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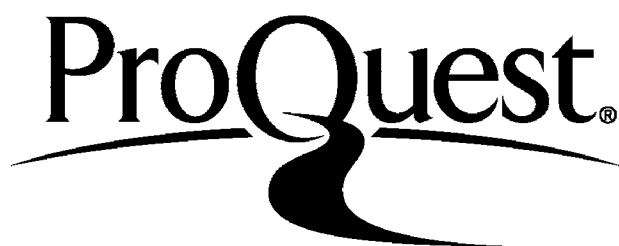
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Abstract of Thesis.

J.W.E. Brading.

The structure of the Polysaccharide from Ulva Lactuca.

The polysaccharide has been purified; after nitric acid oxidation small quantities of mucic and saccharic acids have been obtained. The carbohydrate has been methylated by repeated treatment with Me_2SO_4 , NaOH , the chloroform soluble fraction being further methylated with MeI , Ag_2O to a product with OMe , 31%. Prior treatments to give an acetylated product or sulphate free product, suitable for methylation, were unsuccessful. The properties of fully methylated and CHCl_3 insoluble fractions have been investigated; the former fraction was subjected to methanolysis and the products separated and investigated. Evidence for 2:3:4-trimethyl-L-rhamnose and no other simple end group, partially methylated hexose and rhamnose, an anhydro structure and an ester was obtained.

On autohydrolysis most of the uronic acid and/or pentose is hydrolysed with about 50% of the rhamnose, leaving an unstable macromolecular residue.

THE STRUCTURE OF THE
POLYSACCHARIDE FROM THE
ALGA, ULVA LACTUCA.

The author wishes to express her sincere thanks to Dr. M. Georg for her advice and encouragement during the supervision of this research.

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INTRODUCTION

The investigation of algal polysaccharides has developed during this century and revealed a variety of structural types. Like other polysaccharides, they are hydrolysed to simple sugar units by the action of acids. Early investigators identified some of the sugars present in the hydrolysates by means of derivatives such as osazones and during the same period fairly specific colour reactions with phenols were established for detecting ketoses, uronic acids, etc., and methods were developed for the quantitative estimation of certain sugar types, e.g. by distillation with hydrochloric acid. More precise information, particularly with regard to the linking of the sugars in the macromolecule, followed with the application of methylation techniques to polysaccharides, and the development of periodate oxidation for α -glycol groups and chromatographic analysis of sugars and sugar derivatives, within recent years, provides new and powerful methods for confirming the results of methylation and for elucidating finer details of structure.

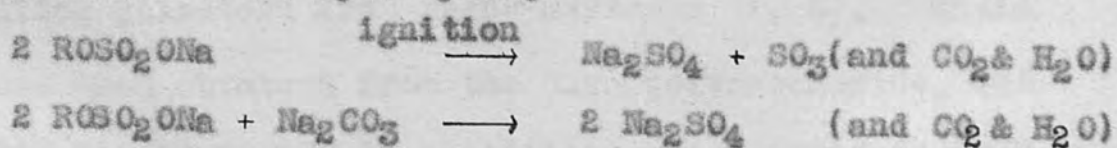
At the present time the survey of algal polysaccharides is very incomplete, the red (rhodophyceae) and brown (phaeophyceae) have been more extensively investigated than the green (chlorophyceae) and blue-green (cyanophyceae) seaweeds, but generalizations are based on scant evidence.

Many of the polysaccharides are acidic, due to the presence of sulphuric acid ester or carboxylic acid groups. Different sugar types occur, the hexoses glucose and galactose, pentoses and methyl-pentoses, although, so far, none of those discovered are exclusive to algal carbohydrates and structural investigation follows along similar lines, and has many difficulties in common with work in other branches of polysaccharide chemistry.

Sulphate esters.

Sulphate esters were shown to be present in the polysaccharides from many red and brown algae by Haas et al. [1, 2]. Qualitative tests showed that the ash from ignition of the polysaccharides consisted mainly of metal sulphate, and was not removed by dialysis of the polysaccharide. The original materials gave ionic reactions for metal but not for sulphate, although these were obtained after hydrolysis with acid or alkali.

When the salts of sulphate esters are ashed alone, only half of the sulphate present is retained in the ash, but all the sulphate is retained if the substance is ignited in the presence of excess sodium carbonate, or estimated in solution after total hydrolysis.



Quantitative estimations of sulphate under these

conditions were carried out and the sulphate in the ash from the salts was found to be approximately half that obtained by the other two methods; this was confirmed independently by Lunde Heen & Oy [5].

Corresponding analyses on the *Ulva Lactuca* polysaccharide [4] showed the presence of sulphate ester, although accurate figures could not be obtained in earlier investigations owing to difficulties in purification, and owing to the presence of carboxylic acid grouping which also retains sodium in the salt form of the polysaccharide.

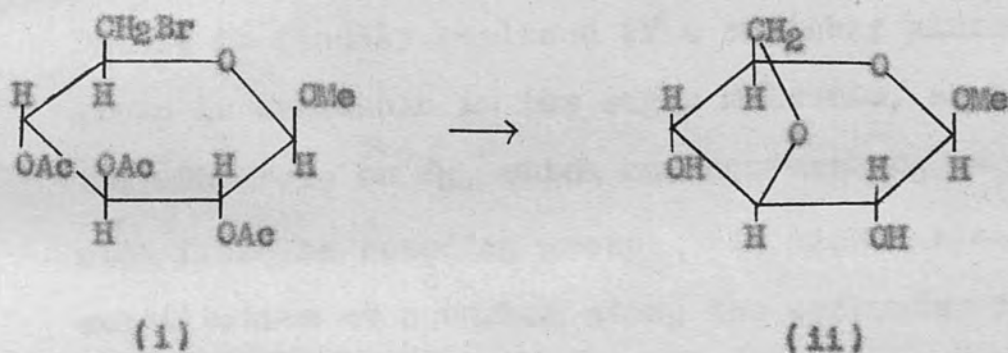
It is noteworthy that galactose is almost always found in polysaccharides containing sulphate ester. In many cases the sulphate has not been proved to be attached to this sugar, but when the carbohydrate consists mainly of galactose, e.g. Agar [5], *Iridaea Laminaroides* [6], the sulphate must be attached to it and the isolation of anhydro-galactose derivatives (see next section) confirms this. *Puccidia* seems to be an exception for ~30% is accounted for as fucose, metals and sulphate [3].[†]

Galactose may be detected by oxidation with 25% nitric acid, when insoluble mucic acid is formed and can be filtered off. Phenylmethyl hydrazine is also used for separating galactose from sugar mixtures [7, 8]. Mucic acid has been obtained from the *Ulva* polysaccharide, but not with the ease, nor in quantities which indicate that 4.1% galactose, 3.3% uronic acid and 1.5% xylose have now been detected [62].

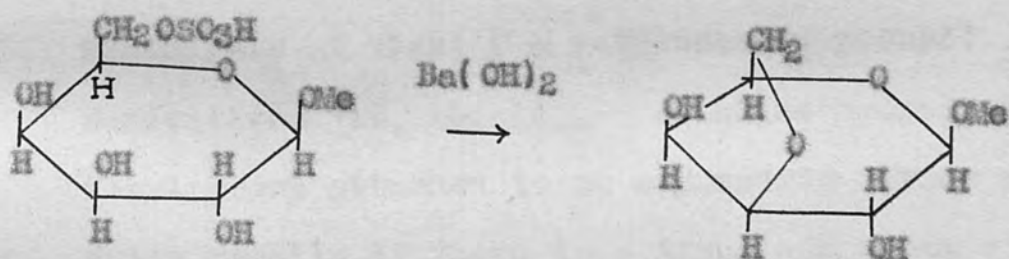
galactose is a major constituent.

Hydrolysis of sugar sulphate esters.

The discovery of unusual derivatives from sulphate ester polysaccharide i.e. 3:6-anhydro galactose derivatives from agar [9] stimulated the investigation of simple sugar sulphates. A 3:6-anhydro compound, methyl-3:6-anhydro- β -D-glucoside, (ii) was first obtained by Fischer and Zach [10] on alkaline hydrolysis of methyl-6-bromotriacetyl- β -D-glucoside (i)



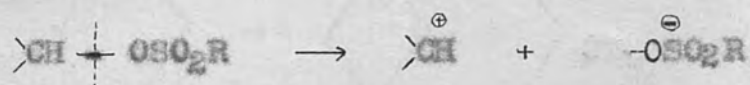
Anhydro compounds are also obtained on hydrolysis of suitable sugar sulphates [11].



(iii) methyl- β -D-galactopyranoside-6-sulphate (iv) methyl-3:6-anhydro- β -D-galactopyranoside

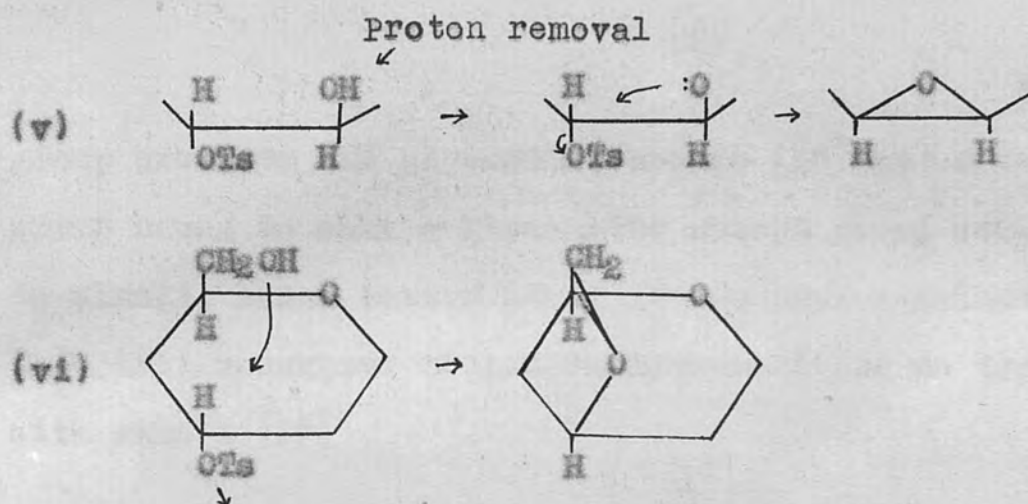
The first stage in the hydrolysis of (i) is the loss of Br^- with the formation of a carbonium cation. When esters

of sulphuric or sulphonic acids are hydrolysed it is known that the oxygen of the ester linkage remains attached to the acid group so that a carbonium cation is formed

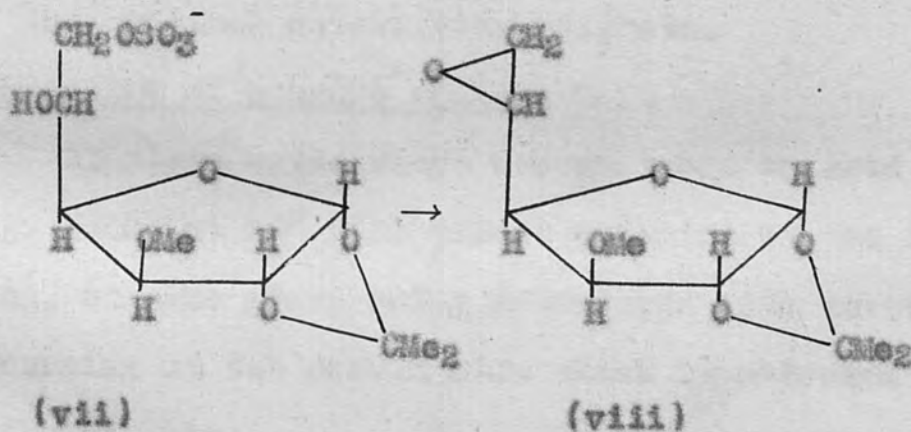


and the mechanism of anhydro ring formation is undoubtedly similar in all these cases.

During hydrolysis there is very little tendency for the lost -ve group to be replaced by a -ve ion from the hydrolysing agent (OH^- from $\text{Ba}(\text{OH})_2$ or OMe^- from NaOMe) But it is readily replaced if a suitably placed nucleophilic group is available in the sugar molecule, such as -O from the -OH group on C_3 , which can approach C_6 on the opposite side from the receding group. In such nucleophilic substitution at a carbon atom, the carbonium ion is never actually free and inversion of configuration occurs. That this mechanism operates in anhydro ring formation has been amply confirmed, mainly from an investigation of the hydrolysis of Tosyl (= p-toluene sulphonyl-) derivatives [12, 13, 14]. Alkaline hydrolysis of a tosyl group attached to an asymmetric carbon atom occurs quite readily if there is a trans -OH group attached to an adjacent carbon atom, when an ethylene oxide ring structure is formed (v), or if there is an -OH group so situated that a hydrofuranol ring can be formed, linked in the position trans to that occupied by the tosyl group (vi)

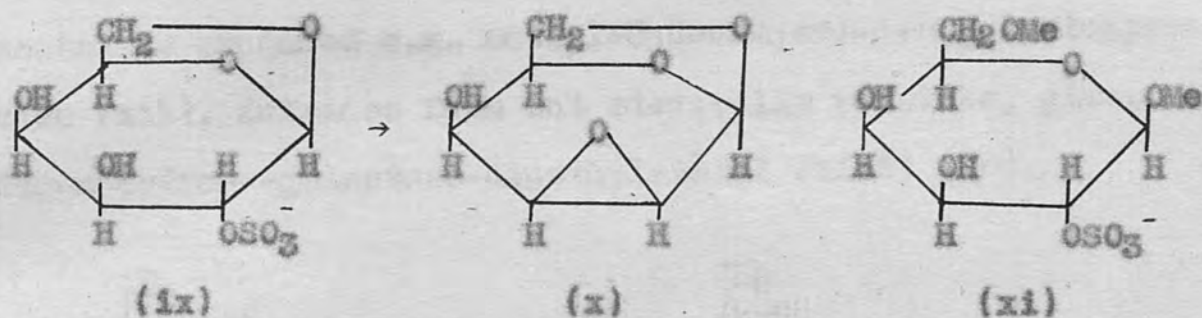


When there is no suitably placed free -OH group, hydrolysis does not occur readily. Percival et al. have shown that the same general conclusions apply to the alkaline hydrolysis of sulphate esters [15, 16, 17]. 3:6-hydro-furanol rings are formed readily e.g. (iv) and ethylene oxides have also been obtained e.g. by treating barium 3-methyl-1:2-monoacetone-glucofuranose-6-sulphate (vii) with NaOMe, at 40°, 1:2-monoacetone-5:6-anhydroglucose (viii) is obtained, substitution at C₃ preventing the formation of a hydrofuranol ring.



The 2-sulphate group of barium 1:6-anhydro- β -D-galactopyranose-2-sulphate (ix) can be hydrolysed yielding a 2:3-anhydro structure (x) when the stable 1:6-anhydro

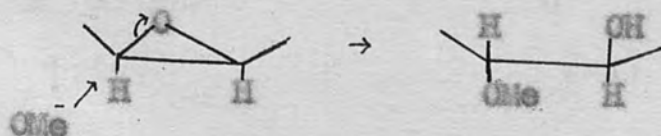
group protects the glycosidic centre [16] but a 2-sulphate group seems to make a glycosidic methyl group very labile to alkali, since barium 6-methyl- β -methyl-D-galactopyranose-2-SO₄ (xi) undergoes extensive decomposition on treatment with alkali [17]



This work has important bearing on the investigation of sulphate containing polysaccharides, relatively facile removal of sulphate by alkali indicating that anhydro ring formation is possible in the macromolecule, and the isolation of anhydro compounds assisting to fix the position of the original esterifying sulphate.

Properties of anhydro sugars.

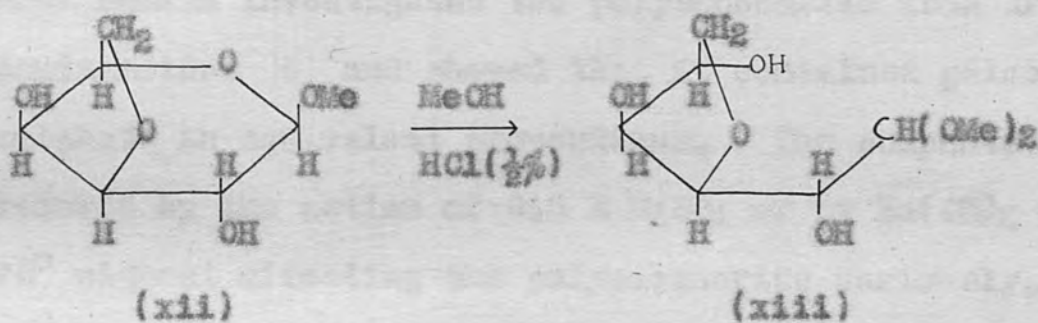
Ethylene oxide rings are unstable to acid and alkali e.g. NaOMe at 90° will effect scission of the threemembered ring, an -OMe group being introduced with inversion occurring on the carbon atom which is attacked by the methoxyl anion.



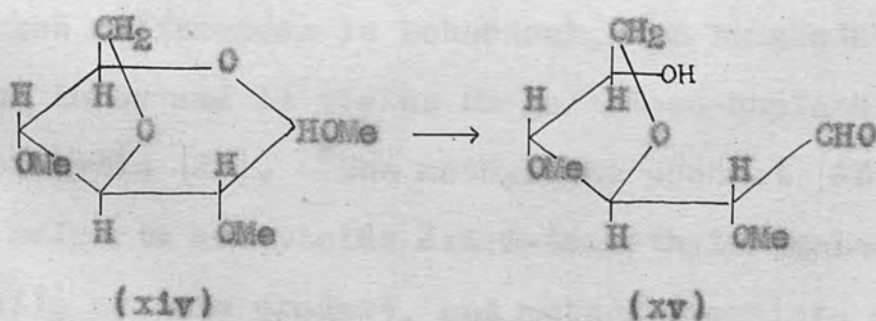
Hydrofuranol rings are more stable; when in

association with pyranose sugar structure, the molecule is slightly strained and changes to the more stable 3:6-anhydro furanose structure if possible; even when methyl glycosides are used, a trace of HCl in various solvents will effect this change [19].

With MeOH/HCl the pyranose ring opens and dimethyl acetal is produced e.g. methyl-3:6-anhydro- β -D-galactopyranoside (xii), furanose form not sterically possible, gives 3:6-anhydro-D-galactose-dimethylacetal (xiii) [20].



Aqueous acid readily hydrolyses the glycoside group with scission of 5 and 6 membered sugar rings to give aldehyde sugars e.g. methyl-3:6-anhydro-2:4-dimethyl-D-glucopyranoside (xiv) yields 3:6-anhydro-2:4-dimethyl-aldehyde-D-glucose (xv)



These products give the reactions of free aldehydes

e.g. restoration of the colour to Schiff reagent [20], a reaction which is frequently used to detect them. Anhydro sugars also give a positive Seliwanoff test (red colour with HCl and resorcinol) which was formerly thought specific for ketoses.

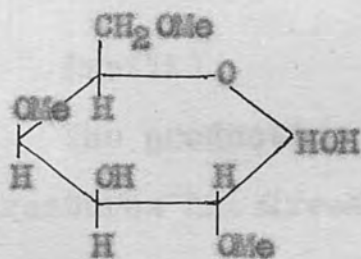
Sulphate ester algal polysaccharides.

Detailed work on the structure of sulphate ester algal polysaccharides has been carried out on products from a few red algae.

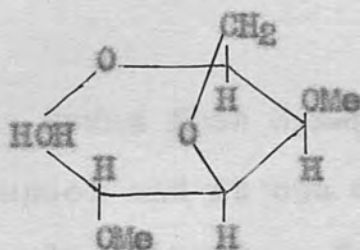
Hassid investigated the polysaccharide from Irideae *Laminaroides* [6] and showed that it contained galactose and sulphate in equivalent proportions. The sulphate was removed by the action of 0.5 N H_2SO_4 or by $Ba(OH)_2$ at 70° , without affecting the polysaccharide seriously. Methylation and hydrolysis of the polysaccharide yielded a dimethyl-galactose and methylation after removal of sulphate gave trimethyl-galactose. Crystalline derivatives were not obtained and there was no suggestion of the formation of anhydro compounds.

Agar is also a polygalactose sulphate [21] but shows marked differences in behaviour, the sulphate content is much lower and it yields DL-galactose-heptacetate by acetolysis [22]. The methylated product [23] contains no sulphate and yields 2:4:6-trimethyl-D-galactopyranose (xvi), as main product, and methyl laevulate, on hydrolysis with aqueous sulphuric acid. Repeated methylation with

Me_2SO_4 and NaOH gave a product of only 30-32 % OMe ; after breakdown by methanolysis the glycoside of (xvi) and 2:4-dimethyl-3:6-anhydro-L-galactose (xvii) were obtained by Percival and Forbes [24] and by Hands and Peat [25].

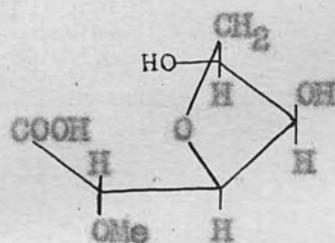


(xvi)



(xvii)

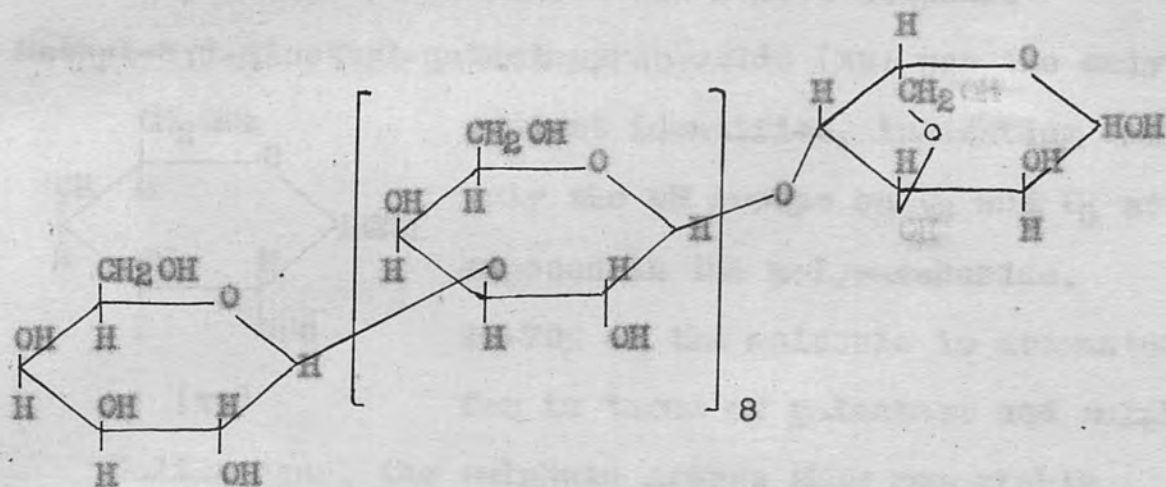
Investigation of the properties of (xvii) showed that it gave a positive Seliwanoff reaction and decomposed to methyl laevulate on treatment with aqueous acid; 3:6-anhydrogalactose was shown [26] to give DL-galactose-heptacetate on treatment with Ac_2O and H_2SO_4 . Thus the earlier observations of Pirie and Percival [28, 23] and the low maximum methoxyl were explained by the presence of 3:6-anhydro-L-galactose units, and the isolation of (xvi) indicated D-galactose to be linked in the 1 and 3 positions. Jones and Peat [27] obtained, in addition, methyl-2:3:4:6-tetramethyl-D-galactopyranoside from the non-reducing end group and 2:5-dimethyl-3:6-anhydro-L-galactonic acid (xviii) from an agar fraction of high ash content, which had been dialysed under acid conditions.



(xviii)

The production of aldehyde forms from 3:6-anhydro structures has already been discussed and it can easily be seen how this galactonic acid could arise from 3:6-anhydro-L-galactose by ring opening under acid conditions and subsequent ready oxidation of the -CHO group. The 3:6-anhydro group presumably being formed on removal of sulphate from C₆.

Quantitative assay of the relative proportions of these methylated derivatives indicated a straight chain structure of 9 D-galactose units, linked in the 1:3- positions, terminated by L-galactose linked in the 4 position (xix) the last residue appearing as 3:6-anhydro, as shown, and L-galactonic acid derivatives, a structure in agreement with the absence of attack by periodate acid [28].



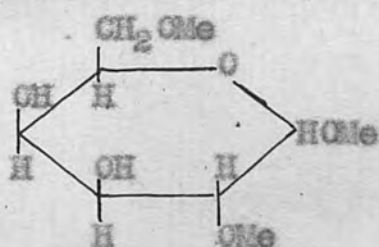
(xix)

Carbohydrate containing galactose and sulphate is also obtained from *chondrus crispus* and *gigartina stellata* (caragheen). In this case sulphate is tenaciously retained and a new acidic derivative has been isolated. The early work of Haas [1, 2] established the presence of sulphate. Percival et al. have shown these polysaccharides to be very similar, except for the presence of glucose in the former; *chondrus crispus* [29], *gigartina stellata* [30, 31].

By acetylating and then methylating the polysaccharide with NaOH , Me_2SO_4 repeatedly, a product of $\sim 20\%$ OMe could be obtained. Direct methylation of the polysaccharide with NaOH , Me_2SO_4 was not successful, nor could the % OMe of the above product be increased with these reagents, or by the EI method. On hydrolysing the product with 0.5N oxalic acid at 100° , 2,6-dimethyl-galactose was obtained.

Methanolysis was difficult and prolonged repeated treatment with MeOH/HCl in the presence of BaCl₂ was necessary to effect breakdown and remove sulphur.

Methyl-2:6-dimethyl-galactopyranoside (xx) was the only



(xx)

product identified, indicating that only the OH groups on C₂ and C₆ are exposed in the polysaccharide.

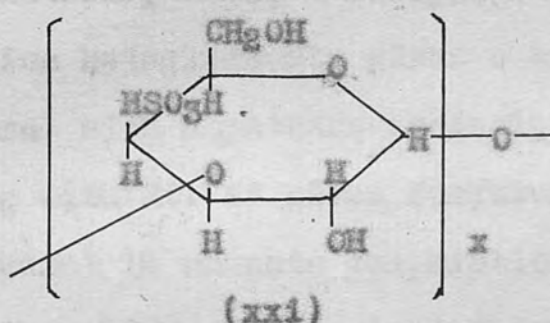
60-70% of the molecule is accounted for in terms of galactose and sulphate.

Unlike agar, the sulphate groups show remarkable resistance to hydrolysis. 72 hours treatment with N NaOH at 100° only removes 62% of the sulphate and practically none was removed during methylation.

By analogy with the behaviour of simple hexose sulphates, Percival assumes that the sulphate group is probably attached to C₄ (if it is linked to galactose and not to the unknown part of the molecule), since any other arrangement would permit the formation of anhydro rings when SO₄ removal would be expected to be much easier. Anhydro derivatives have not so far been isolated, although positive colorimetric Seliwanoff "ketose" reactions given by the unidentified portion may be due to these substances.

It follows from this that the galactose is probably linked through the 1 and 3 positions, as in agar, although the situation of the -SO₄ group is almost certainly

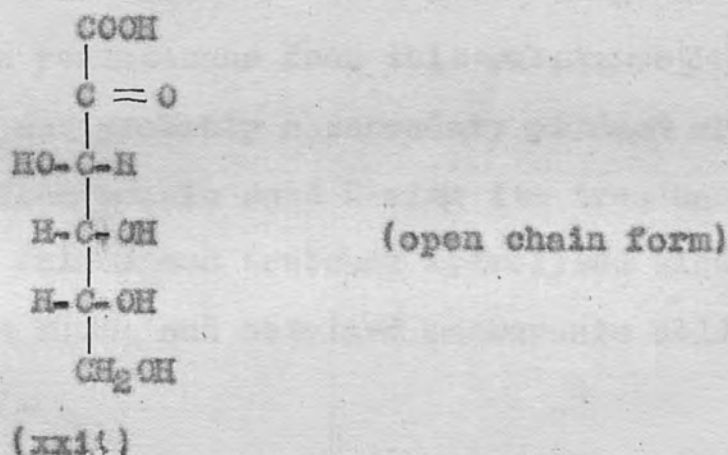
different from that in agar, e.g. (xxi)



Absence of reaction with periodate ion, lends support to this structure [16].

The behaviour of the sulphate group in the *Ulva* polysaccharide seems to resemble that of carageen much more closely than that of agar, comparable difficulties in purification have also been experienced.

Young and Rice [32] have isolated keto-gluconic acid (xxii), via. its diacetone derivative, from *gigartina stellata* polysaccharide after oxalic acid hydrolysis in nitrogen.



The presence of this rare substance has not been confirmed by the British workers, nevertheless the reactions of ketogluconic acid are worthy of note, and

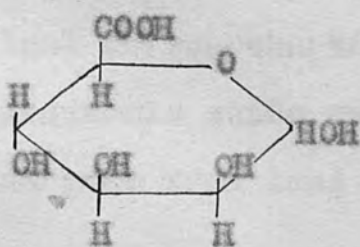
demonstrate the uncertainty which must be attached to colour reactions, etc., when applied to unknown mixtures.

Calcium ketogluconate gives a brownish red (red when impure) with naphthoresorcinol, similar to ketoses. On boiling with HCl it gives furfural (estimated as phloroglucide) in amounts comparable with those obtained from uronic acids; under similar treatment CO₂ evolution was not investigated, but undoubtedly the substance would readily lose CO₂ at the same time.

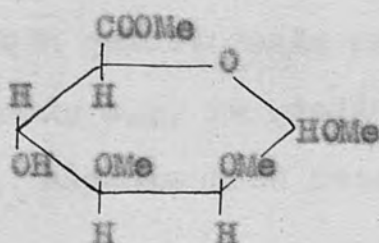
Algal polysaccharides containing Uronic acid.

Uronic acids may be detected by the evolution of CO₂ on boiling with 12% HCl and by colour reactions, such as that with HCl and naphthoresorcinol [7].

Alginic acid, the first algal polysaccharide to be investigated is a polymannuronic acid, and occurs quite widely in brown algae, e.g. *Fucus serratus* *Laminaria* and *Macrocystis* [21]. Hoagland and Lieb isolated pentosazone from this substance [34], but the pentose was probably a secondary product arising by loss of CO₂ from uronic acid during its treatment with hot acid. Nelson and Cretcher hydrolysed alginic acid with cold 80% H₂SO₄ and obtained mannuronic acid (xxiii) [35, 36].



(xxiii)



(xxiv)

Quantitative estimations of equivalent weight and CO₂ evolution with HCl indicated that there was no other major constituent.

The manner in which the simple units are linked in the macromolecule was elucidated by Hirst et al. [37]. Alginic acid itself was found to be extremely resistant to acetylation or methylation by the usual methods but a degraded alginic acid i.e. partially broken down by MeOH/HCl treatment, could be methylated using thallium hydroxide and ethoxide, followed by MeI, Ag₂O. Hydrolysis was exceptionally difficult but 2,3-dimethyl-D-mannuronic ester (xxiv) was obtained after methanolysis with MeOH, HCl.

The extreme resistance to hydrolysis excludes the possibility of furanose ring form so that alginic acid consists of D-mannuronic acid residues linked lineally through positions 1 and 4, the configuration at C₁ is probably β as the polysaccharide shows a large negative rotation and is in agreement with X Ray investigations on alginate fibres.

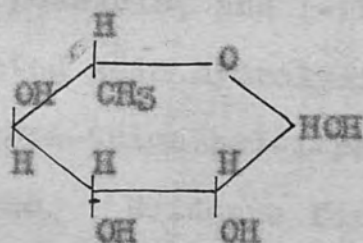
Alginic acid has only recently been partially acetylated using ketene [38].

Mannuronic acid has so far only been obtained from algal polysaccharides but both galacturonic and glucuronic acids occur commonly e.g. in pectic material and gums from land plants. Alginic acid resembles the

other polyuronides in its resistance to methylation and subsequent hydrolysis.

The Ulva polysaccharide is thought to contain uronic (or ketogluconic) acid and is resistant to methylation. The thallium method has been tried by another investigator [39], without success, probably owing to the presence of sulphate.

Alginic acid is often accompanied by polysaccharide containing pentose, most commonly the methyl-pentose L-fucose. Polyfucose also occurs with sulphate containing material. The methyl pentose, L-rhamnose (xxv) has been shown to be



(xxv)

present in the Ulva polysaccharide [4], this is the first instance of the occurrence of Rhamnose in algal polysaccharides, although it is known to occur in gum arabic and mucilages.

SUMMARY OF INVESTIGATIONS ON THE ULVA POLYSACCHARIDE.

No investigation of the polysaccharide material of other green algae has been published. Earlier work on the polysaccharide from the green alga, *Ulva Lactuca*, by M.M.T. Plant and E.D. Johnson [4] involved isolation of an acid polysaccharide as its sodium salt. Attempted fractionation by precipitation into alcohol at different pH values did not yield products which showed marked difference in properties, except those which would be expected from variation in the proportion of acid and salt form. The presence of sulphate ester was established although quantitative assay was not possible, and L-rhamnose was identified in the methylated acid hydrolysis products as the crystalline 2:3:4-trimethyl-L-rhamnose-anilide and -phenylhydrazone. Evidence for uronic acid was inconclusive, ~1000 g. of various fractions yielding 44 g. CO₂. Quantitative estimation of methyl pentose, by distilling with 12% HCl, and weighing methyl-furfural-phloroglucide, assuming uronic acid to be absent, indicated that rhamnose formed 20-25% of the polysaccharide, the proportion being greater in material soluble in 50% acidified alcohol than in the insoluble fraction; these estimations also indicated that small quantities of pentose and/or uronic acid were present in both

fractions but not present in simple hydrolysis products from these fractions.

The isolation of 2:3:4:6-tetramethyl -D-galactose-anilide from methylated hydrolysis products by M.M.T Georg (Plant) [39], established the presence of galactose although it could not be obtained as the phenyl-methyl-hydrazine derivative.

Practically complete purification, except for the separation of a small amount of very tenaciously held protein, has been achieved in recent work by D.M. Hardy [40] who has determined the sulphate and uronic acid content, equivalent weight and periodate uptake of the product. Chromatographic analysis of fractions obtained after methanolysis has established the presence of D-xylose, isolated as crystalline dibenzylidene-D-xylose-dimethylacetal; also uronic acid and sulphate ester, in agreement with other work; and also indicates that glucose, but not galactose, is present in the soluble methanolysis products.

In the present work, purification of the Ulva carbohydrate has been carried out. After nitric acid oxidation mucic acid has been obtained, but not with the ease, nor in quantities indicating simply bound galactose as a major constituent; evidence for saccharic acid was also obtained, in

agreement with the chromatographic work of D.M. Hardy.

The polysaccharide has been methylated by repeated treatment with Me_2SO_4 , NaOH, the chloroform soluble fraction being further methylated with MeI, Ag_2O to a product with OMe, 31%. Prior treatments to give an acetylated product or sulphate free product, suitable for methylation, were not successful. The properties of the fully methylated and CHCl_3 insoluble fractions have been investigated. The former fraction was subjected to methanolysis and the products separated and investigated; evidence for 2:3:4-trimethylrhamnose and no other simple end group, also evidence for partially methylated hexose, rhamnose, anhydro structures and an ester was obtained.

On autohydrolysis biuronic acid seems to be preferentially hydrolysed, leaving an unstable macromolecular residue.

From these results structural features of the polysaccharide are discussed (P.94), calculations based on SO_4 content, Equ. wt., etc., are subject to some error, owing to the contaminating protein, part of which persists throughout the methylation of the polysaccharide.

It also seems probable that the polysaccharide used by E.D. Johnson and in much of the present work is somewhat different from that used by D.M. Hardy and for

the preparation of the second batch of glycosides, particularly with respect to pentose content. Ulva fronds used in the earlier work were long and thin and had been collected from the South of England in early summer; the material used later consisted of broad fleshy fronds and was obtained from Millport, Scotland, in late summer. It is quite possible that the polysaccharide would vary slightly with the habitat and time of year, particularly if one were a haploid and the other a diploid form.

EXTRACTION AND PROPERTIES OF THE POLYSACCHARIDE.

Much of the pigment was removed from the fronds by extraction with aqueous acetone; the acidic polysaccharide was then extracted as sodium salt by boiling the fronds with dilute sodium carbonate, purified by dialysing the extract in "Cellophane" against dilute acetic acid and distilled water, converted to sodium salt, concentrated and precipitated as a white fibrous product by pouring into alcohol.

Properties are described on P. 48

The free acid form obtained at the end of dialysis was not completely free of inorganic material (5 - 6% ash, mainly CaSO_4), but rather than further prolong dialysis it was found more effective to remove the last traces of metallic ions by an ion exchange reaction, using the resin "Zeocarb 215."

The reaction, $\text{Resin H} + \frac{1}{2} \text{Ca} = \text{Resin } \frac{1}{2} \text{Ca} + \text{H i.e.}$ the exchange of metal for hydrogen, is practically stoichiometric up to 0.05 - 0.10 N. [41]. This was carried out by another experimenter [40] and after further dialysis a product of 0.8% ash, pure acid form, was obtained. Fractionation of the product was not attempted at this point since earlier attempts at fractionation [4] had not yielded products which were significantly different in carbohydrate composition.

NITRIC ACID OXIDATION OF THE POLYSACCHARIDE

Nitric acid oxidation of the polysaccharide was carried out since polysaccharides containing galactose or galacturonic acid give mucic acid, and those containing glucose or glucuronic acid give the corresponding glucosaccharic acid which may be isolated as the potassium hydrogen salt.

Initial oxidation attempts gave only slight indication of the presence of mucic acid, owing to the presence of inorganic material, particularly calcium sulphate, which is of similar solubility. Oxidation was repeated on well dialysed acid form carbohydrate (ash ~6%) and a small quantity of mucic acid was obtained.

An organic acid potassium salt was also isolated and had similar properties to KHSaccharate.

METHYLATION EXPERIMENTSAcetylation of the polysaccharide.

It is generally found that when a polysaccharide can be first acetylated, methylation with simultaneous deacetylation can be effected smoothly and easily. In view of the difficulty of methylating the polysaccharide directly the possibility of preliminary acetylation was investigated. The action of pyridine and acetic anhydride in the cold had been attempted [4]; the main difficulty was that the carbohydrate formed a hard intractable solid in pyridine. However, the method of Carson and MacLay [42] (as used for the acetylation of pectin), was tried as it involved a different method for the preparation of the carbohydrate in pyridine, i.e. the water in a swollen aqueous gel was gradually replaced partly by acetone and then by pyridine; but the carbohydrate still formed hard granules in the latter solvent which were not attacked by acetic anhydride, only yielding a product with less than one acetyl group per two anhydro hexose units after repeated treatment. Much material was lost in aqueous washings, as the product was still water soluble, so that it did not seem advantageous to pursue further the possibility of acetylation prior to methylation.

Baryta hydrolysis of the polysaccharide followed by (a) acetylation, (b) methylation.

The product obtained from the polysaccharide after hydrolysis with baryta was submitted to acetylation and

methylation. Varying conditions of baryta treatment were investigated; material could be isolated in 25-30% yield containing ~10% organically bound sulphate, and barium sulphate which could not be completely removed. When acetylated in the manner described earlier, a hard product of ~12% OAc was obtained. Although there was no marked development of reducing power on hydrolysis and the rotation of the product was still negative, it must have been seriously degraded, for after methylating once with NaOH and Me_2SO_4 there was much loss on dialysis, making it apparent that pretreatment with baryta did not yield material suitable for methylation.

Autohydrolysis (P.89) gave an unstable macromolecular residue, unsuitable for methylation.

Methylation of the polysaccharide.Using methyl sulphate and alkali.

For the methylation experiments, crude sodium salt, precipitated in alcohol, centrifuged, but not dialysed, was used. Inorganic and other impurities of small molecular weight should ultimately be lost in the dialyses involved, any traces of protein not hydrolysed by the alkali would not be expected to become soluble in organic solvents by this treatment.

Initially, the addition of cold 30% NaOH and Me₂SO₄ over ten hours, in an atmosphere of nitrogen, was tried; the quantities of reagents being comparable with those employed for the methylation of agar [27]. After seven treatments, when the product had not changed in appearance during three methylations, the product was worked up and it was possible to extract a small amount of chloroform soluble material (30% OMe) from the solid product obtained, but it was obvious that there had been much loss of organic material.

Possible causes of loss were investigated and the methylation procedure varied in order to improve the yield of product. Hydrolysis of unused methyl sulphate at 100° was omitted, but the NaOH was only partially neutralized so that the mixture remained just alkaline to the end of dialysis; although 50% H₂SO₄ was added with ice cooling it was found that the yield

of product was improved if this stage was omitted entirely.

The effect of warming the methylation mixture with reduction in the time of methylation, after the first treatment, was investigated, since after one treatment the polysaccharide should be somewhat more stable. In comparative experiments, at the higher temperature of 35-40° slightly lower yield of alcohol soluble material of 2% higher methoxyl content was obtained; thus warming speeded up the methylation procedure without significant effect on the product.

The methoxyl figure of alcohol soluble product was not increased after six to seven treatments. The carbohydrate was generally given seven treatments, using the procedure described in the experimental section. In this way a hard yellow white solid product was obtained.

Extraction with organic solvents.

Extraction with CHCl_3 : EtOH:: 10: 1 gave material with 26%OMe in 16% yield; this solvent, when acidified, extracted further material and acidified alcohol extracted a third amount, leaving a dull green swollen residue. This suggested that methylated acidic material was present, only becoming soluble when liberated from the salt form.

In order to obtain as much material as possible for

further treatment the crude methylated product was initially extracted with alcohol to which concentrated HCl was added until a faint acid reaction on moist congo paper was obtained, later it was found more satisfactory to extract only the fraction soluble in acidified CHCl_3 for further treatment (see section on methylation with MeI , Ag_2O).

Yields.

The quantity of alcohol soluble material varied as moisture was not rigidly excluded and its presence undoubtedly increased the yield of soluble material, with corresponding lowering of methoxyl figure and increase in inorganic contaminants.

The yield of chloroform soluble product from large scale methylation was 15-20%, OMe, 30-24%.

The organic content of the insoluble fraction was assayed and indicated that the overall recovery of material was ~85%.

All these fractions were hygroscopic.

Further methylation, using methyl iodide and silver oxide.

After treatment of methylated, alcohol soluble material with MeI and Ag₂O it was possible to extract ~45% of the product with CHCl₃. It was not possible to increase this yield significantly by the use of other solvents without heavy contamination by silver, possibly due to complex formation with fractions containing N or SO₄. By using methylated chloroform soluble material for treatment with Furdie reagents satisfactory yields of product were obtained, the methoxyl figure was not increased beyond 31% during five treatments. There was a relatively large increase after one treatment, suggesting esterification, which was confirmed by the production of MeOH on baryta hydrolysis.

Properties of the methylated polysaccharide.

Pale yellow powder. Ash 4% (Na, Ca, SO₄, trace of AgI).

OMe, 31%; corrected for ash.

C, 50.5%; H, 7.56%; N, 2.40%; corrected for ash

(Weiler and Strauss).

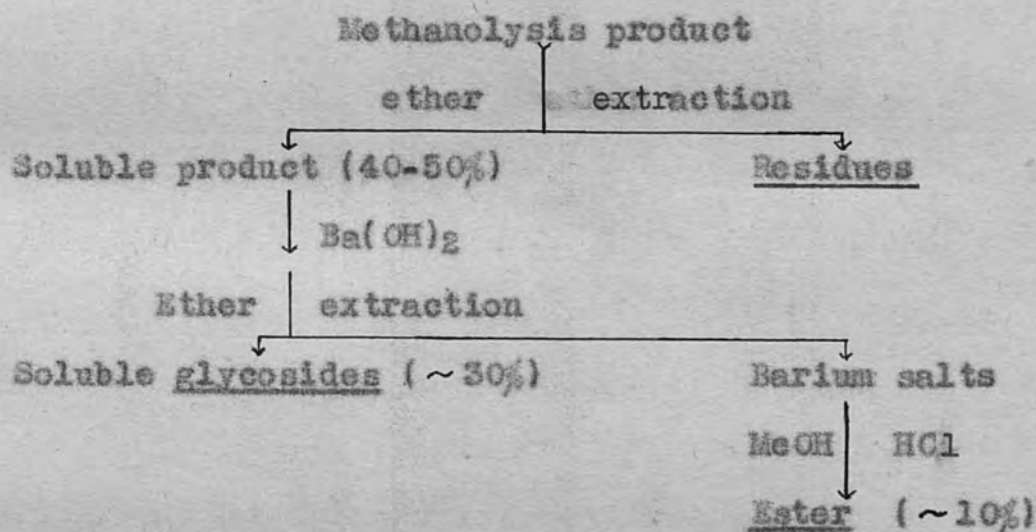
$[\alpha]_D^{16} = -34$ (C = 0.4% in CHCl₃)

Ionic sulphate 0.4%

Total sulphate 3.0%

METHANOLYSIS

Vigorous treatment with hydrogen chloride in dry methyl alcohol was necessary to cause appreciable breakdown of the methylated polysaccharide to ether soluble material; treatment with baryta enabled a barium salt to be separated from glycosides by virtue of its insolubility in dry ether, and the salt was then converted to methyl ester. The separation was carried out according to the following scheme, average results are given.



Rotations could not be observed during methanolysis, but the change in rotation of -20 (starting material) to +20 (ether soluble product) indicated extensive breakdown.

The glycosides did not contain nitrogen and were fractionated in vacuo.

The ester fraction was further purified and investigated separately, it did not contain nitrogen.

The residues were black and extremely viscous;

Nitrogen and silver were present, much of the latter could be precipitated from a solution of residues in MeOH by the action of H_2S .

Investigation of glycosides.

The glycosides were fractionally distilled in vacuo, and the specific rotation, refractive index and methoxyl content of each fraction were measured. Samples were hydrolysed with aqueous acid, losses were heavy where Schiff reactions indicated the presence of anhydro structures, and the detection of laevulinic acid in one such instance agreed with the destruction of anhydro compounds.

In order to identify the reducing methylated sugars, partition paper chromatography and the preparation of anilides was applied, a sample of a major fraction was also fully methylated and investigated. The results showed that all fractions were mixtures; L-rhamnose, isolated as 2:3:4-trimethyl-L-rhamnose anilide after solvent extraction, was the only simple end group present, although anhydro structures, produced by hydrolysis of sulphate, may also form end groups in a branched structure. Evidence for glucose rather than galactose was given by chromatography, but the anilides isolated indicated galactose. Results from chromatography also showed some difference between the two batches of glycosides which were fractionated, the later material probably containing 2:3-dimethyl-xylose.

Investigation of ester.

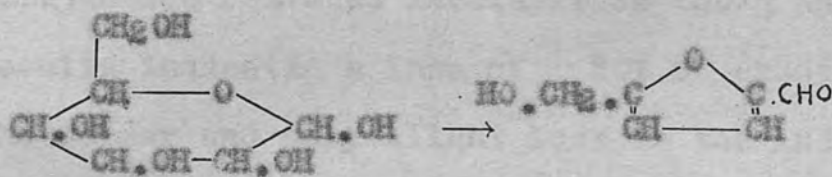
The isolation of ester material confirmed the indications of the presence of -COOH from methylation experiments and furfural estimations. The properties of this fraction suggested a methyl ester of a dimethyl-methyl-hexuronic acid, a crystalline product was obtained, but it was not identical with any established compound of this type; another possibility is that some anhydro hexonic ester is present.

These results are discussed in the last section.

URONIC ACID, FURFURAL AND METHYL FURFURAL ESTIMATIONS.AUTOHYDROLYSIS.

When sugars and many related compounds are boiled with hydrochloric acid, furfural and derivatives may be obtained.

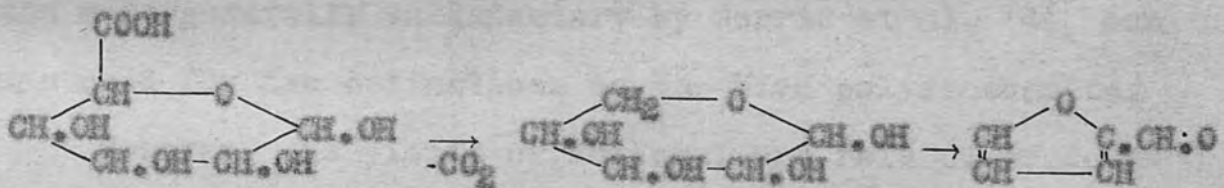
1. Hexoses give very low yields of ω -hydroxy-methyl-furfural.



Hexose

 ω -hydroxy-methyl furfural

2. Hexuronic acids yield carbon dioxide from the carboxylic acid group with probable formation of pentose intermediate which then decomposes to furfural.

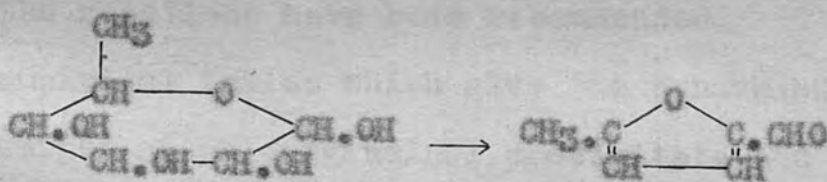


Hexuronic acid

pentose

furfural

3. Pentoses yield furfural (see 2.)
4. Methyl pentoses yield methyl furfural.



Methyl pentose

5-methyl furfural

5. 2-keto hexonic acids yield CO_2 and furfural.

The yield of carbon dioxide in 2. is practically

quantitative and this reaction is used in methods for the estimation of uronic acids. (see P.86).

Determinations on the purified polysaccharide were carried out by D.M. Hardy [41]. The carbon dioxide liberated from fully methylated material and from the methylation residues insoluble in CHCl_3 was determined. Results indicated a loss of ~50% of uronic acid in the former and only slight loss in the latter fraction as compared with the starting material.

Furfural and many of its derivatives give precipitates with solutions of certain reagents, e.g. phloroglucinol, thiobarbituric acid. The former was considered to be the most generally satisfactory by Norris et al. [44] and was used for the estimations on the *Ulva* polysaccharide.

However, the yields of furfural and related products are not quantitative, and the precipitates formed with phloroglucinol are of ill defined chemical composition; hence rigidly standardized conditions for carrying out the reactions have been recommended. Reliable empirical tables which give the equivalence between weight of phloroglucinol precipitate and sugar, under standard conditions, were first drawn up by Krober, Tollens et al., using single sugars (e.g. [45] for methyl pentoses). Later workers have attempted, by discovering the causes of the destruction of methyl furfural for

example, to improve procedure, so that results are less dependent on arbitrary conditions.

Kullgreen and Tyden [46] recommended the addition of salt to 13.15% HCl for the distillation, the salt stabilizing the acid concentrations at this level, for losses of methyl furfural are very dependent on acid concentration [47] which varied during the older procedure of distilling with 12% HCl. Losses due to oxidation can be avoided by working in nitrogen [47]. Quantitative yields of methyl furfural can be obtained by distilling it off in steam at 110° from 13.15% HCl saturated with NaCl [48, 49].

Norris et al. carried out estimations on various sugars and obtained expressions relating weight of sugar to weight of phloroglucinol precipitate, P.

e.g. For anhydro rhamnose, R.

$$R = 1.0433P + 0.0118 \pm 0.0003$$

for 0.04 g. to 0.16 g. Rhamnose.

But these investigators pointed out that the conversion factors are very dependent on conditions and stressed the necessity for investigators to determine the constants for their own conditions.

They also investigated mixtures of two sugar types,

e.g. methyl pentose and uronic acid, such as occur in natural polysaccharides. In general the course of the distillation of one component was affected by the presence of the other, so that expressions obtained for single sugars cannot be applied with accuracy. With uronic acid as one component, a satisfactory relation could be deduced if the uronic acid was independently estimated by CO_2 ^{evolution.} Hexoses increased the weight of phloroglucinol precipitate owing to the production of small amounts of OH-methyl-furfural.

In the present work, the method of Norris was modified for use with smaller quantities of sugars, using rhamnose, arabinose and galacturonic acid. It was clear that the method of Van der Haar [50] for separating methyl furfural phloroglucide precipitate from furfural phloroglucide by virtue of the solubility of the former in 96% alcohol at 60° was unreliable (as stated by Norris) although it was used to give a comparative measure of rhamnose lost and retained in the macromolecule during autohydrolysis.

The appearance and solubility of the phloroglucide precipitates from the Ulva polysaccharide were very like those from arabinose and galacturonic acid, although the

proportion of alcohol soluble material was much greater, confirming the presence of methyl pentose. In the earlier work [4] there was no indication of pentose in the methylated hydrolysis products, and the evidence for uronic acid was inconclusive, but this work suggested that uronic acid and/or pentose was present, and was supported by the present methylation and chromatography studies [40].

Autohydrolysis and furfural estimations.

Since there was evidence that the proportion of methyl pentose in alcohol soluble hydrolysis products was greater than that in the original [4] there was the possibility that it could be preferentially hydrolysed from the macromolecule. Autohydrolysis of the acid form of the *Ulva* polysaccharide was carried out and the product dialysed; the methyl pentose and pentose or uronic acid in dialysable and non dialysable fractions were approximately assayed.

The results indicated a preferential hydrolysis of uronic acid (or pentose), and that di- and possibly tri-saccharide fragments must have been liberated on autohydrolysis, the uronic acid may be liberated as aldobionic acid as is found after the hydrolysis of

various plant gums and mucilages e.g. gum arabic [51].
The non-dialysable portion contained sulphate ester.

EXPERIMENTAL SECTIONEXTRACTION AND PURIFICATION OF THE POLYSACCHARIDE
FROM ULVA LACTUCA.

Ulva Lactuca was obtained from the Marine Biological Station, Millport.

Removal of pigments.

Pigments were extracted from the fronds by covering them with 85% acetone in shallow tanks and allowing them to stand exposed to sunlight for 3-4 days, stirring occasionally. Three treatments yielded pale yellow coloured fronds which were dried in air.

Original extraction procedure.

Initially the procedure developed by E.D. Johnson [4] was employed with slight modification.

i.e. 100 g. dry fronds were heated with 8 L 0.5% sodium carbonate solution on a boiling water bath for 6 hours and the extract filtered off through calico. The fronds were further extracted by three treatments with 8 L 0.25% sodium carbonate, involving 30 hours more heating. The combined extracts were concentrated on a water bath to about 12 L, filtered through fine cloth to separate from a sludge, which was mainly inorganic, and further concentrated to a volume of approximately 600 ml. The polysaccharide was precipitated in a white fibrous form by pouring the dark coloured, thick solution in a fine stream into

5 L. alcohol which was rapidly stirred; the final alcohol concentration being 88-90%.

The only alteration to this procedure was that concentrated hydrochloric acid was added to the sodium carbonate solution during concentration, in order to reduce the alkalinity and hence minimise the hydrolysis of sulphate ester groups known to be present. The solution was kept alkaline to litmus, the total volume of concentrated HCl used initially was ~ 30 ml. equivalent to $\frac{1}{5}$ of the sodium carbonate used.

The product was kept under alcohol until required. It could be worked up to a fairly white solid by centrifuging off the alcohol, triturating twice with alcohol and then with ether.

This crude polysaccharide was found to have a very high inorganic content, ash 40-50% of dry weight. A large part of this inorganic material consisted of sodium chloride and sodium carbonate, which have only slight solubility in alcohol (0.3 gL^{-1} NaCl, 0.2 gL^{-1} Na_2CO_3 in 90% alcohol) but which should be removed by dialysis against water; Ca^{++} and SO_4^{--} were also known to be present.

Dialysis experiments for the purification of the polysaccharide.

"Cellophane" casings were used for dialysis. When dry they are easily damaged by contact with brown paper etc., and so were stored in a damp atmosphere (over 1%

formalin solution) when not in use.

The performance of the casings was first investigated by dialysing a solution containing known amounts of sodium chloride, calcium sulphate, glucose and starch against distilled water. The solution inside the casing and the dialysates were tested for the substances used.

Glucose was estimated by hypiodite titration, the loss involved on concentrating was corrected for by carrying out a comparable concentration of a standard solution; ions were assayed by suitably concentrating and diluting until the limits of sensitivity of the following tests were reached, and by comparing with standard solutions.

Cl' . 0.8 ml. (2 drops) 3% AgNO₃ solution added to 1 ml. Cl' solution, cloud detectable at 1×10^{-3} g. Cl' L⁻¹.

SO₄" 0.8 ml. (2 drops) 12% BaCl₂ solution added to 1 ml. SO₄" solution, cloud detectable at 7×10^{-3} g. SO₄" L⁻¹.

Ca" 2 drops saturated (NH₄)₄.Fe(CN)₆ solution + 1 drop alcohol added to 1 drop Ca" solution, cloud detectable after $\frac{3}{4}$ min. at 5×10^{-5} g. Ca" L⁻¹.

Results showed that the inorganic ions, glucose and a small proportion of the starch (giving a red colour with I_2) dialysed readily and that the remainder of the starch (I_2 colour, blue) was retained through prolonged dialysis.

A solution of the crude polysaccharide, 8 g. organic material, was dialysed against distilled water, and then against dilute HCl of pH 3 (bromphenyl blue), the pH of the acid form of the polysaccharide [4]. The loss of inorganic ions was estimated approximately using the nephelometric tests, P.42.

	After 2 days	2 days	6 days	3 treatments \times HCl (pH 3)		
Cl	0.4 g.	0.015 g.	0.02 g.			
SO ₄	0.69 g.	0.01 g.	0.02 g.	0.016 g.	0.016 g.	0.016 g.
Ca	0.03 g.	0.001 g.	0.001 g.	0.01 g.	0.004 g.	

Final yield 6 g. organic material i.e. ~75%.

The loss of 25% organic material on dialysis is not mainly carbohydrate in nature as Molisch tests on dialysates were only slightly positive. The steady loss of sulphate is probably due to the fact that solid $CaSO_4$ is present and is only removed slowly because of its low

solubility (0.2 at 18°). Dialysis against dilute HCl removed calcium more rapidly but prolonged exposure to mineral acid was not considered safe for the polysaccharide, particularly if it contained uronic acid.

A product worked up as sodium salt gave 22% ash on ignition. A product worked up as acid, by precipitating a solution ^{of sodium salt} at pH 3, in alcohol, gave 11% ash on ignition, but this was probably partly sodium salt, and would contain sodium chloride as this was not removed by dialysis; Ca⁺⁺ and Fe⁺⁺⁺ were also present.

Samples of sodium salt purified in this way, (23% ash) were ignited and the sulphate estimated gravimetrically as BaSO₄.

On ignition without sodium carbonate, 9% SO₄

" " with " " , 13% SO₄

The ash from sodium salt which had only been dialysed for a short period against distilled water was found to contain 22% phosphate (by "molybdenum blue" test, estimated colorimetrically). In view of the presence of calcium and phosphate it was obviously necessary to dialyse against a solution sufficiently acid to dissolve calcium phosphate. Dialysis against acetic acid was therefore tried as this would not affect the carbohydrate.

The concentrated sodium carbonate extracts were not precipitated into alcohol, but the solution was neutralized and dialysed:-

(1) Against rapidly flowing tap water for 24 hours; this removed much of the NaCl and provided that the flow was fast, CaSO_4 did not precipitate in the "cellophane".

(2) Against flowing dilute acetic acid.

(3) Against flowing distilled water.

Samples of the acid form polysaccharide solution were withdrawn at this point and could be concentrated at 80° without charring. On ashing the dry solid, the % ash of the acid could be obtained. Ideally this product should not leave any residue on ignition. The % ash was used as a criterion of improvement in purification as (2) and (3) were varied. In (2) $\text{N}/100$, $\text{N}/50$ and $\text{N}/25$ AcOH were tried and it was found that three days dialysis against $\text{N}/50$ AcOH was necessary for the complete removal of phosphate. In (3) the time of dialysis against distilled water was varied from three to six days, ash figures showed only slight improvement after four days. (see overleaf).

Assistance from Miss D.H.M. Saunders in the experimental work involved in extracting the polysaccharide, etc., is gratefully acknowledged.

e.g.

46.

Batch	HAc dialysis	Distilled water dialysis	Ash from acid form	Ash from sodium salt.
62	N/100 3 days	3 days	5.9% (30%Ca 33%SO ₄ 1.5%PO ₄)	26% pptd pH 9
63	N/50 2½ "	"		21.1% " " 11
	3 "	"	5.5% (PO ₄ = 1% of ash)	
		4 "	5.5%	
69	N/50 3 "	5 "	5.4% (PO ₄ = ½% of ash)	
		6 "	5.4%	

Later batches were dialysed against N/25 HAc for 1 day, N/50 HAc for two days and distilled water for 4 days and gave figures generally varying between 5.5 and 6.0% ash in acid form. This ash consisted mainly of CaSO₄; Ca was removed by the ion exchange resin "Zeocarb"215 in its acid form and the SO₄ by dialysis (D.M. Hardy [40]).

For precipitation the solution was made alkaline with 2 N. NaOH; it was necessary to bring the pH up to 9.5 (Thymol violet) to obtain a precipitate which settled well in alcohol. This required ~35 ml. 2N NaOH. On the basis of a maximum yield of 21g. NaSalt (corrected for CaSO₄ present) and known equivalent weight determinations, 380 g. acid form requiring 23 g. Na for neutralization [40], this is ~5 ml. in excess of the theoretical amount required for neutralization. The carbohydrate separated easily as a white solid when the final alcohol concentration, after precipitation, was 90-94%. The supernatant alcohol

was much less coloured than that obtained with crude material, although it was cloudy; however only a small amount of badly coloured carbohydrate material could be obtained on concentrating the alcohol from three batches; this was not retained.

Final procedure.

100 g. dry pigment extracted fronds were treated with sodium carbonate as hitherto. Each extract was allowed to stand before concentration and addition of HCl, when it could be decanted from a precipitate which was mainly acid soluble CaCO_3 . When the volume of the combined extracts was about 12 L. it was again decanted from a residue which contained less than 30% organic material. During the concentration 12 L. to 600 ml., much more HCl was added so that the total volume (~190 ml.) was equivalent to all the Na_2CO_3 used. The resultant solution, which was still alkaline, was made neutral to litmus and dialysed against rapidly flowing tap water for 24 hr., against flowing $\text{N}/25$ HAc for 24 hr., $\text{N}/50$ HAc for 48 hr. and distilled water for four days.

The solution was neutralized and concentrated to ~ 400 ml., the pH adjusted to 9.5 and the solution poured into ~ 4 L. alcohol so that the final alcohol concentration was not less than 90%. The white fibrous product could be converted to a white powder by trituration with alcohol and ether.

Yield 20g. sodium salt from 100 g. fronds (corrected for CaSO_4)

Ash 21.7%

SO_4 14.2% of sodium salt (" " ")

$[\alpha]_D^{21} = -47$ (C = 0.35% in H_2O)

(Figures varied slightly for different batches).

With iodine solution, the acid form of the carbohydrate gives an intense blue-violet colour, similar to that given by starch, which persists on dilution. The salt form gives only a slight colouration.

NITRIC ACID OXIDATION.

Oxidation of hydrolysis products.

Hydrolysis of the sodium salt form of the polysaccharide was carried out by boiling with 2 N H_2SO_4 for 30 hr. (reducing power, 1 g. = 25 ml. 0.1 N I_2). The acid was neutralized with baryta and the filtrate concentrated.

0.7 g. product was oxidised by 15 ml. 25% HNO_3 (D = 1.5) at 70° , conditions which are used for the oxidation of lactose, but only inorganic crystalline fractions were obtained on concentrating. The hydrolysis product was extracted with 95% alcohol in order to reduce the amount of inorganic material and the extracted sugars were oxidised with HNO_3 at 65° . After slow evaporation two crops of inorganic material were removed, and on standing at room temperature another crystalline fraction which contained organic material was obtained, this darkened at 160° and showed some softening at 190° , but contained inorganic

material as it left an appreciable ash on ignition.

Direct nitric acid hydrolysis and oxidation of the polysaccharide.

Dialysed acid-form polysaccharide (ash 6%) was used for subsequent oxidations as the separation of mucic acid, which seemed to be indicated above, should be easier with less inorganic material present. The procedure was based on that of Doree [52] for the isolation of mucic acid from polysaccharide containing only small amounts of galactose.

2.5 g. acid form carbohydrate was refluxed with 15 ml. 3% HNO₃ for 2 hours. The solution was separated from unattacked material and concentrated in vacuo at 40° with a vigorous air stream, to remove oxides of nitrogen. When the volume was ~8 ml. and ~4 ml. crystalline inorganic material was filtered off. The viscous solution was then heated with 10 ml. 25% HNO₃ at 55° in an evaporating dish. When the volume was ~5 ml. it was allowed to stand at room temperature. The first product to crystallise was mainly inorganic, that which came down slowly contained organic material, (X) More of this solid material was prepared on similar lines and extraction with a small amount of Na₂CO₃ solution followed by acidification with HNO₃ yielded crystalline mucic acid m.p. 217°, mixed m.p. with an authentic sample 216°. The yield was very small.

The solution from (X) was tested for the presence of saccharic acid by adding saturated potassium acetate solution until there was no more precipitation of solid

material, the solution smelled of acetic acid. The solid was filtered off, washed with a little water and recrystallised from hot water. The crystals were very small but some did show trapezoidal facets. on examination under the microscope, and appeared similar to an authentic sample of KH saccharate, prepared from glucose.

The silver salt was prepared by dissolving 0.12 g. of the salt in the minimum volume of water and pouring this solution into a cold solution containing 0.16 g. AgNO_3 . The precipitate was allowed to stand in the dark for 2 hours when darkening occurred, it was filtered, washed with a little cold water and dried in the dark over concentrated H_2SO_4 . There was obvious decomposition during the drying.

On ignition in a platinum crucible the Ag salt was found to contain 58.8% Ag (Theoretical 59.91% Ag), higher figures were also obtained, the Ag did not contain AgCl as there was no loss in weight on extraction with ammonia solution. Decomposition would account for the discrepancy, as also would the presence of some Ag oxalate (71.0% Ag).

Genuine Ag saccharate was prepared and analysed under the same conditions, again there was some decomposition, but it was not so marked. Found 51.08% Ag. Silver tartrate was also less easily decomposed.

Oxalic acid hydrate m.p. 100° was obtained on further concentration of the solution at (X) to $\frac{2}{3}$ volume or less.

The residue which was not attacked in the preliminary HNO_3 hydrolysis (~15% of starting material) was heated

with 30% H_2SO_4 on a water bath at 80° for 30 mins, no appreciable charring took place. Sufficient concentrated HNO_3 was added to make a 15% HNO_3 concentration and the solution was heated for a further hour. After standing and decanting from unattacked solid the solution was made alkaline with $NaOH$ and filtered. It was easier to filter from flocculent material when alkaline, and mucic acid would not be lost; addition of alcohol also aided filtration. The filtrate was acidified with nitric acid, when a small quantity of organic acid material (flocculent, polysaccharide like), was precipitated, the solution was allowed to evaporate at room temperature for two days. Sufficient water was added to dissolve the large crystals of $NaNO_3$ leaving two products, (a) white crystalline material which was partly inorganic, and (b) pale yellow material which adhered to the sides of the dish and filter stick. This hardened on collecting together and washing with a small amount of cold water. m.p. after recrystallizing from hot water 212° .

ACETYLATION OF THE POLYSACCHARIDE.

Sodium salt material, corresponding to 6 gm. dry organic matter, which had been triturated with alcohol, was made into a smooth gel with 38 ml. water, dilute HCl was added until the mixture gave a strong acid reaction to congo and 67 ml. acetone were added. The acid form of the

carbohydrate, as a fine flocculent precipitate, was centrifuged off and 60 ml. pyridine added, the mixture was stirred for 5 minutes and allowed to stand for 3 hours, pyridine was centrifuged off and replaced by 60 ml. fresh, this was repeated three times. The solid was then stirred vigorously with 40 gm. pyridine and 6 gm. freshly distilled acetic anhydride on a water bath at 45°. After an hour, 8 gm. Ac_2O were added and stirring continued for a further two hours. After standing overnight the supernatant liquid was decanted into 150 ml. 3% HCl and the residue, R, was retained. A small amount of flocculent precipitate formed in the HCl, which after standing for 12 hours was centrifuged, washed well with 3% HCl and water and then with alcohol and ether. 0.3 gm. of material with 7% acetyl content was obtained, some product had been lost in the aqueous washings.

The residue R, after washing with pyridine and centrifuging was allowed to stand with 10 ml. water, it swelled to a soft gel, smelling strongly of pyridine; on addition of 30 ml. pyridine with stirring a more flocculent precipitate was obtained. The pyridine was removed by centrifuging and three further pyridine treatments applied, each with 24 hours standing; the rather hard product was acetylated in the same way as before, it did not go into solution. After standing at room temperature for 48 hours the whole was poured into

150 ml. 3% HCl and worked up as before.

Acetyl content 13% ($\sim \frac{1}{2}$ gp per anhydrohexose unit).

Overall yield 45-50%.

Estimation of acetyl content was carried out by stirring a known weight of material with 0.5 N alcoholic NaOH for $2\frac{1}{2}$ hours at 55° in a stream of nitrogen, titrating excess NaOH with 0.5 N HCl using phenol phthalein indicator and comparing with a blank experiment.

e.g. $\frac{1.4400}{0.8094} \times 0.1034$ g. dry wt. of material treated with 10 ml. 0.5 N alcoholic NaOH.

Excess NaOH 10.039 ml. 0.453 N HCl.

Blank 11.075 " " "

1 gm. carbohydrate contains $\frac{1.036 \times 0.453 \times 0.8094 \times 59}{1.4400 \times 0.1034 \times 1000}$

= 0.13 gm. OAc.

BARYTA HYDROLYSIS.

A 5% solution of carbohydrate in 5% baryta was heated at 70° in an atmosphere of nitrogen. Barium salt was precipitated and at no point did all the organic matter go into solution.

Heating time	Reducing power as ml. $\frac{N}{10} I_2 gm^{-1}$
$2\frac{1}{2}$ hours	1.3 ml.
5 hours	2.5 ml.

Baryta concentration increased to 10%

22 hours	2 ml.
----------	-------

On acidification with H_2SO_4 (to conge) the precipitate could be centrifuged off, leaving a dark pungent smelling

solution. Yield, after concentrating and precipitation with alcohol, 30% (including ash).

A similar hydrolysis at 88° showed a reducing figure 3.5 ml. $\frac{N}{10}$ I₂ after six hours which fell to near zero overnight. Fehlings and Seliwanoff tests negative. The solution was retreated with baryta at 80° and worked up as before.

25% recovery of organic material (with 32% ash).

$[\alpha]_{5790}^{20} = -30$ (1% aqueous solution, cloudy).

Baryta hydrolysis at 100° yielded a very viscous degraded product.

Acetylation of baryta treated material, according to procedure given, yielded a small quantity of hard material OAc = 12%.

5 gm. was methylated once with Me₂SO₄ and NaOH at room temperature, very little solid separated out on dialysis. Total yield 30%, containing BaSO₄; 13% OMe.

METHYLATION OF THE POLYSACCHARIDE.

Methylation of the polysaccharide using methyl sulphate and alkali.

Preliminary experiments.

Crude sodium salt form carbohydrate, precipitated into alcohol and centrifuged, was used without further treatment, e.g. drying.

Material corresponding to 6.2 gm. dry dialysed organic

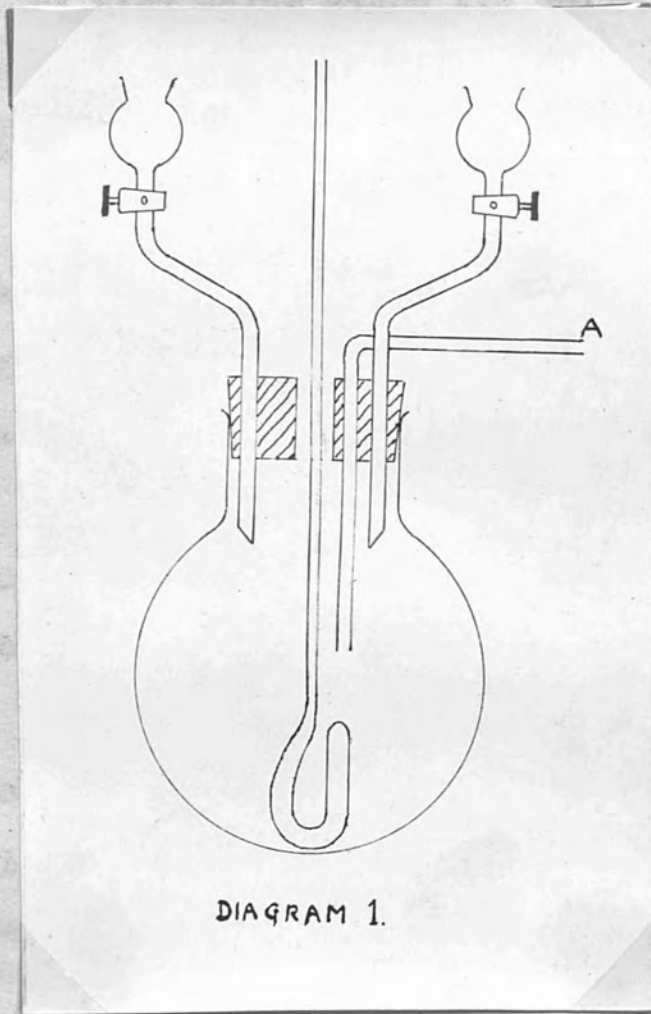


DIAGRAM 1.

material was swollen to a gel with water and methylated at room temperature with 390 ml. 30% NaOH and 140 ml. Me_2SO_4 , using the apparatus shown in diagram 1. The reagents were added slowly, through the two tap funnels, in tenth portions at hourly intervals; a steady stream of nitrogen was passed into the flask through the tube, A, and the mixture was stirred vigorously. At the end of ten hours, any methyl sulphate remaining was hydrolysed by heating on a boiling water bath for 45 minutes, with slow stirring. The carbohydrate darkened on treatment with alkali, more so on heating. After neutralizing with H_2SO_4 the product was dialysed against distilled water for ~ 5 days. The dialysed solution was concentrated at 45° and then remethylated several times, using gradually reduced quantities of reagents with addition of acetone.

Second methylation, as first.

Third to fifth methylations, 240 ml. 30% NaOH,

100 ml. Me_2SO_4 , 80 ml. acetone.

Sixth and seventh methylations, 220 ml. 30% NaOH,

90 ml. Me_2SO_4 , 120 ml. acetone.

The appearance of the product and its insolubility in water did not appreciably alter after 4-5 treatments. The final dialysis was allowed to run for 17 days and on concentrating to dryness 1.7 g. of a light yellow

powder was obtained, which was extracted three times with boiling chloroform.

Yield, 0.27 gm.; OMe, 30%.

Methoxyl figures were estimated by the semi-micro Zeisel method.

Factors causing loss of material.

Material must have been lost during the dialyses, concentration of dialysates from two methylations showed that there was a steady small loss of organic material, and that most of the sodium sulphate was dialysed after 36 hours with distilled water, changed every eight hours. Hence, except for purification in the last stages, much shorter dialysis times were employed (see account of final method employed.)

The action of cold and hot 30% NaOH was investigated. A 2% solution of the carbohydrate in 30% NaOH was stirred vigorously, in an atmosphere of nitrogen, at room temperature, for 10 hours, followed by 1 hour on a boiling water bath. At the times indicated aliquot portions were withdrawn and neutralized, and the reducing power determined by the Willstätter hypiodite method as modified by Baker and Hulton [43]. This is not as accurate as later modifications using carefully buffered slightly alkaline conditions e.g. [53] and was further complicated by the fact that iodoform could be detected after titration, however changes in iodine uptake give an indication of the liberation of

reducing groups.

Hours at 20° in N ₂	$\frac{1}{2}$	1	1 $\frac{1}{2}$	2	2 $\frac{1}{2}$	3 $\frac{1}{2}$	4 $\frac{1}{2}$	5 $\frac{1}{2}$	6 $\frac{1}{2}$	7 $\frac{1}{2}$	8 $\frac{1}{2}$
ml. N/10 I ₂ gm ⁻¹	0.1	0.9	4.0	4.4	4.4	4.7	4.6	4.4	3.0	1.9	1.4
Hours at 100° in N ₂	$\frac{1}{4}$		1		10 hr. at 20° in air.						
ml. N/10 I ₂ gm ⁻¹	2.4		8.0		14.8						

Methylation was repeated, omitting the hydrolysis of Me₂SO₄, only about 25% of the 50% H₂SO₄ required for complete neutralization was added before dialysis (except for seventh methylation) and losses were visibly much less.

The dry product was extracted with CHCl₃:EtOH::10:1.

Yield, 16%; OMe, 26%.

The residual material was treated with the same solvent mixture to which concentrated HCl was added until, after shaking and standing, a faint acid reaction with moist congo red paper was obtained.

Yield, 16%.

Alcohol extracted a further 16%, and the residue had become swollen and green coloured. On standing, the dry material very rapidly absorbed moisture and the methylated carbohydrate was damp at this third extraction stage.

The residue was dried; yield, 52%; OMe, 12%.

Overall yield, 70%.

Acidified EtOH was then used for extraction of material suitable for further treatment.

Effect of warming during methylation.

A trial experiment in which the material was warmed to 40° in the later stages of methylation, (6 treatments), gave a good yield of material of OMe, 24%. Two methylations were carried out simultaneously with identical conditions, except that one was given six 10 hour treatments at room temperature, and the other six, 7 hour treatments, (except the first), at temperatures increasing from 30° (second treatment) to 40° (fifth treatment).

10 hour methylations	49% yield	22% OMe
7 " " "	45% " "	24% " "

There was no significant difference in the colour, etc., of material during the two procedures, the slight difference in yield and % OMe is possibly due to alcohol extracting more material of lower % OMe, owing to the presence of moisture, in the first case.

Variation in the number of treatments.

After four methylations, product of OMe, 19%^{was} obtained.

After six methylations, typical methoxyl figures for alcohol soluble material were:-

Batch	IV	V	VI	VII	VIII
% OMe	24	25	23	20	20
% Yield			47	35	

After seven methylations products with 25.3% and 22.6% OMe were obtained, a sample of the latter, after working up, was given two more treatments, the product had 22.8%

OME with 15% loss. In general material was given seven treatments, particularly as the larger scale procedure, used after methylation VII, did not seem to give as good yields of soluble material.

Rotation $[\alpha]_{5780}^{15} = -10$ $[\alpha]_{5461}^{15} = -13$ (C = 2% in H₂O,
cloudy solution)

At this point, chloroform extraction of material, for methylation by Purdie reagents, was used, and the methylation procedure was only modified in minor ways, described in the next section.

Final methylation procedure.

Crude sodium salt, precipitated into alcohol, was centrifuged and the moisture and ash content estimated; it was ground up to a smooth gel with water, using about 1.5 ml. water for 1 gm. dry weight of organic material. For 15 gm. material 670 ml. 30% NaOH and 250 ml. Me₂SO₄ were run in slowly in tenth portions at hourly intervals, the mixture was well stirred and nitrogen passed into the flask. The product was dialysed in "Cellophane" tubes or bags, to remove sodium sulphate; it was necessary to rock the bags mechanically for efficient dialysis. Distilled water, renewed twice, after 2 and then 3 hours, was first used, this removed much of the free alkali, it was then possible to dialyse against fast running tap water overnight without serious precipitation of calcium carbonate or sulphate on the membrane, and finally the solution

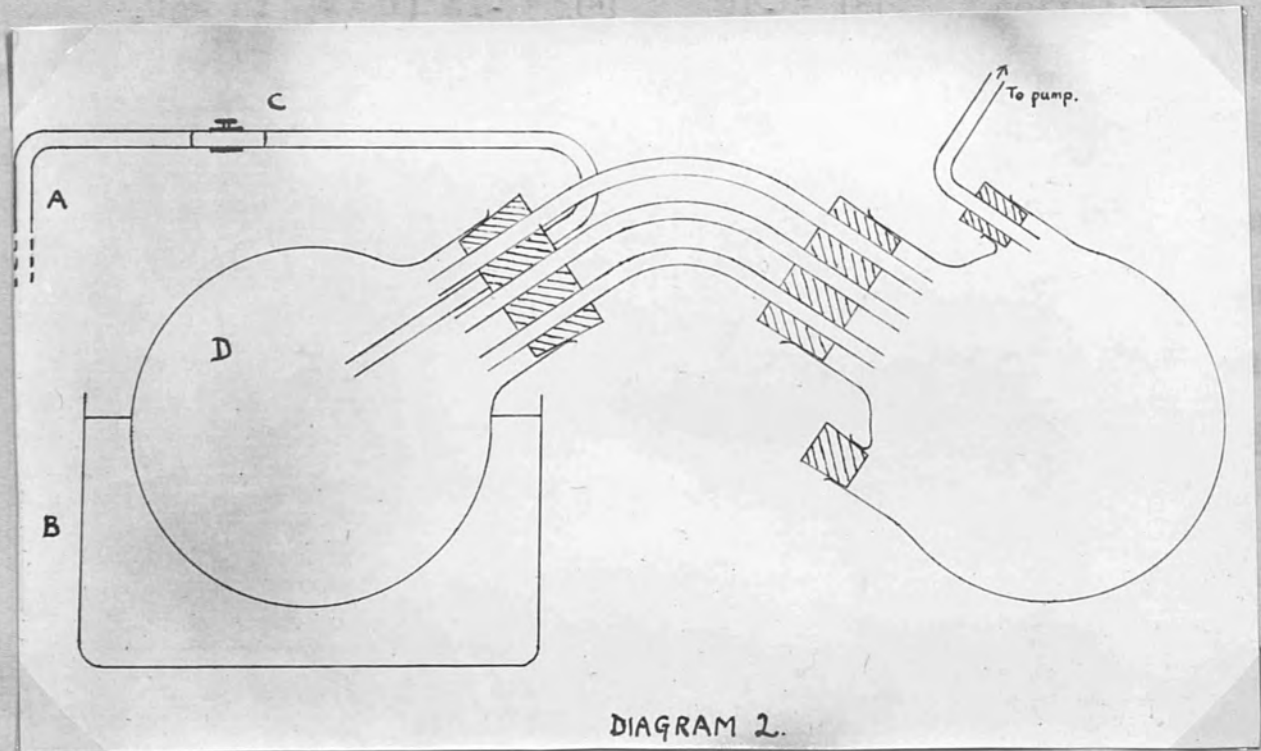


DIAGRAM 2.

was dialysed against distilled water for three hours. In later treatments the reaction mixture was heated; for the fifth to seventh methylations, flowing distilled water was used throughout dialysis to avoid contamination with inorganic material from tap water.

The resulting solution was neutralized, if necessary, and concentrated in vacuo at 45°. Much frothing occurred when aqueous solutions of polysaccharide were concentrated; the apparatus shown in diag. 2. was found suitable for removing water from large volumes of such solutions. The temperature of the heating bath, B, was brought to 40-45° and the bolthead flask evacuated before solution was run into the flask, D, through the tube, A; the rate of entry of liquid was adjusted by the screw clip, C, so that it was approximately equal to the rate of distillation and liquid did not accumulate in D. The receiver was cooled by a fast spray of water.

Much of the organic material settled out as a gel from the solution to be concentrated and was centrifuged off in order to avoid heating it, it was added to the concentrate for further methylation, sufficient 60% NaOH solution being used in methylating to compensate for the water present. After four treatments, much of the organic material separated out very completely and by adjusting the height of the siphoning tube A, could be left in the vessel of solution to be concentrated and was not separated by centrifuging. The product was

re-methylated, seven times in all; gradually increasing amounts of acetone were used, the quantities of methylating reagents were reduced and when the reaction mixture was warmed on a water bath, additions were made at 45' instead of 60' intervals. Quantities of reagents used for the repeated methylation of 14 gm material are shown in the following table.

ml 30% NaOH	ml Me ₂ SO ₄	ml Acetone	Temperature	Duration
600	250	-	Room	10 hr.
530	225	50	30	10 "
530	225	100	40	7 "
360	150	100	40	7 "
360	150	150	40	7 "
275	112	200	40	7 "
275	112	200	40	7 "

After the last methylation the solution was dialysed against flowing distilled water until it gave a negative reaction for sulphate (this took about eight days), and was concentrated to dryness at 40-45°, benzene being added to remove the last traces of water. The solid was extracted four times by boiling with CHCl₃ under reflux, using 300 ml. neutral, 300 ml. + 200 ml + 200 ml. acidified solvent, (concentrated HCl added until faint positive reaction to moist congo-red paper after standing). CHCl₃ was removed in vacuo with good aeration so that any free HCl was driven off.

The product was a light brown glass, difficult to remove from the flask. By precipitating the product from concentrated CHCl_3 solution and triturating with petroleum ether a white powder was obtained more easily. The organic content of the residues was also assayed in some cases. The yields given below show variation, there is a tendency for higher yields to correspond with lower methoxyl figures, as would be expected, the yields have been corrected for known losses, e.g. due to cellophane leaking in dialysis.

	% OMe	% Yield	% overall yield.
XIII	29.9	19	-
XVI	27.8	10	50
XVII	29.7	15	85
XVIII	24	30	100
XIX	31.5	12	85
XX	23.4	20	90
XXI	35.1	12	50
XXII	24.7 (separate batches)		
	29.6		

Properties of methylated material, soluble in acidified chloroform.

(Yellow white powder)

$[\alpha]_D^{20} = -27$ ($C = 0.63$, in CHCl_3); ash, 4%; slight positive reaction for nitrogen by sodium fusion.

Methylated material insoluble in acidified chloroform.

These residues had a high ash content of 22%.

50 gm were dialysed against $\frac{N}{50}$ HAc for $7\frac{1}{2}$ days and distilled water for 8 days; on neutralizing with NaOH and working up 14 g product with 11.4% ash was obtained. Further dialysis yielded material with 10.5% ash; Ca, 22%; SO_4 , 10.6%; CO_3 positive.

The material was a green-brown colour and contained nitrogen. NMe 2.8%.

OMe, 17.2%; organic sulphate 13%.

Uronic acid estimation (see P. 86) indicated one -COOH group present in 1010 g.

It was not possible to prepare a solution suitable for measurement of rotation as the material did not form a homogeneous solution except at very low concentrations.
Further methylation of residues.

Material which was insoluble in EtOH was methylated twice with Me_2SO_4 , 30% NaOH and acetone, at 40° . Extraction with acidified EtOH yielded material of 19% OMe. Thus further treatment yields a little more soluble product but there is no appreciable increase in OMe content.

Material which was soluble in EtOH was extracted with $CHCl_3$ (for methylation with MeI, Ag_2O); the residue (soluble in EtOH, insoluble in $CHCl_3$; $[\alpha]_D^{20} = -8$, $C = 0.63$ in EtOH) was submitted to further methylation with Me_2SO_4 , NaOH; but this was not satisfactory. There was much darkening during methylation, there was loss on dialysis for removing Na_2SO_4 , and material could not be

extracted from Na_2SO_4 in good yield by the use of organic solvents. The second ^{extraction} method yielded a product of 22.3% OMe.

Further methylation, using methyl iodide and silver oxide.

Alcohol soluble material, A, 21% OMe, was used, it was not appreciably soluble in methyl iodide and so 2g was refluxed with 4 ml MeOH, 4 ml MeI and 3 gm Ag_2O , the condenser being closed by a mercury trap. The methyl alcohol was dried by the magnesium methyrate method, methyl iodide was freshly distilled from CaCl_2 , and silver oxide was freshly prepared and dried in vacuo at 45° . Four quantities of 1 gm. Ag_2O were added at two hourly intervals and then 8 ml MeI were stirred in, for the mixture was almost solid. After refluxing for a further three hours the mixture was extracted with dry CHCl_3 and the extract dried with MgSO_4 .

Yield, 45%; OMe, 25%.

Further extraction with MeOH yielded dark material containing Ag.

A was extracted with chloroform, yield, 60%; OMe, 23.5%, and methylated as before; yield, 72%; OMe, 29.0%, readily obtained on CHCl_3 extraction. In this case the fraction insoluble in CHCl_3 is also preserved. The products from both experiments were combined and treated twice more in the same way giving a product with OMe, 29%; yield 82%.

Baryta hydrolysis of product.

The relatively large increase in methoxyl figure on one treatment with MeI, Ag₂O suggested the formation of methyl ester. 0.73 g. was refluxed with 20 ml. 5% baryta at 50-55° for 3 hours and the product distilled carefully. The first ml. of distillate contained MeOH (smell) and gave a strong positive test for MeOH by oxidation to HCHO followed by resorcinol colour test for HCHO.

Dilute H₂SO₄ was added to the residual mixture until it was neutral to congo red. Extraction with CHCl₃ gave 0.67 g. (92%) material 25.4% OMe, containing a very little BaSO₄.

The methoxyl figure of this product, by comparison with that of the starting material (23.5%) indicated that there was some methylation other than esterification.

Two ⁹/₁₀ gm. quantities of CHCl₃ soluble material (ash 4%) were each boiled with 30 ml. MeI, + 4 g. Ag₂O for 2 hours, + 4 g. Ag₂O for 2 hours, + 4 g. Ag₂O for 2 hours, + 10 ml. MeI and 4 g. Ag₂O for 2 hours, + 4 g. Ag₂O for 2 hours. Total heating time ten hours.

The products were extracted with CHCl₃ etc. in the usual way, and retreated with the same quantities of reagents.

Third treatment; Yield, 14.38 g.; OMe, 29.7%

Fifth treatment; Yield, 13.2 g. 79%; OMe, 29.8%, ash 4.1%

i.e. OMe, 31%, correcting for ash.

Thus the methoxyl figure did not seem to be significantly increased after three or four methylations with Purdie reagents.

Properties of fully methylated CHCl_3 soluble polysaccharide.

Pale yellow solid, OMe, 31%; ash 4.1% (Na, Ca, SO_4 ,
trace of AgI)

Analysis C, 50.5%; H, 7.55%; N, 2.40%; corrected
for ash (Weiler and Strauss)

$[\alpha]_D^{20} = -34$ (C = 0.4% in CHCl_3)

Ionic sulphate 0.4%.	Uronic acid, 1 g.e. COOH
Total sulphate 3.0%.	in 2150 g.

Although it seemed unlikely that protein would persist through the methylation procedure, which involved prolonged alkali treatment and dialyses, no positive evidence for the presence of amino sugar in the polysaccharide and difficulty in removing the last traces of protein have been found by another investigator [40]. Accordingly tests for protein were carried out on the methylation product.

Biuret test, negative. After hydrolysis with 20% HCl on a boiling water bath for 6 hours the resultant solution was boiled with charcoal, filtered and neutralised; ninhydrin test, negative. After hydrolysis with 35% NaOH on a boiling water bath for 12 hours the solution was charcoaled, filtered and neutralized; a slight positive ninhydrin test was obtained.

METHANOLYSIS OF METHYLATED POLYSACCHARIDE.

Dry MeOH containing dry HCl gas was used for methanolysis, the methylated polysaccharide readily formed a (cloudy) solution in this solvent. In all experiments exits were closed with Hg traps.

The solution was boiled for the times specified, and after cooling any remaining HCl was neutralized with dry, freshly prepared Ag_2CO_3 . The solution was filtered off through a No. 44 filter paper, which retained much of the semi-colloidal silver, and the residues were further extracted with MeOH. After drying with MgSO_4 , the solvent was evaporated and the product extracted with ether.

11 hours heating in MeOH, HCl (2.5%) gave a hard white solid product, indicating very little breakdown.

47 hours heating in MeOH, HCl (4%) gave a dark viscous product, $[\alpha]_{5790} = +4$, in small yield.

50 hours heating gave 41% yield, and 60 hours, 66% yield of ether soluble material. As HCl was evolved during methanolysis further quantities were passed into the mixture at ~10 hour intervals. It was not possible to follow rotations as the material was very dark coloured at this stage and heating was not further prolonged.

Much of the acid was removed by aeration at ~35' before treatment with Ag_2CO_3 .

from

Material was recovered /AgCl etc. in good yield (~80%)

and was separated into ether soluble and insoluble fractions. The black insoluble residue contained Ag which could be precipitated by H_2S , there should be no free organic acid present at this stage to retain Ag as salt, but possibly a complex was formed with the nitrogen containing material which was isolated in this fraction. The methoxyl content of the ether soluble fraction was 41-43 % when material, OMe, 30 % was used, an increase of OMe % consistent with extensive breakdown. The rotation was positive (+20) as compared with a value ~ -20 for the starting material used in a particular experiment.

At this stage any acidic material would be present as methyl ester, the ether soluble fraction was therefore treated with 3% baryta at 45 -50° for 3 hours; CO_2 was passed into the solution to precipitate excess $Ba(OH)_2$ as $BaCO_3$ and after filtration the solid was washed well with hot water. The aqueous extracts were concentrated and dried well by distilling with dry benzene, leaving a whitish, extremely viscous solid. This material was extracted with dry ether for removal of glycosides; it was important that the ether contained no water or alcohol or Barium salts were partially dissolved, and contaminated the glycosides.

Salts were converted to ester by boiling gently with MeOH, HCl (1½%) for 5 - 6 hours, neutralizing with

with Ag_2CO_3 and extracting with MeOH; the extract was dried with MgSO_4 and MeOH removed. The product contained traces of Ba and Ag salts but could be freed from these by CHCl_3 extraction.

Results.

Methanolysis	I (trial)	II	IIIA	IIIB	IV
% OMe Starting material	24 not Purified	30	27	29	30
Heating time, Hr.	47	60	51	60	66
% Yield ether sol.	9	66	41	56	41
% OMe	40	41	42.8	43.8	-
% Yield glycosides	-	35	35		28
% OMe			43.7		44.0
% Yield ester	-	16	7		8
% OMe	-	33.8	37.8		40.6
% Yield, residues (Ag removed)	-	17	40		58
% OMe	-	32	32		

Changes in rotation

Starting material

I -12

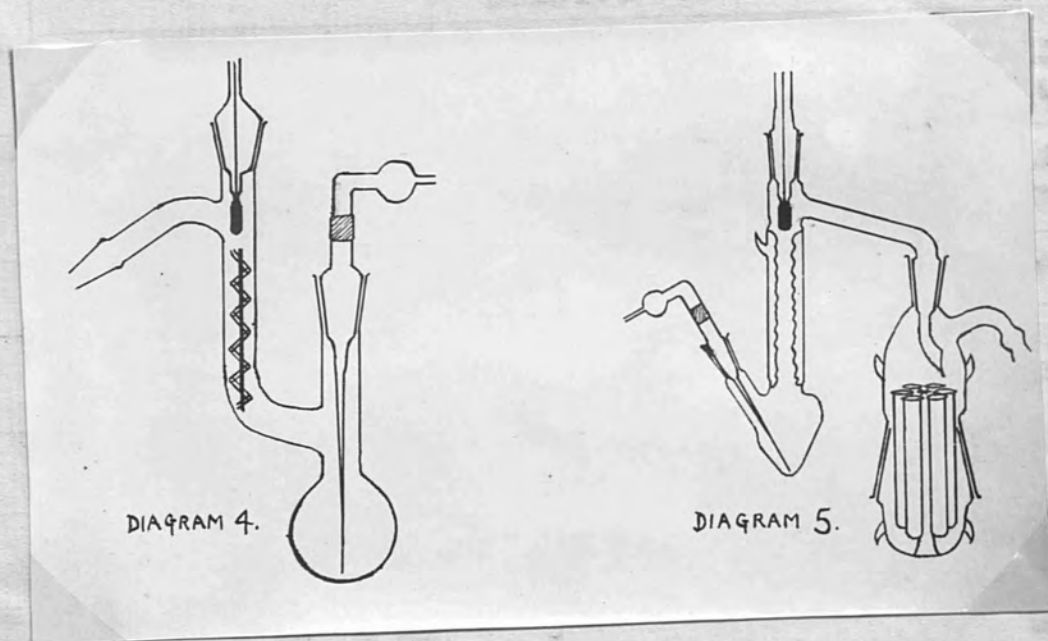
II -20

ether soluble product

I 25

II 20

with a dry and anhydrous salt, the excess was
drifted into a trap and was removed. The product
contained traces of the salt of which it could be freed
from these by drying extraction.



In I, alcohol extracted 74% of the residue, and this fraction contained a crystalline Ag complex 19.5% OMe.

INVESTIGATION OF THE GLYCOSIDES.

Fractionation.

The glycoside fractions from methanolyses II and III were fractionally distilled in vacuo, using a small claisen flask with spiral condenser in the side limb (diag. 4). Temperatures recorded by the thermometer in the flask are not significant as boiling points since ^{the} rate of distillation was slow. Refractive indices were measured with an Abbé Refractometer.

The glycosides from IV were fractionated in the apparatus shown in diag. 5, in which the receivers could be changed without lowering the vacuum. After a preliminary rough fractionation the mixture was refractionated by distilling most of the first fraction before adding the second, as indicated. Addition of fractions involved washing in with ether and removal of solvent.

Glycosides II and III

Frac.	Pres- sure mm Hg	Bath °C	Flask °C	Yield g.	% OMe	n_D^{18}	$[\alpha]^{18}$		C, in CHCl ₃
							5780	5461	
A 1.	0.12	100- <u>104</u> - 105	56- 64	0.633	43.6	1.4503	6	10	1.1
A 2.	0.08	115- <u>117</u> - 118	67- 74	0.880	43.6	1.4524	38	47.4	1.2
A 3.	0.09	133- <u>135</u>	80- 84	2.495	41.6	1.4586	50	56.5	1.3
A 4.	0.06	140- 155		0.136			Intermediate drop.		
A 5.	0.05	158- 172		0.520	40.01	1.4641	66	80.6	1.4
A 6.	Flask allowed to drain			0.053					
Resi- due.				0.273					

A positive Schiff reaction was given by fractions A 2 and A 3, only a very slight reaction was given by A 5.

Glycosides IV.

Pressure throughout both distillations 2×10^{-3} mm Hg.

Fraction	Bath T °C	Yield g.
1.	115 - 120	0.8608
2.	110 - 124	0.0597
3.	140 - 144	0.3019
4.	148 - 152	0.3037

At 148° crystalline material formed in side arm of distilling flask. Residue was allowed to remain in flask.

Fraction	Bath T °C	Yield g.	% OMe	n _D
B.1	106 - 108	0.233	49.1	1.4503 (25°)
Added frac. 2				
B.2	110 - 115	0.053	Intermediate	
Added 3 & 4				
B.3	115- <u>122</u> - 130	0.619	45.3	1.4562 (25°)
B.4	144 - 148	0.199	51.3	1.4633 (18°)
B.5	158 - 168	0.045		
B.6	170 - 175	0.10	44.7	
Residue		1.094		

A positive Schiff reaction was given by fractions B. 3 and B. 4; only a slight reaction, after 24 hours, was given by B. 6. The lower fractions were investigated by hydrolysing and attempting to prepare derivatives from the

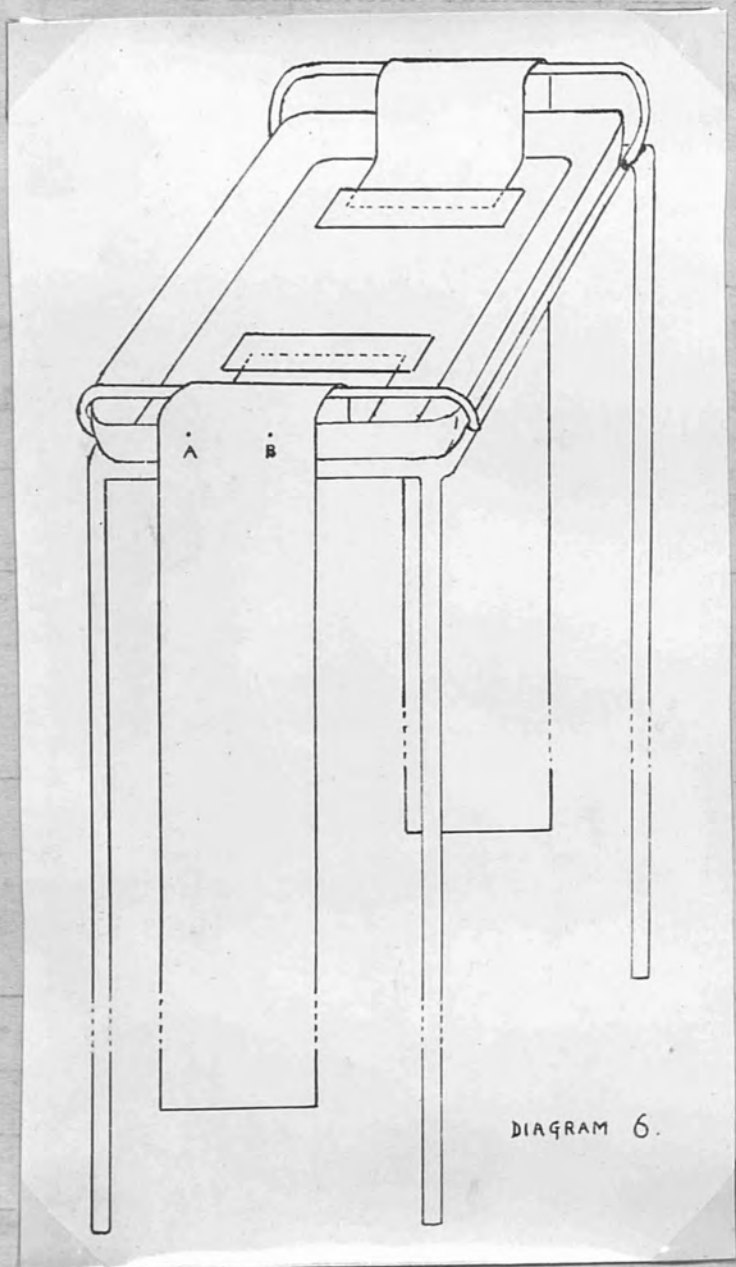


DIAGRAM 6.

partially methylated reducing sugars. Losses on hydrolysis were heavy where the presence of anhydro compound was suggested by the Schiff reaction and the isolation of the dinitrophenyl hydrazone of laevulic acid in one case, also agreed with anhydro derivatives being present initially.

The hydrolysis products were also investigated by the partition paper chromatography technique. This method of analysis was developed by Consden Gordon and Martin [54] for mixtures of amino acids as obtained from proteins and peptides, extended to free sugars by Partridge [55] and to methylated reducing sugars by Hirst et al. [56]. It involves placing a spot of the solution to be analysed, about 8 cm., from the top of a strip of filter paper (Whatman No. 1); the paper is then hung vertically from a trough containing a water-saturated organic solvent, see diag. 6. with the top edge of the paper immersed in the solvent, and passing over a support, 3, to prevent capillary siphoning. The whole is enclosed in a sealed glass vessel so that the atmosphere is maintained saturated with water and organic solvent vapours.

A sharp liquid front advances down the paper and the different constituents of the sugar mixture also move down at varying speeds and hence become separated into discrete spots, their relative positions depending on the

type of solvent mixture used. These positions may be detected by drying the filter paper after the chromatogram has run for a suitable distance, and then spraying with an appropriate reagent: in the present work alcoholic silver nitrate solution was used, and the paper redried at 100° for 3-5 minutes.

The separation of the different sugar derivatives depends mainly on the differences in partition coefficients, since partition of each solute takes place between water bound by the cellulose and the organic solvent moving over the cellulose surface. Adsorption plays some, but a much less significant, part. In general the most satisfactory solvents are those which are only partially miscible with water.

For a given solvent mixture,

R_F values of solutes, where $R_F = \frac{\text{Distance moved by constituent}}{\text{Distance moved by solvent front}}$

remain practically constant and may be used to establish the constituents in a mixture once they have been measured using known compounds. Hirst et al. [56] found that R_F values of sugars varied somewhat with the distance moved and they use the more constant R_g values to characterize sugars and methylated derivatives

$$R_g = \frac{\text{Distance moved by constituent}}{\text{Distance moved by tetramethyl-D-glucose}}$$

A spot of 10% tetramethyl-D-glucose is run alongside

the spot of solution under investigation (A and B in diag. 6.)

R_g values of the constituents of the lower glycoside fractions were measured using the same solvent mixture as these investigators.

Fraction 1.

Hydrolysis.

A sample of fraction B. 1. was hydrolysed by treatment with 0.1 N, followed by N HCl as shown, the hydrolysis was followed polarimetrically by cooling to room temperature (20°) at known time intervals and measuring the rotation of the solution.

	In 0.1 N HCl					Increased to N.			
Time (hr)	0	0.5	1	1.5	2.5	2.5	3.5	5	7
$[\alpha]_D^{20}$		35.4	39.3	41.3	45.1	41.5	42.5	41.5	41.6

After 3.5 hr. heating Fehlings reaction positive (8' at 70°) HCl was removed by passing the solution down a column of resin "deacidite B." This resin was found to be satisfactory for the removal of HCl used for the hydrolysis of a mixture of tri- and tetra-methyl methyl glucosides. The product was taken up in ether to remove traces of material from the resin.

Yield, 93%; Ome. 42.0%;

$$[\alpha]_D^{24} = +46.2$$

Chromatography of hydrolysis product.

Solvent: 40% n-butanol, 10% ethanol, 49% water, 1% ammonia.

Spray: 10% solution of ammoniacal silver nitrate.

A ~10 μ l. spot of a 10% solution of tetramethyl glucose was

used for reference compound and $\sim 10 \mu\text{L}$ spots of a $\sim 20\%$ solution of product was used. Runs were made for 20 hr. at 13°C .

Three constituents were indicated, la, lb, lc, with the following R_f values:-

la	R _f	0.82
lb	R _f	0.86-0.87
lc	R _f	1.01

The R_f value of lb varied over the wide range 0.854-0.88 but consistent values for la and lc were obtained. The R_f value of 1.01 suggested that 2:3:4-trimethylrhamnose was present; 2:3:4:6-tetramethylgalactose and 3:4-dimethylrhamnose are quoted [56] as showing R_f values of 0.88. Additional chromatograms were then run with a third spot of tetramethylgalactose solution in order to compare the position of this compound with lb. 2:3:4:6-tetramethylgalactose was faster moving than lb.

From the relative intensities and areas of the silver nitrate spots, la:lb:lc is roughly 2:2:3.

Preparation of 2:3:4-trimethylrhamnose-anilide.

Since chromatography indicated that trimethylrhamnose was present, 0.1268 g. was extracted with petroleum ether at room temperature in order to separate it to some extent from the two other sugar derivatives; Yield 0.0723 g., 57%. This product was refluxed for 3 hr. with 0.031 g. dry freshly distilled aniline and

3 ml. EtOH. After removal of solvent and trituration with petroleum ether a crystalline product was obtained, which formed feather-like crystals over the walls of the flask.

Yield, 0.032 g. crude, slightly discoloured product; m.p. 107-109°.

Product recrystallized from alcohol:ether:petroleum ether:: 1:1:2, fine white needles; m.p. 110°; the product did not depress the m.p. of 2:3:4-trimethylrhannose anilide; $[\alpha]_D^{16} = 130$ (0.3 in COMe₂); after 14 hr C, 64.8%; H, 8.07%; N, 5.15% (Weiler and Strauss); OMe, 32.6 Theory 64.1, 8.24, 4.98, 33.1.

Fraction 2.

A2. $[\alpha]_{5780}^{10} = +65$ $[\alpha]_{5461}^{20} = +62$ (C = 2.4 in H₂O)

Hydrolysis.

A sample of fraction A2 was hydrolysed with 0.1 N and N HCl as shown, the hydrolysis being followed polarimetrically.

Time (hr)	0.1 N HCl (C = 3.0)								In N HCl			
	0.5	1	1.5	3	5	6	11	14	3.3	5	6.3	8.7
$[\alpha]_{5780}^{10}$	56.7	53.7	51.7	55.0	53.4	50.3	51.2	51.2	-	47	46	47
$[\alpha]_{5461}^{20}$	50.0	57.9	60.4	67	60.5	60.4	60.3	60.3	-	51	47	47.5

After 11 hrs. heating, Fehling's reaction positive (10' at 60°). The product was neutralized with BaCO₃, taken down to dryness in vacuo and extracted with ether.

Yield, 60%, after 0.1 N HCl treatment; (product on subsequent treatment with N HCl recovered in 81% yield).

OMe, 33.5%

$$[\alpha]_{5780}^{12} = +103$$

$$[\alpha]_{5461}^{12} = +106.$$

Preparation of Anilide.

0.100 g. of the hydrolysed fraction was treated with 0.060 g. freshly distilled aniline and 4 ml. alcohol and boiled for 3 hrs., after cooling, triturating with petroleum ether, and allowing to stand for 2 days, a crystalline product was obtained. The product was triturated with cold alcohol filtered and washed with a little petroleum ether M.Pt 126-127°. Yield 20 mg. (1st crop) 0.134 g. treated 0.070 g. aniline gave 30 mg. (1st crop) m.p. 129-132°. The anilide was recrystallized from alcohol petroleum ether (1.1) white needles 1st crop m.p. 130°; 128° OMe, 22.3% 2nd crop m.p. 127°; OMe, 23.7%.

Chromatography of hydrolysed A2.

Procedure, as for fraction B.1.

Three constituents were indicated with the following

R_g values

2a R_g 0.61

2b R_g 0.82

2c R_g 0.87

A chromatogram run with 2:3:4:6-tetramethylgalactose for comparison showed that this compound was faster moving than 2c. 2:3:6-trimethylglucose and 2:3:6-trimethylmannose are quoted [56] as showing R_g values of 0.81; there is evidence from the work of D.M. Hardy that mannose is not

present in the Ulva polysaccharide; spot 2b did move at the same rate as 2:3:6-trimethylglucose in two chromatograms, run for comparison, but moved slightly more slowly in a third.

Fraction 3.

Fraction A3 became yellow after standing for about two days.

A3 $[\alpha]_{5780}^{21} = +65$ $[\alpha]_{5461}^{21} = +73$ (C = 3.7 in H₂O)

Hydrolysis.

0.1 N HCl (C = 2.8)						N H ₂ SO ₄	
Time (hr)	0.5	1	1.75	2.75	5.25		
$[\alpha]_{5780}^{21}$	68.3	70.4	74.3	75.5	75.5	86.8	Constant during 3 hrs. heating.
$[\alpha]_{5461}^{21}$	77.7	80.9	82.8	83.4	83.4	96.8	

Fehlings reaction positive after 5 hr. hydrolysis.

The product was worked up as fraction A2.

Yield, 67% after 0.1 HCl treatment, product on subsequent treatment with N H₂SO₄ recovered in 91% yield.

Ome, 30%. $[\alpha]_{5780}^{20} = +112$ (C = 0.8 in H₂O)

Anilide formation was attempted but only a small amount of very soft crystalline material was obtained on standing, which was lost on triturating with alcohol or petroleum ether.

Isolation of laevulinic acid 2:4-dinitrophenylhydrazone.

By extracting the Barium salts, remaining after ether extraction, with warm water and adding a solution of

2:4-dinitrophenyl hydrazine in HCl, a small amount of yellow precipitate was obtained. This was recrystallized from CHCl_3 , m.p. 205° . Mixed m.p. with the same derivative of laevulic acid prepared from cane sugar, 205° . Molecular weight of A3, was determined cryoscopically in benzene.

0.1200 g. in 17.58 g. C_6H_6 showed a depression of Fz Pt of 0.161. M.Wt. 212.

Further methylation of Fraction A3.

1.032 g. fraction A3 was refluxed with 3 ml. MeI + 1.0 g. fresh dry Ag_2O ; 0.5 g. Ag_2O was added after two hours, 0.5 g. Ag_2O + 2 ml MeI after four hours heating, and 0.5 g. Ag_2O after six and eight hours heating. The product was extracted with CHCl_3 , the extract dried with MgSO_4 , and solvent distilled off.

1st treatment,	yield	0.986 g.	53.5%	OMe
3rd	"	"	0.914 g.	55.46% "
4th	"	"	0.840 g.	56.4% "
5th	"	"	0.652 g.	55.9% "

The product was distilled in vacuo, using a small flask with wide side arm and no fractionating column; 0.523 g. main fraction was obtained, Bath T $96-101^\circ$ / 0.21 mm. Hg. The pale yellow residue in the flask was not volatile at 140° .

$n_{25.5}^D$ 1.4432; OMe, 56.2%.

Hydrolysis.

C = 12.1 in 0.1 N HCl.

Time (hr)	0.25	0.5	1.25	2.0	4.0	6.5	10.5
$[\alpha]_{5780}^{21}$	80	93	93.5	93	93	93	93

Yield 58% (Hydrolysis of methyl tetramethyl glucoside gave 84% yield) 43.9% OMe.

$$[\alpha]_{5461}^{29} = +119 \quad [\alpha]_{5480}^{29} = +110 \text{ (C = 2.8 in EtOH)}$$

$$[\alpha]_{5461}^{29} = -129.6 \quad [\alpha]_{5780}^{29} = +120 \text{ (C = 0.8 in H}_2\text{O)}$$

Formation of anilide from methylated fraction A3.

0.10 g. was refluxed for 3 hr. with 0.06 g. freshly distilled aniline in EtOH. After removal of solvent a small amount of crystalline product was obtained. M pt crude product 165°, after trituration with alcohol petroleum ether (1:2)

Chromatography of A3 and fully methylated A3, after hydrolysis.

Procedure as for fraction B.1.

Three constituents were indicated in fraction A3, with the following R_g values,

3a	R _g	0.47 (trace)
3b	R _g	0.62
3c	R _g	0.82

After methylation the following R_g values were obtained,

3Pa	Rg	0.89-0.91 (trace)
3Pb	Rg	1.0
3Pc	Rg	1.01 (main)

Thus rhamnose and glucose are present although the third spot does not correspond to any value quoted for a fully methylated sugar likely to be present, neither is it due to a trimethyl galactose (2:3:4-anilide, m.p. 168°).

Fraction 4.

Fraction B4 was investigated since this showed a rise of methoxyl figure, suggesting some separation of a more completely methylated biose, which was not apparent in the first fractionation, A. Also crystalline material formed during the preliminary fractionation at bath T. 144°. On triturating a sample of fraction B4 with dry MeOH a small amount of crystalline material was obtained, m.p., after draining on porous tile and recrystallizing once from methyl alcohol, chloroform (1 : 1), 44-45°.

Fraction B4 became yellow on standing for 24 hours.

Hydrolysis.

In 0.2 N HCl (c = 1.3)

In N HCl

Time. hr.	0	0.25	0.5	1	1.75	2.75	-	0.5	1.5	8.5
$[\alpha]_D^{16}$	-	96.8	95	89.2	96.5	93.6	95	93.5	93.5	93.5

The product was worked up as fraction 1B, using "de-acidite" E.

Yield 75% containing a little material from resin.

Chromatography of hydrolysis product.

4a	Rg	0.54
4b	Rg	0.74
4c	Rg	0.86-.87.

INVESTIGATION OF ESTER FRACTION.

Acidic material, after separation as Barium salts was esterified with MeOH/HCl (1.5%), HCl was removed by aeration and Ag_2CO_3 , and the ester extracted with MeOH (P.69).

A sample of the ester from methanolysis II was converted back to Barium salt by refluxing with 4% baryta, the solution was boiled gently and ~ 0.5 ml. distillate collected which contained MeOH (oxidation to HCHO and resorcinol colour test). The solution was made just acid to congo, concentrated somewhat at 40° , and extracted with CHCl_3 . A dark yellow viscous syrup was obtained 23% OMe.

i.e. 0.21 g. 18% OMe	→	0.094 34% OMe	→	0.066 g. 23% OMe
Ba salt		Ester		Free acid + MeOH.

Fractional distillation.

0.40 g. ester from methanolysis III, 37.8% OMe, was fractionally distilled, using a small distilling flask with wide side arm and no fractionating column. 0.136 g. main fraction was obtained, bath T $145^\circ/0.20$ mm. Hg, 40.2% OMe, n_D^{19} 1.4628, $[\alpha]_{5780}^{20} = +49$. ^{C=0.6} in MeOH There was an appreciable residue, not volatile below 180° .

The ester fraction from methanolysis IV was obtained similarly and then reconverted to Barium salt and re-esterified in order to remove glycosides more completely. After re-esterification material was recovered in poor yield (51%) but part of the product crystallized on

standing for 6 days.

Crystalline product.

Mpt crude material 118°.

Mpt after recrystallization from alcohol/petroleum ether 118-119°.

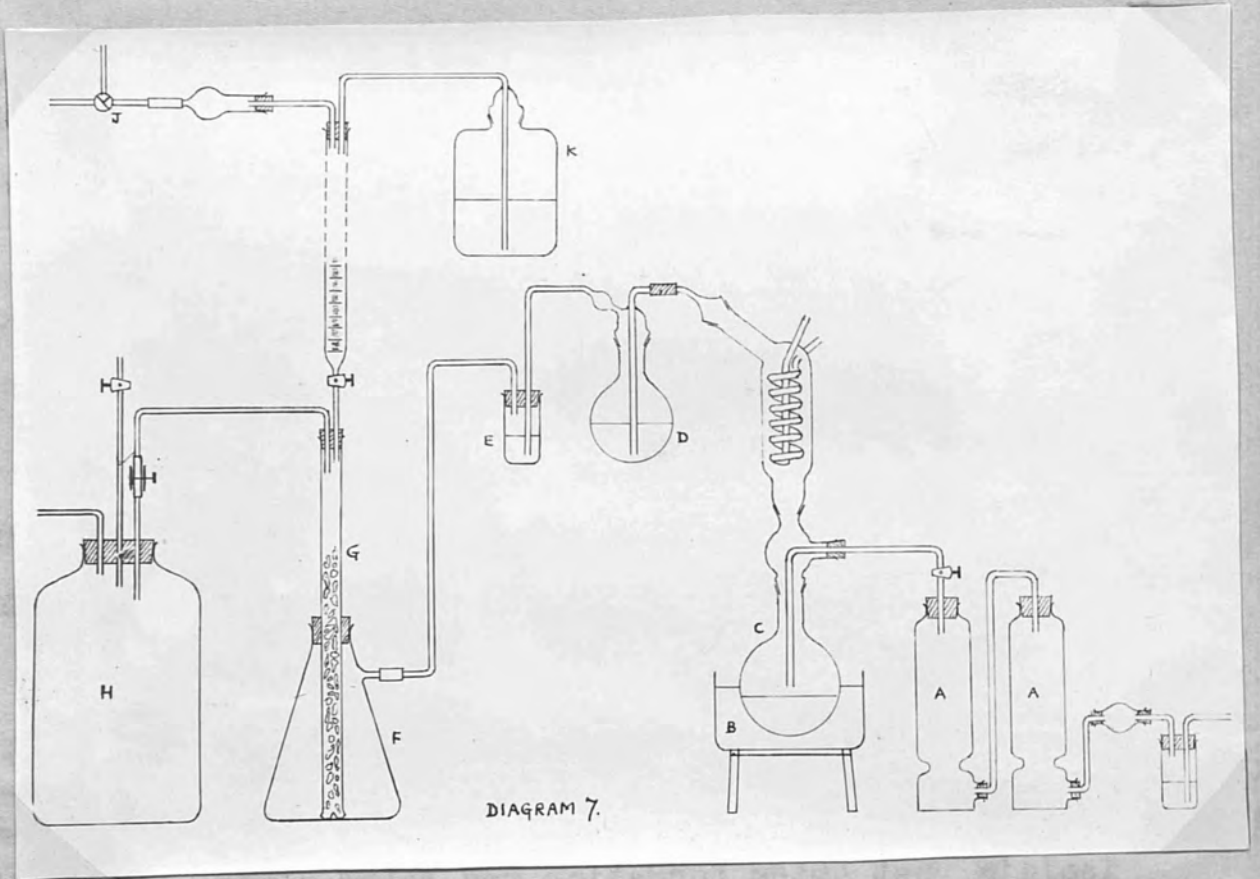
Rotation. The product was apparently insoluble in water, and no optical activity could be detected in an aqueous solution after standing over this crystalline material.

A positive iodoform reaction was given.

Clc, 39.8%.

Attempted preparation of amide.

A solution of 0.063 g. distilled ester in 8 ml. dry MeOH was saturated with dry NH_3 and left at 0° for 3 days. On removal of solvent a syrup was obtained. The treatment was repeated, and after prolonged standing at 2° there was not sufficient crystalline material to isolate, but amide formation had taken place, since after repeated evaporation with MeOH to drive off free NH_3 , the product yielded NH_3 on heating with baryta.



URONIC ACID ESTIMATIONS.

The uronic acid (or keto glyconic) acid) contents of the fully methylated and chloroform insoluble fractions of methylated polysaccharide were estimated by distillation with HCl, according to the procedure of Dickson et al. [57]. The apparatus was modified (Diag. 7) by the use of all glass joints where possible, and the CO₂ absorption involved on introducing baryta in the original method was eliminated by the use of an enclosed siphoning arrangement for drawing baryta into the burette from the reservoir, K, by the three-way tap, J, connected to a pump, to the atmosphere and through a soda lime tube to the burette.

A stream of air, freed from CO₂ by passage through the soda lime towers AA, was drawn through the apparatus by a water pump attached to the safety vessel H. The sample, containing ~0.3 g. uronic acid was placed in the reaction flask C, with 100 ml. 13.15% HCl and porous pot; the height of the oil bath, B, was adjusted so that the acid level was just below that of the oil. The air stream was passed for about twenty minutes, and then heating of the flask commenced. When the acid boiled, approximately 20 ml. 0.2 N baryta was run into the absorbing tower G, filled with glass beads, and the rate of air flow increased. The base of G was flanged outwards, and the upward air stream prevented the baryta running

into the Buchner flask, F. The trap, D, containing aniline, removed furfural and the trap E, containing silver nitrate solution, removed any HCl not refluxed back by the condenser, or removed by PhNH_2 .

Heating was continued for 5 hr. with the temperature of the oil bath maintained at 135-140° and the air flow adjusted to 2-3 bubbles per second. At the end of this period the tower was washed down with CO_2 free water and excess baryta titrated with 0.1 N HCl in an atmosphere of nitrogen, using phenolphthalein indicator.

The chloroform insoluble material was boiled with a little 1% HCl to remove the small amount of free carbonate present and the acid concentration then made up to 13.15% before estimating uronic acid.

Results.

HCl 0.0970 N.

	Direct titration, ml HCl = 10 ml baryta	ml baryta used in tower	ml HCl required to neu- tralize.	ml HCl = CO ₂	
Blank estimation					
	24.78	10.0	24.70	0.08	
	24.78	10.0	24.71	0.07	
Blank correction = -0.03 ml HCl				ml HCl = CO ₂	ml HCl
				corrected for blank	= 1 gm
Check estimation on galacturonic acid					
0.1986 g	24.78	26.24	43.41	21.51	108
				(Theoretical 106)	
Fully methylated material (corr: for ash)					
0.5610 g.	24.78	19.97	43.71	5.63	10.0
0.4314 g.	20.83	19.04	35.50	4.12	9.4
0.5005 g.	20.83	20.32	37.39	4.86	9.7
CHCl ₃ insol. material (corr: for ash)					
0.849 g.	20.83	34.34	67.51	17.48	20.50

∴ 2150 g. Methylated material (30% OMe) contains 1 g.e. COOH,
i.e. 1 acid group per 9 (methylated anhydrohexose) units.

∴ 1006 g. CHCl₃ insoluble material (19% OMe) contains
1 g.e. COOH, i.e. 1 acid group per ~5 units.

AUTOHYDROLYSIS.

Autohydrolysis of a 0.5% solution of acid form polysaccharide, pH 3, was carried out on a boiling water bath in a nitrogen atmosphere. The hydrolysis was followed by the development of reducing power, using the hypiodite titration method of Baker and Hulton [43] and polarimetrically when the solution had cleared sufficiently. Both methods indicated that a steady state was reached after 45-50 hr. heating.

Time (hr.)	1	1.25	8.25	14	20	32	40.5	43.25	50
$[\alpha]_{5780}$	-	-	-72	-65	-59	-47	-34	-36	-36
ml. 0.1N I_2 gm^{-1}	6.5	15.3	20.9	24.7	34.3	45.8	53.2	55.2	55.2

The resulting solution was yellow brown and contained a light-weight brown suspension. It was concentrated to about one third volume in vacuo at 45° in N_2 ; a little $CaSO_4$ was precipitated. The solution, containing 2.1 g. organic matter, was dialysed against three 500 ml. volumes of distilled water over a period of 2½ days. During this time the material inside the dialyser darkened considerably. The dialysates were concentrated at 45° in N_2 and gave a positive naphthoresorcinol reaction for uronic acid, there was no marked reaction from the non-dialysable material, although its dark colour made results inconclusive. Aliquot portions of both fractions were boiled with HCl to assess the distribution of methyl pentose and uronic acid (and/or pentose).

EXPERIMENTAL

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 a ... The ... by the
 development of ...

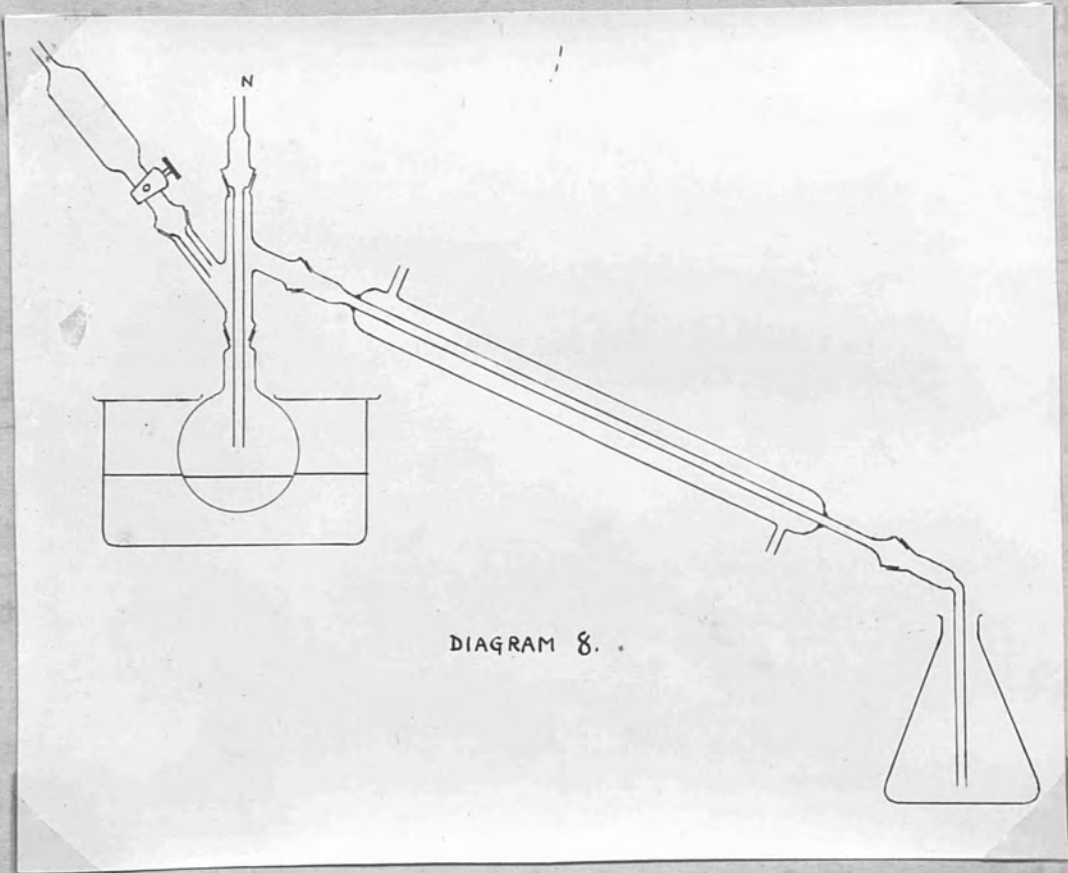


DIAGRAM 8.

... was ...
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Furfural and methyl-furfural Estimations.

The all-glass apparatus used is shown in diagram 8. A sample of the material under investigation, containing ~ 0.02 g. L-rhamnose was placed in the 100 ml. round flask together with 20 ml. 13.15% HCl, saturated with NaCl. The flask was immersed in an oil bath to the level of the liquid and a steady stream of nitrogen was passed through the apparatus before and during the heating period; the nitrogen inlet, N, did not dip below the surface in order to avoid blocking.

The oil bath was heated to 170-175° and maintained at this temperature. When the contents of the flask boiled 30 ml. 13.15 HCl, saturated with NaCl, was added through the tap funnel, three more similar additions were made at 15 minute intervals and the liquid boiled for a total of 1½ hours. During this time the volume stayed in the range 20-50 ml.

The rate of distillation was not fast enough to further shorten the heating time; increased heating time did not yield increased weights of phloroglucide precipitate from a fixed quantity of rhamnose hydrate. The liquid remaining in the flask was only slightly discoloured when rhamnose was used and contained much solid NaCl.

Phloroglucinol solution was prepared according to Browne & Zerban [7]. 11 gm AnalaR phloroglucinol was dissolved in 300 ml boiling 12% HCl and poured into

1200 ml 12% HCl; after standing at least a week the solution was filtered before use.

10 ml phloroglucinol solution was added to the distillate and the total volume made up to 130 ml with 13.15% HCl, if necessary. The solution darkened and a precipitate formed slowly, it was filtered off in a Grade 4 sintered glass crucible after standing for 24 hours. The precipitate was washed, using 50 ml water, dried in an air oven at 100° for an hour and weighed.

There was a negligible blank correction.

The precipitate was then extracted with three 6 ml. portions of 96% alcohol at 60°, the crucible being heated in a beaker immersed in a water bath at suitable temperature.

Using rhamnose, the precipitate was light brown in colour and was soluble in 96% alcohol except for a very small darker residue (~1%). Using arabinose, the phloroglucide precipitate was black and partly soluble in alcohol (~10%), under the conditions specified. The alcohol extract was dark green in colour. The appearance and solubility of the phloroglucide precipitate from *D*-galacturonic acid was similar to that obtained when using arabinose.

In appearance, the phloroglucide precipitate from the Ulva polysaccharide was like that obtained when using arabinose, but a greater proportion of it was soluble in 96% alcohol.

Results

Mass of L- rhamnose hydrate, R.	Mass of phloroglucinol ppt, P.	Proportion insol in 96% EtOH at 60°.
0.01764 g.	0.00996 g. (mean of 4 results.)	1%
0.03296 g.	0.02145 g. (mean of 4 results.)	

$$\therefore R = 1.332P + 0.00432$$

$R' = 1.273P + 0.00418$ where R' = mass of anhydrous rhamnose.

0.02194 g. L-rhamnose hydrate gave 0.01320 g. ppt., in agreement with this expression (0.01320 g. ppt. from 0.2190 g. rhamnose hydrate).

0.02052 g. arabinose gave 0.01620 g. ppt. 10% soluble in alcohol.

Estimations on autohydrolysis products.

2.1 g. carbohydrate material present before dialysis.

Dialysable material.

1.01 g. carbohydrate; naphthoresorcinol reaction, positive.

On distillation with $HCl^{5/46}$ of this material gave 0.05158 g. phloroglucinol ppt. 0.02154 g. insoluble in 96% alcohol at 60°, indicating

0.365 g., 36%, methyl pentose = 44 ml. 0.1 N I_2

0.27 g., 27%, pentose, or

0.34 g., 34%, uronic acid = 43 ml. 0.1 N I_2

Total reducing power of dialysate = 56 ml. 0.1 N I_2 .

This discrepancy is probably due to the fact that the fragments hydrolysed from the macromolecule are of the di (and tri?) saccharide order.

Nondialysable material.

This solution, which had become very dark, was concentrated; samples were used for boiling with HCl and the rest worked up to a hard dark solid by trituration with alcohol and ether. Yield 0.85 g., allowing for sampling; there were known losses on working up, estimate 1.0 g. non-dialysable material.

On distillation with HCl ^{5/101} of this material gave 0.01363 g. phloroglucinol ppt. 0.00204 g. insol. in 96% alcohol at 60°, indicating

0.38 g., 38% methyl pentose

0.06 g., 6%, pentose or 0.08 g., 8% uronic acid.

Rotations and calculations of % are all based on ash free organic carbohydrate material.

These figures are approximate as the distillation of standard mixtures of methyl pentose, pentose and uronic acid with HCl was not investigated, and for calculation they have been assumed to distil independently. CO₂ estimations for assay of uronic acid were not performed. But the results show that ~50% of the carbohydrate is broken down to dialysable fragments on autohydrolysis and that this portion contains practically all the pentose or uronic acid whereas the distribution of methyl pentose is not significantly

different in the two cases.

The instability of the residual macro molecule after autohydrolysis suggests that it would not be suitable for methylation.

DISCUSSION OF STRUCTURAL FEATURES.

The following results, obtained by D.M.Hardy on the purified polysaccharide [41], are also considered

Total SO_4 in sodium salt, 16.3%; on ashing Na salt alone, SO_4 retained, 12.3%.X

After baryta hydrolysis,

Total SO_4 in sodium salt, 13.8%; on ashing Na salt alone, SO_4 retained, 9.4%.

Free acid polysaccharide, Equ.Wt., 386; 44 g. CO_2 from 896 g.; reaction with 1 g. mol IO_4^- by 820 g., formic acid liberation very slightly positive.

The sulphate content of purified starting material as used for methylation, 14.2%, and the above, indicate 1 SO_4 group to 3 (16.3%) to 4 (13%) units (unit = anhydro

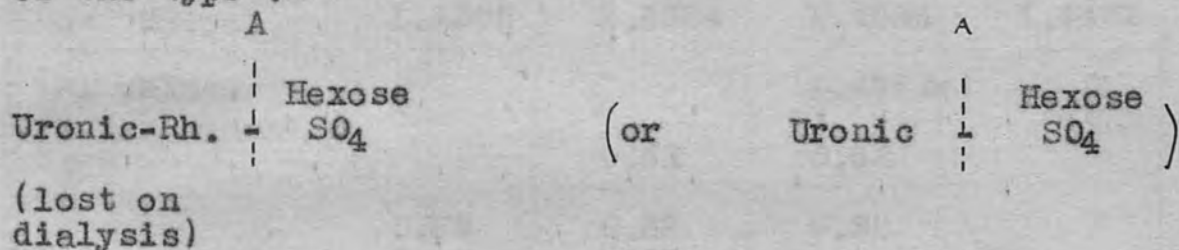
hexose); results varied on different batches, probably owing to small differences in extraction procedure.

The 13% SO_4 content of methylated material insoluble in CHCl_3 , shows no change compared with the starting material, allowing for the introduction of 17.2% OMe, there is therefore no concentration of a sulphate containing polysaccharide in this portion. The almost complete absence of sulphate and evidence of anhydro structures in fully methylated material suggests that some sulphate removal has occurred during methylation; the losses on hydrolysis of glycoside fractions giving a Schiff reaction are compatible with the original 1 in 3 SO_4 ester units having given a corresponding amount of anhydro derivatives. But it is difficult to reconcile this with the retention of most of the sulphate on heating with baryta, and the absence of any Seliwanoff reaction from the products. Possibly during methylation breakdown occurs (also indicated by dialysable fragments, p.63) making available a suitably situated -OH group for anhydro ring formation; $-\text{SO}_4$ on C_2 renders galactosidic groups labile (p.7).

The uronic acid in the polysaccharide appears to occur in 1 in 4-5 units (protein^{not} will introduce a significant error in CO_2 estimations) and the quantity of uronic acid in insoluble methylated material is only

slightly less, 1 in 5 units (P.88). The ratio of SO_4 to COOH agreeing with the SO_4 figures, X, on P.94. In the fully methylated material only 1 in 9 units yield CO_2 with HCl , allowing for introduction of OMe .

Thus the insoluble material does not appear to be significantly different from the starting material i.e. if the latter were a mixture of polysaccharides, no particular component has been fractionated. Consequently, as overall losses on methylation are not heavy ($\sim 15\%$), the soluble, completely methylated product ($\sim 20\%$) must also be related to the starting material; it is degraded, SO_4 has been removed and \sim half the uronic acid lost, possibly by fissure at point A in a part of the structure of the type :-



It is not possible to decide whether all the units which carried sulphate form anhydro structures. The difficulty of removing sulphate makes it impossible to compare methylated de-sulphated material with these products.

Of the other constituents 37% appears to be rhamnose (P. 92), confirmed in other work of D.M. Hardy; nitric acid oxidation indicates that only a small proportion of other sugar is glucose or galactose, or their corresponding uronic

acids; glucose is confirmed by chromatography, but if significant quantities of galactose are present it must presumably be as sulphate, so linked to SO_4 and other residues that SO_4 is not easily hydrolysed.

A possible repeating unit could be 3 hexose sulphate, 2 uronic acid, 3 rhamnose, and other sugar. M.Wt., 1624; Eq.Wt., 333; CO_2 from 812; SO_4 , 16.6% of NaSalt; rhamnose 30.3% of acid form polysaccharide.

Summary of the properties of the lower glycoside fractions.

Fraction.	B1	A2	A3	A3 fully methyltd	B4
B.Pt.(bath)	106	117	135	100	144
% OMe	49.1	43.6	41.6	56.2	51.3
% OMe after hydrol.	42	33.5	30.0	43.9	
η_{sp} before hydrol	1.4503	1.4524	1.4586	1.4432	1.4633
Rg values after hydrol.			0.47(tr)		0.54
		0.61	0.62		0.74
	0.82	0.82	0.82		
	0.87	0.87			0.86-0.87
				0.9(tr)	
				1.0	
	1.01			1.01	

The more volatile constituents are similar in fractionations A and B, but significant differences appear in the 130 - 140 b.pt. region, second fractions show a rise in % OMe and chromatography shows different constituents. As there was very little evidence for pentose in earlier work of E.D. Johnson and now xylose

has been detected by D.M.Hardy, it seems probable that spot Rg 0.74 is due to 2:3-dimethyl-D-xylose (Rg = 0.74 [56]).

In fraction B1, 2:3:4-trimethyl-L-rhamnose is definitely present, confirmed by anilide, to the extent of 50% from chromatography. The second constituent is probably 3:4-(or other)dimethyl-L-rhamnose; 3:4- is the only dimethyl rhamnose investigated. A high proportion of rhamnose is in agreement with the S.F., methoxyl content, increase in the positive sense of rotation in water as compared with that in organic solvent, and the changes in rotation on hydrolysis [57, 58]. The observed value of n_D is high for trimethyl- plus dimethyl-methyl-rhamnoside plus other fully methylated sugar. But the presence of trimethyl-hexose would account for the observed high value of n_D . Trimethyl-glucose seems probable from chromatography, spot Rg 0.82, and this constituent is present in A3, which gives fully methylated glucose on treatment with Purdie reagents. Trimethylglucose would also contribute the additional positive rotatory power shown by this fraction as compared with rhamnose derivatives alone.

Physical constants of the pure methylated glycosides under consideration are shown overleaf.

	%OMe	%OMe after hydrol.	n_D before hydrol.	$[\alpha]$ after hydrol.
50% 2:3:4-methyltrimethyl-L-rhamnoside	56	45	1.4415	20
25% 3:4-methyldimethyl-L-rhamnoside	45	32	1.45	19
25% 2:3:6-methyltrimethyl-D-glucoside [54]	52	42	1.4583	70
Mixture	52	41	1.45	42

figures agreeing well with those found. Thus a rhamnose end group forms ~5% of the glycosides. The probable 3:4-dimethyl-L-rhamnose has been obtained from various mucilages where 2-D-galacturonido-L-rhamnose occurs eg. [50]; oxidation by Br₂, for the isolation of 3:4-dimethyl-L-rhamnonolactone has not yet been tried.

Although its properties all suggest material more fully methylated than dimethylhexose, the anilide from fraction A2 was similar to the dimethylgalactose anilide, m.p. 130°, prepared by Robertson and Lamb [61], and thought to be the 2:3-dimethyl compound. Since the anilide from A3 purified had a m.p. corresponding to 2:3:4-trimethyl-D-galactose anilide, it seemed that galactose was present. However authentic 2:3-dimethylgalactose anilide has now been synthesised by D.J. Bell and does not possess the properties of the compound obtained by Robertson and Lamb; neither can any evidence of dimethyl hexose in A2, or tri- or tetra-methylgalactose in A3 purified, be obtained by chromatography. 2:3-dimethylrhamnose and 2:3:6 trimethyl-

glucose do not form crystalline anilides, so that the anilide from A2 is probably not a mixture, confirmed by lack of fractionation in repeated crystallizations.

Rotations of A3 before and after hydrolysis indicate the absence of significant quantities of di- and trimethyl-L-rhamnose, in agreement with the absence of spots Rg 1.01 and 0.87 on chromatography. Rg 0.82 is presumably trimethyl glucose and one of the other constituents is a rhamnose derivative since rhamnose is indicated by chromatography after complete methylation. The properties of fully methylated A3, except rotation, suggest that it is mainly methylated rhamnose, but as tetramethylmethylglucoside is present there must also be a fully methylated compound with high positive rotation.

In B4 the constituent Rg 0.86-0.87 is probably due to the same trimethyl glucose, persisting through the other fractions. Rg 0.74 is possibly due to 2:3-dimethyl-D-xylose, quoted Rg 0.74 [56], xylose is known to be present in the carbohydrate [41]. The rise of % OMe at this b.p. suggests fully methylated biose; Rg 0.54 is not due to dimethylhexose or monomethylrhamnose, (Rg values of this order quoted) for the % OMe of B4 is too high. It is more likely to be due to unhydrolysed biose, but fully methylated sugar should appear from hydrolysis of this constituent.

Since there are many methylated sugars (e.g. dimethyl-

rhamnose other than 3:4) which have not yet been investigated by paper chromatography, other conclusions concerning the glycosides are possible and none will be reliable until comparison with authentic compounds has been carried out in more than one solvent mixture.

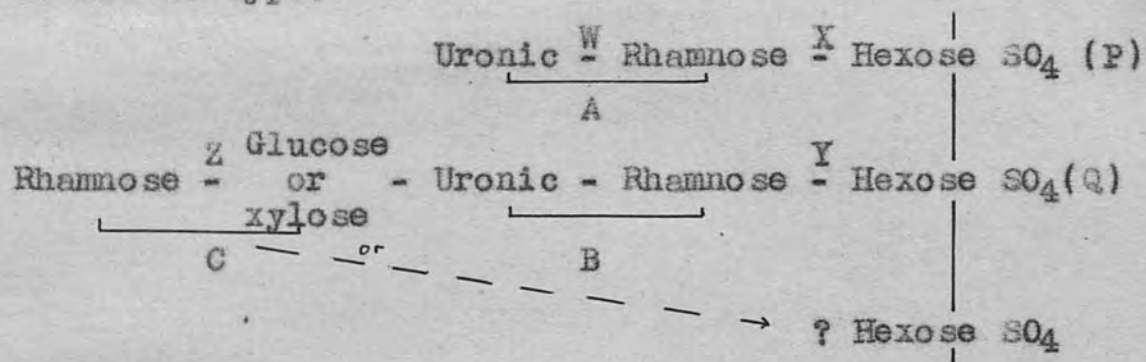
Concerning the anhydro compound, a methylated anhydro-glycoside (B.p. $\sim 90^\circ$) or corresponding dimethyl acetal (B.p. $\sim 110^\circ$) could be formed on methanolysis, both being likely at the strength HCl used, if the structures are comparable with 3:6-anhydrogalactoses [20]. Dimethyl-acetal would show a higher % OMe and fall in % OMe on hydrolysis than is found; a monomethyl-3:6-anhydro-methyl-hexoside seems probable. Such a structure would be expected to give the corresponding aldehyde compound on 0.1 N and hydrolysis, without extensive decomposition, but oxidation can easily occur and may account for the losses during the hydrolysis of fractions A2 and A3.

The crystalline material of the ester fraction could be a dimethyl-anhydro-hexonic ester (39.1% OMe) although the galactose compound of this type [20] does give an aqueous solution with positive rotation; a methylated saccharic ester is not likely. The properties of the distilled portion suggest a mixture of the methyl esters of methyl-mono- and di-methylhexuronic acid (39.4% OMe) but not fully methylated compound. Physical

constants do not permit of any distinction to be made between glucuronic and galacturonic acids. The undistilled residue is probably a bionic ester (fully methylated rhamnobionic ester has 49.5% OMe).

The fractions of the fully methylated material not so far investigated cannot have very much lower methoxy content and are probably merely less completely hydrolysed.

It is not possible to draw conclusions as to the structure of the macro molecule. A repeating unit might be of the type:-



fragment A being lost in dialysis by fissure at X during Me_2SO_4 , NaOH methylation. A biose fragment is probable owing to the known stability of links of type W, this making possible a loss of SO_4 from P with formation of anhydro structures e.g. by liberating $\text{C}_3\text{-OH}$ if $\text{C}_6\text{-OSO}_3\text{H}$ were present. After methanolysis, B would yield uronic and biuronic acid. Some of the link Y might be broken during extraction with acidified CHCl_3 , but subsequent conditions are not those in which Q would be expected to form an anhydro structure. The rhamnose end group and methylated glucose and xylose and biose come from C. In

view of the isolation of galactose by M. Georg [39] the hexose, PQ, is possibly galactose.

It is not possible to reconcile this structure, nor even the methylated fragments (if 3:4-, 2:3- and 2:3:6-methylated compounds) with the periodate uptake of the polysaccharide; the rhamnose end group should give significant quantities of H.COOH, which was not found [41]. If links W and Z are 1:3- and not 1:4- the theoretical periodate uptake (2 molecules) is in better agreement with that found.

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