by Koch (1882) as a solidifying agent in his study, may not have been one of the agars, since the material he used was obtained from Malaysia, and Malayan agar was extracted from Eucheuma speciosum, which is a carrageenan-producing seaweed. The erronious use of carrageenan as agar has often occurred in the history of agar, and in fact still does occur. Commercial agars, such as British agar and Danish agar were obtained from

carrageenan-producing algae (see page 7). The U.S. Pharmacopoeia (1970) and many other sources state that agar can be obtained from Gelidium species and related red algae (Rhodophyceae), without specifying the genera of agar-producing plants. Carrageenan is also extracted from red algae, and is gel-forming. Thus, the definition of agar should include its chemical composition and it could possibly be defined as "those phycocolloids derived from the Rhodophyceae which consist of a basic repeating structure of (1 → 4)-linked α-L-galactose and (1 → 3)-linked β-D-galactose units masked to a certain extent by 3,6-anhydro-L-galactose, sulfate and pyruvate, and which will melt above 85°C and gel below 45°C."

Genera Gelidium, Gracilaria, Ahnfeltia, Phyllophora, Pterocladia, Acanthopeltis, Campylaephora and Ceramium species have been classified as Agarophytes (Tseng, 1945). Genera Digenia and Gelidiella were shown in this study to be agarophytes (Fig. 16). Other genera of the Rhodophyceae may also be agarophytes, however, a thorough screening with regard to the chemical nature of the polysaccharides in this group has not been reported. A simple technique has been described in this thesis to study the chemical nature of the polysaccharides, extracted from Rhodophyceae, by means of enzymic hydrolysis. The enzymic hydrolysis is specific and the oligosaccharides produced can easily be recognized by TLC (Duckworth and Yahph, 1970b).

Agar-digesting bacteria have been isolated from seawater, marine sediments, soil and from marine algae. Their importance as a group (Angst, 1929)

has been indicated in that they are not limited to the use of agar as carbon source, but have the potential to use a wide variety of other substances (Humm, 1946), such cellulose, starch, chitin and many other polysaccharides. Because of their great potential to utilize substances other than agar, the distribution of agar-digesting bacteria in the environment, and in particular the marine environment, may not necessarily be abundant on the agarophytes. On the other hand, the presence of these bacteria may be ecologically significant with regard to the rate of turn-over of organic substances in the marine environment. All the studies to date have dealt with the degradation of organic substances, in particular the algal polysaccharides, which may not represent the natural state of these materials, as present in the marine environment. 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 bacteria in nature. The role of agar-digesting bacteria in the marine environment can only be evaluated by studying the actual degradation of natural algal material. In nature, it is a common phenomenon that polymer degradation is limited to a small member of microorganisms, while the ability to utilize the products of enzymolysis of the polymers is attributed to a wide variety of microorganisms. For example, Reese and Mandels (1964) have reported that a limited number of soil fungi possess mycodextranase ability. However, the presence of enzymes capable of hydrolyzing the mycodextran oligosaccharides was found to be more wide spread. A similar situation may exist in the marine environment, where agar-digesting

bacteria produce agarases to hydrolyze agar, yielding the oligosaccharides which may furnish other non-agar-digesting microorganisms with nutrients. This situation may be further supported by the fact that all the agarases reported (Araki and Arai, 1956; Yaphe, 1957; Duckworth and Turvey, 1969a) appear to be extracellular enzymes.

The localization of enzymes produced by microorganisms has been a matter of long-standing interest. Pollock (1962) classified enzymes, according to their location, into: (a) cell bound (truly intracellular or surface bound) and (b) extracellular. According to this classification, the agarases of P. atlantica and strain GJ1B are thought to be extracellular since the amount of enzyme found in the medium increased with increase in the cell population. The strain 3A agarase found in the medium did not increase with the cell growth, and maximum enzyme production in this case was found after 3 days of incubation, whereas maximum cell growth was reached within 24 hr. It is therefore probable, that the 3A agarase is a cell bound enzyme. However, the localization of an enzyme is by no means absolute. For example, Cheng et al. (1971) have reported that alkaline phosphatase of P. aeruginosa can be surface bound or extracellular depending on the Mg^{++} concentration or pH, or both.

The crude and A/S agarase preparations were contaminated with oligosaccharidases, even though the P. atlantica and strain 3A enzymes were harvested during the exponential phase of growth. A β-tetrasaccharidase from P. atlantica, which hydrolyzes neoagarotetraose to yield neoagarobiose, has been shown to be

located in periplasmic region (Ross, 1970). Leakage of this enzyme into the culture medium was always observed. An α-disaccharidase, which hydrolyzes neoagarobiose to galactose and 3,6-anhydrogalactose, has been isolated from the cell sonicates of P. atlantica (Papenburg and Yaphe, unpublished results; Yaphe, 1966) indicating that the enzyme is either located in the periplasmic region or intracellularly. The presence of oligosaccharidases in the culture medium of P. atlantica may indicate that a small number of cells were lysed during the exponential phase of growth. A similar explanation may also be applied to the strain GJ1B preparation; whereas in the strain 3A preparation, lysis of cells was expected. This enzyme was harvested 2 days after the exponential phase of growth. Araki and Arai (1956) obtained their agarase from a one-week old culture medium of P. kyotoensis, and used this crude enzyme without further purification. Their enzyme preparation may thus have contained a mixture of oligosaccharidases in addition to the β-agarase. Fortunately, they were able to use this unpurified enzyme preparation with success in the isolation of neoagarobiose and -tetraose in the elucidation of the structure of agar. Purification of the agarase by ion-exchange chromatography has been reported by Yaphe (1966) for the P. atlantica enzyme and by Duckworth and Turvey (1969a) for the Cytophaga species enzyme. However, the criteria of purification were not discussed. In this study, the purpose of the purification was to separate the oligosaccharidases from the agarase, and the criterion of purification was that the purified enzyme no longer hydrolyzed neoagarotetraose. This tetrasaccharide (Fig. 10) contains two α- and one β-

linkages and is thus vulnerable to hydrolysis by both the α- and β-oligosaccharidases (Ross, 1970; Papenburg, 1972, unpublished results).

The great loss of enzyme activity during the purification process of the GJ1B agarase (21.5% recovery) and the strain 3A agarase (22.3% recovery), may be attributed to their lability. The majority of enzyme activity was lost in the treatment with the DEAE-cellulose. The enzyme, however, was stabilized in the presence of the Ca^{++} ion, and thus it would probably be better to use calcium hydroxyapatite instead of DEAE-cellulose for the purification, if large quantities of the enzymes are required. It is interesting to note that in the P. atlantica enzyme purification process, the total recovery of the enzyme after DEAE-cellulose treatment was increased (from 57.5 to 75.9%). This may be due to the presence of inhibitor(s) in the ammonium sulfate preparation, which was (were) removed during the DEAE-cellulose step.

The role of calcium on the activity of strain GJ1B agarase may be best expressed as a stabilizing, and not a catalytic, effect. A catalytic reaction, by definition, is not affected by the concentration of the catalyst, i.e., it is an all-or-none reaction. In the absence of a catalyst, no reaction should be observed. The agarase from strain GJ1B, when mixed with washed agar in the absence of calcium (Fig. 21), reduced the viscosity of the substrate to a small extent, and addition of calcium to the digest during the progress of the reaction did not seem to enhance the enzyme activity, indicating that all the enzyme was inactivated within a short period of time at 42°C. The enzyme preparation, after Ca^{++}-treat-

ment, followed by dialysis against Tris-buffer containing no calcium ions, presumably contained no free calcium ions, and yet it exhibited a far greater enzyme activity than a non-calcium treated preparation (Fig. 21). It would seem that the enzyme is capable of binding or chelating calcium ions. Dialysis in Tris-buffer does not appear to remove the bound calcium. However, addition of EDTA to the digest during the progress of reaction stops the reaction immediately. Should the added EDTA inactivate the enzyme activity directly, there would not be any enzyme activity remaining after incubation with EDTA, even in the presence of calcium (Fig. 22). EDTA appears to be a much stronger chelator than the agarase. An increase in the calcium concentration of the reaction mixture increases the enzyme activity (Fig. 23). An equilibrium would seem to exist between the calcium concentration and enzyme-calcium complex at 42°C. Therefore, an increase in the calcium concentration favors the formation of enzyme-calcium complex and hence the enzyme becomes more stable and the activity increases. The dissociation constant of this enzyme-calcium complex may be so low at 4°C that dialysis for 3 hr. would not remove an appreciable amount of calcium from the complex. Enzymes of this kind have been reported by Bissell, Tosi and Gorini (1971), who showed that the only function of calcium in the proteinase of a Sarcina strain (Coccus P), was to stabilize the active structure of the enzyme molecule; and by Hsiu et al. (1964) who proposed that the calcium requirement of a variety of α-amylases played a structural role in the production of effective catalytic activity.

The exact way in which calcium is involved in the stabilization is not immediately apparent. It would seem rather unlikely that the calcium participates directly in the formation of the enzyme-substrate complex, or that it is directly involved in the cleavage of the glycosidic bond. To interpret the effect brought about by the calcium on the agarase activity, the following explanation is proposed. Calcium confers to the agarase molecule the structural rigidity required for biological activity, by forming a tight, intramolecular, calcium chelate structure. Many calcium-dependent enzymes, such as the α-amylase from B. subtilis (Junge et al., 1959), the neutral protease from Streptomyces griseus and the proteinase from a Sarcina strain (Bissell, Tosi and Gorini, 1971) possess no disulfide bridge. It would seem possible, therefore, that calcium takes the place of the missing disulfide linkages. Pollock and Richmond (1962) have found that bacterial exo-proteins, in general, contain no or a low cyst(e)ine content. The disulfide linkages in a protein are the most important if not the only, covalent bonds contributing to the rigidity and stability of the tertiary structure of protein molecules (Boyer, 1959). A protein lacking disulfide bonds would have to rely mainly on the weaker and more easily ruptured hydrogen bonds and metal- and salt-linkages, to preserve its folded state. Based on this reasoning, Pollock and Richmond (1962) proposed that bacterial exo-protein might have more than the usual degree of flexibility and be more easily unfolded and refolded than the proteins which contain disulfide bonds. In order for these enzymes to pass through the cell envelope more freely, the enzymes might have evolved a degree of flexibility through the elimination of

disulfide bonds. However, it should be noted that the site of synthesis of bacterial extracellular enzymes is still not known. It is proposed that the effect on the enzyme activity by addition of EDTA and the calcium may represent, respectively, unfolding and refolding of the enzyme molecule. The disulfide content of the GJ1B agarase was not determined.

The neoagarobiose series of oligosaccharides obtained with the β-agarases from P. atlantica and strain 3A was separated, yielding chromatographically pure sugars up to DP 16. It appeared from a preliminary examination, using the wedge TLC technique (Fig. 24) that oligosaccharides having DP 18 and 20 could also have been isolated. However, this was not carried out. The pure oligosaccharides could be used not only as a tool to study the mode of action of the agarases, but also as a substrate for the α-agarase, in order to produce oligosaccharides having an odd number of hexose units. Furthermore, these oligosaccharides, especially the high molecular weight sugars (DP 16, 18, 20), are thought to be of importance in the study of the gelling mechanism of agarose.

The powdery precipitate, formed when an agarose hydrolysate is kept at 4°C overnight, was found to remain as a precipitate on warming to 25°C, although it had been in solution at 25°C before the cooling treatment. However, the precipitate redissolved at 55°C. This phenomenon may be likened to that of agarose which dissolves on boiling, but gels at 40°C. The gellation process can be considered to be a process of precipitation, whereby an association of chain segments to form "junction zones", which are joined into a network by chains

that run through two or more junction zones, results in gel formation (Rees, 1969). Smith-degraded agarose, i.e., agarose which has been cleaved at the "kink", having an average DP higher than the oligosaccharides obtained at achroic point, also formed a powdery procipitate, and not a gel, which dissolved on reheating. However, incorporation of a galactomannan into this "kink-cleaved" agarose resulted in gel formation (Dea, McKinnon and Rees, 1972). The Smith-degraded agarose undergoes a change in conformation, presumably to yield secondary and tertiary structures, as porposed by Rees (1972), without gel formation, because the chain length of this agarose is not long enough to form cross-links to yield a network and hence a gel. On the addition of the galactomannan, the smooth section of the galactomannan binds to the agarose helices, the rest of the molecule acting as the cross-links to form a network thus producing a gel. Although the Smith-degraded agarose and the agarose hydrolysate obtained at achroic point appeared to have similar properties, whether or not the agarose hydrolysate will gel in the presence of a galactomannan has not been determined. However, the author feels that it will most likely form a gel with a galactomannan, if Rees' gelling mechanism (1972) is correct. The question then arises as to how long a chain of agarose is required to form a critical nucleus for helix formation. Enzymic hydrolysis of agarose is possibly the easiest, if not the only way to obtain the various oligosaccharides, required for this type of study.

The isolation of sugars which contain 3,6-anhydrogalactose as end sugar strongly indicates that the agarase from strain GJ1B is an α-agarase, which

specifically hydrolyzes α-1,3 linkages of agarose to yield the agarobiose series of oligosaccharides which have 3,6-anhydro-L-galactose as the end sugar. Although the nomenclature of oligosaccharides of this type was proposed by Araki and Arai in 1957, the isolation of the agarobiose series of oligosaccharides has not been achieved up till now (with the exception of agarobiose which can be obtained by the partial acid hydrolysis of agar), because of the lack of a proper method for production of these sugars from agar. Before this scheme of nomenclature was introduced, an enzymolysis of agar with a bacterial agarase prepared by Ishimatsu et al. (Ishimatsu and Maitani, 1953; Ishimatsu, Kibesaki and Maitani, 1954; Ishimatsu and Kibesaki, 1955) produced a disaccharide which was named agarobiose. This disaccharide, however, differs from the agarobiose reported by Araki (1944) in the following aspects: the melting point of its phenylosazone, its chromatographic pattern and its methanolytic products. Thus the disaccharide is probably neoagarobiose rather than agarobiose, the enzyme used being a β-agarase and not an α-agarase.

The α-agarase producing bacterium, strain GJ1B, exhibits different properties to those of the β-agarase producing bacteria (P. atlantica, strain 3A, P. kyotoensis, Cytophaga species) as far as their action on the gel state of agar is concerned. The β-agarase producing bacteria, when grown as colonies on an agar surface, make small depressions. In some cases, no visible change to the agar surface was noted. When iodine was flooded over the surface, however, a clear zone arround the colonies was observed, indicating that the β-agarase

had hydrolyzed the agar and changed the structure of the agar. When strain GJ1B was grown as a colony on the surface of an agar gel, it bored a hole through agar gel, liquefaction was observed and a clear zone was obtained arround the hole when the agar surface was flooded with iodine. The liquefaction of an agar gel by strain GJ1B may be associated with the nature of the α-agarase or perhaps with another enzyme which is able to unwind the agar helices, thus making agar molecules available for attack by the α-agarase. Experiments to demonstrate the liquefaction of agar have been tried with the purified α-agarase, but no conclusive evidence can be given at the moment.

There are two general classes of polysaccharidases: (a) endo-enzymes, which hydrolyze the polymer substrate in random fashion and (b) exo-enzymes, which remove products sequentially from the one end of the polymer chain. In addition, it is possible for each of these classes to exhibit one of three types of mechanism during the digestion of their substrates; single chain, multichain and multiple attack (see Introduction). All the agarases used in this study were shown to catalyze the hydrolysis of agarose by an endo-mechanism, since the rapid reduction in the viscosity of the agarose was accompanied by the release of only a small amount of reducing sugar. A plot of the reducing values versus the specific viscosity (Fig. 43) for each enzyme shows that when equal viscosity units of enzyme are used, the agarase of strain GJ1B yields a higher reducing value than the agarases from strain 3A and P. atlantica. The difference may be interpreted as a difference in the degree of multiple attack. The degree of

multiple attack was calculated from (r - 1), where r is the ratio of the total reducing sugar in the digest, to the reducing sugar of a 75% ethanol precipitate of the digest (Robyt and French, 1967). The total increase in reducing sugar represents the sum of two types of bond cleavages: (a) primary attack on a polysaccharide molecule to give two polymer fragments, and (b) secondary attack on the newly formed end of one polymer fragment to low molecular weight oligosaccharides. The polymer may be easily separated from the oligosaccharides by ethanol precipitation. The number of new reducing groups in the polysaccharide fraction is equal to the number of effective encounters, and the number of new reducing groups in the oligosaccharide fraction is equal to the number of bonds broken in the secondary phase of multiple attack. Therefore, r is the total number of bonds broken divided by the total number of effective encounters (i.e. the total bonds broken per effective encounter), and r - 1 is the average number of subsequent bonds broken, after the first, and thus gives the degree of multiple attack. In this analysis, it must be assumed that the formation of oligosaccharides through the primary attack is insignificant, since the mechanism of action is random and the possibility of attack to yield low molecular weight oligosaccharides is very small, when one consider a polymer such as agarose which has a molecular weight of the order of 120,000 (Hickson and Polson, 1968). The degree of multiple attack of the GJ1B agarase is 1.58, i.e., for every effective encounter, the agarase breaks one to two addition bonds and releases two moles of low molecular weight oligosaccharides. The degree of multiple attack of the agarases from strain 3A and P. atlantica is

0.08 and 0.54, respectively. These agarases are considered to exhibit a multi-chain mechanism, since analysis by TLC of the supernatant fluid, after precipitation with 75% ethanol showed that the low molecular weight oligosaccharides appeared after 20 min. of incubation in both cases. If these agarases had catalyzed the hydrolysis by a multiple attack mechanism, the oligosaccharides would have appeared immediately after incubation, as is found in the case of the GJ1B agarase. Thus, in studies on the mechanism of action of a polysaccharidase, comparison of the reduction in viscosity and the release of reducing sugar only indicates the type of encounter between the enzyme and substrate to form an active complex.

The degree of multiple attack obtained for these agarases is only true when it is determined under the stated conditions, i.e., pH 7.2 (Tris-buffer, 0.01 M) at 42°C. Robyt and French (1967) have shown that by changing the pH of the buffer, the degree of multiple attack of porcine pancreatic α-amylase changed. They interpreted this phenomenon as possibly being due to a change in the ionization state of the catalytic groups, or of the binding sites, or being the result of gross changes in protein conformation so that only a small fraction of enzyme is enzymatically active.

The smallest oligosaccharide molecule which was hydrolyzed by the P. atlantica agarase is neoagarohexaose, and the bond broken in this case being the β-1,4 linkage nearest to the reducing end (Fig. 39). The agaropentaose produced by the hydrolysis of agaro-octaitol and agarodecaitol with the P. atlantica agarase

does not appear to be further broken down by the enzyme, indicating that the enzyme requires at least four monosaccharide units, i.e., two neoagarobiose units, to the left of cleavage site for the catalysis to occur. Although agarohexaitol (Fig. 41 A) and neoagaropentaitol (Fig. 41 C) each contain a neoagarotetraose unit to the left of the cleavage site of the enzyme, these oligosaccharides are not hydrolyzed, suggesting that the enzyme requires not only a neoagarotetraose unit to the left of cleavage site, but also a neoagarobiose unit to the right of cleavage site, in order to make the catalysis occur. This would explain why neoagarohexaose is the smallest molecule hydrolyzed by the P. atlantica enzyme. It would also explain why the enzyme hydrolyzes oligosaccharides such as neoagarohexaose, neoagaro-octaose, agaro-octaitol and agarodecaitol at the β-1,4 linkage closest to the reducing but never the one nearest to the non-reducing termini.

It has been shown, for the first time, that an α-agarase produced by the strain GJ1B specifically cleaves the α-1,3 linkages of the agar molecule, yielding the agarobiose series of oligosaccharides which cannot be obtained by chemical methods. The combined use of both α- and β-agarases in the hydrolysis of agarose, would be expected to produce a series of oligosaccharides with an odd number of monomeric units (Fig. 41). These oligosaccharides, together with the neoagarobiose series of oligosaccharides should be the ideal materials for the study of the gelling mechanism of agar.

The advantage of enzymolysis over chemical analysis, is its specificity. Each enzyme hydrolyzes the agar molecule by cleaving only one specific

type of linkage, whereas, chemical hydrolysis is non-specific. Controlled hydrolysis of agarose with the agarases may be used to produce polysaccharide fragments similar to those obtained by a Smith-degradation of agarose. However, with the latter method, the chain length of the polysaccharide fragment is non-controllable and the products obtained are not uniform. Enzymolysis of agar can also be applied to the detection of agar; an agarophyte can easily be identified by extracting the algae with boiling water, the extract then being hydrolyzed with agarase. The presence of oligosaccharides in the hydrolysate is an indication that the algae is an agarophyte. Thus, a simple enzyme kit consisting of an agarase preparation and TLC could be devised for botanists for use in field studies. The quality of the agar obtained from an agarophyte can be determined by fractionation of the agar on DEAE Sephadex A-50 (Cl^-), the resultant fractions being hydrolyzed with agarase. This method is particularly useful in the development of agar resources. The relationship between the algae and agar production with regard to seasonal variation, nutritional condition and other ecological aspects can be studied in a similar fashion.

Although the evidence available is consistent with the occurrence of the same basic repeating structure throughout the agar, namely, a chain of alternating D- and L-galactose, linked α-1,3 and β-1,4, it should be pointed out that no methods have yet been proposed which can rigorously prove this structure. The most compelling evidence that has been obtained in agar, has been the isolation of the repeating disaccharide and other oligosaccharides in 40 to 75%

yield after controlled fragmentation (Anderson, Dolan and Rees, 1965). In other words, the basic structure of agar is known, but the fine structure is still somewhat in doubt, particularly in the highly sulfated fractions. The fine structure of agar should be further studied in order to shed light on the inter-relationship between the masking groups on the agar, as well as to form a basis for the study of the biosynthesis of agar. Knowing the mode of action of the α- and β-agarases, the combined use of both enzymes should prove to be a very useful tool to study the fine structure of agar.

The need for standarization of agar and agarose becomes obvious when precise biochemical experiments are being performed. For the past few decades, erratic results have been obtained by the scientific community, due to the non-uniformity of agar or agarose. The achievement of standards for agar and agarose for scientific use requires the co-operation of the scientific communities and industrial organizations. More information about agar must be obtained, such as the species and ecology of the algae, and the method of purification used in the production of agar and agarose. Standards could then be set which would guide the industrial producer to manufacture an agar and agarose to meet the requirements for scientific purposes.

VI. SUMMARY

1. Algae from five major groups of agarophytes have been investigated for their agar components. The properties of the agars varied from species to species. It was shown that agar is not composed of agarose and agaropectin, but consists of a complex mixture of polysaccharides being masked to a varying degree with different groups.

2. Three bacterial agarases have been purified from the oligosaccharidases, by precipitation with ammonium sulfate followed by DEAE-cellulose ion-exchange chromatography.

3. Purified bacterial agarases can be classified into two major groups, being specific for either the β or α linkage in agarose. The β-agarases are further differentiated by the mechanism of hydrolysis of neoagaro-octaose. P. atlantica β-agarase preferentially hydrolyzes the central linkage, yielding two molecules of neoagarotetraose, while the β-agarase from a soil bacterium, strain 3A, hydrolyzes the terminal linkage forming neoagarohexaose and neoagarobiose.

4. An extracellular α-agarase has been isolated from a culture of the bacterium, strain GJ1B, growing on agar. The α-agarase hydrolyzes agarose specifically at the α-1,3 linkages, yielding the agarobiose series of oligo-

saccharides. Two of these oligosaccharides have been identified as agaro-hexaose and agaro-octaose.

5. All the purified agarases have an endo-action, producing a rapid decrease in viscosity of agar solution with little development of reducing power. However, the α-agarase from strain GJ1B catalyzes the hydrolysis of agarose by a multiple attack mechanism, whereas the β-agarases from P. atlantica and strain 3A hydrolyze agarose by a multichain mechanism.

6. Oligosaccharides having an odd number of monomeric units, such as neoagarotriose, neoagaropentaose and agaropentaose, can be obtained through the combined hydrolysis of agarose with α- and β-agarases.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

The author makes the following claims to originality:

1. The isolation, purification and studies on the mode of action of an α-agarase which catalyzes the hydrolysis of agarose specifically at the α-1,3 linkages.

2. The agarobiose series of oligosaccharides have been isolated from hydrolysis of agarose with the α-agarase.

3. The combined use of α- and β-agarases has been shown to yield members of both the agarobiose and the neoagarobiose series of oligosaccharides, which contain an odd number of monomeric units.

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 0.3% solution
Aged sea water to	1000 ml

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

(2) H-1 Medium

NaCl	75.0 g
K_2HPO_4	0.3 g
$MgSO_4 \cdot 7H_2O$	15.0 g
KCl	3.0 g
$CaCl_2 \cdot 2H_2O$	0.6 g
Casamino acids	7.5 g

$FeSO_4 \cdot 7H_2O$	30 ml of a 0.3% solution
Distilled water to	3000 ml

Solid medium is prepared by adding 20 g of the washed agar per liter of medium. Semi-solid medium is prepared by adding 2 g of the washed agar per liter of medium. The medium is adjusted to pH 7.2 with 0.1 N NaOH, and is dispensed into 500 ml Erlenmeyer flasks, 200 ml per flask. The media are sterilized at 15 pounds pressure (121°C) for 15 minutes.

(3) H-2 Medium

NaCl	75.0 g
$MgSO_4 \cdot 7H_2O$	15.0 g
$CaCl_2 \cdot 2H_2O$	0.6 g
KCl	0.3 g
$NaNO_3$	6.0 g
Na_2HPO_4	0.3 g
$FeSO_4 \cdot 7H_2O$	30 ml of a 0.3% solution
Distilled water to	3000 ml

Solid and semi-solid media are prepared with the washed agar as described for H-1 medium.

(4) Alexander Salt Medium

Proteose peptone	15.0 g
NH_4NO_3	1.5 g
K_2HPO_4	2.4 g
KH_2PO_4	0.6 g
$CaCl_2 \cdot 2H_2O$	0.3 g
$MgSO_4 \cdot 7H_2O$	0.6 g
$FeSO_4 \cdot 7H_2O$	30 ml of a 0.3% solution
Distilled water to	3000 ml

Solid and semi-solid media are prepared with the washed agar as described for H-1 medium.

B. REAGENTS

(1) Sulfate reagent

This reagent is prepared by dissolving 25 mg of 4-amino-4'-chlorodiphenyl hydrochloride in 50 ml 0.1 N HCl. The solution is then filtered and stored at 4°C.

(2) I_2/KI

Iodine	50 g
KI	100 g
Distilled water	950 ml

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

(3) 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

The above solution is diluted to 2400 ml with CO_2-free distilled water, 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

The above solution is diluted to 600 ml with CO_2-free distilled water and allowed to stand overnight. The solution is then filtered into a glass-stoppered bottle, and stored in the dark. Four volumes of Solution I plus one volume of Solution II are mixed immediately prior to use.

(4) Nelson's chromogenic 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 to 48 hr. at 37°C. The reagent is stored in a brown bottle at 4°C in the dark.

(5) 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°C. Before use, two parts of Solution I are mixed with one part of Solution II.

(6) Aniline hydrogen phthalate reagent

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

(7) Ammoniacal silver nitrate

To a 5% (w/v) silver nitrate, ammonia was added dropwise until the brown precipitate disappeared.

(8) Silver nitrate-sodium hydroxide

(a) Saturated silver nitrate solution 0.1 vol

Acetone 20 vol

(b) 0.5% sodium hydroxide in ethanol

The chromatogram is sprayed with reagent (a) and air dried, and then with reagent (b), air dried in the dark.

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