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ELIZABETH DENT JOHNSON.

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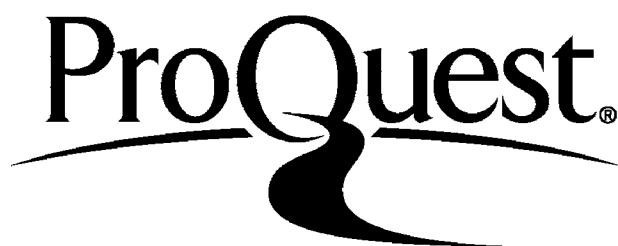
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Abstract of Thesis submitted for the Degree of
Doctor of Philosophy in the University of London
by Elizabeth Dent Johnson.

AN INVESTIGATION OF THE CARBOHYDRATE OBTAINABLE
FROM THE GREEN ALGA ULVA.

The carbohydrate obtainable from a member of the chlorophyceae has been investigated for the first time.

This polysaccharide is of an acidic nature and its extraction from the alga has been effected with hot dilute sodium carbonate solution.

Uronic acid residues do not appear to be present and the acidity of the material has been shown to be dependent on the presence of organically bound sulphur. It is suggested that such sulphur exists as a sulphuric acid ester group of the type $R.O.SO_2.OH$, but complete quantitative confirmation of this view is difficult to obtain.

The polysaccharide has been shown to consist in part of methyl pentose units, which are apparently removed preferentially during dilute acid hydrolysis, since the simple sugar fractions from such hydrolyses are richer in methyl pentose than the original material. Methylation of simple hydrolysis products has led to the isolation of a fully methylated methyl pentose and the identification of

the sugar as (1) rhamnose, the presence of which in algal carbohydrates has not previously been reported. Little evidence has been obtained as to the constitution of the remaining part of the carbohydrate complex but it apparently contains a certain amount of highly unstable material.

An arbitrary separation of the polysaccharide extract into two fractions according to their solubility in 50% alcohol has been effected. The fractions differ appreciably in their equivalent weights but in view of their very similar sulphate figures it appears that the higher equivalent weight of the more soluble fraction is due to the presence in it of a certain amount of sodium salt. There is a well-defined difference in the rhamnose contents of the two fractions, suggesting that a more profitable separation into a rhamnosan and a rhamnose-free polysaccharide might be possible.

AN INVESTIGATION OF THE CARBOHYDRATE

OBTAINABLE FROM THE GREEN ALGA ULVA.

The author wishes to express her sincere
thanks to Dr. M.M.T. Plant for directing
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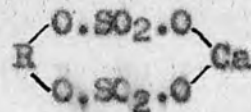
INTRODUCTION.

Complex carbohydrate material was extracted from certain algae as early as 1883(1). Although the acidic nature of the polysaccharide was noticed and it was given the name alginic acid no further investigation of its chemical composition was made and the material was merely used for commercial purposes.

Kylin in 1913(2) reported that alginic acid on acid hydrolysis produced only pentose sugars and Hoagland and Lieb (3) two years later showed that the alginic acid from *macrocystis pyrifera* after hydrolysis gave rise to a sugar with a phenyl osazone corresponding to either *d*-xylose or *d*-lyxose. In 1926 Atsuki and Tomoda(4) attributed the acidic properties of alginic acid to the presence in it of uronic acid residues since, on boiling with hydrochloric acid, carbon dioxide was evolved.

Nelson and Cretcher(5) have isolated a polysaccharide from the brown algae *laminaria aghardii*, *fucus serratus* and *macrocystis pyrifera* by extraction with sodium carbonate solution and subsequent acidification and precipitation into alcohol. They have proved conclusively in all three cases that it is a polyuronic acid which on hydrolysis yields mannuronic acid. This result was confirmed by Bird and Haas(6) in 1931 in the case of *laminaria*.

Polysaccharides, apparently of a somewhat different nature, have been isolated by various workers largely but not entirely from members of the Rhodophyceae. It had been known for some time that the carbohydrate extracted by water from *Chondrus crispus* (carrageen) left considerable quantities of calcium sulphate on incineration and the removal of this ash could not be effected by dialysis. Haas(7) in 1921 suggested that this calcium sulphate was not pre-existing but was produced from a calcium ethereal sulphate group in the polysaccharide, possibly represented by some formula such as:-



His evidence in support of this view was that, although the original material gave tests for the calcium ion, sulphate tests could only be obtained after hydrolysis or ashing and the percent sulphate in the ash was approximately one-half of that in the hydrolysate.

Russell Wells(8) has shown that the carbohydrates of other red algae contain ethereal sulphate groups and Butler(9) has investigated further the material obtainable by water-extraction of carrageen and has confirmed the presence of a sulphate ester group but considers that the cation contains other metals besides calcium, notably potassium. Apparently little was known of the nature of

the carbohydrate moiety but the presence of some galactose and of very small quantities of pentosan was reported(8).

By heating the red alga *Iridia laminariodes* with water, concentrating the extract and precipitating into alcohol, Hassid(10) has isolated a polysaccharide which he has investigated in some detail and shown it to be the sodium salt of a sulphate ester of a galactan. Unlike the ethereal sulphate salts from *chondrus crispus*, the sodium could apparently be removed easily by electro dialysis, leaving the sulphuric acid ester of the galactan which had a lower pH than the original salt and a somewhat different physical form, being flocculent, whereas the salt was fibrous. The ethereal sulphate group could be removed by acid or alkali hydrolysis leaving a galactan which was investigated by methylation. Hassid found no evidence for the presence of either uronic acid or pentose sugar residues and considered the polysaccharide to be made up entirely of galactose units. This does not, however, preclude the possibility of other carbohydrate material being present in the alga but not being extractable by water.

Bird and Haas(6) have shown that ethereal sulphate groups are also present in the mucilaginous material which exudes from certain seaweeds such as *laminaria* and *fucus*. This mucilage has long been known as fucoidin and Kylin(2) as early as 1913, reported the presence in fucoidin of the

methyl pentose fucose. Bird and Haas(6) confirmed the presence of fucose and also claimed that fucoidin contained a small percentage of uronic acid. Lunde, Heen and Öy(11) have made a detailed investigation of fucoidin. They have confirmed the presence of ethereal sulphate groups and have estimated the fucose quantitatively. They conclude that the whole of the carbohydrate cannot be accounted for as fucose but they disagree with Bird and Haas' statement that uronic acid residues are present and suggest that the product used by these workers was impure.

AIMS OF THE INVESTIGATION.

The foregoing account summarises the main lines which the investigation of algal carbohydrates has followed. It would appear that in general such polysaccharides are of a complex type, often containing other than simple sugar residues. Nevertheless, the subject has been treated by no means exhaustively and since the carbohydrate reserves of the green algae do not appear to have been studied, it was decided to investigate a member of the chlorophyceae from this point of view. A consideration of secondary importance was that it might eventually be possible to correlate in some way photosynthetic product with the algal pigment. The seaweed *Ulva* was chosen for this investigation since it could be obtained in quantity.

EXTRACTION AND FRACTIONATION OF CARBOHYDRATE.

By analogy with the methods of extraction used in the researches on other algae, already described, it seemed that it might be possible to effect an aqueous extraction of carbohydrate from the Ulva. The pigment extracted alga was therefore heated on the water-bath with distilled water. That material was being extracted by this method was obvious since, on straining off the fronds, a slightly viscous liquid was obtained which gave a strong Molisch-reaction. This process of extraction was repeated once, after which further extraction produced only very dilute solutions. When the combined extracts were evaporated to a small volume and poured into alcohol an almost white curdy precipitate was formed. The precipitated material was worked up by trituration with alcohol, the final product being a white powder. The water-extracted fronds were then subjected to further similar extractions using 0.5% sodium carbonate solution instead of water. On acidifying the concentrated carbonate extracts and pouring into alcohol a precipitate, resembling that from the aqueous extract, was obtained in appreciable yields. Hence it was concluded that the whole of the carbohydrate present in the alga cannot be directly extracted with water. It seemed that the extracting power of the sodium carbonate might be due either to the formation

of a soluble sodium salt (of an acid polysaccharide) from a pre-existing insoluble salt or to the actual disruption of the cells of the alga by the alkali and the consequent freeing of their contents for extraction.

Until the properties of the two extracts could be further elucidated it was considered desirable to treat the "aqueous" and "carbonate" extracts separately.

A preliminary investigation of the conditions under which the aqueous-extracted material could be hydrolysed showed that, on heating, hydrolysis occurred whenever the pH of the solution was less than 7. The aqueous-extract itself was, however, slightly acidic, a 1% solution having a pH of approximately 5 and on heating with water alone hydrolysis was shown to take place slowly. It was obvious therefore that extraction with water and subsequent evaporation of the aqueous solution was undesirable since the prolonged heating necessary would almost certainly cause some degradation. The decision was therefore made to extract entirely with sodium carbonate and the alga was treated once with 0.5% sodium carbonate and then three times with 0.25% sodium carbonate. When the combined extracts, after evaporation to a small volume were poured into alcohol so that the alcohol/water ratio was 4:1, a somewhat intractable fibrous precipitate was produced. If, however, the

concentrated carbonate extract was acidified and then poured into alcohol a more tractable, flocculent precipitate resulted. This, together with pH values mentioned later, almost undoubtedly indicated the presence of an acidic group in the polysaccharide, the material obtained after acidification being the free acid corresponding to the sodium salt isolated in the first case. This difference of physical form appears to be not unlike that observed by Hassid(10) in the case of the sulphuric acid ester of galactan and its sodium salt. Approximate pH values, obtained by the use of a series of indicators, showed that aqueous-extracted material had a pH of approximately 5 and carbonate-extracted acidified material (quite free from mineral acid) a pH of approximately 3.

The precipitated material, whether 'acid' or 'salt', was generally of a greenish-brown tinge, probably due to the extraction of any remaining pigment by the carbonate solution. This impurity was readily removed by solution and re-precipitation. The procedure adopted was, therefore, to precipitate as the sodium salt, swell it to a smooth gel by heating with a small volume of water and then to acidify with concentrated hydrochloric acid and re-precipitate the acidic material with alcohol. It was noticed, however, that during such acidification there was a great decrease in viscosity

and at the same time the solution became opaque, indicating the presence of water insoluble material. This suggested a possible method of fractionating the product. The acid solution was centrifuged but the supernatant liquid was still opaque. On adding alcohol, however, till the alcohol/water ratio was rather less than 1:1 and re-centrifuging a good separation was effected. The solid product was worked up by trituration with alcohol and designated Fraction I. The mother liquors, from which Fraction I had been precipitated, on pouring into sufficient alcohol to make the alcohol/water ratio 4:1, gave a further quantity of white flocculent precipitate designated Fraction II. It was later found that the addition of some water to the mother liquors before precipitation of Fraction II was advantageous since it reduced the quantity of sodium chloride thrown out along with this fraction.

To what extent this method of fractionating the carbohydrate extract is suitable will more profitably be discussed when all the information obtained about the two fractions has been presented.

PURITY OF EXTRACTED MATERIAL.

When the carbohydrate extract was subjected to the usual protein tests, negative results were obtained. Since

the tests had been shown to be applicable in the case of a starch-protein mixture containing as little as 0.1% of the protein constituent it was assumed that if the extract contained any protein at all it was present in negligible quantity.

The material had, however, a very appreciable ash content (15-40%). Examination of the residue after incineration indicated the presence of sodium, calcium, iron and sulphate ions. The removal of this inorganic matter by electro-dialysis across a parchment membrane was attempted. The colloidal suspension to be dialysed was placed between two membranes, a flow of distilled water being established on the other side of each membrane. Current densities of the order of 0.001 amp/cm² were used. It was found that some reduction in mineral content could be achieved by this method, but the results were disappointing as, even after dialysing for several days, the ash contents of the products isolated were high. Recovery of the material after dialysis was also difficult as precipitation of the acid was not easily effected.

The chief experimental difficulties encountered were:-

- (a) the maintenance of a flow of water on each side of the dialysing membranes at such a slow rate that the current did not fall below the value required. This was partially remedied by the

substitution of very dilute hydrochloric acid for distilled water, when it was possible to use a greater flow.

- (b) the prevention of material from settling to the bottom of the dialyser as a sludge, which caused a clogging of the membrane-surface. This difficulty was, to a certain extent, overcome by bubbling nitrogen through the dialysing solution.

Meanwhile it was found possible to remove a part of the inorganic matter from the carbohydrate extract in a much simpler manner. A considerable proportion of the ash was evidently calcium sulphate and some of this was seen to separate out during concentration of the extracts. This suggested that a part of the mineral impurity should be capable of removal by filtration before precipitation of the carbohydrate. This removal of calcium sulphate was effected by filtration, at the highest concentration practicable, first through cloth and then through filter papers and by this means the products were freed from the greater part of the insoluble inorganic matter present. The possibility of removing the remaining, largely soluble, ash was not pursued further although it is conceivable that dialysis might proceed more readily after the removal of the calcium sulphate. The presence of such impurity was not necessarily a serious hindrance in the investigations of the carbohydrate complex to be described, as the necessary ash correction could be made. In methylation experiments, from which a

considerable amount of information has been gained, the inorganic matter was automatically lost during the methylation processes so that ash-free products were obtained.

PROPERTIES OF THE EXTRACTED MATERIAL.

Fraction I was a brownish-white amorphous powder which swelled readily with water, in which it was slightly soluble to give a frothy colloidal solution. A 1% solution had a pH of approximately 3.

The greatly differing water solubilities of this acid and its sodium salt was easily shown by the addition of caustic soda solution to an aqueous suspension of the acid. After shaking for some time a solution sufficiently clear for optical use was obtained and the specific rotation in sodium hydroxide solution was determined:-

$$[\alpha]_{578}^{20^{\circ}} = -74^{\circ} (c = 0.88).$$

The physical form of Fraction II was very similar to that of Fraction I except that Fraction II was almost white. Fraction II exhibited a similar swelling effect with water, but its solubility was, as anticipated, very much greater. The solution obtained was, however, never sufficiently clear for the specific rotation to be determined, so, as with Fraction I, observations were made after treatment with alkali:-

$$[\alpha]_{546.1}^{20^{\circ}} = -76^{\circ} (c = 1.08)$$

The pH of a 1% solution of Fraction II was approximately 5.

The inorganic residues remaining after incineration of the two fractions were examined comparatively and the results may be summarised thus:-

Fraction I.

Fraction II.

SO₄ ++
Fe ++
Ca +
Na +?

SO₄ ++
Fe +?
Ca +
Na ++

(ash does not fuse) (ash fuses)

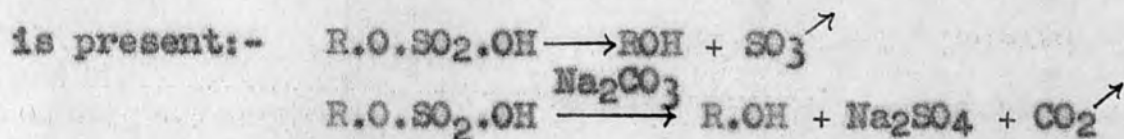
No test for the chloride ion could be obtained on the ash of either fraction, indicating the complete removal of sodium chloride during the precipitation and trituration of the products. That the removal of excess hydrochloric acid was also complete was shown by the fact that tests for the chloride ion, after ashing in the presence of sodium carbonate, still gave negative results.

SULPHATE ESTIMATIONS AND EQUIVALENT WEIGHTS.

In view of the reported occurrence of ethereal sulphate groups in the carbohydrates of various algae, the acidic nature of the Ulva product and the apparently high sulphate content of its ash, it seemed desirable to investigate this sulphate content more exhaustively.

A sample of Fraction II material was ashed in the usual way in a platinum crucible and another sample was ashed

similarly but in the presence of sodium carbonate. If an ethereal sulphate group were present there should be a greater quantity of sulphate retained in the ash when sodium carbonate is present:-



On testing for sulphate on the two ashes dense precipitates were obtained in both cases and these were very difficult to compare.

When barium chloride was added to an aqueous solution of the original Fraction II material only a very faint precipitate was produced, but on adding a drop of concentrated hydrochloric acid and heating, a dense white precipitate of barium sulphate was immediately thrown down. This seemed to indicate that the sulphate ions present in the ash were not pre-existing in the material before incineration or hydrolysis.

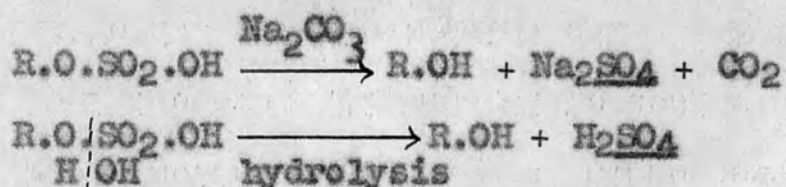
Quantitative estimations of the sulphate present after hydrolysis with 5% hydrochloric acid were made on corresponding samples of Fraction I and Fraction II material and values of 12% and 15% were obtained respectively.

The sulphate in the ash of this Fraction II material was found to be:-

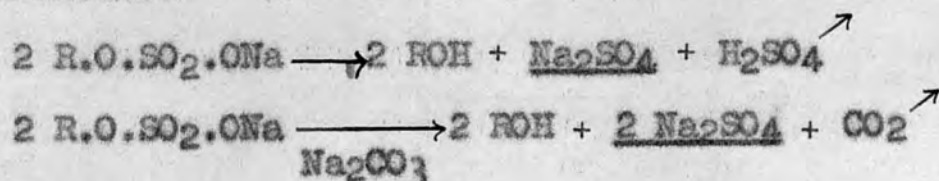
7% ashed alone
13% ashed with sodium carbonate)

These figures suggest that part at least of the sulphate must be organically bound.

There is reasonably good agreement between the percent sulphate present after hydrolysis and that after ashing with sodium carbonate, which would be the case were the source of the sulphate an ethereal sulphate group, since in both cases the whole of the sulphur would be estimated:-



The ratio of total sulphate to sulphate in ash is seen to be approximately 2:1 and this ratio has been confirmed in subsequent experiments. Were the Fraction II material undoubtedly a salt this ratio might be taken as quantitative evidence of the presence of a simple ethereal sulphate group of the type R.O.SO₂.OX (X = metal) such as Hassid(10) considers to be present in the carbohydrate of *Iridiae laminariodes*:-



Fraction II was, however, shown to be distinctly acidic and to have a pH approximately 5 (1% solution). Equivalent weight determinations by titration against decinormal alkali, showed that different samples of Fraction II material had equivalent weights varying from 1,000 to 1,400 and the Fraction II, on which the sulphate figures quoted above were

obtained, had an equivalent weight of 1,100. If the acidity were due entirely to sulphuric acid ester groups a total sulphate content of 13% would correspond to a lower equivalent weight than 1,100 (actually to an equivalent weight of 730). It seems, therefore, not unreasonable to suppose that Fraction II contains sodium salt as well as free sulphuric acid ester, since it would then have a higher equivalent weight for a given percent sulphate. The presence of a certain amount of sodium salt in Fraction II was also in agreement with the fact that the ash of Fraction II contained a very considerable quantity of sodium, which could not be due to sodium chloride. (The existence of sodium salt in Fraction II, which was, as already described, precipitated in distinctly acid solution, is not easily understood. The shifting of the equilibrium $\text{NaX} + \text{HCl} \rightleftharpoons \text{NaCl} + \text{HX}$ in the direction \leftarrow in the presence of alcohol might possibly be one of the causes.) At the same time, even if Fraction II is partially sodium salt, it is difficult to understand why so much sulphate is retained when it is ashed alone.

Sulphate determinations on the inorganic residue after incineration of a sample Fraction I gave:-

6% ashed alone
10% ashed with sodium carbonate)

The total percent sulphate, obtained when the material was ashed with carbonate (10%), is in fair agreement with that

quoted for sulphate in hydrolysate (12%). The equivalent weights of various samples of Fraction I material were found to be decidedly lower than those of Fraction II and to vary from 450-850. Assuming Fraction I to contain only free sulphuric acid ester and no sodium salt, a total sulphate content of 12% corresponds to an equivalent weight of 800. On the other hand, if the material is acid and not salt, it is not clear why sulphate should be retained during ashing without sodium carbonate. This unexpected retention of sulphate during the ashing of both Fractions I and II might, however, be due to the presence of oxides of other metals or to small quantities of inorganic sulphates not detected by the sulphate tests on the material before incineration on account of the large quantity of colloidal material present.

Hydrolysis of a sample of Fraction II material by heating with $N/5$ baryta for 7 hours gave a product with a very much higher equivalent weight viz. 12,000 and quantities of barium sulphate, indicating the removal of an acidic sulphur containing group.

The general inferences from the various sulphate-estimations appeared to be:-

- (1) that organically bound sulphur was undoubtedly present in the carbohydrate, since material originally giving no sulphate test gave strong tests after ashing and after hydrolysis.

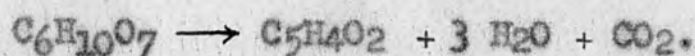
- (2) that the sulphur containing group (possibly an ethereal sulphate group of some kind) was responsible for the acidity of the material, since the removal of this group by baryta-hydrolysis left a non-acidic polysaccharide.

It is impossible to establish with any certainty the ratio of total sulphate/sulphate in ash unless material which is purely a salt of the supposed sulphuric acid ester (i.e. containing no free acid) can be obtained, since if any free acid is present there is always the possibility of "sulphate in ash" being low due to the escape of sulphuric acid. The preparation of a salt suitable for this estimation would offer considerable difficulty as the conversion of acid to sodium salt by addition of sodium carbonate is by no means accurate. The point at which the exact equivalent of sodium carbonate has been added and there is excess of neither free acid nor sodium carbonate cannot be determined until more is known about the material. It is obvious that the presence of excess sodium carbonate would also render the sulphate figure on the ash spurious since it would cause the retention of too much sulphate.

INVESTIGATION OF MATERIAL FOR THE PRESENCE OF URONIC ACID.

The extracted material was examined for the presence of uronic acid residues according to the method described by Dickson et al(12) with very slight modifications.

This determination is dependent on the practically quantitative yield of carbon dioxide from a uronic acid when it is boiled with 12% hydrochloric acid:-



The method consisted essentially in boiling the substance under investigation with hydrochloric acid in a current of carbon dioxide-free air and absorbing any carbon dioxide evolved in a measured volume of standard baryta solution. By back-titration of the baryta the weight of carbon dioxide given off from a known weight of the carbohydrate could be determined.

Determinations were carried out on a number of different samples of material and the results obtained may be summarised thus:-

Sample	Wt. of carbohydrate giving 44 gm carbon dioxide
(a) Aqueous extracted	(1500)
(b) " "	1200
(c) Carbonate extracted	936
(d) Partially dialysed	920
(e) 25% hydrolysed	960
(f) Fractionated - Fraction I	1220 (Equivalent weight = 620)
(g) " - Fraction II	1200 (" " = 1020)
(h) Fairly simple hydrolysis product (soluble in 85% alcohol.	1180

The fact that the carbohydrate contained an acidic group of some kind and undoubtedly evolved carbon dioxide under the prescribed conditions seemed at first to indicate the presence of uronic acid residues.

Examination of the figures quoted above does, however, reveal that the weight of material yielding 1 gm molecule of carbon dioxide shows surprisingly little variation considering the range of products examined. Another remarkable feature is that fractionation apparently causes no appreciable difference in carboxyl content even when the equivalent weights (determined by alkali titration) are widely different.

Again, attempts to isolate a salt of a simple glycuronic acid, after acid-hydrolysis of the polysaccharide, have always failed (see later section).

These facts, although not necessarily conclusive, all seemed to indicate that the source of the carbon dioxide measured might not be a uronic acid group. More definite evidence against the presence of such groups was obtained from furfuraldehyde estimations (described in detail later). Material giving evidence for uronic acid by carbon dioxide evolution was shown to yield no furfural but only methyl furfural resulting from methyl pentose residues. The sample (h) in the table of results given can be quoted as an

example. In a furfural estimation (h) gave a soluble brown methyl furfural phloroglucide, corresponding to 25% methyl pentose but no black insoluble furfural phloroglucide.

Hirst, Campbell and Young(13) conclude that the evolution of small quantities of carbon dioxide on boiling carbohydrate material with hydrochloric acid does not necessarily indicate the presence of uronic acid residues and they show that the yield of carbon dioxide from starch is of the same order as that from maltose or glucose (0.5%). They give values for the percent carbon dioxide evolved from a large number of simple sugars (0.3%-0.9%) and infer that the effect is in some way dependent on the reducing group of the sugar since, with mannitol, there was no evolution of carbon dioxide.

The amount of carbon dioxide evolved from the material under investigation was certainly decidedly greater than the values quoted by Hirst, etc., for the various simple sugars - being of the order of 4%. There has, however, been very definite evidence in both hydrolysis and methylation experiments of the presence of labile, easily oxidisable residues in the polysaccharide. It is, therefore, suggested very tentatively, since there is no direct evidence for the supposition, that this labile material may be responsible for the evolution of the small quantities of carbon dioxide estimated.

ACID HYDROLYSIS OF THE POLYSACCHARIDE.

In order to gain information as to the nature of the constituent units of the polysaccharide it was desirable to effect a breakdown to simpler material and the standard method of degradation of a polysaccharide by hydrolysis with mineral acid was applied.

Preliminary trial experiments showed that when the extracted material was heated on the water-bath with acid of moderate strength (about 2N) hydrolysis proceeded very rapidly and after only 1 hour the solution had charred considerably, exhibited a strong reducing action on Fehling's solution and readily absorbed alkaline iodine.

With more dilute acid ($N/25$, $N/50$ and $N/100$) small scale experiments indicated that a less vigorous hydrolysis occurred which could be followed by withdrawing portions of solution and titrating with iodine (method of Baker and Hulston⁽¹⁴⁾). The rate of hydrolysis was evidently dependent on the strength of the acid. On heating with water alone there was a slow hydrolysis, the rate of which was about half that when $N/100$ acid was used. This was no doubt due to the acidic nature of the material itself, since on heating an aqueous solution, to which alkali had been added till the pH was 7, no hydrolysis occurred and a similar result was obtained with a solution which was definitely alkaline.

Larger scale hydrolyses were then attempted with the object of isolating the products, partially or wholly degraded. In the first experiments of this kind aqueous extracted material was heated for 24 hours with $N/50$ sulphuric acid. After neutralising with baryta, filtering and pouring the concentrated filtrate into alcohol, a white precipitate not unlike the original material was obtained. The alcoholic mother liquors on evaporation left a very viscous syrup. It appeared, therefore, that material of varying complexity was produced and this was supported by the fact that the iodine-absorptions/gm. of the two products were markedly different. The mean of these iodine absorptions was, however, very considerably lower than that of the final hydrolysis mixture and this loss of reducing power was confirmed in subsequent similar experiments. That reducing power was lost during evaporation of the neutralised hydrolysis mixture was shown by iodine titration before and after concentration and in an experiment of this kind almost 70% of the reducing power was found to be lost during concentration to a third of the volume. When a hydrolysis-mixture was concentrated in an atmosphere of nitrogen there was only a very small decrease in the iodine-absorption/gm., suggesting that the previously observed loss of reducing power was due to an oxidation of labile material of some kind. In all

subsequent hydrolyses both the initial treatment with acid and concentration of the neutralised hydrolysis mixture were carried out in nitrogen and the results indicated the retention of reducing power.

Applying these precautions a further hydrolysis with $N/50$ acid was attempted and heating was continued until on plotting iodine-absorption/gm. against time it appeared that the rate of hydrolysis had become very small. On working up the hydrolysed material and fractionating by alcohol-solubility products of varying complexity were again isolated. The simplest of these products had, however, an iodine absorption/gm. of only 40 cc. $N/10$ (cf. absorption/gm. for glucose = 111 cc. $N/10$) indicating incompletely degraded material. Hence it was inferred that $N/50$ acid was capable of causing only a partial break-down of the polysaccharide and the observation that no monosaccharide was isolated pointed to the fact that this degradation was not brought about by the scission of simple sugar residues.

In order to effect a more complete degradation the extracted material was treated with very much more concentrated acid (N). It appeared, however, that this treatment was not satisfactory since, although a fraction of fairly high iodine-absorption (65 cc. $N/10$) was isolated, the yield of material recovered from the hydrolysis mixture was very low

and there was a considerable loss of reducing power. At the same time the simplest fraction had no measurable optical rotation. After conversion of this fraction to methyl glycoside, when the methoxyl content indicated that it was largely monosaccharide, it still had no apparent optical rotary power.

Since treatment with N acid appeared to result in a too drastic breakdown of the carbohydrate and $N/50$ acid to cause only a slight degradation, an acid of intermediate strength was next employed, viz., $N/5$ and hydrolysis with acid of this concentration has been shown to give satisfactory results. The loss of yield and of reducing power was not appreciable and in all cases some material was isolated, which from its solubility in 99.4% alcohol and its high iodine-absorption, was evidently almost entirely monosaccharide. Furthermore, the products of such hydrolyses had measurable specific rotations.

Hydrolyses of various samples of material were effected with $N/5$ acid, the reactions being followed by iodine titration and it appeared that the course of the hydrolysis was very similar whatever material was used (aqueous extracted, Fraction I or Fraction II). When the iodine-absorption/gm. was plotted against time it could be seen that in all cases there was an initial fairly rapid

reaction, the rate of which gradually decreased so that, after 20 hours, continued heating caused very little change, the iodine-absorption then being of the order of 45 cc. N/10 per gm.

From such a hydrolysis-mixture the degraded material was isolated by neutralising with baryta and barium carbonate, evaporating and fractionating by alcohol-solubility. Titration of samples of the various fractions with iodine showed that the iodine absorption/gm. rose regularly with increasing alcohol-solubility. This is not readily understood if uronic acid residues are present in the polysaccharide since barium salts of simple glycuronic acids would presumably then be found in the hydrolysis mixture and these would have a low alcohol-solubility but high iodine-absorption. Furthermore, attempts to regenerate free glycuronic acids from hydrolysis fractions with moderately low alcohol solubility were unsuccessful.

Since salts of glycuronic acids appeared to be absent from the neutralised hydrolysis mixture, the evidence was that fractionation of such mixtures was resulting in a crude separation of the products according to their complexity. Observations of the optical rotations of the fractions from N/5 acid-hydrolysis showed that in all cases there was a regular increase in specific rotation, in the positive sense,

from the most complex to the simplest product and whilst the fractions with lowest alcohol solubility were laevorotatory the 99.4% alcohol soluble material was dextrorotatory

$[\alpha]_{546.1}^{20^{\circ}}$ being of the order of $+16^{\circ}$. When a hydrolysis of

Fraction II material with $N/10$ acid was followed polarimetrically and arrested whilst the optical rotation was still very definitely negative, the products being isolated as described previously, a very large proportion of material recovered was found in the more complex fractions, having very appreciable negative specific rotations. There was, however, an extremely small quantity of 99.4% alcohol soluble product, apparently very similar to that obtained in $N/5$ acid-hydrolysis. On attempting to hydrolyse further by the use of more concentrated acid the various fractions from this comparatively mild-hydrolysis there was a considerable loss of material and at the same time, very little increase in iodine-absorption and it appeared that a complete breakdown was taking place.

Apart from the observation that the more complex fractions from $N/5$ acid-hydrolysis mixtures were characterised by negative specific rotations, which showed a definite correlation with the iodine-absorptions of these products, very little further information was obtained as to the nature of such fractions. Some comparative figures, resulting from

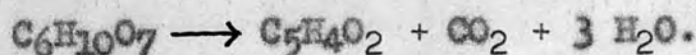
furfural estimations (see next section) on the fractions from hydrolysis mixtures were however obtained and these gave some indication as to the course such a hydrolysis follows.

More detailed investigation was made of the nature of the simple hydrolysis product, which was a fairly mobile syrup and had $[\alpha]_{20}^{20} +15 - +18^{\circ}$. That this material was almost entirely monosaccharide was shown from its high iodine-absorption/gm. and furfural estimations indicated that it contained up to 45% methyl pentose, the presence of which has been amply confirmed in methylation experiments. It seemed that the remainder of this fraction consisted of hexose sugars, there being no indication of it containing pentose. Evidence was therefore sought for the presence of one or more of the hexose sugars most usually occurring in natural products, viz. glucose, mannose, galactose or fructose. There was, however, no evidence of fermentation when an aqueous solution of the sugar mixture was treated with yeast at 30°C , the yeast having previously been shown to ferment a solution of glucose. Of the above-mentioned sugars glucose, mannose and fructose are readily fermentable whilst galactose ferments slowly. It is possible that the presence of galactose might not be detected by fermentation unless galactose-pretreated yeast was used. Likewise failure to

obtain crystalline mucic acid when the sugar mixture was oxidised with 30% nitric acid might not necessarily indicate the complete absence of galactose since no special refinements were employed. It seemed at this point more profitable to attempt to identify the constituents in the mixture of simple sugars after complete methylation and fractional distillation of the methylated sugars. The results obtained from methylation experiments will be mentioned in a later section. Hydrolysis experiments can, therefore, be said to have consisted mainly in determining the most suitable conditions for obtaining simple sugar material from the polysaccharide. Apart from the observation that such material exhibited a small positive specific rotation of the order of $+16^\circ$, further information as to its nature was only obtained as the result of the investigations described in the two sections following.

FURFURALDEHYDE ESTIMATIONS.

Qualitative colour tests appeared to indicate that furfuraldehyde was evolved when the carbohydrate extract was boiled with hydrochloric acid. Some evidence had already been obtained that the material contained uronic acid residues, which would yield furfural under these conditions:-



Quantitative estimations were therefore made with the object of finding if the furfural evolved from any given sample was in excess of that anticipated from the supposed uronic acid content, in which case the presence of pentose sugars would be suspected.

The method employed was that of Norris and Resch(15) and it consisted in boiling a known weight of material with 12% hydrochloric acid and precipitating the evolved furfural as the phloroglucide.

In several experiments of this kind the weight of phloroglucide precipitate obtained was considerably in excess of that expected from the uronic acid thought to be present and a value for the pentose content was calculated by using a formula suggested by Norris and Resch(16):-

$$A = 0.9942P - 1.9081C + 0.01313$$

where A = anhydropentose
P = phloroglucide
C = uronic carbon dioxide.

Later when a phloroglucide precipitate was examined according to the procedure described by van der Haar^r(17) it was found to have a distinctly brownish tinge and to be partially soluble in 96% alcohol at 60°. Furfural phloroglucide resulting from pentose or uronic acid is black and insoluble under these conditions, whilst methyl furfural phloroglucide from a methyl pentose is brown and soluble in 96% alcohol. The presence of methyl pentose

residues in the ulva product was therefore suggested.

When, however, simple material produced by hydrolysis of the polysaccharide was examined in this way it was found to give a phloroglucide precipitate all of which was soluble in 96% alcohol, indicating the presence of methyl pentose but the complete absence of either pentose or uronic acid. When this material was examined for uronic acid, by the method described in a previous section, it was shown to evolve carbon dioxide in the same percentage as other products. This seemed to prove beyond doubt that the source of such carbon dioxide was not a uronic acid group. All subsequent pentose-methyl pentose figures were therefore calculated on the assumption that uronic acid residues were absent.

Comparative furfural-methyl furfural estimations on a number of samples of Fraction I and II material revealed that the two fractions showed a well-defined difference as far as their pentose-methyl pentose content was concerned - the range for Fraction I being 16-25% and for Fraction II 27-38% (the wide range of values is not due to difficulty in obtaining consistent analyses but is no doubt dependent on the actual fractionation being somewhat indefinite). Both Fraction I and Fraction II original extract contained small quantities of pentose but when the products of their $H/5$ acid hydrolysis were examined it was found that pentose was only

present in the most complex fraction. At the same time the methyl pentose content increased from the most complex to the simplest fraction so that the percentage of methyl pentose in the simple sugar fraction was considerably greater than in the original material (e.g. 28% on a sample of Fraction I and 43% Fraction II). Methylation experiments, described in the section following, in which simple hydrolysis products were fully methylated, have confirmed the presence of methyl pentose in these proportions.

This would appear to suggest that during such acid-hydrolysis of the polysaccharide there is a preferential removal of methyl pentose residues, although other sugars are undoubtedly split off at the same time.

METHYLATION.

ATTEMPTED METHYLATION OF THE POLYSACCHARIDE.

Attempts were made to effect a methylation of the polysaccharide by means of dimethyl sulphate and alkali, the conditions at first employed being similar to those described by Haworth and Learner(18) in the methylation of inulin. A very concentrated aqueous gel of the material was treated with dimethyl sulphate and 35% caustic soda at 50°C, a small volume of acetone being added during the reaction. No chloroform-soluble methylated material could be isolated when the methylation-mixture was worked up,

neither did methylation appear to be effected when considerable volumes of methyl alcohol were added throughout the reaction nor when dimethyl sulphate and potassium hydroxide were used in the cold.

Likewise efforts to obtain a methylated derivative of the polysaccharide by de-acetylation and methylation of an acetate were unsuccessful since attempts to introduce acetyl groups into the polysaccharide failed.

METHYLATION OF SIMPLE HYDROLYSIS PRODUCTS.

More successful results have been attained in attempts to prepare fully methylated derivatives of simple hydrolysis products (designated for convenience H.S.).

In a small scale trial experiment H.S. material (soluble in 85% alcohol) from $N/5$ acid hydrolysis of Fraction I was first converted to methyl glycosides by treatment with methyl alcoholic hydrogen chloride. The methoxyl content at this point indicated that the material must be largely monosaccharide. Successive treatments of the glycosides with the Purdie reagents resulted in the production of chloroform soluble material and a gradual increase in methoxyl content. After five treatments the methoxyl was 50.1% and this was unaltered in two further methylations. The yield of methylated product was 85% which was fairly satisfactory considering the small

quantities being employed. Fractional distillation of this material gave two fractions of methoxyl 54.2% and 44.9% respectively.

In a somewhat larger scale experiment glycosides resulting from Fraction II H.S. on two treatments with dimethyl sulphate and caustic soda gave a product of moderately high methoxyl content (45%) but in yield of only 66%. After treating this product three times with the Purdie reagents a methoxyl content of 52.8% was attained and this was unaltered in a fourth methylation, the total loss of material in the Purdie treatments being only 4%. The methylation product on fractional distillation yielded two major fractions, the more volatile having OCH_3 49.1% and the less volatile OCH_3 52.9%. Since the undistillable residue would undoubtedly have a still lower methoxyl content it appeared that methoxyl was lost in the fractionation. That these products were by no means pure was revealed when samples of each were hydrolysed with $\text{N}/10$ acid for the removal of the glycosidic methoxyl group. The hydrolyses were followed polarimetrically and in both cases discontinuous curves resulted when $[\alpha]_{546.1}^{20}$ was plotted against time. Such curves could scarcely represent the removal of glycosidic methoxyl from single methylated sugars, nor were the methoxyl figures on the products easily

interpreted, their order of magnitude now being reversed (39.1% and 37.5% respectively).

The hydrolysed and unhydrolysed portions of each fraction were combined and separately treated again with the Purdie reagents. Since the methoxyl contents of each were now found to be increased above their previous values the Purdie treatments were continued until it again appeared that no more methoxyl could be introduced (OCH_3 55.0% and 53.2% respectively).

When the two fractions were combined and refractionated two major products of OCH_3 55.1% and 53.2% were obtained. There were, however, considerable losses during this fractionation and the yield of the higher boiling fraction was very small.

Hydrolysis of a sample of the first fraction ($\text{OCH}_3 = 55.1\%$) was effected by boiling with $N/10$ hydrochloric acid and was followed polarimetrically. The hydrolysis curve was smooth and indicated that after about 30 hours a constant specific rotation was attained ($[\alpha]_{546.1}^{20^\circ} \Delta 20^\circ$). The product was isolated in 80% yield and had $\text{OCH}_3 = 44.3\%$. The methoxyl contents before and after hydrolysis appeared to be in reasonably good agreement with those of a trimethyl-methyl pentoside and a trimethyl-methyl pentose (viz. 56.3% and 45.1%).

Attempts to effect a crystallisation of the supposed trimethyl-methyl pentose from ether-petroleum ether were unsuccessful but a crystalline anilide was prepared by boiling the methylated sugar with dry freshly-distilled aniline in ethyl alcohol solution. When recrystallised from petroleum ether the anilide was deposited in rosettes of almost colorless needle-shaped crystals which melted sharply (with slight decomposition) at 113°C . This agreed reasonably well with the melting point quoted by Irvine and McNicoll(19) for the anilide of trimethyl rhamnose. Similarly the specific rotation of the final hydrolysis mixture, although of necessity only approximate, was in agreement with the values quoted for trimethyl rhamnose. More conclusive evidence for the presence of fully methylated rhamnose in such methylation mixtures was obtained in larger scale methylations.

Hence, methylation by the agency of dimethyl sulphate followed by methyl iodide treatment gave indication that the methyl pentose constituent of the Fraction II H.S. (about 40%) was rhamnose but little evidence as to the nature of the remaining part of the hydrolysis product was gained. There were considerable losses throughout this methylation, those in the methyl sulphate treatment being outstanding. The fact that there was no such serious loss of yield during

the small scale trial methylation using the Purdie reagents only, seemed to suggest the presence of labile material unable to withstand the alkali-treatment. Other indications of the presence of such labile material had been obtained in uronic acid determinations and during hydrolyses. In the latter case it had been shown that the greater part of such breakdown could be avoided by hydrolysis in an atmosphere of nitrogen.

When a control dimethyl sulphate methylation of some Fraction II H.S. was carried out in nitrogen, the loss of material in two such treatments was found to be very little different from that in the previous dimethyl sulphate methylation, a product of OCH_3 45% again being isolated in 70% yield. It seemed, therefore, that although dimethyl sulphate methylation was effective in giving rise to a product of relatively high methoxyl, there was a large and unavoidable loss of material during such treatment and in subsequent methylations the Purdie reagents were employed throughout and it was hoped that by this means it would be possible to gain some information as to the nature of the non-rhamnose portion of the simple hydrolysis product.

In a larger scale methylation using the Purdie reagents only a sample of Fraction II H.S. (methyl pentose content 42%) gave after 8 treatments a product with OCH_3 54.2%.

The loss of material during these treatments was only 8% and most of this occurred during the first methylation. When the methylation mixture was fractionally distilled the products indicated in the table below were isolated.

<u>Fraction</u>	<u>Yield</u>	<u>Bath Temperature</u>	<u>Pressure</u>	<u>OCH₃</u>	<u>n_D^{22°}</u>
A	2.54 gm.	73°	0.22 mm.	55.0%	1.4443
B	0.43 "	94°	0.22 mm.	55.7%	1.4456
C	1.09 "	105°	0.25 mm.	57.8%	1.4506
D	0.65 "	110-170°	0.30 mm.	56.9%	-

EXAMINATION OF FRACTIONATED METHYLATED II H.S.

Fraction A.

This fraction boiling at 44°C/0.22 mm. had OCH₃ 55.0%. When the glycosidic methoxyl group of this product was removed by hydrolysis a hydrolysis-curve was obtained which was similar to that reported by Hirst(21) for the hydrolysis of a mixture of α and β methyl rhamnosides and the methylated sugar isolated after hydrolysis had OCH₃ 44.4%. Determination of the specific rotation of this material in water and alcohol gave $[\alpha]_{D}^{20} = +24.0^{\circ}$ (water) and

$[\alpha]_D^{20} = -6.5^{\circ}$ (alcohol) (both determined as $[\alpha]_{546.1}^{20}$). The

values quoted by Hirst and Macbeth(21) for trimethyl rhamnose are $[\alpha]_D^{20} = +24.9^{\circ}$ (water) and $[\alpha]_D^{20} = -9.0^{\circ}$ (alcohol). The

rotation in water of the methylated sugar from the ulva product was thus in reasonably good agreement with that reported for trimethyl rhamnose. The value of the rotation in alcohol is probably low in the negative sense on account of the alcohol not being entirely water-free.

A phenyl hydrazone of the supposed trimethyl rhamnose was prepared by the method described by Purdie and Young(20). After recrystallisation from ether the hydrazone was deposited in light yellow prisms having melting point 136°C . This melting point is higher than that quoted by Purdie and Young(20) but micro-analysis figures on the product agreed more closely with the theoretical values for trimethyl rhamnose phenyl hydrazone than do those of Purdie and Young.

A crystalline anilide was again prepared and had melting point 113°C , the value quoted by Irvine and McNicoll(19) for the anilide of trimethyl rhamnose being $111-113^{\circ}\text{C}$. The anilide exhibited muta-rotation in acetone solution(22), the muta-rotation rapidly proceeding to completion on the addition of a trace of hydrogen chloride. The final specific rotation

was $[\alpha]_D^{20} = +45.5^\circ$ as compared with a value $+46.9^\circ$ quoted by Irvine and McNicoll.

The evidence was, therefore, that the methyl pentose constituent of Fraction II H.S. was rhamnose.

Fractions B, C and D.

The Zeisel figures on all these fractions of the methylation mixture were higher than those of the fully methylated rhamnose fraction being 55.7%, 57.8% and 56.9% respectively. These fractions were therefore combined and refractionated in the hope of separating fully methylated hexose. The major product of this second fractionation, which distilled over a very small temperature range, when boiled with $N/10$ acid gave a smooth hydrolysis curve with a final $[\alpha]_D^{20} \approx +83^\circ$, suggesting that the sugar might be tetramethyl glucose ($[\alpha]_D^{20} = +83^\circ$) (23). The methylated sugar was isolated in good yield but attempts to effect a crystallisation, using authentic tetramethyl glucose as nucleus, were unsuccessful. On combining this material with the unhydrolysed part of the same fraction and with the other products of the latest fractionation and re-treating with the Purdie reagents there appeared to be some decomposition as the product isolated was much darker and had a very pungent smell, these properties again being present after a further Purdie treatment. The product was isolated in 90% yield and

the methoxyl content was found to have fallen to 53.1%. Fractional distillation of this material gave a major fraction of OCH_3 53.2%. It seemed possible that this loss of methoxyl together with the pungent odour developed might be due to an oxidation. A somewhat similar observation was made with the higher boiling constituents of methylated Fraction I H.S.

Time did not permit of the further investigation of this material so that little is known of the non-rhamnose constituent(s) of Fraction II H.S. but it would seem to contain material which is undoubtedly unstable. The apparent decrease of methoxyl content during Purdie treatments may be due to the complete loss of highly methylated material by breakdown and it is conceivable that this or some similar effect is responsible for the fact that during the initial Purdie treatments of the sugar mixture it was never possible to obtain a product of OCH_3 greater than 55%.

A comparative methylation of Fraction I H.S. (methyl pentose content 26%) was carried out using the Purdie reagents only. A methoxyl content of 54.0% was attained after 8 methylations. There was, however, a very considerable loss during such treatment, 12% of material being lost during conversion to methyl glycosides and almost 24% during the first Purdie treatment. Since this loss was very

much greater than that occurring during the methylation of Fraction II H.S., already described, and as the two methylations were carried out under exactly similar conditions, it appeared that Fraction I was much richer in the unstable constituent, the presence of which has already been postulated.

Fractionation of the mixture of methylated sugars gave the products tabulated below:-

<u>Fraction</u>	<u>Yield</u>	<u>Bath Temperature</u>	<u>Pressure</u>	<u>OCH₃</u>
(a)	0.95 gm.	55°	0.17 mm.	56.0%
(b)	1.16 gm.	90°	0.30 mm.	58.2%
(c)	0.21 gm.	91-160°	0.30 mm.	-

Removal of the glycosidic methoxyl group from Fraction (a) was effected by boiling with decinormal hydrochloric acid, the hydrolysis curve resembling that of fully methylated rhamnose. The methylated sugar, having OCH₃ 45.0% was identified as trimethyl rhamnose through formation of the anilide.

Fraction (b) showed no alteration in optical rotation when boiled with decinormal acid and with 2N acid there was only a very small rotation change. Attempts to

increase the methoxyl content of fractions (b) and (c) by further Purdie treatments led, as in the case of the higher boiling fractions from methylated Fraction II H.S., to the production of dark colored, pungent smelling material with a lower methoxyl content. Here again it was not possible to continue the investigation further so that only the rhamnose constituent was identified.

SUMMARY.

Polysaccharide material of an acidic nature has been extracted from the green alga *Ulva* by the agency of very dilute sodium carbonate solution.

Uronic acid residues appear to be absent and it is suggested that the acidity of the material is due to the presence of a sulphuric acid ester group of the type $R.O.SO_2.OH$. Complete quantitative confirmation of this view is at present difficult to obtain.

Methyl pentose residues have been shown to constitute a part of the polysaccharide structure and such residues are apparently removed preferentially during dilute acid hydrolysis, since the simple products from such hydrolyses are richer in methyl pentose than the original material. Methylation of simple hydrolysis products has led to the isolation of a fully methylated methyl pentose and the identification of the sugar as rhamnose (hitherto unknown in algal products). Little is known as to the constitution of the remaining part of the carbohydrate complex but throughout the investigation there has been evidence of the presence of a decidedly unstable constituent.

A somewhat arbitrary separation of the extracted material into two fractions, according to their solubility in 50% alcohol, has been effected. Although the fractions show

a definite difference in their equivalent weights this may be due merely to the presence of a certain amount of sodium salt in the 2nd fraction. The two fractions do, however, differ very appreciably in their rhamnose content, which suggests that the method of fractionation employed is not fundamental and that it might be possible to effect a separation into a rhamnosan and a rhamnose-free complex.

EXPERIMENTAL.

Immediately after collection the alga was washed several times with water to free it from adhering impurities and then dried thoroughly. In this state it could be stored for an indefinite period.

Before extracting the carbohydrate it was found advantageous to remove the greater part of the pigment by treatment with cold 80% aqueous acetone over a period of some few days, preferably with exposure to sunlight.

EXTRACTION PROCESSES.

1st Method employed.

25 gm. of the pigment-extracted alga was heated for 12 hours on the water bath with 2 litres of water (temperature attained = 80°C). The extract was then strained off from the fronds which were again subjected to the same treatment. The combined aqueous extracts, after evaporation to about 200 cc. were poured with continuous stirring into 650 cc. alcohol, when a white curdy precipitate was formed. This precipitate was separated from the supernatant liquid by centrifuging and was then triturated twice with alcohol. After drying in vacuo at room temperature the product was in the form of a fine white powder (yield \approx 18% weight of dry fronds).

The water-treated fronds were twice extracted with 0.5% sodium carbonate solution under the same conditions. When the combined carbonate extracts had been evaporated to 200 cc. 2N hydrochloric acid was added until there was free mineral acid present and the product was obtained by precipitation into alcohol and worked up in the same way as the aqueous extract. The yield was about 10% the weight of the fronds and the material was very similar in appearance to the first product. There was, however, a definite difference in the pH values of 1% solutions of the two extracts, viz.

aqueous extract pH \approx 5)
carbonate " pH \approx 3).

2nd Method using Sodium Carbonate only.

100 gms of the pigment-extracted ulva together with 8 litres of 0.5% sodium carbonate were heated at 80°C for 4 hours. This treatment was followed by 3 extractions with 0.25% sodium carbonate lasting 6, 12 and 12 hours. The combined extracts after evaporation to a volume of about 6 litres were filtered through fine cloth for the partial removal of calcium sulphate. A further quantity of calcium sulphate was removed by slow filtration through filter papers when the extract had been evaporated to a volume of 3 litres. After further concentration to about 1 litre, when the extract was in the form of a fairly stiff gel, concentrated

hydrochloric acid was added with constant and rapid stirring until most of the excess sodium carbonate was removed. On pouring the slightly alkaline extract into 4 litres of 95% alcohol a fibrous precipitate of sodium salt was formed. This product, after removal of the supernatant alcohol, was broken up and swollen to a smooth gel by heating on the water bath with approximately 150 cc. of water. After cooling to room temperature concentrated hydrochloric acid was added until free mineral acid was present. This resulted in the solution becoming completely mobile and at the same time turbid. Addition of 150 cc. absolute alcohol caused a settling out of the precipitate, which was centrifuged off from the supernatant liquid and worked up by alcohol trituration (Fraction I). The mother liquors, from which Fraction I had been precipitated, were diluted with 150 cc. of water and poured into 1 litre of absolute alcohol when Fraction II was precipitated.

Fraction I obtained in yield of $\approx 8\%$ weight of the fronds, was a slightly brownish powder having an ash content of 4-12% and containing about 10% moisture. It was slightly soluble in water, giving a frothy colloidal solution. The pH of a 1% solution was ≈ 3 . Measurement of the specific rotation in sodium hydroxide solution gave:-

$$[\alpha]_{578}^{20^{\circ}} = -74^{\circ} \quad (c = 0.88)$$

Fraction II. The yield of this fraction was \approx 12% weight of the fronds, the material being in the form of an almost white powder, with a moisture content of about 10% and containing 8-16% ash. Fraction II was water soluble and a 1% solution had pH \approx 5. The specific rotation in sodium hydroxide solution was:-

$$[\alpha]_{578}^{20^{\circ}} = -75^{\circ} (c = 1.08)$$

The extracted material gave no indication of the presence of protein when tested with Millon's reagent or by the xanthoproteic test, both tests having been shown capable of detecting 0.1% protein in a starch-protein mixture.

ELECTRODIALYSIS.

All attempted dialyses were carried out in the earlier stages of the investigation on samples of material from which calcium sulphate had not been removed by filtration before precipitation. The ash contents of such material were very high, being 20-40%.

In preliminary experiments, samples of aqueous-extracted material with ash contents of the order of 20% were used in about 1% solution and dialysed against distilled water. The electrolysers employed was as shown in Figure I and current densities of the order of 0.001 amp/cm² were maintained.

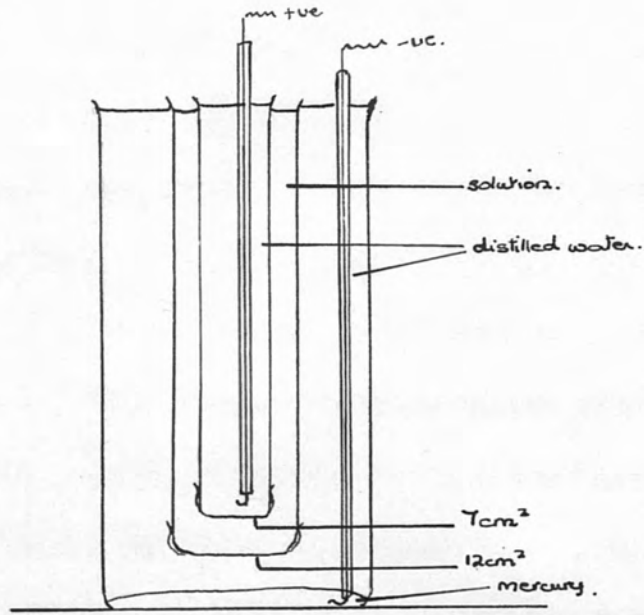


Fig. 1.

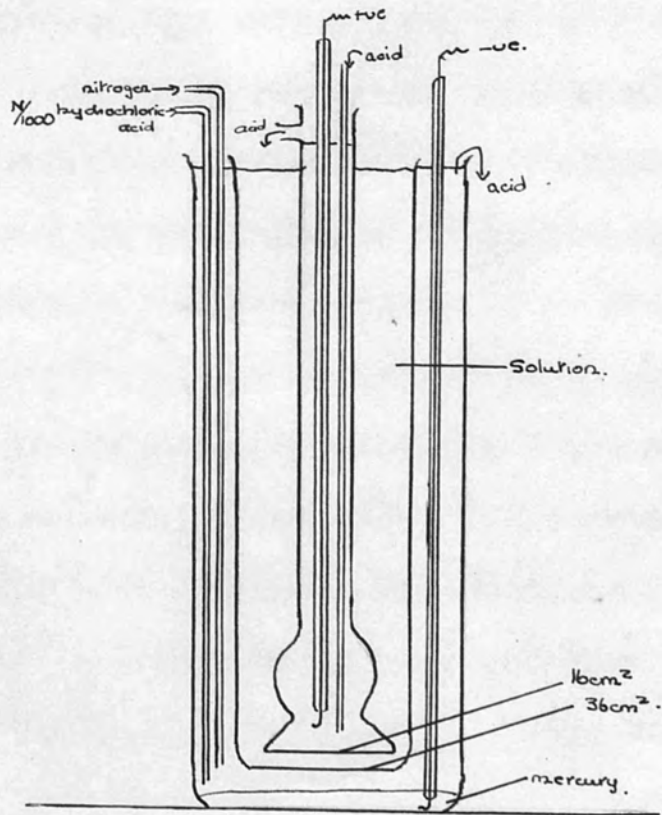


Fig. 2.

Attempts were made to estimate the reduction in ash content during dialysis by withdrawing from the solution in the central compartment aliquot portions and evaporating and incinerating them. This method was, however, not satisfactory, no doubt due to the settling of solid material to the bottom of the vessel. Dialysis was continued for about ten days, the distilled water being changed at frequent intervals. On working up the dialysed solution by evaporation and alcohol precipitation, very poor yields were obtained and the products isolated had ash contents not very different from the original material.

Running distilled water was next employed with a larger dialyser. The maintenance of a flow of water at such a slow rate that the current did not fall below the required value was, however, a matter of considerable difficulty. In order to permit of a somewhat greater flow the inner membrane was increased in size and the distance between the electrodes was decreased as shown in Figure II. Even with these improvements it was found impossible to adjust the water flow so that a sufficiently large current was obtained. $N/1000$ hydrochloric acid was therefore substituted for distilled water in the reservoir and with this modification a steady current could be maintained. At the same time, a stirring device was introduced to prevent sludge from settling out

from the solution onto the dialysing membrane. This stirring was effected by bubbling nitrogen through the solution.

Several dialyses were attempted with the modified apparatus using samples of carbonate-extracted (unfractionated) material of ash content of the order of 40%.

In a typical experiment 10 gms of such material (ash content = 40%) together with 700 cc. N/1000 hydrochloric acid was placed in the central compartment of the dialyser (Figure II) and a flow of N/1000 hydrochloric acid was established on either side of the membrane. The total membrane-area was 52 cm² and the currents used were of the order of 0.05 amp. After dialysing for 600 hours the material was recovered in 40% yield and had an ash content of 21%.

SULPHATE ESTIMATIONS.

(1) Sulphate in Hydrolysate.

Fraction I. To 0.2063 gm of Fraction I in 80 cc. 5% hydrochloric acid an excess of barium chloride solution was added. There being no initial precipitate the mixture was heated for 5 hours on the water-bath, after which time it appeared that the precipitation of barium sulphate was complete. The barium sulphate was collected on a fine, ashless filter paper, dried, ignited with the addition of a little

concentrated sulphuric acid and weighed = 0.0475 gm. whence SO₄ = 10%.

Fraction II. The total sulphate was estimated as for Fraction I, 0.1300 gm. giving 0.0399 gm. barium sulphate. Hence SO₄ = 13%.

(2) Sulphate in Ash.

Fraction I. 0.2259 gm. Fraction I was heated carefully in a platinum dish until only inorganic matter remained. The ash was extracted three times by heating with water on the water-bath and the sulphate precipitated by acidifying with hydrochloric acid and adding barium chloride. The barium sulphate, after collection in a small Gooch crucible was dried, ignited and weighed = 0.0348 gm. Hence SO₄ = 6%.

In the Presence of Sodium Carbonate.

In a determination of the total sulphate by ashing with sodium carbonate 0.3005 gm. Fraction I together with about 1 gm. of anhydrous sodium carbonate were ignited as in the previous estimation and the barium sulphate precipitated from the acidified extract weighed 0.0712 gm. Hence SO₄ = 9%.

Fraction II. With similar experimental procedure a sample of Fraction II material gave the following figures:-

without carbonate 0.5272 gm. \rightarrow 0.0905 gm. barium sulphate.
Hence $\text{SO}_4 = 7\%$.

with carbonate 0.1810 gm. \rightarrow 0.0556 gm. barium sulphate.
Hence $\text{SO}_4 = 13\%$.

HYDROLYSIS WITH BARYTA FOR REMOVAL OF SULPHURIC
ACID ESTER GROUP.

About 2 gm. of a sample of Fraction II material in 20 cc. water was warmed gently until a colloidal solution was formed. This was kept at 70°C and carbon dioxide free air passed through whilst 20 cc. 10% baryta solution was added through a filter (There was an immediate precipitation of a white bulky solid, most probably a barium ethereal sulphate of the polysaccharide and this made it difficult to see when barium sulphate was being precipitated and also undoubtedly accounted for the poor yield of product.).

The mixture was heated to 70°C for 2 hours, after which time precipitated barium sulphate (together possibly with some barium ethereal sulphate) was removed by centrifuging. Heating was then continued and the removal of barium sulphate was carried out at intervals until it appeared that there was no further deposition. After neutralising the excess baryta with sulphuric acid, the product was isolated by evaporating the solution and precipitating into alcohol. After triturating and drying a white powder very similar to the original material was obtained in 50% yield.

This had $[\alpha]_{546.1}^{20^{\circ}} = -54^{\circ}$ and the equivalent weight by alkali titration was 12,000.

EQUIVALENT WEIGHT DETERMINATIONS.

Although the carbonate-extracted, fractionated material was appreciably acidic it could not be titrated to a definite end-point with caustic soda. This was probably due to slow acid-base exchange and the difficulty was effectively overcome by adding a measured volume of aqueous sodium hydroxide to the material, leaving it to stand for 10-15 minutes and then back-titrating the excess caustic soda with dilute hydrochloric acid, using phenolphthalein as the indicator.

In an estimation of this kind a sample of Fraction I material, having ash content 6% and moisture content 9% gave the following results.

- (1) 0.3120 gm. reacted with 3.28 cc. 0.1228 N sodium hydroxide. ∴ Equivalent weight = 660.
- (2) 0.4342 gm. of the same sample reacted with 4.70 cc. 0.1228 N alkali. ∴ Equivalent weight = 640.

Likewise 0.6271 gm. of a sample of Fraction II material, with ash 12% and moisture 11%, reacted with 3.93 cc. of alkali of the same normality. The equivalent weight was therefore 1020.

In general it was found that the equivalent weights of samples of Fraction I material were 450-850 and those of Fraction II material were 1000-1400.

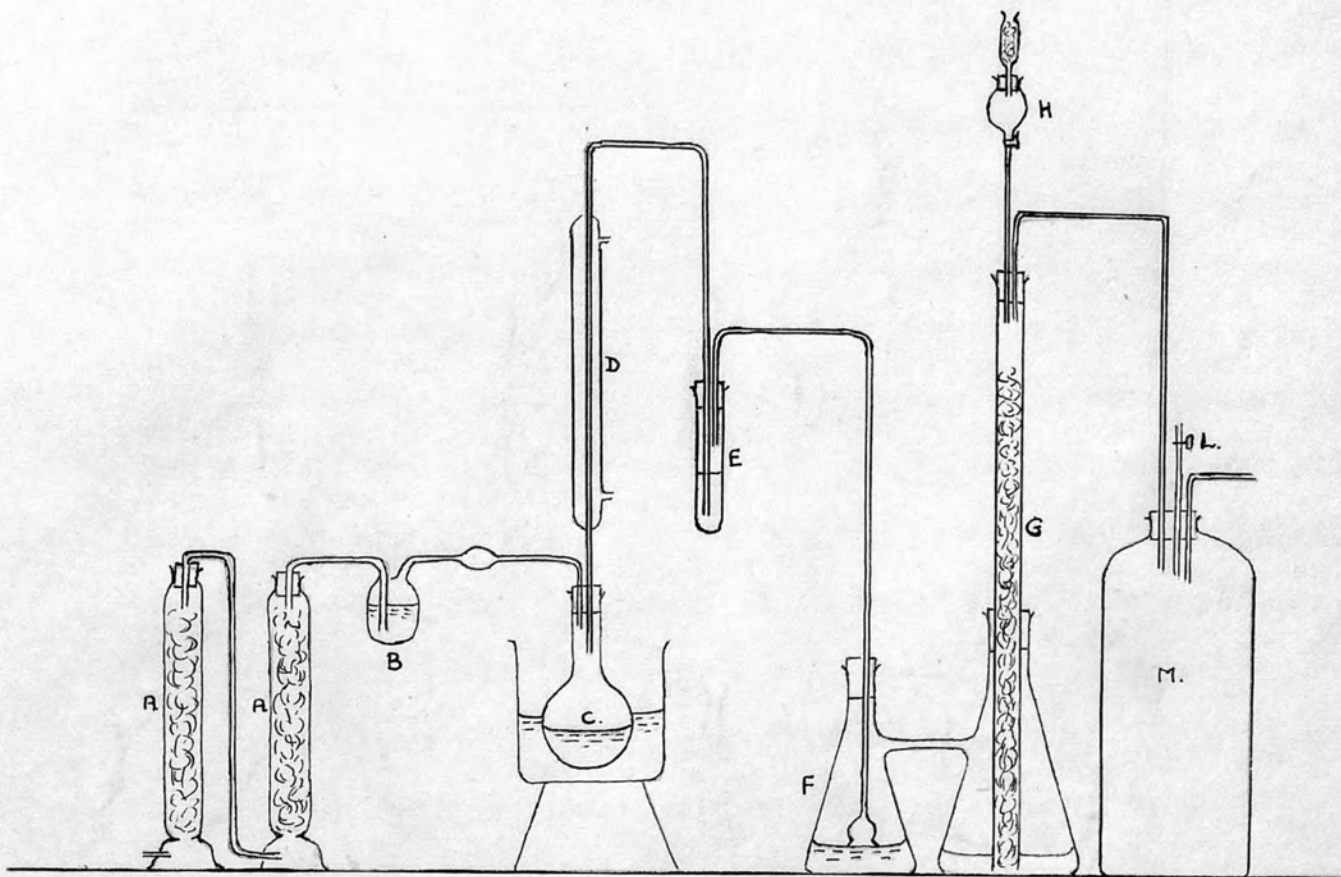


Fig 3.

EXAMINATION OF MATERIAL FOR URONIC ACID RESIDUES.

The apparatus employed in examining the extract for uronic acid was as shown in Figure III. The 500 cc. round flask C contained the sample of material under investigation together with 100 cc. 19% hydrochloric acid. The absorption tower G was filled with small glass beads and the tap-funnel H was charged with a known volume of $N/5$ baryta. Air, free from carbon dioxide by passage through the soda lime towers A, was drawn through the apparatus for twenty minutes so that all carbon dioxide was removed. C was then heated on an oil bath which was eventually kept at 135-140°C. When the solution in C just began to boil baryta was introduced into G from H and the air current, which was regulated by the screw-clip L and measured by the bubbler B, was at this point increased somewhat to prevent carbon dioxide or liquid being sucked back from C into B. When the solution was boiling gently L was adjusted so that 2 or 3 bubbles passed through B per second (E was an aniline trap for the removal of furfuraldehyde and F contained 10% silver nitrate to ensure that no hydrochloric acid was carried over to the baryta tower.).

After 4 hours the heating was discontinued and the tower was disconnected from F and M. The baryta in the tower was quickly washed into the flask with carbon dioxide free water and back titrated with $N/10$ hydrochloric acid using

phenolphthalein as indicator. The weight of carbon dioxide evolved from the sample was then calculated. It was necessary to carry out a blank experiment to ascertain whether the apparatus was leak-proof and to obtain a value for the small correction to be applied to the calculations due to carbon dioxide absorption during charging and titrating.

In an estimation of this kind 0.634 gm. of ash and moisture-free material gave carbon dioxide equivalent to 13.4 cc. $N/10$ hydrochloric acid, whilst in a blank experiment a titration indicating that carbon dioxide equivalent to 0.2 cc. of acid of the same strength was obtained. The weight of carbohydrate giving 1 gm. molecule of carbon dioxide was, therefore, given by the expression:-

$$\frac{2 \times 0.634 \times 10^4}{13.2} = 960.$$

A summary of the results obtained from such determinations is given in the theoretical section.

ACID HYDROLYSIS OF THE POLYSACCHARIDE.

Preliminary experiments having indicated that the extracted material exhibited an increasing iodine absorption when heated on the water-bath with water or dilute acid but that the products isolated after such treatment had iodine absorptions considerably lower than that of the final hydrolysis mixture an investigation of this peculiar effect was necessary.

For this purpose 4 gms of material together with 500 cc. $N/50$ sulphuric acid were heated under reflux on a boiling water-bath. At intervals suitable portions of solution were pipetted from the reaction flask and titrated as follows. 20 cc. of $N/10$ iodine were run into the test sample and then about 30 cc. of $N/10$ NaOH was added. After 3 minutes the solution was acidified with N sulphuric acid and the excess iodine titrated with $N/20$ thiosulphate.

Time (hours)	Absorption of $N/10$ iodine/gm.
0	3.5 cc.
12	8.8 "
21	10.6 "
36	16.0 "

The hydrolysis mixture after neutralisation with baryta and removal of barium sulphate was concentrated at $40^{\circ}\text{C}/30$ mm. to 150 cc. and the iodine absorption was again determined and found to have fallen to 5.5 cc./gm.

Investigation of the iodine absorption of the distillate showed that this only corresponded to about 1 cc $N/10$ /gm.

When, however, 4 gms of material were hydrolysed with 500 cc. $N/50$ sulphuric acid, a stream of nitrogen being passed through the solution both during hydrolysis and

concentration, the following results were obtained:-

Time (hours)	Absorption of $N/10$ iodine/gm.
0	0.2 cc.
2	4.5 "
4	6.5 "
11	11.0 "
25	20.0 "
36	23.5 "
51	25.3 "

After neutralisation and concentration of the hydrolysis mixture to 100 cc. at $45^{\circ}C/30$ mm. the iodine absorption per gm. had only fallen to 21.8 cc. $N/10$.

The hydrolysis mixture was fractionated as the table below indicates and the mean iodine absorption of the products was in fair agreement with that of the mixture.

Fraction	Method of Precipitation	Yield	Abs ⁿ of $N/10$ iodine/gm.	$[\alpha]_{546.1}^{20^{\circ}}$
1	75% alcohol	0.8 gm.	17	-
2	80% "	0.6 "	20	-25°
3	90% "	0.5 "	35	-20°
4	Unprecipitated with 90% alcohol.	0.6 "	37	-12°

HYDROLYSIS WITH N SULPHURIC ACID.

10 gms of material were hydrolysed for 9.5 hours in an atmosphere of nitrogen with 500 cc. N sulphuric acid. The iodine absorption per gm. was then 80 cc. N/10.

The mixture was neutralised and evaporated in nitrogen to 100 cc., the iodine absorption having fallen to 38 cc. The following products were isolated from the mixture:-

Fraction	Precipitation	Yield	Iodine Absorption	$[\alpha]^{200}$ 546.1
1	75% alcohol	1.0 gm.	16.6 cc	-
2	Residue	2.36 "	65 cc.	0

0.96 gm. of the second fraction from the hydrolysis mixture was converted to methyl glycosides by heating under reflux for 4 hours with 3% methyl alcoholic hydrogen chloride. After neutralisation of the acid with silver carbonate the methyl alcohol was removed by distillation to leave a dark syrup weighing 0.89 gm. and having OCH₃ 19.0%. The product had no measurable optical rotatory power.

HYDROLYSIS WITH N/5 SULPHURIC ACID.Aqueous Extract.

5 gms of aqueous extracted material (moisture 8%, ash 14%) was hydrolysed with 55 cc. N/5 sulphuric acid in an atmosphere of nitrogen, the course of the hydrolysis being

followed by iodine titration.

<u>Time (hours)</u>	6	16	21
<u>ccs N/10 Iodine absorbed/gm.</u>	31.5	45.5	46.1

A small quantity of suspended solid in the hydrolysate was removed by centrifuging and the clear liquid resulting was neutralised with baryta and barium carbonate.

After evaporation of the neutralised mixture, at 40°/20 mm., to a small bulk the products were isolated as indicated in the table below:-

Fract ⁿ	Yield	Nature	Ash	Mois- ture	I ₂ abs ⁿ /gm.	[α] ^{20°} 546.1
1	0.35 gm.	Solid suspen- sion remaining after hydrolysis	67%	7%	27	-
2	1.25 gm.	Precipitated by 80% alcohol	15%	9%	24	-18°
3	0.17 gm.	Precipitated by 92% alcohol	9%	10%	43	-23°
4	0.85 gm.	Precipitated by 99.4% alcohol	-	-	80	+9°
5	0.36 gm.	Soluble in 99.4% alcohol	-	-	83	+12°

Fraction I Material.

10 gms Fraction I extract after hydrolysis with 120 cc. N/5 sulphuric acid for 17 hours had an iodine absorption of 48 cc. N/10/gm. and this showed no appreciable

alteration on heating for a further 3 hours.

The hydrolysis mixture was very dark and there was a considerable quantity of suspended solid matter, which was removed by centrifuging and the remaining clear liquid was neutralised with baryta and barium carbonate, concentrated and fractionated as indicated in the appended table.

Fraction	Yield	Nature	Ash	I ₂ abs ^N / gm.	Pentose Content	Methyl Pentose Content	[α] ^{20°} 546.1
1	1.5 gm	Solid suspension after hydrolysis	13%	20cc. ^N /10	3%	3%	-
2	1.6 gm	Precipitated by 85% alcohol	8%	55 "	2%	10%	-7.0°
3	2.4 gm	Precipitated by 99.4% alcohol	-	83 "	0	21%	+11.5°
4	1.5 gm	Soluble in 99.4% alcohol	-	96 "	0	27%	+17.2°

The original Fraction I extract had 2% pentose, 15% methyl pentose.

Fraction II Material.

9.0 gm. Fraction II extract were hydrolysed with 150 cc ^N/5 sulphuric acid, the course of the hydrolysis being followed polarimetrically.

<u>Time (hours)</u>	2	6	8	20	24
$[\alpha]^{20^\circ}$	-60°	-20°	-11°	+11°	+12°
546.1					

At the end of the reaction the iodine absorption was determined and found to be 49 cc. $N/10/gm.$

The products tabulated below were isolated in the manner described previously.

Fraction	Yield	Nature	I ₂ abs ⁿ per gm.	$[\alpha]^{20^\circ}$ 546.1
I	0.16 gm.	Insoluble material removed after 1 hour's hydrolysis	-	-
II	1.63	Precipitated by 75% alcohol	16 cc $N/10$	-20°
III	1.1	Precipitated by 90% alcohol	36 "	+3°
IV	0.4	Precipitated by 99.4% alcohol	72 "	+8°
V	2.7	Soluble in 99.4% alcohol	93 "	+12°

HYDROLYSIS WITH $N/10$ SULPHURIC ACID.

5 gms of Fraction II extract were hydrolysed in 120 cc. $N/10$ sulphuric acid, the course of the reaction being followed polarimetrically. The hydrolysis was arrested whilst the optical rotation was still very definitely negative so that incompletely degraded material might be isolated.

<u>Time</u> (hours)	$[\alpha]^{20^\circ}$ 546.1	<u>I₂ absorption/gm.</u>
0	-100°	4.3 cc. N/10
1	-93°	10.6 "
4	-77°	13.3 "
7	-68°	18.7 "
14	-47°	32.0 "

The products isolated as in the previous hydrolyses were:-

Frac- tion	Yield	Ash	Mois- ture	$[\alpha]^{20^\circ}$ 546.1	I ₂ absorption per gm	Nature
I	1.85 gm	21%	13%	-67°	18 cc. N/10	Insoluble in 83% alcohol.
II	0.91 "	20%	-	-16°	34 "	Insoluble in 99.4% alcohol.
III	0.50 "	15%	-	+9°	74 "	Soluble in 99.4% alcohol.

ESTIMATION OF PENTOSE AND METHYL PENTOSE IN EXTRACTED MATERIAL AND ITS HYDROLYSIS PRODUCTS.

The sample of material to be examined was weighed into a 500 cc. flask and covered with 100 cc. of 12% hydrochloric acid. The reaction flask was fitted with a tap-funnel and a condenser, at the end of which was an adapter dipping into a conical flask.

The reaction flask was immersed in a paraffin oil bath maintained at a temperature of 170°C and when the hydro-

chloric acid reached boiling point a further 60 cc. of acid of the same strength was added, similar additions being made at 20 minute intervals for 2 hours. By this time about 400 cc. of distillate had collected in the receiver and to this was added 20 cc. of a solution of phloroglucinol in 12% hydrochloric acid (11 gm/230 cc.). The mixture was allowed to stand overnight and the phloroglucides precipitate was then filtered through a sintered glass crucible (Grade G.b.4), washed with 100 cc. distilled water, dried at 100°C for 3 hours and weighed.

The crucible and contents were then placed in a small beaker with 20 cc. 96% alcohol and heated to 60°C in a water bath for 10 minutes, after which the alcohol was sucked off at the pump and the extraction process repeated twice. During this treatment brown methyl-furfural phloroglucide, resulting from methyl pentose residues went into solution leaving the insoluble black phloroglucide of furfural formed from pentose or uronic acid. The crucible and remaining precipitate was dried again for 2 hours and re-weighed.

A sample of Fraction II extract gave the following figures:-

0.2094 gm (ash 13%, moisture 10%) → 0.0445 gm phloroglucides
↓
0.0045 gm (after alcohol extraction)

Hence methyl pentose content = 27.1%

and pentose content = 3.0%.

0.2375 gm of the simple hydrolysis product of the above material (ash 8%, moisture zero) 0.0892 gm phloroglucide
(Entirely soluble in 96% alcohol)

Hence methyl pentose content = 41.2%.

A comparable sample of Fraction I extract had methyl pentose content 16.2% and pentose content 2.1% and its simple hydrolysis product contained 28.5% methyl pentose.

METHYLATION OF POLYSACCHARIDE.

Methylation of 1.5 gm. of the polysaccharide was attempted using dimethyl sulphate and alkali. The carbohydrate extract was treated with a small volume of water so that a stiff gel was formed and to this the methylating agents, 20 cc. dimethyl sulphate and 50 cc. 30% caustic soda solution, were added in 10 portions at intervals of $\frac{1}{4}$ hour. The reaction mixture was kept at 50°C throughout the additions and was stirred vigorously. 25 cc. acetone was added after 1 hour and when all the reagents had been added the temperature was raised to 100°C and kept at this value for $\frac{3}{4}$ hour so that the excess dimethyl sulphate was removed. The mixture was then made just acid with concentrated sulphuric acid and filtered. The precipitate was extracted twice with boiling chloroform, but on removal of the solvent

there was no residue indicating that no chloroform-soluble, methylated material had been produced. A similar negative result was obtained after remethylation with the same quantities of reagents.

In a similar experiment in which caustic potash was substituted for caustic soda and the reaction mixture was kept at room temperature, no chloroform soluble material was produced. Nor was methylation of the polysaccharide effected when frequent additions of methyl alcohol were made to the methylation mixture.

ACETYLATION OF THE POLYSACCHARIDE.

Several attempts were made to introduce acetyl groups into 3 gm. portions of the extracted polysaccharide using various quantities of pyridine and acetic anhydride.

In one such experiment 3 gms carbonated extracted (unfractionated) material were heated to 80°C with 20 cc. pyridine and maintained at this temperature for 1 hour to effect a swelling of the material. The mixture was then cooled and 12 cc. acetic anhydride added slowly, after which the temperature was raised to 50°C, at which value it was kept for 20 days. The excess reagents were then removed by standing the mixture under water, which was repeatedly decanted off and renewed, until free from water soluble acid. The mixture was then centrifuged and the solid product was

washed very quickly with alcohol and ether and dried. The alkali-absorption was determined by treatment with $N/2$ caustic soda for 3 hours at 50°C , after which the excess alkali was back-titrated. In an estimation of this kind 0.234 gm. material was found to absorb only 2.9 cc. $N/2$ alkali, which is comparable only with the acidity of the original material.

Similar results were obtained when the method of acetylation was varied by alteration of the relative quantities of the reagents and the temperature and when undried material was employed.

METHYLATION OF SIMPLE HYDROLYSIS PRODUCTS.

PRELIMINARY EXPERIMENTS.

(1) Small-scale Methylation using Purdie Reagents only.

The material used for this methylation was part of Fractions 3 and 4 from the $N/5$ acid hydrolysis of Fraction I extract described on page 60. All the material was soluble in 85% alcohol.

1.3 gm. fraction 3 and 0.5 gm. fraction 4 were converted to methyl glycosides by heating under reflux for 4 hours with 50 cc. dry 4% methyl alcoholic hydrogen chloride. After neutralisation of the hydrogen chloride with dry, freshly prepared silver carbonate and removal of the silver chloride by filtration the methyl alcohol was distilled off under reduced pressure and the product isolated in 98% yield.

The methyl glycosides together with 10 cc. dry, freshly distilled methyl iodide and 5 cc. dry methyl alcohol were heated on the water-bath, at 45°C, under slight pressure for 6 hours, 10 gms dry, freshly prepared silver oxide being added in portions at 1 hour intervals. After methylation the mixture was repeatedly extracted with hot methyl alcohol and the product was isolated in 96% yield and had OCH₃ 25.2%. This partially methylated material was remethylated using 7 cc. of methyl iodide, 2 cc. of methyl alcohol and 7 gms of silver oxide, after which the methoxyl content had risen to 34.0%. The product was now soluble in methyl iodide so that in the five further methylations to which it was subjected no methyl alcohol was employed but the quantities of methyl iodide and silver oxide were unaltered and after each methylation the material was extracted with boiling chloroform. The methoxyl content could apparently not be raised above 50.1% and the final yield was 80%.

Fractional distillation from a flask with no fractionating column gave the following products:-

Fraction	Yield	OCH ₃	Mol. Wt.	Bath Temp.	Press.
1	0.58 gm.	54.2%	≈ 200	71°C	0.03 mm.
2	0.25 gm.	44.9%	≈ 200	166°C	0.03 mm.

The approximate molecular weights were determined by Rast's freezing point method with camphor as the solvent.

(2) Methylation using Dimethyl Sulphate and Alkali followed by the Purdie Reagents.

7.3 gms of simple material (soluble in 85% alcohol) from $N/5$ acid hydrolysis of 15 gms of Fraction II extract were converted to methyl glycosides by treatment with boiling 3% dry methyl alcoholic hydrogen chloride for six hours.

The glycosides, isolated in 95% yield, had OCH_3 16.2%.

6.95 gms of glycosides were dissolved in 10 cc. water together with 28 cc. acetone and to this solution the methylating agents, 48 cc. dimethyl sulphate and 32.5 gms sodium hydroxide in 120 cc. aqueous solution, were added in 10 equal portions at intervals of 12 minutes. The reaction mixture was kept vigorously stirred throughout and immersed in a water-bath, the initial temperature of which was $45^{\circ}C$, this being raised slowly to $50^{\circ}C$. After the addition of all the reagents the bath was heated very cautiously to $80^{\circ}C$ and kept at this temperature for 45 minutes to decompose the excess dimethyl sulphate. The reaction flask was then cooled to $0^{\circ}C$ and the mixture made just not acid with concentrated sulphuric acid, after which final acidification was effected by bubbling carbon dioxide through the mixture. Sodium sulphate was removed by filtration and the filtrate extracted

twice with 90 cc. of chloroform in the cold. The aqueous solution was re-filtered and evaporated to 30 cc. when a further quantity of sodium sulphate was filtered off. To the filtrate was added an equal volume of acetone and re-methylation was effected using 30 cc. dimethyl sulphate and 20.3 gms sodium hydroxide in 75 cc. water, the procedure being similar to that in the first methylation. After neutralisation of the alkali the methylation mixture was extracted twice with chloroform in the cold and the aqueous layer was evaporated to dryness, sodium sulphate being removed at intervals. After thorough drying of the residue a further extraction with hot chloroform was carried out. The combined sodium sulphate were likewise dried and extracted with chloroform in the hot. When the chloroform extracts were together evaporated to dryness the yield of methylated sugars was only 4.6 gms (or 66%). The methoxyl content was 45.0%.

(Since this poor yield suggested that material of low methoxyl content and small chloroform-solubility might have been left unextracted in the residues all the residues were re-extracted with methyl alcohol. Removal of sodium sulphate from these extracts was difficult and the combined extracts together with some remaining sodium sulphate were re-methylated using 24 ccs dimethyl sulphate and 16.2 gms sodium hydroxide in 60 ccs water. Extraction of the

methylation mixture with hot chloroform gave, however, only 0.03 gm. material.)

Further methylation was effected with dry silver oxide and methyl iodide. 4.6 gms partially methylated material dissolved in 16 cc. methyl iodide was refluxed at 45°C, under slight pressure, for 6 hours with 15 gms dry silver oxide, which was added in 6 portions at 1 hour intervals. The product was extracted with boiling chloroform and after removal of the solvent it was again methylated with the same quantities of reagents and isolated as before in yield of 4.6 gms. The methoxyl content was 51.5%.

After 2 further treatments with 9 cc. methyl iodide and 8 gms silver oxide 4.5 gms of methylated material were isolated having OCH_3 52.8%. The methoxyl content was unaltered after another treatment with the same quantities of reagents.

The product was fractionated and re-fractionated as indicated in the tables below. The fractionating apparatus employed in both cases being a small Quick-fit flask provided with a Dufton column.

Fraction	Yield	Bath Temp.	Internal Temp.	Pressure
1	2.19 gm.	130-131°C	83-85°C	14 mm.
2	0.87 "	50-130°C	70-75°C	0.06 "
3	0.05 "	150-160°C	60°C	0.06 "

Refractionation:-

Fraction	Yield	Bath Temp.	Internal Temp.	Pressure	OCH ₃
(a)	1.64 gm.	139-141°C	105-106°C	24 mm.	49.1%
(b)	0.88 "	92-93°C	45-50°C	0.06 mm.	52.9%
(c)	0.18 "	110-125°C	65-70°C	0.06 mm.	< 41%

Hydrolysis of Fractions (a) and (b).

Fraction (a).

0.39 gm. material was dissolved in 25 cc. $N/10$ hydrochloric acid. The initial optical rotation was determined and the solution was heated under reflux on the boiling water-bath until the polarimetric readings indicated that hydrolysis was complete:-

Time (hrs)	0	1	2	4.5	7.5	14	19	25	29	38.5
$[\alpha]_{20}^{546.1}$	-10.5°	-4.5°	0	+3.1°	+4.7°	+10.7°	+22.2°	+28.9°	+31.1°	+31.1°

The solution was neutralised with solid barium carbonate, filtered and evaporated to dryness. The dried material was extracted 3 times with boiling ether and after

removal of the ether a thin syrup, with a very pungent odour, was isolated. This product, weighing 0.38 gm. reduced Fehling's solution and had OCH_3 39.2%.

Fraction (b).

0.40 gm. of Fraction (b) was hydrolysed with 25 cc. N/10 hydrochloric acid in a manner similar to that employed for fraction (a). The optical rotations recorded were:-

Time (hours)	0	0.75	2	4	8	17.5
$[\alpha]_{20}^{20}$	+22.1°	+16.6°	+18.0°	+18.0°	+35.3°	+35.3°
546.1						

0.39 gm. of hydrolysed material was isolated as a thin syrup with a strong odour and the methoxyl content was 37.5%.

Further Methylation of (a) and (b).

The hydrolysed and unhydrolysed portions of both fractions (a) and (b) were given further treatments with the Purdie methylating agents:-

1.56 gm. of fraction (a) was dissolved in 5 cc. methyl iodide and refluxed for 6 hours at 45°C with 5 gms of dry silver oxide, added in portions at intervals of 1 hour. The product was extracted with hot chloroform and after removal of the solvent the methylation process was repeated with the same quantities of reagents. After 3 such treatments the methoxyl content was 55.1% and this could apparently

not be increased further. 0.9 gm. of product was isolated
 0.80 gm. of hydrolysed and unhydrolysed fraction (b)
 was similarly treated three times with the same quantities
 of methylating agents after which 0.51 gm. of material with
 OCH₃ 53.2% was isolated.

Combination of fractions (a) and (b) followed by
 refractionation from a flask without a column gave the
 following products:-

Fraction	Yield	Bath Temp.	Pressure	OCH ₃
A	0.95 gm.	55-56°C	0.07 mm.	55.1%
B	0.16 "	69-72°C	0.07 mm.	53.5%
Residue	0.05 "	-	-	-

Hydrolysis of Fraction A.

0.55 gm. of the major fraction (A) was dissolved
 in 20 cc. N/10 hydrochloric acid and after observation of the
 initial specific rotation, the solution was heated under
 reflux on a boiling water-bath until the polarimeter
 readings indicated that hydrolysis was complete:-

Time (hours)	0	0.5	1.0	1.5	2.5	4.0
$[\alpha]_{546.1}^{20^\circ}$	+5.0°	0	+2.5°	+3.7°	+6.1°	+11.0°

Time (hours)	6.75	9.25	14.25	19.5	23.5
$[\alpha]_{546.1}^{20^\circ}$	+12.5°	+15.2°	+19.5°	+20.0°	+20.0°

The hydrolysed material was isolated by neutralising with solid barium carbonate, filtering, evaporating to dryness and extracting with boiling ether. The yield was 0.43 gm. and the product had OCH_3 44.3% suggesting that it was a trimethyl methyl pentose.

Preparation of Anilide of Hydrolysed Fraction A.

0.4 gm. of the methylated sugar was dissolved in 5 cc. of dry ethyl alcohol containing 0.4 cc. dry, freshly distilled aniline. The mixture was boiled under reflux for 3 hours, after which the alcohol was distilled off under reduced pressure and the traces of excess aniline were removed by steam distillation to leave a mass of almost colourless needle-shaped crystals. When recrystallised from petroleum ether the anilide melted sharply at 113°C , which was taken to indicate that Fraction A was fully methylated rhamnose.

Preparation of Phenyl Hydrazone of Lactone of Hydrolysed Fraction A.

0.24 gm. of the methylated sugar was treated with 20 cc. bromine-water, containing 0.6 gm. bromine. The mixture was maintained at 30°C for 48 hours, after which the acid formed was neutralised with silver oxide. The solution was filtered, the precipitate being washed well, after which hydrogen sulphide was passed through the combined filtrate and washings, the precipitated silver sulphide being removed by filtration. The filtrate, after evaporation to dryness, was extracted with ether. Removal of the ether left a very viscous syrup weighing 0.11 gm. The small yield of lactone made purification by distillation impossible and an attempt was made to prepare the phenyl hydrazone directly by refluxing with the exact equivalent of phenyl hydrazine for $\frac{1}{2}$ hour in the presence of ether and then for 3 hours in the absence of solvent. The phenyl hydrazone failed to crystallise when the resulting syrup was triturated with ether.

INVESTIGATION OF LOSS OF MATERIAL DURING DIMETHYL SULPHATE/ALKALI METHYLATION.

In view of the very considerable loss of yield during the previous dimethyl sulphate methylation a second methylation was carried out with special precautions to reduce such loss to a minimum.

2.5 gms of Fraction II H.S. was converted to methyl glycosides by refluxing for 6 hours with 2% methyl alcoholic hydrogen chloride. The glycosides, isolated in 96% yield, had OCH_3 13.8%.

2.4 gms glycosides were dissolved in 40 cc. 75% acetone and to this solution were added 16 cc. dimethyl sulphate and 10.8 gms caustic soda in 40 cc. water, in 10 portions at 12 minute intervals. The mixture was stirred vigorously throughout the reaction and the initial temperature of 35°C was raised to 45°C after the first 3 additions of reagents and finally maintained at $45\text{-}50^\circ\text{C}$. After 1 hour a further 20 cc. acetone were added.

When the addition of the reagents was complete the reaction flask was heated slowly to 80°C , at which temperature it was kept for $\frac{3}{4}$ hour. Throughout the entire reaction a slow stream of nitrogen was passed through the solution.

After cooling, the mixture was made just not acid with concentrated sulphuric acid, the final acidification being effected by passing carbon dioxide through the solution. Sodium sulphate was removed by filtration, after which the mixture was twice extracted with chloroform in the cold. The aqueous layer was evaporated in an atmosphere of nitrogen to about 30 cc. when a further quantity of sodium sulphate was filtered off. This sodium sulphate was washed

carefully with 50% aqueous acetone and after evaporation of the filtrate and washings to 10 cc. a further 30 cc. of acetone was added and the mixture remethylated with 10 cc. dimethyl sulphate and 7.2 gms sodium hydroxide in 27 cc. water, nitrogen being passed throughout the reaction.

The neutralised methylation mixture was twice extracted with cold chloroform and the aqueous layer was evaporated, in nitrogen, to a syrup and this together with the combined sodium sulphate residues was dried thoroughly and extracted with hot chloroform. On removal of the solvent from the combined chloroform extracts a mobile syrup with OCH_3 45.3% was isolated in yield of 1.7 gm. (69% of theory).

METHYLATION USING THE PURDIE REAGENTS ONLY.

(A) FRACTION II. SIMPLE HYDROLYSIS PRODUCT.

9.0 gms of Fraction II H.S. (soluble in 85% alcohol), having a methyl pentose content of 41%, were converted to methyl glycosides by refluxing for 24 hours with 260 cc. 1% dry methyl alcoholic hydrogen chloride. After neutralisation of the mixture and removal of methyl alcohol by distillation under diminished pressure 8.3 gms of syrup were isolated, having OCH_3 16.3%.

PURDIE METHYLATION OF GLYCOSIDES.

8.3 gms methyl glycosides of II H.S. were dissolved in 20 cc. dry methyl iodide and 10 cc. dry methyl alcohol and

refluxed at 45°C under slight pressure, 20 gms dry silver oxide being added in portions at intervals of 1 hour. The partially methylated glycosides were extracted with boiling methyl alcohol and removal of the solvent left 7.8 gms of viscous syrup, which was remethylated with 14 cc. methyl iodide, 3 cc. methyl alcohol and 14 gms silver oxide. The product was extracted with boiling chloroform and weighed 7.7 gms. After 4 further methylations with 14 cc. methyl iodide and 14 gms silver oxide 7.7 gms of a very mobile syrup resulted in having OCH₃ 53.8%. Two more methylations with the same quantities of reagents gave 7.6 gms of product with OCH₃ 54.2%.

The methylation mixture was fractionated as indicated in the tables following:-

Fraction	Yield	Bath Temp.	Pressure
(a)	3.04 gm.	70-72°C	0.70 mm.
(b)	1.35 "	84°C	0.30 mm.
(c)	0.68 "	101-103°C	0.18 mm.
(d)	0.55 "	118°C	0.18 mm.
Residue	0.76 "	-	-

(Distillation without fractionating column)

Refractionation (from Quickfit apparatus with Dufton fractionating column)

Fraction	Yield	Bath Temp.	Internal Temp.	Pressure	OCH ₃	$n_D^{22^\circ}$
x	0.1 gm.	< 70°C		0.4 mm.		
A	2.54 gm.	72-73°C	43-44°C	0.25 mm.	55.0%	1.4443
B	0.43 gm.	93-94°C	41-45°C	0.25 mm.	55.7%	1.4456
C	1.09 gm.	104-105°C	49-54°C	0.22 mm.	57.8%	1.4506
D	0.65 gm.	110-170°C	≈ 70°C	0.30 mm.	56.9%	-
Residue	0.13 gm.	-	-	-	-	-

Hydrolysis of Fraction A (Fully methylated methyl pentose)

2.2026 gms in 20 cc. $N/10$ hydrochloric acid were heated on a boiling water-bath under reflux until hydrolysis was complete.

Time (hours)	0.5	1.25	3.25	8.75	13.0	16.0
$[\alpha]_D^{20^\circ}$	-0.5°	+4.2°	+10.4°	+17.0°	+20.3°	+20.4°

The hydrolysed material was worked up as described previously and had OCH₃ 44.4%. The yield was 2.0 gms.

The final specific rotation of this material in water was $[\alpha]_D^{20^\circ} = +24.0^\circ$ ($c = 9.2$) and in alcohol $[\alpha]_D^{20^\circ} = -6.5^\circ$

($c = 4.0$).

The phenyl hydrazone of the hydrolysed Fraction A was prepared by adding to 0.4 gm. of the methylated sugar the exact equivalent of phenyl hydrazine in 50% acetic acid solution. The sugar dissolved and an oil was precipitated. This failed to solidify on standing in ice overnight but on removal of the acetic acid and water by distillation and the addition of a further quantity of water light yellow prismatic crystals separated slowly. The product after recrystallisation from ether was isolated in 75% yield and had melting point 136°C . Micro-analysis gave the following figures C 60.95%, H 7.967%, N 9.7% and OCH_3 30.2%. Trimethyl rhamnose phenyl hydrazone $\text{C}_{15}\text{H}_{24}\text{O}_4\text{N}_2$ requires C 60.74%, H 8.18%, N 9.45%, OCH_3 31.2%.

The anilide of hydrolysed Fraction A was prepared by dissolving 0.5 gm. of the material in 5 cc. dry ethyl alcohol containing 0.5 cc. dry, freshly distilled aniline. After boiling the mixture under reflux for 3 hours, the alcohol was removed by distillation and the last traces of aniline by steam distillation. The anilide was isolated in 60% yield after recrystallisation from petroleum ether and was in the form of needle shaped crystals melting sharply at 113°C . The muta-rotation in acetone solution was observed.

Time (hours)	0	7	16	Trace of hydrogen chloride added. $+45.5^{\circ}$
$[\alpha]_{\text{D}}^{20}$	$+140.0^{\circ}$	$+136.0^{\circ}$	$+129.5^{\circ}$	

EXAMINATION OF FRACTIONS B, C AND D.

0.37 gm. B

0.96 gm. C

and 0.62 gm. D were combined and refractionated.

Fraction	Yield	Bath Temp.	Internal Temp.	Pressure
(a)	0.16 gm.	$\approx 80^{\circ}\text{C}$	40°C	0.20 mm.
(b)	0.25 gm.	$85-100^{\circ}\text{C}$	$42-43^{\circ}\text{C}$	0.24 mm.
(c)	1.01 gm.	120°C	$60-63^{\circ}\text{C}$	0.21 mm.
(d)	0.14 gm.	$124-170^{\circ}\text{C}$	70°C	0.20 mm.
Residue	0.12 gm.	-	-	-

0.584 gm. of the major fraction (c) was hydrolysed with $\text{N}/10$ hydrochloric acid in the manner described previously. The specific rotation was found to alter with time as indicated below:-

Time (hours)	0	0.5	1.5	2.5	4.5	7.5	13.0
$[\alpha]_{20}^{\text{D}}$	$+56^{\circ}$	$+63^{\circ}$	$+67^{\circ}$	$+72^{\circ}$	$+77^{\circ}$	$+80^{\circ}$	$+83^{\circ}$

The hydrolysed material was isolated in the usual manner and weighed, 0.57 gm. An attempt to crystallise the product by nucleation with tetramethyl glucose failed. Since it was thought that the failure of the material to crystallise

might be due to the presence of small quantities of incompletely methylated sugar, this product together with the unhydrolysed portion of fraction (c) and fractions (a), (b) and (d) was remethylated with the Purdie reagents. After two such treatments a dark coloured product with a very pungent odour was isolated in 90% yield. The methoxyl content was found to have fallen to 53.1%.

Refractionation gave 2 products, but the fractionation was not very sharp as the table below shows:-

	Fraction	Yield	Bath Temp.	Internal Temp.	Pressure	OCH ₃
1.37 gm. fractionated	(1)	0.40 gm.	110-120°C	45°C	0.25 mm.	53.2
	(2)	0.42 gm.	125-170°C	54°C	0.25 mm.	-
	Residue	0.20 gm.	-	-	-	-

Loss during fractionation = 0.35 gm.

(B) FRACTION I. SIMPLE HYDROLYSIS PRODUCT.

5.6 gm. of Fraction I H.S. (soluble in 85% alcohol) having a methyl pentose content of 26%, were converted to methyl glycosides in the manner described under Fraction II H.S. 5.0 gm. of glycosides were isolated, having OCH₃ 16.2%.

PURDIE METHYLATION OF GLYCOSIDES.

5.0 gms methyl glycosides of I H.S. were dissolved in 15 cc. dry methyl iodide and 8 cc. dry methyl alcohol. The mixture was refluxed for 6 hours at 45°C, under slight pressure, 15 gms dry silver oxide being added in portions at 1 hour intervals. The product was extracted 3 times with hot methyl alcohol and on evaporation of the solvent 3.8 gms of a rather viscous syrup were obtained. A further methylation of this material was effected with 9 cc. methyl iodide, 2 cc. methyl alcohol and 9 gms silver oxide and the product extracted with boiling chloroform. After 4 more methylations with 9 cc. methyl iodide and 9 gms silver oxide the yield of methylated sugars was 3.7 gm. and the methoxyl content 51.3%. After a seventh methylation with the same quantities of reagents the methoxyl content had risen to 54.0%, this being unaltered by an eighth treatment. The final yield was 3.7 gms so that practically all the loss of about 24% occurred during the first methylation (cf. loss of 8% during first Purdie treatment of Fraction II H.S.).

The methylation mixture was fractionated as shown in the following tables:-

Fraction	Yield	Bath Temp.	Internal Temp.	Pressure	(Distilled from Quickfit apparatus with Dufton fractionating column.)
A	1.03 gm.	74-76°C	42°C	0.22 mm.	
B	0.30 gm.	91-93°C	42°C	0.20 mm.	
C	1.32 gm.	108°C	58°C	0.22 mm.	
D	0.32 gm.	120-180°C	≈ 60°C	0.30 mm.	
Residue	0.38 gm.	-	-	-	

Re-fractionation.	Fraction	Yield	Bath Temp.	Pressure	OCH ₃	(Distilled from flask without fractionating column)
	(a)	0.95 gm.	54-55°C	0.17 mm.	56.0%	
	(b)	1.16 gm.	85-91°C	0.30 mm.	58.2%	
	(c)	0.21 gm.	91-160°C	0.30 mm.	-	
	Residue	0.12 gm.	-	-	-	

HYDROLYSIS OF FRACTION (a) (OCH₃ 56.0%).

0.92 gm. of (a) was hydrolysed with 10 cc. N/10 hydrochloric acid in the manner described previously. The hydrolysis was followed polarimetrically and was apparently complete after 19 hours:-

Time (hours)	1.5	5.5	9.0	16.0	19.0
$[\alpha]_{20}^D$	+5.2°	+12.5°	+15.8°	+21.0°	+21.0°

The hydrolysed material was worked up as in previous hydrolyses and was obtained in yield of 97%. The methoxyl content was 45.1% and the anilide prepared in the same way as that of II H.S.A. crystallised in rosettes of needles melting at 113°. The mixed melting point observed with a mixture of this product and the anilide of II H.S.A. was likewise 113°, indicating that the methyl pentose constituent of Fraction I H.S. was (1) rhamnose.

ATTEMPTED HYDROLYSIS OF FRACTION (b) (OCH₃ 58.2%).

When a sample of (b) was heated on the water-bath with N/10 acid there was no measurable alteration in the specific rotation. Treatment with 2N acid resulted in the specific rotation falling from +66° to +58° in 8 hours and the material isolated after this treatment had OCH₃ 48.2%.

The remainder of Fraction (b) together with Fraction (c) was given 2 further treatments with the Purdie reagents after which a dark-coloured product was isolated in 90% yield and having a pungent odour. The methoxyl content was only 56.1%.

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