"STUDIES ON GALACTANS WITH PARTICULAR REFERENCE TO AGAR".

-by-

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STUDIES ON GALACTANS WITH SPECIAL REFERENCE TO AGAR.

INTRODUCTION.

"Agar-Agar" is the name loosely applied to a class of commercial gel-forming carbohydrate products, extracted from the red algae of the family Rhodophyceae. The name was originally a Malayan term meaning "seaweed", and was restricted to the edible weed Eucheuma spinosum, which flourishes abundantly near the South Sea Islands.(1) In Japan the substance is known as "kanten", meaning "cold sky", since frost is necessary for its preparation.(2) Two further synonyms, Sauvageau's "gelose", and "Japanese isinglass", are descriptive of its characteristic gelling properties.

Sources of Supply.

Before the 1939-46 War, Japan supplied nearly 95% of the World's agar, and between 1930 and 1940 maintained an average production of 2,476 metric tons per annum. Since the largest importers of Japanese agar during this period were the United States the United Kingdom, Germany and France, the industry declined very rapidly during the War years, so that in 1945 only 170 out of 536 plants were working, and production had fallen from 2,934 metric tons in 1940 to 716 metric tons in 1945.(1)

Among the nations allied against Japan the United States had the only sizeable existing agar industry.(3) This industry, established in Tropico (now Glendale) California, had been unsuccessfully competing with the Japanese production since 1920, but at the outbreak of War the industry was greatly expanded/

expanded and reached a peak production of 165,954 lb. per annum.

The need for agar for vital bacteriological work in preparing protective vaccines for their armed forces was met in other countries in a variety of ways, from the production of a high grade agar to the boiling up of unidentified red seaweeds, which happened to be locally available. South Africa, India, Russia, Malaya, Ceylon, Portugal, Spain, Italy and Eire were among the countries which made small quantities of agar.(4) China began an industry but the area was subsequently cut off by the Japanese. New Zealand, Australia and Britain set to work on the botanical aspects of the agar problem as well as on the making of a high grade product. It is clear from the diversity of methods and materials employed in these countries that the constitution of commercial agar assumed an even more heterogeneous character during this period, and the following table, showing weeds utilized by various nations, exemplifies this point.

Species of Seaweed used in Japan

Manufacturers Term	Botanical Name	Manufacturers Term	Botanical Name
	Gelidium amansii Lmx		Gracilaria confervoides
	G. Pacificum		Gr. gigas
	G. divaricatum		Gr. chorda
	G. Crinale	Ogo	Gr. compressa
	G. Liatulum		Graulana
Tengusa	G. Pusillum		Blodgetti
	G. Subfastig-iatum		Graulana lichenoides
	G. vagum		Gelidium linoides
	Ptero-cladia capillacea		
	Pt. tenuis		
	Pt. densa		Ceramium rubrum
	Pt. nana	Igisu	Cer. Boydenii
			Cer. crassum
	Gelidialla acerosa		
Oni	Gelidium Japonicum		Gelidium subcostatum
		Hirakusa	G. tenue
			G. planisculum
Toriashi	Acanthopeltes Japonica		
			Eucheuma muricatum
			Eu. gelatinae
		Kirinso	Eu. amakusaensis
Ego	Ceramium Hypnaeoides		Eu. crustaeforme
Itaniso	Ahnfeltia plicata		

Species of weeds used:-

In America:- Gelidium cartilagineum, Gracilaria confervoides, Gelidium arborescens. Gelidium ruedifrons, Gracilaria foliifera. Gelidium densum, Gelidium ramuliferum.

In Britain:- Chondrus crispus, Gigartina stellata.

In Australia:- Gracilaria confervoides, Eucheuma muricatum, Hypnea musciformis, Pterocladia lucida.

In New Zealand:- Pterocladia lucida.

In Russia:- Ahnfeltia plicata, Iridaea and Phyllophora.

Utilization and Industrial Applications of Agar.

In addition to its inclusion in the staple diet of the Japanese and Chinese population, who regard it as a great delicacy, commercial agar is a valuable industrial colloid. Many of its applications may be ascribed to its strongly hydrophilic nature and to its high gel strength.

It is curious and interesting to note that the eastern races appear to obtain more nourishment from seaweed products than peoples of the western hemisphere, who are unable to assimilate algal proteins and carbohydrates.[13] This may be due to the Japanese technique of preparation of algal food which involves partial fermentation, thus breaking down the constituents to some extent. Another interesting explanation is due to E.J. Ferguson Wood,[2] who suggests that the individual Japanese, since he is fed on seaweed from infancy, may develop an intestinal flora which is capable of dealing with algal material. However our western deficiencies have been turned to good account in the use of agar as a bulk-producer in certain types of breakfast foods and special bakery products for victims of constipation.

Agar has many other uses in the food industry. It is used to stabilize icings and ice-cream and in making chiffon pies, meringues and fillings. It is used as a thickener in jams and sauces and in theconfectionery trade for the production of jelly candies and marshmallows. Malted milks and acidophilous milks are stabilized with agar. It is incorporated in fruit cakes and some types of bread to keep them moist. In the wine and vinegar industries agar acts as a fining agent. Mayonnaise and some vegetable pastes often contain agar as the stabilizing agent. The canning

industry makes considerable use of agar for such commodities as canned tongues, galantines, canned rabbit and poultry, and in other foods where appearance is improved by a firm, white jelly, or where it is desirable to remove excess moisture from the meat while retaining it in the can. Agar has definite advantages over gelatine in canning practice since it is usual to heat the cans at 230°-240°F. and 7-10 lbs. pressure for over an hour. At such temperatures and pressures gelatine tends to hydrolyse, and consequently does not set on cooling. Agar is not hydrolysed appreciably under two hours at 266°F. and 26lb. pressure. Moreover, gelatine melts and remains liquid in the vicinity of 77°F, while agar sets between 87° and 122 F. and does not melt until 176°-203°F. In the tropics, where temperatures of 90°F are usual, agar is obviously much preferable to gelatine. A further point in the favour of the use of agar is its greater stability to acids. Agar is relatively stable to a pH of 4.5 at boiling-point, and lower in the cold, whereas gelatine tends to break down below pH.6. This is important in packing acid foodstuffs such as fruit juices.

The medical uses of agar are many and varied. Many laxatives consist of liquid paraffin as a base, with agar, benzoic acid or lactic acid, and possibly gum acacia or gum tragacanth.(7) Although in some of these preparations agar is used as a laxative, it is usually present in too small amounts to be effective for this purpose, and it mainly acts as an emulsifying agent. An interesting use in medicinal preparations is in "seal-ins" for pills, a type of coating which regulates the rate of solution of the capsule, and consequently/

consequently the time of its opening. The agar is added in tiny particles and distributed in the waxy material of the coating. By virtue of its water absorption, agar assists in the release of the coated medicinal preparation in the desired place. Agar is also used in the coatings of certain gentian violet capsules employed in the treatment of infection with Oxyuris vermicularis. It is also a constituent in a preparation for the treatment of Coccidioides infection in chickens. On account of its anti-coagulant properties agar is used in surgical dressings and in greaseless dressings.

The best-known use of agar is in the preparation of solid microbiological culture media, and as such it is an indispensable material in the routine sanitary analyses of water and milk. Its importance in public health work and in medical and scientific research is so significant that, during World War II, the United States Government had to control the supply of agar so that the nation's health would not be impaired by lack of this seaweed colloid.

Agar was first used as a base for bacteriological media in 1881 by Dr. Walter Hesse.(8) It was found that gelatine, which had been used previously, has several drawbacks.

1. It is difficult to sterilize, owing to its hydrolysis at sterilizing temperatures, and it cannot be incubated at 37°C (98°F), the optimum temperature of most human pathogenic bacteria.

2. Also it is liquified by a number of common bacteria, including many of those occurring in the human intestine.

3. Agar has none of these drawbacks. It is certainly liquified by a few bacteria of marine or soil origin, but these organisms have an optimum growth temperature below 25°C/

25°C (78°F), and are not pathogenic to man. Silica gels are displacing agar for some bacteriological uses, but must be prepared before the bacterial inoculum is introduced, whereas agar can be mixed with the bacterial suspensions. If bacteria are sown into an agar medium they will produce colonies, which are held stationary and can be counted. This is the principle used in "plate counts" or "viable counts". Certain vaccines are prepared by growing cultures of the required organisms on agar surfaces with suitable nutrients, and then washing off and formalizing the growth. Agar is preferable to broth for this purpose because the growth on agar is on the surface, and little food material is washed off with it. This is desirable since protein from the nutrient in the medium may produce a reaction (anaphylaxis) in the patient. Again, agar is used for counting bacteria in milk, water, sewage etc., and as a substrate in the diagnosis of scarlet fever, meningitis, and many other diseases. Agar also possesses certain growth factors which promote the growth of organisms.(9)(10) It is probably for that reason that Itano and Tsugi(11) find that Azotobacter chroococcum, Bacillus subtilis, and Saccharomyces cerevisiae, grow better on lower gade agar.

In scientific laboratories agar serves as an embedding medium for microtome sectioning, as a vehicle in the standard Avena test for plant growth hormones, and as a coagulant for barium sulphate precipitation. It is used in some types of pH determinations, studies in diffusion, electrochemistry and research on the properties of gels and sols.

In/

In agriculture agar is useful as an insecticide activator and carrier. Sharp suggested the use of agar as a dispersion medium for nicotine sprays, and found that one part agar to five hundred by weight gave these sprays three times the normal lethal effect. Agar is also used for making coatings for nitrogen bacteria cultures for agricultural purposes,[15] and it has been recommended that shredded agar should be incorporated in small amounts in tobacco, to retard excessive evaporation of moisture.

Agar is extensively used in prosthetic work[14] as the basic material in most dental impression compounds. For this purpose the hysteresis (lag between liquifying and setting temperatures) is used. The concentrated agar solution is heated to liquify, placed in the cavity, and allowed to set. A low setting point agar is preferable for this purpose.[12] In highly critical work, agar-based compounds are practically the only materials used.

Many greaseless cosmetics, including creams and soaps, have an agar base, and it has been incorporated in some brands of toothpaste and hand-lotions. Manufacturers of shoe stains have utilized agar in their products, and the raw weed is used in Australia for glazing upper leather. In the textile industry agar is used as a size, and as a thickener in the dyeing and printing of textiles. In the paper industry it is used for waterproofing and for duplicator rolls, and is the emulsifying agent in many water paints.

The electrical industry employs agar in welding fluxes and/

and in the drawing of tungsten lamp filaments, for which a lubricant of agar gel and powdered graphite has been found more efficient and more economical than "aquadag".

Agar has also been used in the electroplating of lead, and in the manufacture of marine storage batteries.

Although agar alone is unsuitable for use in certain photographic materials because of its tendency to stick to gelatine, and its insolubility in organic solvents and alkaline solutions, the esters of agar are soluble in a number of organic solvents, and can therefore be employed as coatings or backings for photographic films, from which they may later be removed by means of alkali. These backings keep the films flat, and may be used to carry coloured materials for minimizing halation. Photographic film has been made from aqueous solutions of agar treated with formaldehyde, and it was found that these need only one-eighth of the thickness of gelatine films.(16)

The building industry has benefitted from the use of agar in films for windows, and as a constituent of high-grade adhesives for the manufacture of plywoods. The residual weed from agar extraction plants has been processed for wall-board.

The above survey of the industrial uses of agar gives some indication of the versatility of this colloid. The relative importance of the many applications listed may to some extent be gauged from the following table published in an article by Tseng(17) in 1944, which shows the uses of agar in the United States about that period.

Industry	lb. per annum.
Laxative	100,000
Microbiology	100,000
Bread etc.	100,000
Confectionery	100,000
Dental Impressions	75,000
Meat Packing	50,000
Emulsification	50,000
Cosmetics	25,000
Miscellaneous	50,000
	650,000

It is clear that this seaweed colloid is of considerable economic value, and it is important to review the methods whereby it is extracted in order that some judgment may be made of its homogeneity and state of purity for use in these many fields.

Commercial Harvesting, Extraction and Purification Methods.

(1) In Japan.

There is a Japanese legend which tells how a mountain peasant entertained an Emperor to a choice banquet of seawoed jelly. Excess food had to be thrown out - an unprecedented act for the impoverished peasant! During the night it froze on a shrub, and later, as it thawed, the jelly collapsed, the water drained away and only a shrunken papery mass remained. The ingenious householder found that on heating this with water and cooling the solution, the original jelly was restored. In this way, says the legend, arose the great agar industry of Japan.

In Japan, to this day, operations are conducted much like those credited to the mountain peasant. Many kinds of seaweeds are used, each with its special purpose, and to obtain a uniform product it is considered necessary to blend seaweeds from different areas, and of different varieties.

This/

This blending produces an agar with a low setting point and viscosity, and a high gel strength. It is also important to regulate the harvesting season since seaweed from the first growth collected in May and June has a greater agar content than that of the second growth. The following table indicates the most desirable proportions of various weeds in one batch for agar extraction.(1)

Japanese Group Name	Percent	Pounds
Tengusa	45	225
Oni	10	50
Toriashi	5	25
Ego	10	50
Ogo	15	75
Igisu	5	25
Hirakusa	10	50
	100	500

The seaweed is gathered by fishermen and their families and by deep-sea divers. The method of gathering depends upon the local customs, the depth of the water, and the formation of the ocean bed. Generally seaweed growing in shallow water is gathered either by means of long-handled rakes, with which it is scraped off the rock, or by the labours of women divers, who operate to a depth of 30ft., with goggles as their only mechanical equipment. In gathering seaweed from rocks where the ocean depth is greater than ten fathoms, men divers, equipped with diving apparatus, are employed. The quality of weed from shallow waters is inferior to that from the deeper beds, and a fairly turbulent environment produces the best growths.

After collection the seaweed is spread on bamboo racks along the beach to dry, and to partially bleach by the action of sun and rain. The dried material is stored in barns in

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a similar manner to that adopted for hay, and is then transported, by rail, to the mountain processing areas, where advantage is taken of the frosty nights and clear sunny days of December, January and February, when most of the processing is carried out. Ideal weather conditions include freezing at night with the temperature at -7°C or -8°C, and clear sunny days with light southerly winds. Strong winds would blow dust and soot onto the drying colloid resulting in an impure product.

On arrival at the processing plant the dried weed is placed in cement bins, standing in a sunlit position, and is covered with cold soft water at a temperature below 10°C. The material is stirred frequently, in order to free it from stones and shells, and to give it the maximum exposure to the sunlight, which completes the bleaching. This preliminary soaking continues from twelve to twenty four hours, depending on the variety of the weed.

After soaking, the weed is washed and pounded with mallets to soften it prior to extraction. Washing machines have been employed, but do not provide sufficient mechanical crushing for the harder weeds. Extraction is then carried out in wooden tanks heated by wood fires. The water is allowed to boil before the weeds are added and the hard weeds are the first to be extracted. When boiling is resumed, after the addition of the hard weeds, sulphuric acid is added to help break down the cell walls and to bring the pH to the region of 6.3. During the subsequent simmering the softer weeds are added, and the whole material is extracted for twelve hours. The plant is run on a "feed-back" principle and dilute liquors from a previous batch/

batch are added to each boiling. Prior to the last war, it was customary to add two pounds of sodium dioxide to the tanks to assist the bleaching. This was usually added shortly before the extraction was completed.

After extraction the weed is allowed to settle in the tanks and the mother liquor is dipped off and strained through 20 mesh cloth filters into sloping collection tanks. Here it is again allowed to settle, so that impurities sink to the bottom and the clear jelly can be drawn off into dehydration trays. These are stacked and left in the fields for two or three weeks to freeze and thaw alternately, until the moisture content has been reduced to 22%, and most of the impurities have been drained away. It is then sorted, tested, and graded for home consumption, or export. The yield is about 23% of the dried weight of seaweed. Modernisation of the Japanese industry has been attempted, but in view of the supply of plentiful cheap labour in Japan, it was not found to be an economic proposition.

2. Industrial Processing in the United States.

In the U.S.A., however, mechanization of the agar extraction processes was inevitable, since climatic conditions in California, where the first industry in the U.S. was started, were not conducive to the natural field dehydration of the jelly.

Thus the U.S. industry is now equipped with pressure steam digestors, large filter presses, shearing machines, artificial refrigeration tanks, rotary ice crushers and filters, special hot-air dehydrators and all the paraphernalia of a modern/

modern factory. Yet the basic item - the raw material - is much less carefully chosen than the heterogeneous, but balanced mixtures of Japan. To quote from an article by Robertson on the "agar Industry in California"[18] "The agar industrialists are at a loss to identify some of the moss offered for sale. It is likely that there are varieties quite unknown in Japan".

PHYSICAL AND CHEMICAL PROPERTIES.

It is now desirable to examine some of the physical and chemical properties of this indiscriminate extract.

In the discussion of the uses and manufacture of agar many of its physical properties have been mentioned. The most important of these, gelation, hysteresis, and syneresis, have aroused considerable interest among physical chemists, and have been the subject of much research.

Gelation.

Krishnamurti[19] believes that agar is a lyophilic colloid which is capable of dispersion in water to give negatively charged micelles, or particles, each of which is an aggregation of agar molecules. Between these he supposes to exist a true solution of agar in water. Each of the micelles is surrounded by a hydration layer of water molecules held by electrostatic attraction. He suggests, that when an agar sol is cooled the true solution becomes supersaturated, and the agar molecules condense on the outside of the hydrated micelles. The aggregation of these complex structures is thought to constitute the gel. Bungenberg de Jong[20] believes that gelation occurs by the direct aggregation of the micelles, and subsequent redistribution of/

of charge.

Some evidence supporting Krishnamurti's theory has been provided by studies of the Tyndall effect in agar gels by Hatschek,[21] and by the work of Lipatov and Morozov.[23] The latter workers claim to have separated agar into a soluble or "poorly associated" and an insoluble or "highly associated" fraction and to have shown that the former exercises a stabilizing effect on the latter during dehydration and gelation. Gelation and the swelling are stated to reside in the insoluble fraction.

Takahashi and Shirahama[27] found that the treatment of agar in an autoclave at 130°C gave two distinct degradation products - "hydrate kanten δ" which formed a gel with water and "hydrato kanten λ" which was soluble in water. Percival and Thomson repeated this work but could find no significant difference between the fractions after acetylation and methylation. Additional degradation may however have occurred during this process.

(b) Hysteresis.

Some work has been done on the hysteresis of agar but the results admit of considerable variation in behaviour depending on time, concentration and previous history. Banerji and Ghosh[23] found that it was possible to completely eliminate the hysteresis effect if several days were allowed to elapse between melting and setting of agar. Ghosh[24] could find no regular correlation between concentration and hysteresis in a given time interval, but showed that the phenomena could be considerably reduced by added electrolytes. This is to be expected in a lyophilic colloid.

(c)/

(c) Syneresis.

Syneresis is considered to be due to a decrease in surface area of the internal phase, the cause of which is not known. It usually is accompanied by a transfer of salts from the gel to the syneresis liquid.[25] This fact is utilized in the freeze-thaw technique for the purification of agar.

(d) Viscosity.

Many viscosity measurements have been made on agar and its derivatives, but these figures show a wide diversity, and obviously depend upon the previous history of the specimen under investigation.

Historical Survey of Agar Chemistry.

With a few notable exceptions, all the workers who have investigated the chemical composition of agar, have utilized the commercial material, which, as we have seen, is of a very heterogenecus nature. Consequently, it is not surprising that the reported constituents of the material have become ridiculously numerous, and although recently much excellent work of a more discriminating nature has been carried out, a satisfactory solution to the structural problem has not yet been offered. It is, however, of interest to review the advances made in this study, which has excited the interest of chemists since 1859.

Early Work.

In that year Payen showed that agar contained a structural principle of anhydro galactose units, to which he assigned the empirical formula $C_6H_{10}O_5$. He called this substance "gelose", and believed that it was similar to pectin/

pectin, and gave to agar its characteristic gel-forming properties. Anderson has classified agar as an "acid" hemicellulose, which fills up the cellular tissue in the algae, and is thus similar to pectin in its physiological function. He has, however, been careful to note that some "acid" hemicelluloses give only sugars on hydrolysis, so that the fact that agar has not been proved to contain a uronic acid does not preclude it from this classification.

Reichardt[30] was the next recorded worker. He identified the carbohydrate principle as "parabin" - a substance which had already been found in carrots and beetroots. This material, he believed, contained pentoses and methyl pentoses, and had the empirical formula $C_{12}H_{22}O_{11}$.

The empirical formula of Payen was supported by Bauer,[51] who identified the carbohydrate residue as a galactan already obtained from lucerne seeds and certain non-starchy plants, and for which the formula $C_6H_{10}O_5$ had been proposed.

Greenish[38] obtained no less than seven carbohydrates, including glucose, arabinose and xylose, by various treatments of agar. He found that the original material was non-reducing to Fehling's solution, and that the hydrolysate was not fermentable by yeasts. He favoured the formula $4.C_6H_{10}O_5.H_2O$.

In 1880 Morrin[31] treated "gelose" with dilute nitric acid and detected oxalic and mucic acids in the hydrolysis products.

This was the basis of the estimation of 33% galactose in agar, which was reported by König and Bettels[39] in 1895. This figure was later(1929) substantiated by Ludtke[40] (30-40%). In addition to the mucic acid method he also estimated/

estimated the galactose as the methylphenylhydrazone.

The multiplicity of sugars suggested by Greenish and Reichardt received ample support from later workers. In 1900 Seber[41] confirmed Reichardt's pentoses, and in 1916 Takao[42] and Matsui[42] again reported pentose sugars, and showed the presence of a ketose in the molecule. A uronic acid was stated to be present by Furuichi[40] in 1927, but this was later denied by Ludtke, 1929, and by Takahashi and Shirahama[44]. Ludtke[40] had reported a ketose and laevulinic acid among the hydrolysis products of agar.

Meanwhile, Leroide and Tassidy,[32] in 1906, had opened up a new field in the search for the constituents of agar, by reporting the presence of traces of arsenic in the material. Fellers,[33] in 1916, no doubt correctly ascribed this to the presence of arsenic in the sulphur dioxide used for bleaching, but the element hunt had already won more workers.

In 1913, Forbes, Beagle and Mensching, had further complicated the position, by reporting the detection of sulphur, calcium, magnesium, sodium, potassium, chlorine and phosphorus in agar. Later workers added boric acid,[36] zinc,[35] iodine,[37] iron, aluminium, manganese, phosphoric acid and carbonate[45] to the list of impurities, or constituents, in the agar molecule.

Research on the Chemical Significance of the Agar Gel.

It is clear from the above review that, up to the second decade of this century, the constitution of agar-agar had appeared to be of the utmost complexity, and each piece of fresh evidence had only served to complicate the position. The/

The most characteristic and economically important feature of agar - its gelling power - was the subject of several of the early theories on its structure.[46,47,48,49,50] The first attempts at some comprehensive correlation of the evidence were made in 1921, by Samec and Ssigevic,[46] and Neuberg and Ohle,[47] who, working independently, put forward the following theories. Neuberg and Ohle showed that agar contained organically bound sulphur, since hydrogen sulphide was evolved by bacterial action, and sulphuric acid was set free on hydrolysis. Samec and Ssijevic put forward the consistent theory that agar-agar was a sulphuric ester of gelose, just as amylopectin was a phosphoric ester of the amyloses, and that the high viscosity of agar was due to the relatively high concentration of the sulphate ion. They later showed[48] that the chief electrolyte in agar ash was sodium sulphate. This could not be removed by electrodialysis from the untreated agar. On autolysing agar the sulphuric acid was dialysable but the solution would no longer gel, so that a causal connection between the sulphur content and the gelling power of agar was deduced.

This deduction was questioned a year later by Fairbrother and Mastin,[49] who showed that agar breaks down in the presence of even dilute acids, and that almost all the sulphur is liberated as sulphuric acid during this hydrolysis. They analysed agar ash and found in it calcium sulphate, magnesium sulphate and silica, as well as traces of other salts. They showed that the calcium was replaceable by other metals, and claimed that by heating agar alternately with/

with dilute hydrochloric acid and with water they had produced an almost ash free material which did not gelatinize after reheating and cooling. On the basis of these studies they suggested that agar was the calcium salt of an acid sulphuric ester, and that the ash-free material was the free agar acid, which underwent autohydrolysis on heating, so that the resultant material was no longer capable of forming a gel. Hoffman and Gortner[50] found that the calcium could be removed by electrodialysis, and that subsequent neutralisation of the free agar-acid resulted in the formation of a firm gel. They stated that the setting of agar was the gelation of a salt, not that of a complex polysaccharide.

However, during this time, evidence was appearing that agar consisted of two polysaccharides. In 1912 Cran[52] working with bacterial cultures, showed that one portion of agar, which was used as a nutrient by bacteria, produced a violet colour with iodine, whilst the remainder gave no colour reaction with the same reagent.

As mentioned above, Takahashi and Shirahama[53] showed that on treating agar with water in an autoclave at 130°C two distinct degradation products were obtained. One of these, "hydrato-kanten δ", formed a gel with water, contained 0.3% sulphuric and no uronic acid. "Hydrato-kanten λ" was water soluble, contained 2.1% sulphur and a small quantity of a pentose. Both fractions were said to contain 32-39% of a carbohydrate. On the basis of these experiments they suggested, for gelose, the formula $H_1 - H_2 - (O.SO_2.O)M$ where M denotes metals, chiefly calcium, and they believed that the linkage of the two carbohydrate residues, H_1 and H_2, was the main factor in the gelling of the material.

Three/

Three years later Neuberg and Schwietzer[54] reported a similar separation of agar, by simple extraction with water at room temperature. They obtained a soluble portion which made up 10% of the total polysaccharide, and contained most of the sulphate residues (5% sulphur) of the original material. The insoluble portion (90%) was almost sulphur-free and behaved similarly to the untreated material.

Most of the following work was carried out on washed industrial agar, which was presumed to consist mainly of the insoluble portion of Neuberg and Schwietzer.

Recent Work on Chemical Structure.

Pirie[55] in 1936, was the first worker to make a serious study of the chemical as opposed to the physical structure of agar. Previous workers had held the mechanism of the agar gel as the primary motive of their investigations. Pirie attempted to degrade agar by chemical means, in order to investigate its organic constituents more carefully. He experienced great difficulty in his attempts to acetylate the material, but succeeded in acetolysing it with acetic anhydride and sulphuric acid. From this experiment he obtained crystals of hepta-acetyl-D,L-galactose, from which he assumed ~~showing~~ that a portion (12%) of the galactose in agar existed in the L - form. He found, however, that if he first hydrolysed the agar, and then acetylated, he could only get crystals of the penta-acetyl-D,L-galactose. He explained this result by postulating that, in agar, the D.L. galactose occurred as the aldehydic or straight chain modification, and not in the usual pyranose ring form. He confirmed the presence

of/

of L - galactose in the agar molecule by feeding galactose-trained yeasts on the hydrolysis products of agar, but his yields were low and unsatisfactory (0.8%).

In the same year, Percival and Sim,[55] working in Edinburgh, had succeeded in acetylating agar with acetic anhydride and pyridine, and had obtained a chloroform soluble product (OAc 39%), which contained no sulphur. By treatment of this material with caustic soda and dimethyl sulphate simultaneous deacetylation and methylation was effected, and a chloroform soluble material (OMe 32%) was obtained.[57],[58] This was hydrolysed with 6% sulphuric acid, and methanolysed with methyl alcoholic hydrogen chloride to yield a syrup of carbohydrate glycosides. This syrup was fractionally distilled under reduced pressure to give three fractions; firstly, an optically inactive mobile ester (16%) which was recognised as methyl/laevulinate, secondly a crystalline trimethyl methyl galactoside (65%) which was proved to be the 2:4:6-trimethyl derivative by oxidation methods, and by comparison with an authentic sample synthesised by D.J. Bell,[59] and finally a syrup (14%) which appeared from its reactions to be a ketose derivative. From this evidence Percival and Somerville suggested that the main unit of agar was comprised of D-galactopyranose units linked by positions 1 and 3, at that time a unique linkage in a natural product. From the strongly negative rotations of acetylated and methylated agar, they deduced that β-linkages must predominate in the structure. The stereochemistry of such an arrangement necessitated ascribing to agar a structure comprising a zig-zag chain, or a closed loop of six units.

The unidentified syrup from the above experiments was further/

further examined by Forbes and Percival.[60][61] After methylation, they were able to show that it was a 2:4-dimethyl-3:6-anhydro-α-methyl galactoside, and by comparison of the physical constants of their material and a synthetic sample of 2:4-dimethyl-3:6-anhydro α-methyl D-galactoside, they showed that the material obtained from agar was the enantiomorphous L-derivative. The low acetyl and methoxyl contents of agar acetate and methylated agar suggested that the anhydro-L-galactose was preformed in agar, and since further methylation was required to produce the dimethyl derivative, it was adjudged to be linked by at least two points in the molecule.

Simultaneously with the above-mentioned experiments of Forbes and Percival in Edinburgh, Hands and Peat in Birmingham had been investigating the syrupy residue from methylated agar hydrolysates, and had independently reached the same conclusion as to its identity.[62] Thus it would seem to be firmly established that, after methylation, a 3:6-anhydro-L-galactose derivative could be isolated from agar. Whether this was preformed in the original molecule, produced during methylation, or elaborated by the plant itself during growth, from an originally simpler constituent, remained undecided.

During a series of interesting experiments in 1941, Duff and Percival[63] showed that a 3:6-anhydro ring could be formed by alkaline treatment of methyl galactoside-6-sulphate, and this led to new speculations concerning the formation of an anhydro sugar in the agar molecule, which, in its native state, was known to contain a small proportion of sulphur.

When/

When the presence of an anhydro-L-galactose derivative in agar was first detected, it was held to form consistent evidence with Pirie's isolation of hepta acetyl-D,L-galactose from the material. However, in 1942, Percival and Cottrell[64] showed that the acetolysis of 3:6-anhydro-β-methyl-D-galactopyranoside gave the same D,L-galactose derivative, and that the racemisation was due to a rearrangement in the special symmetry of the galactose series.

An extensive investigation of the properties of these 3:6-anhydro sugars was carried out by Haworth, Jackson and Smith,[65] who found that, owing to the stereochemistry of the system, the 3:6-anhydro-ring was more resistant to attack than the pyranose sugar ring, which could be opened quite easily to give the free aldehydo-form of the 3:6-anhydro-galactose. This evidence, together with that concerning the formation of anhydro-rings from galactose sulphates, was utilized in 1942 by Jones and Peat,[66] in their attempt to formulate a definite structure for the agar molecule.

These workers[66] isolated 2:5-dimethyl-3:6-anhydro-L-galactonic acid from methylated agar, which had been dialysed in acid solution, remethylated, and hydrolysed. They suggested that this was produced by atmospheric oxidation of the anhydro sugar, in its aldehydo-form. At the same time Percival and Thomson also isolated this acid, by the acetolysis of methylated agar followed by oxidation, methylation and hydrolysis of the mixture of disaccharide esters produced. This offered certain proof that the 3:6-anhydro L-galactose residues were linked through $C_{(4)}$ to the main carbon chain.

During/

During these investigations both pairs of workers sought for a non-reducing end group of tetramethyl galactose, but were unable to detect any significant quantity of this sugar.

As a result of this study,[66] Jones and Peat proposed for agar a structure made up of repeating units of nine D-galactopyranose units, linked in a chain through positions one and three, terminated by an L-galactopyranose residue linked through $C_{(4)}$ and carrying a sulphate group on C_6. This L-galactose residue was envisaged as the precursor of the 3:6-anhydride.

Various objections have been made to this simplified structure. These have been mainly based on the estimated sulphur content of natural agar, which is far too low to account for the 1.8% sulphur required by the Jones-Peat structure. Barry and Dillon[67] found only 0.36% sulphur in Gelidium latifolium agar, and Percival[68] recorded figures of (0.43 & 0.47%) sulphur for agars from Gracilaria confervoides, and Gelidium crinale. Barry and Dillon also sought to prove that the anhydro-galactose was not an artefact but a constituent of the agar molecule, by demonstrating that the original polysaccaride was invulnerable to attack by periodic acid. However, Percival has pointed out[68] that there is no evidence that the sulphate groups remaining in agar after isolation, are situated on C_6 of the L-galactose residue. They could equally well be situated on C_3, and still hydrolyse to yield an anhydride, whilst providing no α-glycol grouping to react with the periodate.

Thus the precise significance of the anhydro-galactose residue/

residue and the relationship, if any, between it and the sulphur content of the agar molecule, have not been satisfactorily settled.

Original Aims of the Present Work.

The present investigation was undertaken with the intention of using a purified extract of a single species of an agar-bearing weed, in contrast to the earlier work on the commercial product, which has been shown to be of very variable composition. By using the modern technique of chromatography it was hoped to obtain a more accurate separation and estimation of the expected anhydro sugar constituent, and to thus determine more clearly its relationship to the structure of the polysaccharide, both before and after methylation.

DISCUSSION OF EXPERIMENTAL WORK.

I. Work on the Unmethylated Extract.

The material used in these investigations was prepared from specimens of washed, bleached Gelidium latifolium, by aqueous extraction without the addition of acids or bleaching agents. Thus it may be assumed that the polysaccharides obtained are not artefacts but constituents of a unique cell-wall material.

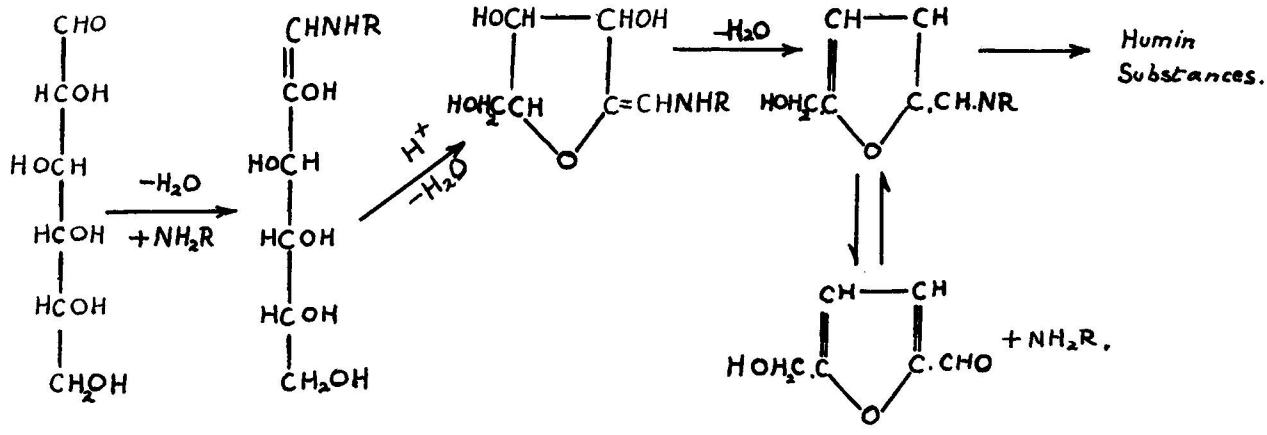
Estimation of Sulphur and Nitrogen in the Extract.

Sulphur determinations on samples of the extract indicated that, although the crude material often contained 1% of sulphur, it was possible by prolonged dialysis to reduce this figure to a minimum of 0.36% sulphur, for a representative sample. Attempts were made to remove this residual sulphur, by treatment with sodium hydroxide, under methylation conditions, but these were not successful. Since the fully methylated material does not contain sulphur it must be presumed that one treatment with alkali is insufficient to remove this residual percentage.

Tseng,[1] speaking of agar, defines it as "the dried amorphous gelatine-like non-nitrogenous extract from Gelidium and other red algae....." However, nitrogen estimations on the extract from Gelidium latifolium, showed a constant proportion of this element, which, in terms of protein, amounted to over 5% of the original material.

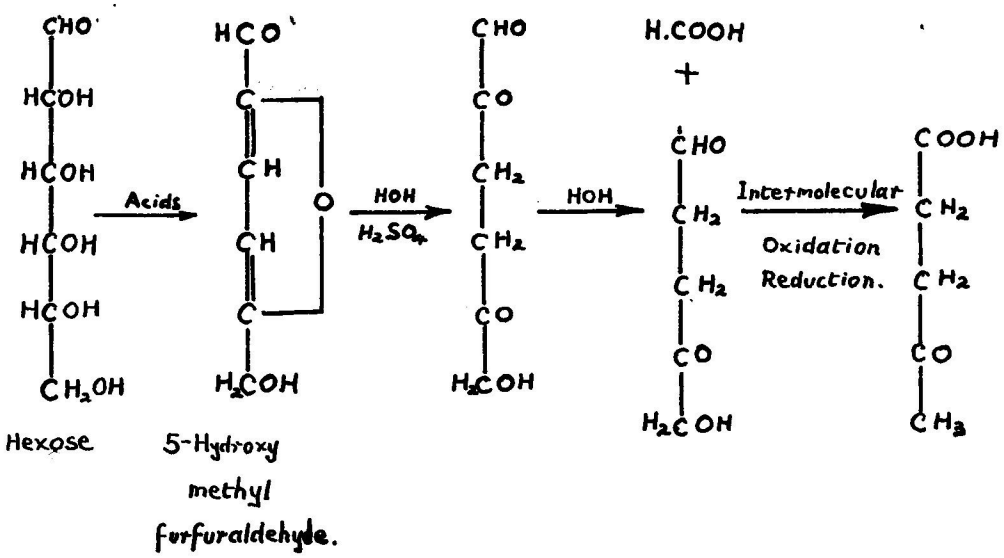
In an article in "Advances in Carbohydrate Chemistry", Stacey[2] discusses the properties of a class of "muco polysaccharides containing up to 1% nitrogen which is not present/

present as an amino sugar constituent." He considers that the nitrogenous constituent is a "vestigial remnant", a protein, in combination with the polysaccharide, possibly originating from polysaccharide synthesizing enzymes. The physical characteristics of this class of material are interesting. Their aqueous solutions show a very high viscosity and they tend to become insoluble in water after intensive drying. Saponification treatment is usually required to remove the protein before a normal solution is obtained. Partridge,[3] ("Nature" April 1950), suggests that galactose, or any sugar, may form an N-glycoside with amino acids, leading to a brown or yellow colour on heating with alkali (Sugar/Protein Browning Effect).[18][19][20][21] These N-glycosides yield 5 hydroxy-methyl-furfuraldehyde on heating with dilute mineral acid. They also give a colour reaction with dimethyl amino benzaldehyde.



With these points in mind the algal extract was submitted to various tests. Its physical properties, of very high viscosity, and tendency to insolubility after dehydration, seemed consistent with those of typical muco-polysaccharides/

polysaccharides. On warming the polysaccharide gel with 30% sodium hydroxide, a deep yellow colour developed immediately, and the gel appeared to be broken. It gave the characteristic purple colour with para-dimethyl-amino benzaldehyde. 5-hydroxy-methyl-furfuraldehyde was isolated from the polysaccharide hydrolysate by chloroform extraction, and was identified by comparison of its properties with those of a synthetic specimen, and by preparation of the naphthylamine derivative. Although it is possible to produce 5-hydroxy-methyl-furfuraldehyde from hexoses, by treatment with dilute mineral acid, it is unlikely that this mechanism was responsible for the production from the algal polysaccharide, since specimens of pure hexoses, hydrolysed under identical conditions, produced no 5-hydroxy-methyl-furfuraldehyde. Laevulinic acid has been reported among the hydrolysis products of commercial agar (Ludtke[4] Percival and Somerville[3]). It is possible that this is produced from 5-hydroxy-methyl-furfuraldehyde (Pummerer and Gump[6][7]).



It is interesting to note that L-galactose which has now been shown to occur in the algal polysaccharide (see below) also occurs naturally in a mucopolysaccharide of animal origin:- the ~~mucin~~ extract of Helix pomatia, from which the free polysaccharide is isolated with 30% potassium hydroxide (Bell).[6]

Attempts to hydrolyse the protein present and analyse the amino acids by chromatography were not successful. The only amino acid with an R_G value corresponding to that found (0.62) is tryptophane, but this acid is normally destroyed by mineral acid and the syrup failed to react to the tryptophane-aldehyde test with formalin, sulphuric acid and mercuric sulphate.

Chromatographic Search for the Anhydro Sugar in the Extract

Since much of the recent controversy on the structure of the marine polysaccharide "agar" has centred on the subject of the preformation of 3:6-anhydro-galactose,[7][8][9] it seemed of considerable interest to attempt to identify this sugar among the hydrolysis products of the unmethylated polysaccharide, by paper chromatography. This, however, led to unexpected complications. It was found that although anhydro sugars of the hydrofuranol type, such as 3:6 anhydro glucose, gave clearly defined spots on a paper chromatogram, solutions of 3:6-anhydro-galactose produced an indefinite streak extending almost two-thirds of the length of the solvent flow. Intensive purification from salts by pyridine extraction,[14] and with the aid of ion exchange resins, did little to localise the sugar on the chromatogram.

Haworth/

Haworth, Smith, and co-workers[10][11] have investigated the stability of the dicyclic systems in anhydro sugars of the hydrofuranol type, and they conclude that the hydrofuranol ring assumes the character of the principal ring, to which the sugar ring (pyranose or furanose) is subsidiary. Peat[12] summarizes their conclusions as follows:-

"The combination of a 3:6-anhydro ring and a furanose ring in glucose or mannose represents a stable, strainless structure; in galactose, however, the establishment of the two rings is sterically prevented. Where it is not possible for the two five membered rings to exist together, as in galactose, or 4-methyl glucose, an alternative strainless form is the open chain aldehydo-form."

In the light of these facts it was thought possible that the 3:6-anhydro-galactose, if it pre-existed in agar, was oxidised to the 3:6-anhydro-galactonic acid during hydrolysis and chromatography. On this assumption chromatograms were run in the solvent recommended by Lugg and Overell[13] for sugar acids, but little localisation of the streak was obtained. A pure sample of the 3:6-anhydro-galactonic acid was prepared and this was run in an acidic solvent with the agar hydrolysate; on development with bromophenol blue both specimens gave disperse yellow streaks. Thus it appears to be very difficult to identify 3:6-anhydro-galactose by paper chromatography under the usual conditions.

Existence of L-galactose in the Extract.

It has been proposed by Jones and Peat[15] that L-galactose exists in the structure of unmethylated commercial "agar"/

"agar" as a sulphate ester linked through C_4. It has been previously noted that in the purified polysaccharide isolated from Gelidium latifolium there is insufficient sulphur to account for this, but it was decided to investigate the possibility of the existence of L-galactose among the hydrolysis products of this polysaccharide.

A proportion of the total galactose present in the polysaccharide hydrolysate was isolated as the methyl-phenyl-hydrazone, and from this material the penta-acetyl derivative was prepared. This compared exactly with a synthetic specimen prepared from pure D-galactose in all respects save rotation. The rotation of the material isolated from the polysaccharide hydrolysate indicated the presence of 8.5% of the L-derivative. This point is of considerable interest since L-galactose has also been detected in the hydrolysis products of other carbohydrate extracts from the red algae.[16]

Methoxyl Estimations in the Extract.

During the isolation of 5-hydroxy-methyl furfuraldehyde attention was drawn to the persistent occurrence of spots of high R_G value on the developed chromatograms. This observation initiated an investigation of the possible methoxyl content of the unmethylated polysaccharide. These methoxyl estimations were carried out very carefully with rigid controls and there is little possibility that they were erroneous. However, no explanation can yet be offered for the determined methoxyl content of 1.5% of the unmethylated, purified, polysaccharide.

Fractionation/

Fractionation of the Extract.

Dr. F. Isherwood, of the Low Temperature Research Station, Cambridge, has developed an interesting theory concerning the polysaccharides of plant cell walls.[17] He suggests that each polysaccharide contains only one sugar (not including aldobionic acids) and that if arbitrary fractions of the polysaccharide material from a plant cell wall are selected and hydrolysed, then each sugar in the hydrolysis products represents only one polysaccharide, unless there is evidence to the contrary; for example, in pear cell wall both cellulose and starch give glucose in the hydrolysate.

This theory served as a useful working hypothesis for experiments using the Tiselius electrophoresis technique in separating the polysaccharide constituents of arbitrary fractions extracted from pear cell wall material. It clearly cannot be applied to plant gums since Hirst, et alia, have shown that this class of substance usually contains several sugars.

Since galactose, glucose and xylose occur among the hydrolysis products of the algal polysaccharide, on this theory three separate polysaccharides might be supposed to exist in the extract, and it was considered of interest to attempt to separate these postulated constituents.

Partial fractionation was achieved by continuous repeated extraction of the algae with hot water. Examination of three fractions produced in this way indicated considerable variation in the proportions of the constituent sugars, which appeared/

appeared to indicate the existence of at least three polysaccharides. Further evidence in support of this view was obtained from an examination of methylated agar. Although xylose occurs in the unmethylated material it does not appear among the hydrolysis products after methylation. Glucose, however, does appear as methylated derivatives, but the galactan, which appears to be the primary polysaccharide component could be freed from the glucosan by fractional precipitation of the methylated material.

In support of this assumption of the primary importance of the galactan, it is interesting to note that galactans are very common as constituents of the species of red algae which have been examined up to the present time. They occur in all varieties of Gelidium, and in all other species such as Pterocladia and Gracelaria which have been used in the production of commercial agar. Dillon[23] found that the chief constituent of Dilsea edulis and of Dumontia incrassata was a galactan sulphuric ester, and investigation on extracts of Chondrus crispus and Gigartina stellata also led to the identification of a galactan sulphuric ester. According to Hassid,[24] the polysaccharide from Iridea laminariodes contains a galactan sulphate and work now in progress at Edinburgh on extracts of Polysyphonia fastigiata indicates that once again galactose and sulphur play an important part in the polysaccharide structure.

In contrast to this unanimity of opinion concerning the basic importance of galactan sulphuric esters in these structures, little mention has previously been made of the presence/

presence of significant quantities of xylose and glucose among the hydrolysis products from these sources. Yet chromatographic evidence indicates their presence in most of the "agar producing" algae, and in the case of the weed under investigation, Gelidium latifolium, derivatives of glucose have been isolated and identified from the methylated extract.

It was hoped to achieve qualitative fractionation of the unmethylated polysaccharides by means of the Tiselius electrophoresis apparatus, and during a visit to the Low Temperature Research Station at Cambridge, an attempt was made to examine agar electrophoretically. However, owing to the mechanical difficulties of dealing with a polysaccharide with strong gelling tendencies, little satisfactory information was gained on the homogeneity of the material by this method.

Periodate reactions of the Extract.

Barry and Dillon[22] studied the action of sodium para periodate on "agar", and by measurement of the periodate uptake they came to the conclusion that no terminal groups containing the α-glycol grouping existed in agar, and that the 3:6-anhydro sugar was therefore probably preformed. These periodate experiments were repeated with the intention of estimating any formic acid liberated. Using similar quantities to those employed by the Irish workers, the findings of Barry and Dillon were confirmed, since the solution remained neutral during the twelve days of the experiment, and attempts to assess any non-reducing end groups by production of formaldehyde in this solution were also of no avail. However, in the light of later experiments indicating the presence of one per cent of end group/

group, the weight of agar used was increased ten-fold and some reaction was obtained after 24 hours shaking. During a further 36 hours agitation, the solution was found to be neutral and remained neutral during the remaining seven days of the experiment. A calculation based on the only obtainable reading indicated an end group of 0.87%, which is in fair accord with the value of 1% obtained from fractional extraction experiments, but in view of the unusual behaviour of the polysaccharide under these conditions, this figure should not be regarded as entirely reliable.

Chromatograph Search for Anhydro-Galactose.

Since the presence of derivatives of 3:6-anhydro-galactose has been definitely established among the hydrolysis products of methylated commercial agar, it was of some importance to verify whether 3:6-anhydro-galactose could be detected chromatographically among the hydrolysis products of extracts from other algae used in the production of commercial agar. Although many algae from various sources were examined, no clear indication of the presence of 3:6-anhydro-galactose was obtained. Several of them contained the streak-producing constituent previously mentioned, and the presence of galactose, glucose and xylose among the hydrolysis products, was almost universal.

II. WORK ON THE METHYLATED EXTRACT.

The polysaccharide was methylated by the standard techniques of Haworth and of Purdie and Irvine, and a methoxyl content of 38% was obtained for a representative sample. This figure is very low for a galactan of straight chain configuration, and lends support to the postulated existence of a 3:6-anhydro-residue in the molecule. The methylated material was methanolysed, hydrolysed and the mixture of partially methylated sugars thus obtained was separated by partition chromatography on a cellulose column.

Partition Chromatography of Methylated Sugars.

The results of the first of these column separations indicated the existence of 8% of tetra-methyl galactose end group in the methylated polysaccharide. This evidence was not supported by periodate experiments on the unmethylated material, and was contrary to the findings of other workers on commercial agar. Two later experiments in which the column separation was repeated, failed to reveal any of the 2:3:4:6-tetra-methyl galactose, so it must be concluded that some degradation occurred during the methylation of this first quantity of material, and that the end-group found was therefore an artefact and not part of the original polysaccharide structure.

The quantitative estimation of the 2:4:6-trimethyl galactose was complicated by the presence of 2:3:6-trimethyl glucose, from which it was not possible to separate the galactose derivative under the conditions employed. The dimethyl/

dimethyl galactose derivatives present suffered from similar contaminations with dimethyl glucose. Since separation of the mixtures of sugars after hydrolysis was impractical, further attention was given to the possibility of removing the glucose containing material from the methylated polysaccharide by physical methods. It was found possible to obtain a glucose free material by repeated fractional precipitation of the methylated polysaccharide, and by additional methylation the methoxyl content of this fraction was raised to 40.1%. On hydrolysis it appeared to consist entirely of 2:4:6-trimethyl galactose units, but a structure on these lines would require a methoxyl of 44%. It was therefore decided to repeat the technique of previous workers on a small sample of the material, by methanolysing it and attempting to isolate an anhydro sugar by fractional distillation in high vacuum. On distillation only 2:4:6-trimethyl-methyl galactoside was identified in a yield corresponding to 72% of the original material. The residue appeared to be at least partially decomposed into 5-hydroxy-methyl furfuraldehyde. In order to avoid the production of this substance, a further attempt to isolate an anhydro sugar from the methanolysis products was made on a larger scale by fractionally extracting an aqueous solution of the methanolysed polysaccharide with petroleum ether of two distinct boiling point ranges. Again no anhydro sugar was detected, but the interesting observation was made of the existence of a proportion of fully methylated galactose in the first of these extracts. This extract was therefore hydrolysed/

hydrolysed and examined by quantitative chromatography. The proportion of tetra-methyl galactose was determined as 21-23% in this fraction, giving a figure of 1% for the polysaccharide as a whole. This may be compared with a figure of 0.87% obtained from periodate experiments on the unmethylated material. Estimations of trimethyl galactose based on fractional extraction followed by fractional distillation gave a figure of 68% as compared with 72% obtained from the simple distillation experiments. In both these experiments, a non-distilling residue remained which has not been fully investigated, so that absolute figures for the proportions of constituent sugars cannot be given, but it appears extremely unlikely that this particular agar from Gelidium latifolium contains any of the anhydro sugar found in the commercial products.

On the present evidence it is impossible to postulate any completely substantiated structure for the polysaccharide from Gelidium latifolium, but it seems likely that it is a polymer of D and L-galactose units in the proportion of 10:1 linked through carbon atoms one and three. This is similar to the structure proposed for commercial agar in 1942 by Jones and Peat.[15] However, the sulphate content of this polysaccharide is too low to allow of one sulphate residue on each L-galactose unit as proposed in the Jones-Peat agar structure.

It is also suggested that the polysaccharide contains about 1% of end group giving a chain length of 100 units and molecular weight of about 16,000, which is in fair agreement with that calculated from the viscosity of methylated/

methylated agar (M_v = 15,500) and with the value of 16,000 calculated from the iodine number of the unsubstituted polysaccharide by Percival & Thomson.[9]

The author wishes to express her sincere thanks to the many people who directly or indirectly have assisted and directed the course of this work.

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The assistance of the Marine Biological Station, Millport, and the Staff of University College, Galway, in the collection of the algae, was of considerable importance, and the advice of members of the Institute for Seaweed Research at Musselburgh was always welcomed.

The supervision of Professor Hirst has been a source of inspiration and encouragement, but to the late Dr. E.G.V. Percival must fall the main proportion of gratitude. His unfailing interest, tolerant/

tolerant understanding, frequent advice and buoyant humour over the depressing stages from which all research must occasionally suffer, made this investigation more than just prosaic research, rather, a stimulating exploration into the intricacies of a natural product.

EXPERIMENTAL.

Collection and Preparation of the Algae.

Specimens of Gelidium corneum (var. latifolium) were obtained through the Marine Biological Station at Millport. A second sample of the same algae was collected by the author near Mutton Island, Galway Bay, Eire. Thanks are due to Miss Smith, of the Institute for Seaweed Research, Musselburgh, and to Miss De Valera, of University College, Galway, for their assistance in the identification of these specimens.

The weeds were well washed to remove sand and shells, and then carefully sorted to ensure that the samples did not contain fronds of other algae such as Chondrus crispus or Corallina sp. The washed weed was sun-bleached for ten days before extraction. Samples of the washed, bleached weed were analysed for ash and sulphur content. Specimens were analysed again for sulphur after two weeks dialysis against running tap water.

Specimen I: 9.8% Ash, 1.4% Total Sulphur, 0.57% Sulphur after dialysis.

Specimen II: 9.2% Ash, 1.3% Total Sulphur, 0.54% Sulphur after dialysis.

Extraction and Purification of the Polysaccharide.

The prepared weed (40gm.) was softened by soaking in cold water for 4 hours. The weed was then boiled vigorously in tap water (300ml.) for two hours, and allowed to simmer on the electric hot-plate for a further 16 hours, as recommended in the Japanese agar industry.[1] The solution was cooled slightly and pressed through muslin into a large crystallising/

crystallising dish, which was immersed in an ice/salt freezing mixture overnight. The following day the frozen gel was allowed to thaw on wire gauze. This material proved unsatisfactory since the metal discoloured the product, and in subsequent extractions muslin was substituted in its place. It was also found, during a series of experimental extractions, that syneresis was most complete if the freezing temperature was less than -5°C, and the frozen gel was partially crushed before thawing. Drying temperatures of over 20°C turned the polysaccharide brown and resulted in a rather horny material, which was difficult to redissolve. After several preliminary experiments it was found possible to produce a colourless, transparent material by the above technique, in a yield of 32% (dry weight) of the dry weight of weed extracted. Moisture, ash and sulphur estimations were carried out on representative samples.

Found:- Sample I: Moisture 5%, Ash 4.7%, Sulphur 0.5%.
Sample II: Moisture 4%, Ash 4.1%, Sulphur 0.51%.

Moisture contents were obtained by drying to constant weight at 60°C, in vacuo, over phosphorous pentoxide. Ash contents were determined by incineration in a platinum crucible. Sulphur estimations were carried out by heating with hydrochloric acid or by igniting the material with sodium carbonate and sodium peroxide in an iron crucible followed in both cases by precipitation as barium sulphate and weighing.

The syneresis liquors were evaporated to small volume (20ml.) under reduced pressure, and tested for polysaccharide by/

by the addition of absolute alcohol (60ml.). A small precipitate was obtained which proved to be of negligible weight (0.03gm.) on drying. The remaining liquors were analysed for sulphate.

Found:- Sample I: 0.47% Sulphur; Sample II: 0.49% Sulphur.

Purification of the Polysaccharide (Method II).

A more dilute solution (weed 20gm.) in water (500ml.) was used for this extraction, which was carried out exactly as before. After extraction the dilute liquors were filtered through glass wool directly into absolute alcohol (1000ml.) which was vigorously stirred. Attempts were made to coagulate the gelatinous product by high speed centri-:fuging, but these were unsuccessful. It was found necessary to add another 1000ml. of absolute alcohol to harden the precipitate, before filtration was possible. The product was a coarse white powder which was obtained in a yield of 25% (dry weight).

Found:- Moisture 43%, Ash 4.7%, Sulphur 0.87%.

The high sulphur appeared to be due to the lack of purification by syneresis. The comparatively low ash to sulphur ratio can be attributed to the finer filtering media. This purification method appeared to possess no advantages over the freeze-thaw technique, and was very extravagant in solvents.

Attempted Removal of Sulphur by Dialysis.

Samples of the polysaccharide (Sulphur content 0.50%) were dialysed, in cellophane bags, against running tap water, for 8 days, at a concentration of 2%. At this concentration/

concentration a gel was formed, which became increasingly weak, owing to dilution during dialysis. Some agar (10%) appeared to be lost during this process. The residual material, after drying gave the figures:-

Found:- Ash 4.0%, Sulphur 0.46%.

To eliminate the possibility that inorganic sulphate was mechanically held by the gel during dialysis, a similar process was carried out using ~~extremely~~ dilute solutions (0.4%), which did not form a gel. After ten days dialysis under these conditions, Ash 3.8%, Sulphur 0.32% was found.

Attempted Removal of Sulphur by Alkaline Treatment.

The polysaccharide (0.98gm.) was dispersed in water (46ml.) containing sodium hydroxide (13.8gm.). The solution was vigorously stirred, and gradually neutralised by the dropwise addition of concentrated nitric acid (16ml.) over a period of ten hours. These conditions imitated those of a methylation without introducing extra sulphur into the constituents. The residual polysaccharide was precipitated from solution by the addition of a large excess of alcohol (250ml.), filtered off, dried and redissolved in distilled water (400ml.) to give a non-gelling dilute solution. This precipitation technique was used to avoid colloidal material which might retain inorganic sulphur. The dilute solution was dialysed for five days against running distilled water, the solution was evaporated under reduced pressure, and the residue was analysed for sulphur. A sulphur figure of 0.34% was found, which is the same as that of the untreated polysaccharide after dialysis. Blank controls/

controls were run in conjunction with this experiment and with others of a similar nature which varied slightly in technique. No sulphur was detected in the reagents.

Protein Estimations on the Polysaccharide.

Protein was estimated by the Kjeldahl technique. A series of estimations was carried out in each case with control blanks. A typical set of results is shown below.

Sample	Alkali (0.0565N) For back titration (mls.)	% Nitrogen	% Protein
I (135.1mg.)	6.0	0.82	5.1
II(291.24mg.)	4.2	0.86	5.4
Blank I	7.4	-	-
Blank II	7.4	-	-

LUCO POLYSACCHARIDE TESTS.

(I) A sample of the polysaccharide was heated with 30% caustic soda solution for a few minutes. A deep yellow colouration was produced, and the solution appeared to have lost its characteristic gelling properties.

(II) A sample of the polysaccharide was subjected to intensive drying at 60°C, under reduced pressure. On attempting to redissolve this material in water considerable difficulty was experienced. For this reason all samples were normally used with a known moisture content, and were not completely dried, unless the experiment involved other anhydrous reagents.

(III) On warming a sample of the polysaccharide with para dimethylamino benzaldehyde, a pink colouration, was produced.

(IV) A sample of the polysaccharide, on treatment with Millon's reagent (mercurous nitrate and mercurous nitrite) gave/

gave a white precipitate which did not turn brick red on warming.

(V) The xanthoproteic test with concentrated nitric acid was carried out on the polysaccharide. A white precipitate was obtained which turned yellow, and finally orange on addition of ammonia.

Search for amino acids among the Polysaccharide Hydrolysis Products.

The dialysed polysaccharide (100mg.) was hydrolysed for 4 hours, at 95°C, in 2N sulphuric acid (10ml.). The solution was neutralised with barium carbonate and centrifuged to give a clear, supernatant liquid. Two samples of this liquid were run on a one dimensional paper chromatogram for 28 hours in a collidine/water solvent. On exposing the dried chromatogram to ultra-violet light two distinct fluorescent spots were observed. On development with ninhydrin only one clearly defined spot of Rg.0.62 and greenish-blue in colour was obtained. A test on a sample of the liquid used on these chromatograms showed no purple colouration with formalin.

Complete Hydrolysis of the Polysaccharide with Sulphuric Acid.

The dialysed polysaccharide (10mg.) was heated with N/2 sulphuric acid (0.5ml.) in a sealed tube at 85°C, for 4 hours. After neutralising the solution with barium carbonate it was centrifuged, and the supernatant liquid was applied to a chromatogram with controls of pure samples of galactose, glucose and xylose. On developing the paper with ammoniacal silver nitrate, distinct evidence of the presence/

presence of all three sugars was obtained. An indefinite streak, extending two-thirds of the length of the solvent front, was also observed. Another chromatogram of the polysaccharide hydrolysis products was run, with an additional control of 3:6-anhydro-D-galactose. A similar streak to that obtained from the hydrolysis products of the polysaccharide was observed from this control sugar.

Production of a Salt Free Sample of 3:6 Anhydro-D-Galactose.

3:6-anhydro-α-methyl-D galactoside (0.3165gm.) was hydrolysed at room temperature for 16 hours, with 0.1N sulphuric acid (30ml.). The solution was neutralised, filtered and the filtrate evaporated under reduced pressure to yield a pale yellow glass (0.2980gm.). This was redissolved in distilled water (10ml.) and passed consecutively through anion and cation resin exchange columns. The solvent was removed under reduced pressure, and the colourless glass thus obtained was placed on a paper chromatogram as before. This salt-free specimen still produced a disperse streak on the paper after development. A pure specimen of 3:6-anhydro-glucose gave a well-defined spot under the same conditions.

Preparation of 3:6-Anhydro Galactonic Acid.

The salt-free 3:6-anhydro galactose (0.2gm.) was dissolved in distilled water (5ml.) to which was added bromine (0.5ml.) and lead carbonate (2gm.). The mixture was gently agitated for 16 hours, the bromine was removed by vigorous aeration, and the lead bromide was filtered off. The residual lead was precipitated with hydrogen sulphide/

sulphide and the solution, after filtration, was treated with hydrogen sulphide neutralised with silver oxide, filtered and evaporated under reduced pressure, to yield a colourless glass (0.18gm.). This glass was acid to Congo Red and did not crystallize on cooling. Chromatographic examination, using aniline oxalate as developing reagent, revealed an indistinct brown smear which was not appreciably localised by the use of acidic solvents and sprays.

Chromatographic Tests.

Samples of the polysaccharide hydrolysate were chromatogrammed in butanol/ethanol/water solvent, and developed with a solution of diphenylamine trichloracetate. Galactose, glucose and xylose showed up plainly as blue spots, and no streak was visible, but a clear yellow spot, R_G 0.90, appeared before the paper was heated.

Extraction of the Material, R_G 0.90, from the Polysaccharide Hydrolysate.

The dialysed polysaccharide (50gm.) was hydrolysed for 8 hours at 80°C, with N/2 sulphuric acid (250ml.). The solution was filtered and the brown residue was again hydrolysed with N/2 sulphuric acid (50ml.) at 80°C, for a further 12 hours, after which time very little solid matter remained. After filtration the solution was neutralised and evaporated to a small volume (30ml.) under reduced pressure. After a second filtration to remove suspended matter the solution was evaporated to give a partially solid sugar mixture (40gm.), Rotation $[\alpha]_D^{20'} = 42°$(c, 0.3 in water). Successive portions of this hydrolysate (10gm.) were dissolved/

dissolved in water (20ml.) and extracted in a continuous extraction apparatus with chloroform (150ml.) for 48 hours. The total chloroform solutions from four such extractions were combined and again extracted with chloroform (200ml.) to free the resulting syrup from a scummy deposit. A golden-yellow glass (1.32gm.) was obtained, which could not be crystallised. Yield: 3.3% of the hydrolysed polysaccharide.

Found:- C, 53.3%; Hydrogen, 4.1%.

Calc. for $C_6H_6O_3$; C, 57.1%; H, 4.8%.

Investigation of the Chloroform Extracted Syrup.

The material was spotted on a paper chromatogram with galactose controls, run in butanol/ethanol/water solvent and developed with diphenylamine trichloracetate. No galactose was detected, but two yellow spots, R_G 0.90 and R_G 1.03, appeared before the paper was heated.

The syrup was reducing to Fehling's solution, and gave positive reactions in the Selivanoff and iodoform tests.

Rotation $[\alpha]_D^{19°}$ = -6° (c, 0.1 in chloroform)

Attempts were made to separate the two constituents of the syrup on a cellulose column, using a solvent of 50% butanol and 50% (100-120°C B.P.)petrol, saturated with water, but these were not successful.

Preparation of 5-Chloro-Methyl-Furfuraldehyde[2]

Cane sugar (30gm.) dissolved in distilled water (20ml.) was stirred with carbon tetrachloride (250ml.), and hydrogen chloride gas was bubbled through the solution for one hour at room temperature, and a further four hours at 40°C, with addition of carbon tetrachloride to maintain the volume of the/

the solution at 250ml. The resultant liquids were allowed to settle for 12 hours, the carbon tetrachloride layer was then separated off and dried with sodium sulphate. The syrup was again treated as above. The combined carbon tetrachloride fractions, from the two treatments were neutralised with sodium bicarbonate, dried with magnesium sulphate, and evaporated under reduced pressure to yield a white, waxy solid (5gm.).

Preparation of 5-Hydroxy Methyl Furfuraldehyde.(2)

The 5-Chloro-methyl furfuraldehyde (5gm.) was boiled for five minutes with distilled water (50ml.). The solution was extracted five times with ethyl acetate, the combined extracts were decolourized with charcoal and evaporated under reduced pressure to yield a golden glass (3gm.) which did not crystallise on cooling.

Found:- C, 52.2; H, 4.3%.

Calc. for $C_6H_6O_3$; C, 57.1; H, 4.8%.

A sample of this material was run on a chromatogram with a sample of the chloroform extract from the polysaccharide, and on development with diphenylamine trichloracetate both gave identical yellow spots of R_G 0.90 and R_G 1.03.

Preparation of the -Naphthylamine Derivative of 5-Hydroxy Methyl Furfuraldehyde.(3)

(1) Using the Chloroform extracted syrup.

The purified syrup (0.2gm.) was dissolved in ethyl-acetate (10ml.) and β-Naphthylamine (0.3gm.) was added with a small quantity of anhydrous potassium carbonate (0.1gm.). The mixture was boiled for five minutes, filtered and the filtrate/

filtrate evaporated under reduced pressure to give a light brown solid. This was redissolved in benzene and reprecip-:itated with petrol (B.P.100-120°) to give a yellow solid (0.29gm.). After five successive recrystallisations from petrol (B.P.100-120°), yellow wedge-shaped crystals were obtained (0.21gm.), melting point 129°.

Found:- C, 76; H, 6.15; N, 5.86%.

Calc. for $C_{16}H_{13}O_2$; C, 81; H, 5.5; N, 5.6%1

(11) Using the synthetic specimen.

The glass (0.71gm.) was dissolved in ethyl acetate (30ml.) and treated as above with β naphthylamine (1gm.) and anhydrous potassium carbonate (0.3gm.). After working up yellow wedge-shaped crystals were obtained (0.81gm.) m.p. 130°; m.mp. with (1) above 129°C.

Found:- C, 75; H, 5.0; N, 5.22%.

Calc. for $C_{16}H_{13}O_2$; C, 81; H, 5.5; N, 5.6%.

Attempted Removal of Galactose from the Polysaccharide Hydrolysis Products.

The residual syrup (2.9gm.) from which the 5-hydroxy-methyl furfuraldehyde had been extracted, was dissolved in distilled water (50ml.). $[\alpha]_D^{20}$ = 43°(c, 0.3 in water). To this solution was added redistilled assymmetric methyl-phenyl hydrazine (12.5gm.) and glacial acetic acid (1.5ml.) in alcohol(50ml.). The mixture was warmed at 30°C for 20 hours and then allowed to stand at 0°C for a further 24 hours. On filtration large pale yellow crystals of galactose methyl-phenyl hydrozone were obtained (1.03gm.). The filtrate was evaporated under reduced pressure to a volume/

volume of 20ml. and a further crop of crystals was isolated. The combined yield of galactose methyl-phenyl hydrazine was (1.24gm.) giving a figure of 27% for galactose in the original syrup. The yellow crystals were recrystallised from alcohol after decolourizing with charcoal. Glistening white crystals of sharp m.p. 190° (with decomposition) and $[\alpha]_D^{21°}$ = 0° (c, 0.2 in pyriding) were obtained.

After isolation of the second crop of crystals the filtrate was boiled under reflux for five hours with the addition of redistilled benzaldehyde (0.20ml.) in absolute alcohol (50ml.). The mixture was cooled in ice for 15 hours and the benzaldehyde methyl-phenyl-hydrazone was precipitated by the addition of distilled water 30ml. The residual liquors were evaporated under reduced pressure and the solid thus obtained was extracted several times with chloroform. The residue, after this extraction, was dissolved in water, decolourized with charcoal and again evaporated under reduced pressure to yield a pale yellow syrup. This was reducing to Fehling's solution and gave negative results when subjected to the iodoform and Selivan-:off reactions. On chromatographic analysis it was found to contain xylose, glucose and galactose. The streak-producing constituent appeared to be still present. The experiment was repeated on this syrup in an attempt to remove the galactose, but even after this further treatment the material was not chromatographically galactose-free.

Attempted Estimation of Glucose Present, as Glucosazone.

A portion of the syrup (0.5gm.) dissolved in distilled water/

water (15ml.) was treated with sodium acetate (0.3gm.) and phenyl-hydrazine hydrochloride (0.5gm.) for five minutes at boiling point. No precipitate was formed. On standing overnight an amorphous powder was deposited which appeared to possess the characteristics of galactosazone. It was produced in too small a yield (30mg.) to invite further investigation.

Attempted Column separation of the Constituents of the "Galactose Free" Syrup.

An attempt was made to separate the constituents of the above syrup on a hydrocellulose column, using a butanol/ethanol/water partition solvent and a tracer dyestuff, Methyl Red R_G 0.38, to follow the progress of the sugars. This did not prove satisfactory and no appreciable separation was achieved. The attempt was repeated using a partition solvent of benzene (10 parts) pyridine (30 parts) butanol (50 parts) and water (30 parts). In this case no dyestuff indicator was used as it was found to give a streaky effect in this solvent. No separation of the sugars was achieved.

Preparation of Penta-acetyl Galactose Methylphenyl hydrazone.[4]

(A) Using galactose methyl-phenylhydrazone prepared from the Polysaccharide.

The galactose methyl-phenyl-hydrazone crystals (160mg.) were dissolved in pyridine (2ml.) and acetic anhydride (1ml.) was added. The mixture was allowed to stand at 0°C for 48 hours, subsequently filtered and the filtrate poured into ice-water (25ml.). The pale yellow precipitate was/

was filtered off, redissolved in alcohol and decolourized with charcoal. It was recrystallised four times from alcohol to give glistening, white, rhombic crystals (115mg.); microscopic mp. 137.5°.

$[\alpha]_D^{21°}$ = 21.7° (c, 0.2 in chloroform).

Found:- C, 53.6; H, 5.7; N, 5.61%.

Calc. for $C_{23}H_{30}N_2O_{10}$; C,55.8; H, 6.0; N,5.70%.

(B) Using the Synthetic Sample of D-galactose Methylphenylhydrazone.

This sample (130mg.) was dissolved in pyridine (1.5ml.) and treated with acetic anhydride (0.75ml.) under identical conditions to those used in (A). After isolation and purification, pure crystals were obtained (154mg.); microscopic mp. 139.0°. $[\alpha]_D^{20°}$ = 26.2° (c, 0.3 in chloroform)

Found:- C, 53.8; H, 5.7; N, 5.63%.

Calc. for $C_{23}H_{30}N_2O_{10}$; C,55.8; H,6.0;N,5.70%.

Use of Amino-Guanidine Sulphate in Attempted Detection of 3:6 Anhydro Galactose.(5)

A solution of amino-guanidine (0.1%) in 50% sulphuric acid was warmed with the following materials which all gave the required red colouration.

1. Authentic 3:6 anhydro L-galactoside.
2. Gelidium polysaccharide hydrolysis products.
3. Pure Galactose.
4. Hydrolysate of B.D.H. Agar.
5. Hydrolysate of Ahnfeltia Agar.

This reaction is clearly too general to be of analytical application.

Methoxyl Estimations on Unmethylated Polysaccharide.

Two methoxyl estimations were carried out on samples of/

of the purified polysaccharide. Blanks were run with these but showed no precipitate.

Results:-

Estimation	Wt. of Polysaccharide	Wt. of ~~Methyl~~ Silver Iodide	% Methoxyl
I	9.72 mg.	1.14 mg.	1.5
II	10.32 mg.	1.37 mg.	1.7

A larger scale estimation was carried out in conjunction with a blank.

Result:-

Wt. of Polysaccharide	Wt. of ~~Methyl~~ Silver Iodide	% Methoxyl
91.54 mg.	6.85 mg.	1.05

Attempted Fractionation of the Polysaccharide.

Gelidium latifolium (20gm.) which had been carefully washed and bleached was extracted for two hours at 100° with distilled water (100ml.):- Extract I.

The residue, after filtration, was again extracted for one hour at 100° with distilled water (50ml.):- Extract II.

This residue, after filtration was extracted for a third time for four hours at 100° with distilled water (50ml.):- Extract III.

The three extracts were then examined for sulphate, and by quantitative chromatography for the three sugars, galactose, glucose and xylose. The latter analyses were rendered somewhat inaccurate, by the presence of the usual streak on the paper, but may be used for comparative purposes.

Fraction	Weight	Sulphate	Galactose	Glucose	Xylose
I	3.7gm.	0.70% after dialysis	68%	14%	12%
II	1.0gm.	0.95%	42%	40%	18%
III	0.4gm.	1.07%	31%	51%	18%

A sample of the polysaccharide was dried with alcohol, and then with ether, and it was noticed that a portion of the material (circa 2%), was soluble in the aqueous alcohol, and was reprecipitated by the ether. The material was isolated as a white powder very sparingly soluble in cold water, (Solubility Ca SO_4 is 0.2gm./100ml. at 18°C). After hydrolysis with 2N. Sulphuric acid, for two hours, at 85°C, only xylose, and glucose, could be detected chromatographically.

Partial Hydrolysis of the Polysaccharide with Oxalic Acid.

The purified polysaccharide (10gm.) was hydrolysed for twelve hours at 85°C, with N. oxalic acid (100ml.). Chromatographic investigation of the neutralised extract, showed faint disaccharide spots, galactose and glucose, a little xylose, but the usual indefinite streak was not present.

An attempt to methanolyse the polysaccharide with 2% methanolic hydrogen chloride, for four hours, at 80°C, was not successful.

Attempted Separation of Constituent Polysaccharides by Tiselius Electrophoresis Apparatus.[6]

2% solutions of agar, in N and N/10 caustic soda, were found to be partially gelled after centrifugation, and were thus unsuitable for use in the apparatus. Solution was attempted in N and 2N solutions of both potassium and lithium hydroxides, without success. A solution was made up in glacial phosphoric acid at room temperature. After shaking for four hours, this was found to be fairly fluid, and on dilution to four times its volume with water, gave

b/

a clear solution. However, on investigating the free sugar concentration, with the Somogyi reagent, the polysaccharide was found to be considerably degraded. The experiment was repeated at 0°C, in an attempt to avoid degradation, but at this temperature the polysaccharide would not go into solution, even after 36 hours shaking. In a further attempt to obtain a non-gelling solution, of the intact polysaccharide, a 2% solution of the material was warmed until fluid, and 50% of formic acid was added to it. The whole was rapidly cooled to 0°C. The resulting mixture remained clear and fluid, with no signs of discolouration, but investigation showed that some degradation had again occurred. Glacial acetic acid in 50% concentration, under the same conditions as the formic acid, was not so efficient in preventing gel formation. Urea and formamide were used in 50% concentrations, in attempts to break the gel, but were not really effective. The experiment was therefore abandonned.

End Group Investigation by Periodate Oxidation[7] (Formic Acid ~~Liberation~~ Estimation).

The purified polysaccharide (0.4019gm.) dispersed in distilled water (100ml.) was shaken in diffused light with potassium periodate (0.4551gm.) for 24 hours. A blank sample was similarly treated. Both samples were centrifuged and a portion of the supernatant liquid (10ml.) was treated with ethylene glycol (1ml.) and a few drops of methyl red in order to titrate them with 0.1 N. sodium hydroxide. Both the blank and the polysaccharide sample were neutral and both remained neutral during 12 days agitation.

Periodate/

Periodate Oxidation:- Formaldehyde Investigation.

Samples from the above periodate oxidation, taken from the polysaccharide suspension, and the blank control, were tested, after 4 days, for formaldehyde, together with a true formaldehyde control solution. The solutions were centrifuged, the excess periodate was destroyed with dilute hydrochloric acid, and sodium arsenite solution, and to the liquids were added, two crystals of potassium ferricyanide, recrystallised phenyl-hydrazine hydrochloride (0.1gm.) and concentrated hydrochloric acid (1ml.). No ~~wine~~ red colour was observed in the blank solution, or in the polysaccharide solution. The formaldehyde control reacted normally.

Periodate Experiments on increased quantities.

In the original periodate experiments the quantity of material used was based on the erroneous figure of 8% of end-group obtained from the first column separation of methylated agar. A second experiment based on the assumption of 1% end-group was carried out as follows:- Specially purified agar dried at 60°C, weight 4.1004gm., was placed in a dark glass bottle with 0.5gm. sodium meta periodate, potassium chloride (2gm.) and distilled water (150ml.). A blank control was similarly arranged. The bottles were mechanically shaken for 24 hours, then 10ml. portions of the supernatant liquid were withdrawn, treated with ethylene glycol and methyl red, and titrated with standard caustic soda. The blank control remained neutral and the agar required 0.882ml. of 0.0111N caustic soda to neutralise it. The bottles were shaken for a further

36 hours but on withdrawing samples again they were both found to be neutral. A calculation based on the only available figure was therefore made as follows.

Normality of formic acid = $\frac{0.882}{10.0}$ x 0.0111 N.

Molecular weight of anhydro residue = 162.

Equivalent of formic acid = 0.0087 per anhydro residue

% end group = 0.87%.

Chromatographic Investigation of Polysaccharides from Various Algae.

Algae from various sources, which have been utilised in the production of commercial agar, were extracted, the polysaccharides were hydrolysed, and the products of hydrolysis were examined by paper chromatography. The following table indicates the results of these investigations.

Algae	Source	Gelling Power	Galactose	Glucose	Xylose
Gracilaria confervoides	Plymouth	Good	+	+	+
Gelidium crinale	Ayrshire	Good	+	+	+
Ahnfeltia plicata	Northumberland	Fair	+	+	+
Hypnea musciformix	Australia	Good	+	+	-
Laurencia sp.	Australia	Good	+	+	+
Gracilaria confervoides	Australia	Good	+	+	+
Gracilaria speciosum	Australia	Fair	+	+	+
Eucheuma speciosum	Australia	Fair	+	+	+
Eucheuma murecatum	Australia	Poor	+	+	+
Gigartina stellata	Galway,Eire	Good	+	+	+
Chondrus crispus	Galway,Eire	Good	+	+	+
Pterocladia sp.	New Zealand	Fair	+	+	+
Desmerentia sp.	Aberdeen	Poor	+	+	+
Polysiphonia fastigiata	North Berwick	-	+	+	+

Methylation of the Polysaccharide[8] (Method I)

The purified polysaccharide (9.2gm.) was dissolved in warm water (300ml.) and to this solution was added sodium hydroxide (138gm.) in water (160ml.). The mixture was vigorously stirred at room temperature and dimethyl sulphate (125ml.) was added, dropwise, to the solution, over a period of ten hours. After completion of the addition, the viscous liquid was neutralised with 15% sulphuric acid, and made slightly alkaline with 2N caustic soda. The alkaline mixture was boiled for one hour, at 95°, on the water bath. A flocculent grey precipitate appeared, which was filtered off through four layers of muslin. On washing the precipitate with hot water, to remove sodium sulphate, it became very slimy and difficult to handle. The washings were boiled up and centrifuged to give more of the grey precipitate. The total solid was dissolved in acetone (200ml.), sodium hydroxide (138gm.) in water (250ml.) was added and the mixture was again methylated with dimethyl sulphate (125ml.) for ten hours, at room temperature. The acetone was removed under reduced pressure, the material was neutralised, made alkaline, and filtered, to give a grey stringy material. This was again methylated in acetone, and the product was reprecipitated from a 1:10 mixture of alcohol and chloroform, with light petroleum b.p. 60°- 80°. The white powder thus obtained, (8.7gm.) was dried, and its methoxyl content determined, as 31.3%. However, the material smelt strongly of acetone, and it was feared that further methylation in this solvent might lead to undesirable addition products. Therefore/

Therefore, after a second reprecipitation, with light petroleum from dioxan, the partially methylated polysaccharide was combined with a second sample, which had received the treatment noted below.

Methylation of the Polysaccharide (Method II)

The purified polysaccharide (7gm.) was subjected to a double methylation, without intermediate isolation of the product from the aqueous solution. In this way, the mechanically difficult stage of methylation was avoided, and the product, a white powder (6.6gm.) was combined with the purified material from Method I. Further methylations, by the Haworth technique, were carried out, using dioxan as the initial solvent for the partially methylated material. It was found necessary to distil the dioxan from sodium prior to use in order to remove undesirable aldehyde constituents. After six further methylations, the material was isolated as a white powder, which was considerably contaminated with sodium sulphate. It was partially purified by precipitation from chloroform solution with light petroleum, and was then dialysed in aqueous suspension, against running distilled water, for 7 days. A portion of the total product (8.2gm.) was examined for ash, sulphate and methoxyl content.

Found:- Ash, 0.24; Sulphate, 0.05; OMe, 35.1%.

Purdie Methylation[9] of the Partially Methylated Material.

A. Small Scale Trial Experiment:

Partially methylated polysaccharide (1gm.) was refluxed with methyl iodide (10ml.) and dry silver oxide (5gm.) was added/

added in portions, over a period of 25 hours. The addition of 10% of dry methanol was found of assistance in keeping the material in solution. After cooling, the mixture was filtered, the residue was extracted five times with hot chloroform, and these extracts were added to the filtrate. Further Soxhlet extracts of the residue were made, but the total yield was only 8%. After purification, by reprecip-:itation from chloroform with light petroleum, the material showed - Ash, 0.21; OMe, 36.7%.

Purdie methylations were then carried out on the bulk of the material, using the same technique. After four Purdie methylations, the material was purified by reprecip-:itation, and dialysed for 6 days, against running distilled water. The dialysed material was dissolved in chloroform, the solution was dried with anhydrous sodium sulphate, and fractionally precipitated with petroleum (b.p. 40/60°), to yield the following fractions. Note variation in $[\alpha]_D$ of fractions.

Fraction	Weight gm.	Methoxyl %.	Sulphate	Rotation in($CHCl_3$)	Ash %.
I	3.96	38.1	-	$[\alpha]_D^{20}$ -83° (c,0.84)	0.18
II	1.04	35.4	-	$[\alpha]_D^{20}$ -56° (c,0.54)	0.22
III	0.23	31.6	-	$[\alpha]_D^{20}$ -29° (c,0.84)	0.28

Methanolysis and Hydrolysis of Fraction I.

Fraction I of the methylated polysaccharide (3.2271gm.) was methanolysed for 70 hours, with 2% methanolic hydrogen chloride. During the last 30 hours of this period, it is probable that the observed changes in rotation were due to the α-β equilibrium, and that the methanolysis was complete after approximately 40 hours ($[\alpha]_D^{18°}$ = 57°). The solution was/

was clarified during this period, by the addition of 10% of chloroform. The resultant liquid was neutralised with silver carbonate, filtered, and evaporated under reduced pressure, to yield a pale yellow syrup (3.7531gm.). This syrup was hydrolysed for 12 hours, with N/2 hydrochloric acid, to give a solution of constant rotation $[\alpha]_D^{20} = 46.2^\circ$ (c, 0.2). The solution was neutralised with silver carbonate, the excess silver removed with hydrogen sulphide, and the liquid decolourized with a little animal charcoal. It was evaporated at 35° under reduced pressure, to give a pale yellow, partially crystalline, solid (2.9550gm.).

Paper Chromatography of Methylated Polysaccharide Hydrolysate.

A small quantity of the methylated polysaccharide hydro-:lysate was chromatogrammed, with appropriate control sugars, in butanol/ethanol/water solvent, and after drying the paper, was developed with a saturated solution of aniline oxalate. The following sugars were detected.

R_G		Probable identity of constituent.
0.92	...	5 Hydroxy. Methyl Furfuraldehyde
0.88	...	2:3:4:6 Tetramethyl Galactose
0,80	...	2:3:6 Trimethyl glucose
0.67	...	2:4:6 Trimethyl galactose
0.51	...	3:6 Dimethyl glucose
0.40	...	2:4 Dimethyl galactose

Separation of the Methylated Constituents on a Cellulose Column.

A chromatography column[10] (3.5 x 40cms.) was constructed from powdered cellulose, obtained by the fine milling of Whatman No.1 filter paper clippings. Before use this column was thoroughly washed with water, butanol, and with the/

the chromatographic solvent, (50% n. butanol, 50% petrol (100-120° b.p.) saturated with water). The petroleum ether had been previously purified from unsaturated compounds, by shaking for 12 hours with concentrated sulphuric acid, neutralising with solid sodium hydroxide, and fractional distillation in the required boiling point range.

The partially crystalline sugar mixture (2.9550gm.) was dissolved in distilled water (3ml.) and the solution was placed on a plug of cotton wool, on the top of the cellulose column, and covered by a similar plug, to minimise back diffusion of the material. 50% n-butanol and 50% purified petrol 100-120°b.p. (100ml.) was run through the column, before the water saturated solvent, in order to avoid the possible formation of a two phase system. The solvent was then run through the column. 100ml. were allowed to flow through without fractionation, the eluate was then collected by means of fraction cutter which changed the collection tubes at regular time intervals. The liquid from every tenth collection tube was evaporated, and the residue was applied to a paper chromatogram, with an appropriate control sugar for identification. The following table indicates the progress of the separation.

Tube Number	Chromatographic Analysis	Weight after Purification	
100ml. (not fractionated)	-	-	
1 - 60	-		Fraction I
60 - 80	5-Hydroxy methyl furfuraldehyde	0.1448gm.	
80-170	2:3:4:6 Tetramethyl galactose	0.2580gm.	Fraction II
170 - 200			
200 - 230	2:3:6 Trimethyl glucose	0.1179gm.	Fraction III
230 - 310	2:3:6 Trimethyl glucose + 2:4:6 Trimethyl galactose	0.5290gm.	Fraction IV
310 - 320	2:4:6 Trimethyl galactose	1.5163gm.	Fraction V
320 - 600, 500ml. solvent, 500ml. water	3:6 Dimethyl glucose + 2:4 Dimethyl galactose	0.1040gm.	Fraction VI
		Total = 2.6700gm.	
		% Recovery = 90.4%	

Tubes containing homogeneous samples were combined, and evaporated under reduced pressure, to give impure syrups. These were purified with "Filter-cel" and charcoal, and again evaporated, to yield the weights of purified material shown in the above column. After tube 600, a volume of the solvent, 500ml., was run through the column without fractionation. The column was then removed from the tube, powdered, and extracted with 500ml. of distilled water. These two extracts were evaporated with tubes 320-600 as shown above.

Examination of the Fractions.

Fraction I.

This fraction was a pale yellow syrup which appeared to be chromatographically pure and represented 4% of the methylated hydrolysate. It was reducing to Fehling's solution, and gave positive Selivanoff and iodoform tests.

It/

It gave a yellow colouration in the cold with diphenylamine trichloracetate $[\alpha]_D^{20}$, 0°(c, 0.1 in $CHCl_3$); OMe, 0%.
R_G in butanol/ethanol/water = 0.91; Found: C,54.1; H,4.4%.

Calc. for 5 Hydroxy-methyl furfuraldehyde ($C_6H_6O_3$);
C, 57.1; H, 4.8%.

Fraction II. This fraction appeared on the chromatogram to contain a trace of 2:4:6-trimethyl-D-galactose, but was predominantly 2:3:4:6-tetramethyl-D-galactose (RG 0.88). Physical data indicated considerable impurities.
$[\alpha]_D^{18}$ = 95°(c, 0.6 in water) (2:3:4:6-tetramethyl galactose has 119°); OMe, 42%, (a tetramethyl-hexose required 52.6%). The material could not be induced to crystallize by seeding. On distillation in high vacuum it yielded 0.1997gm. of partially crystalline 2:3:4:6-tetramethyl-D-galactose, OMe, 51.0%; $[\alpha]_D^{21}$ = 107°(c, 0.9 in water) mp. 70°
Found: C, 51; H, 8.5%.

Calc. for $C_{10}H_{20}O_6$; C, 50.9; H, 8.48%.

70mg. of the sugar were boiled under reflux with 25mg. of redistilled aniline and 3ml. of absolute alcohol for 2 hours. After removal of the solvent the residue was recrystallised from dry ethyl acetate to give the crystalline anilide (40mg.) mp. 195°; m.mp. with authentic 2:3:4:6-tetramethyl-D-galactopyranose anilide 193°.
$[\alpha]_D^{21}$ -72°(c, 0.8 in acetone).
Found: C, 61.0; H, 8.1; N, 4.3%

Calc.for $C_{16}H_{25}O_5N$; C,61.8; H, 8.0; N, 4.5%.

The residual material (45mg.) after distillation of the trimethyl galactose was hydrolysed by boiling for 2 hrs. with/

with 2N hydrochloric acid and the hydrolysate worked up to give 30mg. of pale yellow syrup which by paper chromatography appeared to be a mixture of 2:4:6-trimethyl galactose and the tetramethyl galactose in approximately equal proportions. No separation of these sugars was attempted.

Fraction III. On recrystallisation from a mixture of light petroleum and ether, white crystals were obtained of mp. 82°C, OMe, 40.1%; mixed mp. with authentic 2:3:6-trimethyl glucose 81°C.

Fraction IV. This fraction (0.5290gm.) had a methoxyl content of 40.1% and was completely crystalline $[\alpha]_D^{20^\circ} = 98^\circ$ (c, 0.3 in water), mp. very indefinite 80-90°C. Paper chromatographic analysis showed it to contain two constituents corresponding to 2:3:6 trimethyl glucose (R_G 0.80) and 2:4:6 trimethyl galactose (R_G 0.67). Attempts were made to separate these two sugars by fractional distillation, by chromatographic elution from a narrow cellulose column and by methylation followed by fractional crystallisation but these methods were not successful.

Fraction V. This main fraction of the hydrolysate (1.5163gm.) equivalent to 56% of the total recovered, was partially crystalline and appeared by paper chromatographic analysis to be pure 2:4:6-trimethyl galactose. However its methoxyl content was 37%, where a trimethyl hexose required 41.8%. Rotation of this fraction $[\alpha]_D^{20^\circ} = 50^\circ$ (c, 7.5 in water). Attempts were made to purify it by recrystallisation from petroleum/

petroleum ether and 0.1383gm. of crystalline material were obtained OMe 40.7% and mp. 99°- 100°C (later raised by further recrystallisation to 103°C; mixed mp. with authentic 2:4:6 trimethyl α -D-galactose 97°C) $[\alpha]_D^{21'} = 100°$ (c, 1.0 in water) falling to 60°. This is at variance with that for 2:4:6-trimethyl α -D-galactose $[\alpha]_D^{20} = 93°$ (c, 0.9) (equilibrium value). Analysis Found:- C, 48.0; H, 7.3%;

Calc. for $C_9H_{18}O_6$; C, 48.7; H, 8.1; OMe, 41.8%.

2:4:6-trimethyl methyl-D-galactoside was obtained in quantitative yield by treating the crystals with 0.7% methanolic hydrogen chloride followed by neutralisation and removal of the solvent. After recrystallisation from light petroleum the product had mp. 64°C $[\alpha]_D^{18'} = 99°$ (c,0.5 in water). Again this rotation is lower than that of a pure specimen of the galactoside $[\alpha]_D^{18'} = 107°$(c,0.4 in H_2O). Mixed mp. with the authentic specimen 58°C.

The non-crystallising syrup after the extraction of the crystalline 2:4:6 trimethyl galactose weighed 1.2655gm. It was expected that if any monomethyl anhydro sugars were present in the hydrolysate, they would be contained in this fraction as chromatographically they could be reasonably expected to behave as trisubstituted hexoses. Therefore it was hoped by methylation and fractional distillations to separate any such constituents. The syrup was twice methylated by the Purdie technique in 10ml. of methyl iodide with the addition of 5gm. of silver oxide over a period of 24 hours. The mixture was filtered and the residue repeatedly extracted with hot water. These extracts/

extracts, together with the filtrate were evaporated under reduced pressure to yield a pale yellow syrup (1.38gm.). This syrup was fractionally distilled at 0.5mm. pressure to give two fractions:- (1) b.p. 69-71°C, white crystals

(2) b.p. 101-104°C, white waxy material.

Fraction (1) had mp. 94°C and OMe 51.1%.

Fraction (2) had mp. 72°C and OMe 51.2%.

Since the methoxyl contents appeared to correspond closely to that required for trimethyl methyl galactoside (OMe 52.5%), a small quantity of each fraction was hydrolysed and examined by paper chromatography. Only 2:4:6 trimethyl galactose could be detected. Therefore the Purdie methyla-:tion was repeated on the combined fractions, the syrup was extracted and redistilled under reduced pressure to give two fractions:-

Fraction (1) b.p. 98-102°C (at 0.1mm.) white waxy material

mp. 71°C OMe 51.0°C.

Fraction (2) Not distilled yellow syrup; OMe, 51.3%.

Portions of fractions (1) and (2) on hydrolysis and chromatographic investigation showed only 2:4:6 trimethyl galactose.

<u>Fraction VI</u> (from column) This fraction (0.1040gm.) was a pale yellow syrup (OMe 29.8%) which could not be crystallised and appeared to consist of a mixture of dimethyl galactoses and dimethyl glucoses. In view of its complex nature, this mixture was not further investigated as attempts to separate the constituents on a small chromatographic column had proved unsuccessful.

Further/

Further Work on Chromatographic Separation.

A second chromatographic separation of the methylated hydrolysate was attempted on a syrup having OMe 38.2% and $[\alpha]_D^{20} = 50°$(c, 0.2 in water). A considerably longer column was used, 2.5cms. x 65cms., in an attempt to effect some separation between the methylated glucoses and galactoses, but owing to some mechanical defects in the packing of this column the flour became irregular and little separation was obtained after the elution of the 2:3:6-trimethyl glucose. No fully methylated galactose was detected. A third attempt at chromatographic separation was made using a long column (2.5cms. x 65cms.) of hydrocellulose in the hope that this might show a greater variation in adsorption between glucose and galactose derivatives than the cellulose previously used. However, no improvement was obtained as is shown by the following table of results. Original weight of syrup, 1.400gm. It will be noted that again no tetramethyl galactose appeared among the hydrolysis products.

Tube Number	Chromatographic Analysis	Weight
1-70	-	
70-110	2:3:6-trimethyl glucose	20mg.
110-240	2:3:6-trimethyl glucose 2:4:6-trimethyl galactose	0.1614gm.
240-340	2:4:6-trimethyl galactose	1.0192gm.
340-360	2:4:6-trimethyl galactose 3:6-Dimethyl glucose	0.1127gm.
360-480	3:6-Dimethyl glucose 2:4-Dimethyl galactose	0.0193gm.
	Total...	1.3126gm.
	% Recovery = 93%.	

Further Purification of Methylated Agar.

Since no success had been obtained in attempts to separate/

separate the glucose and galactose derivatives after hydrolysis of methylated agar, further efforts were made to eliminate the glucose containing fraction from the methylated polysaccharide.

10gm. of the methylated agar (OMe 38.2%) were dialysed for 10 days against running distilled water. The mixture was evaporated, dried at 35°C in a desiccator and dissolved in dry chloroform from which it was repeatedly fractionally precipitated with 60°-80°C b.p. petroleum ether to give finally three fractions of fine white powder.

Fraction A: 1.96gm. Fraction B: 7.74gm. Fraction C: 0.121gm.

Hydrolysis, followed by paper chromatographic examination of these fractions indicated that fraction A contained the usual mixture of methylated galactose and glucose derivatives. Fraction B appeared to contain only dimethyl and trimethyl galactoses and Fraction C showed only trimethyl galactose. A methoxyl estimation on this fraction showed OMe 40.1%, the highest figure yet obtained. Fraction B was methylated 4 times by the Purdie technique to give 6.21gm. of a white powder OMe = 39.1%. This was dissolved in chloroform and repeatedly fractionated by precipitation with petroleum ether to give two fractions - Fraction D: 0.82gm. and Fraction E: 5.20gm. Fraction D had OMe 38.8% and appeared to contain both trimethyl and dimethyl galactose on hydrolysis and chromatographic analysis. Fraction E had OMe 40.1% and showed only trimethyl galactose on the chromatogram. Therefore fractions C and E were combined to give 5.32gm. of methylated agar OMe 40.1% and appearing to consist entirely of 2:4:6-trimethyl galactose residues.

Investigation/

Investigation of Methylated Polysaccharide by Methanolysis followed by High Vacuum Distillation.

Methylated agar (OMe 40.1%) (0.781gm.) was heated with 2% methanolic hydrogen chloride for 40 hrs. The solution was neutralised with silver carbonate, filtered, treated with hydrogen sulphide and evaporated to give a pale yellow syrup 0.723gm. This syrup was distilled under reduced pressure (0.03mm.) and the distillate collected in a vessel, cooled in solid carbon dioxide. Almost all of the material distilled at 98-100°C to give a white crystalline sugar (0.53gm.), OMe 51.0%. Trimethyl methyl galactoside requires OMe 52.5% and $[\alpha]_D^{20'} = 107°$. The rotation of its distillate was $[\alpha]_D^{18°} = 92°$ and mp. 60°C, m.mp. with authentic 2:4:6-trimethyl methyl D-galactoside 56°C.

Authentication of 2:4:6-Trimethyl methyl galactoside.

The crystalline sugar (0.5gm.) was twice methylated with methyl iodide and silver oxide. The residues were extracted in a Soxhlet extractor, evaporated and distilled in high vacuum to yield 0.32gm. of a colourless syrup. This was hydrolysed for 2 hours with 7% hydrochloric acid, the fully methylated sugar was isolated and treated with aniline to yield 0.2gm. of crystalline tetramethyl galactopyranose anilide mp. 190°C, $[\alpha]_D^{17°} = -68°$ (c, 0.4 in acetone), m.mp. with authentic specimen 189°C.

Treatment of Residue from High Vacuum Distillation.

The residue (0.14gm.) after removal of the trimethyl galactoside was a dark brown solid OMe 40%. It was proposed to methylate this and attempt to distil any dimethyl anhydro sugar/

sugar which might be present. The material was therefore methylated twice by the Purdie technique, extracted with chloroform in a Soxhlet and the resulting syrup was again subjected to high vacuum distillation at 0.03mm. A very small quantity of white waxy material distilled off at 100°C and was collected in solid CO_2. This was recfystallised from 60-80° b.p. petrol and appeared to be more trimethyl-methyl galactoside. The residue in the microdistillation apparatus was a dark brown solid $[\alpha]_D^{11°} = 12°$(c, 0.4 in $CHCl_3$) which gave a strong Selivanoff reaction and appeared on chromatographic examination after hydrolysis to be mainly 5-hydroxy methyl furfuraldehyde and 2:4:6 trimethyl galactose.

Fractional Extraction of the Methylated Polysaccharide after Methanolysis.

Methylated agar (OMe 40.1%) (4.54gm.) was heated for 24 hours with 2% methanolic hydrogen chloride, neutralised and the residues extracted with chloroform in a Soxhlet apparatus and worked up to give 4.24gm. of a pale yellow syrup. This was dissolved in 20ml. of distilled water. A small residue of undissolved material, 0.12gm., remained; presumably the methanolysis had not been quite complete. The solution was continuously extracted in a "Quickfit & Quartz" liquid extractor for several 4-day periods, first with 38-40°C b.p. petroleum ether, followed by 60-80°C b.p. petroleum ether and finally the solution remaining was evaporated, this giving three fractions.

Fraction I: 0.1848gm.; Fraction II: 0.7926gm.; Fraction III: 2.8474gm.

Hydrolysis of portions of each fraction followed by chromatographic/

chromatographic examination showed that Fraction I appeared to contain a small percentage of tetramethyl galactose in addition to the trimethyl galactose. Fraction II was entirely 2:4:6-trimethyl galactose and Fraction III contained dimethyl galactose and trimethyl galactose.

Treatment of Fraction III.

This fraction was distilled in high vacuum (0.03mm.) to yield 0.8460gm. of crystalline trimethyl methyl galactoside recrystallised from petroleum ether, mp. 72°C $[\alpha]_D^{17} = 54°$ (c, 0.2 in water). The residue of 2gm. of dark brown solid gave no Selivanoff test and was subjected to three further high vacuum distillations to yield a further 1.27gm. of trimethyl methyl galactoside. These were recrystallised and combined with the first distillate and with fraction II from the extraction experiment to yield 2.90gm. of crystalline 2:4:6-trimethyl methyl galactoside from 4:24gm. of original syrup, i.e. 68%. The residue in the distillation apparatus was examined chromatographically and appeared to contain more of the 2:4:6-trimethyl methyl galactoside, but this could not be removed by further distillation.

Treatment of Fraction I. (from the liquid extraction experiment)

It was decided to hydrolyse this fraction and estimate the tetramethyl sugar by quantitative paper chromatography. The syrup (0.1348gm.) was hydrolysed for 12 hours with N/2 sulphuric acid, neutralised with barium carbonate and worked up to give 0.1273gm. of free sugars. These were treated with "Amberlite" resins to remove ions; it was found necessary/

necessary to repeat the resin treatment several times before non-streaking chromatograms were obtained. These chromatograms indicated the presence of trimethyl galactose, tetra-methyl galactose and small amounts of tetra-methyl glucose and dimethyl galactose. It is suspected that the latter was produced by demethylation during hydrolysis as it seems unlikely that it would be extracted by 38-40°b.p. petroleum ether. The estimations were carried out by the method described by Flood, Hirst & Jones, J.C.S. 1948, 1679, and the following results were obtained:-

	Ml. of Thiosulphate	Paper Correction	Corrected Volume	Ratio of Iodine used
Tetra-Me-Glucose	9.70	0.06	9.76	0.01
Tri-Me-Galactose	8.36	0.12	8.48	1.29
Tetra-Me-Galactose	9.17	0.06	9.29	0.48
Di-Me-Galactose	9.25	0.06	9.25	0.46
Water Blank	9.77	-	9.77	-
Paper Blank	9.71	-	9.71	-

Ignoring the Tetramethyl glucose

$$\frac{\text{Tetra Ga}}{\text{Tri Ga}} = 38\% \qquad \frac{\text{Di Ga}}{\text{Tri Ga}} = 36\%$$

$$\frac{\text{Tetra Galactose}}{\text{Total Sugars}} = 21.8\%$$

Syrup under examination = 4.8% of the total weight of methanolysed sugars.

% Tetra in total weight of methanolysed sugars is 1.04%.

A second estimation gave the following results:-

Percentage Tetramethyl Galactose in hydrolysed syrup is 23%

Therefore percentage in Total methanolysed sugars is 1.1%.

SUMMARY OF EXPERIMENTAL WORK.

1. Methods for the small scale extraction and purification of an agar from Gelidium Lastifolium were evolved.
2. The polysaccharide thus obtained was analysed for ash, sulphate, organic nitrogen and methoxyl content.
3. Attempts were made to remove the sulphate by prolonged dialysis, and by treatment with alkali, but a small percentage remained in the polysaccharide.
4. The polysaccharide was completely hydrolysed with sulphuric acid. No amino acids were identified in the hydrolysate. Paper chromatography indicated the presence of galactose, glucose and xylose in the hydrolysate. There was a disperse indication of another substance.
5. A small percentage of 5-Hydroxyl methyl furfuraldehyde was isolated from the hydrolysate by liquid extraction with chloroform. It was identified by preparation of the β -Naphthylamine derivative and comparison with an authentic sample.
6. Attempts were made to remove the galactose from the hydrolysate with methyl phenyl hydrazine and to estimate the glucose present as its ozazone but these were not successful.
7. Preparation of the penta-acetyl derivative of the galactose methyl phenyl hydrazone indicated that 10% of laevo-galactose was probably present.
8. An attempt to separate the free sugars, in the polysaccharide hydrolysate, on a cellulose column was unsuccessful.

9. Precipitation technique indicated the possible existence of more than one polysaccharide in the original extract, but an attempted separation of these by electrophoresis proved impractical.

10. Periodate oxidation experiments gave some evidence of the presence of about 1% of end group in the unmethylated polysaccharide.

11. The polysaccharide was methylated to give a material containing 38% methoxyl which, on subsequent hydrolysis and analysis by paper chromatography, appeared to contain fully and partially methylated derivatives of galactose and glucose.

12. The separation of this material by partition chromato-:graphy on a cellulose column yielded an appreciable percentage of fully methylated galactose, but this was not confirmed by subsequent experiments. 2:3:6-Trimethyl glucose and 2:4:6-Trimethyl galactose were separated and identified but accurate estimates of proportion could not be made owing to the difficulty of separating closely similar derivatives of glucose and galactose.

13. A new sample of methylated agar was prepared and analysed in a similar manner by partition chromatography. A longer column was used in an attempt to improve the separation. The results were the same as those of the first separation except that no fully methylated galactose was obtained.

14. A third attempt at separation was made using hydrocellulose in place of cellulose, but the results confirmed those of the second separation.

15. A partial fractionation of the methylated polysaccharide was achieved by fractional precipitation and a material with a methoxyl content of 40% was obtained. This appeared to contain only 2:4:6-Trimethyl ~~methyl~~ galactoside residues.

16. Fractionation of this material by high vacuum distillation of the methanolysis products gave only the expected 2:4:6-trimethyl methyl galactoside and an unidentified residue.

17. The methanolysis products of the methylated polysaccharide (OMe 40%) were partially separated by fractional liquid extraction with petroleum.

18. One of these fractions (Fraction I) appeared to contain a small proportion of fully methylated galactose. Fractions II and III yielded 60% of crystalline 2:4:6-trimethyl methyl galactoside and further quantities of this material remained in the residual syrup but could not be separated by distillation.

19. Fraction I was hydrolysed and analysed by paper chromatography. It was found to contain 22% of tetramethyl galactose giving a figure of 1% of this sugar in the original methanolysed syrup.

20. On this evidence no completely verified structure can be given for agar. It is suggested that the polysaccharide is a polymer of D and L galactose in the ratio of 10:1, linked through carbon atoms one and three. It is also suggested that the polysaccharide contains about 1% of end group giving a chain length of 100 units and a molecular weight of about 16,000. The role of the inorganic nitrogen and the small proportion of methoxyl/

20(Cont'd)/

methoxyl groups in the unmethylated polysaccharide cannot be indicated on the available evidence. No anhydro sugar residues have been detected in this polysaccharide.

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