UNIVERSITY OF EDINBURGH

ANALYTICAL STUDIES ON THE CARBOHYDRATES
OF THE PHAEOPHYCEAE

by

ALAN G. ROSS., B.Sc.

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The investigations described herein were undertaken under the auspices of the Scottish Seaweed Research Association, the aim and object of the research being to work out convenient and, if possible, rapid methods for the routine determination of certain constituents present in the seaweeds found round the Scottish coasts. The seaweeds found most abundantly in this area belong to the group Phaeophyceae or brown seaweeds which, along with the Chlorophyceae or green seaweeds, Rhodophyceae or red seaweeds and Cyanophyceae or blue-green seaweeds form the main bulk of marine algae. (1)

Although the red and brown seaweeds have received some study, very little is known of the polysaccharide constituents of the other two groups. Of the red seaweeds little more need be said here, except to note that these algae are the source of the well-known carrageenin derived from the genera Gigartina and Chondrus commonly known as Irish Moss. Other genera, found mostly off the coast of Japan, provide the important polysaccharide, agar. The Phaeophyceae contain mainly laminarin, alginic acid and fucoidin as the principal polysaccharide constituents. It is only this last section with which this investigation is concerned.
Several genera of seaweed occur in this latter division, notably *Laminaria*, *Fucus*, *Macrocystis* and *Sargassum* but of these, the most important are *Laminaria* and *Fucus*, although several others occur in the Scottish area, such as *Himanthalea* which is referred to in a later section as a source of fucoidin.

The bulk of the material in Scottish seaweeds is, apart from water, carbohydrate in nature along with inorganic matter, notably iodine, and smaller quantities of nitrogenous bodies such as protein and pigments, fats, etc. The carbohydrate portion consists mainly of laminarin (a polyglucoside with 1:3 linkages), alginic acid (a polyuronide) and mannitol, with fucoidin and algal cellulose in smaller amounts. The percentage of a given component varies very considerably with a number of factors and, in general, may be as high as 30% to the exclusion of others, or practically zero.

The Scottish Seaweed Research Association have undertaken a broad investigation into the variation of composition with the factors mentioned above, with a view to the future utilisation of the information for commercial purposes. It is obviously of the utmost importance to know the best location, time of year, type of seaweed, etc. for harvesting to obtain the optimum yield of the desired material in the purest
state. The Association have made analyses of seaweeds (mainly Laminariales) collected at monthly intervals from various sources, the fronds and stipes being treated separately. Their results have shown the influence of the following factors on the chemical composition.

1. Time of year of sampling.
2. Depth of immersion in the water.
3. Exposure to the air due to tides or wave action.
4. Variation from frond to stipe.
5. Open sea or sea loch sampling.

The following table gives a typical example of the results obtained. All the analyses are carried out on samples air dried to a moisture content of less than 10% and ground in a Christy and Norris mill to a fine powder. The seaweed used is Laminaria cloustoni collected monthly from November 1945 to November 1946 and the table shows the variation in mannitol content, the figures being based on the anhydrous weight.

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</thead>
<tbody>
<tr>
<td>Frond</td>
<td>12.5</td>
<td>14.5</td>
<td>10.0</td>
<td>5.5</td>
<td>4.9</td>
<td>6.0</td>
</tr>
<tr>
<td>Stipe</td>
<td>9.2</td>
<td>11.0</td>
<td>9.7</td>
<td>7.5</td>
<td>7.0</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>May</td>
<td>June</td>
<td>July</td>
<td>Aug.</td>
</tr>
<tr>
<td>Frond</td>
<td>5.0</td>
<td>16.8</td>
<td>27.0</td>
<td>25.7</td>
<td>22.3</td>
<td>22.0</td>
</tr>
<tr>
<td>Stipe</td>
<td>4.6</td>
<td>7.6</td>
<td>7.7</td>
<td>6.9</td>
<td>7.4</td>
<td>10.0</td>
</tr>
</tbody>
</table>
Methods of analysis have already been worked out for laminarin, mannitol and alginic acid. In the case of laminarin, hydrolysis to glucose and determination of the latter by a micro-copper reagent (Somogyi) is used. Mannitol is readily estimated by its rapid reaction with periodic acid solution. Alginic acid is not quite so straightforward and must be determined by titration with standard alkali after an extraction process. Part of the present research has been devoted to an attempt to apply a colorimetric reaction with carbazole to the estimation of alginic acid and is described later.

The remainder of the research is concerned with fucoidin and algal cellulose. Fucoidin is a carbohydrate sulphuric ester in the form of a metallic salt, the main sugar present being L-fucose, a methylpentose. Owing to the presence of other sulphate radicles in the seaweed a method of analysis for fucoidin was necessarily based on a fucose estimation after hydrolysis. Since the constitution of the polysaccharide was as yet undetermined, further investigations were carried out on fucoidin itself after isolation from the seaweed in order to find the percentage of fucose present.

Algal cellulose has been investigated from the point of view of its estimation and also as to its
constitution with particular reference to its identity with normal land plant cellulose.

Methods for the analysis of seaweeds for alginic acid (colorimetric), fucoidin and cellulose have been worked out and applied to seaweed samples and information obtained as to the constitution of the two latter substances. The thesis has, therefore, been divided into three parts:-

I  Cellulose.
II  Alginic Acid.
III  Fucoidin.
1. Newton.    A Handbook of the British Seaweeds (1931) IX.


3. Cameron, Ross and Percival.

PART I

CELLULOSE
INTRODUCTION

The term algal cellulose first appears in the literature about 1885 in a report by Stanford\(^1\) on alginic acid in which he claims to have obtained a pure cellulose, suitable for making a tough, transparent, non-fibrous paper and representing about 10% of the air-dried plant. Following this isolated instance little research appears to have been done on algal cellulose for some years. It is of interest to survey firstly the various methods since used to prepare algal cellulose.

Methods of Preparation.

In 1915, Kylin\(^2\) carried out some work on the subject from a quantitative point of view. By the method now used generally for determination of crude fibre in animal feeding stuffs, etc., i.e., by successive boiling with sulphuric acid (1.25%) and sodium hydroxide (1.25%), he obtained an insoluble residue which he called cellulose. The yields obtained for a number of species were as follows, the results being expressed as percentages of the dry weight content. This is the usual custom and is followed throughout this thesis.
Species | Percentage
---|---
Fucus vesiculosus | 1.6
Laminaria digitata | 5.3
" saccharina | 4.4
Ascophyllum nodosum | 1.7

The above treatment of seaweed with acid and alkali is the general basis of all later attempts to isolate the algal cellulose, although several minor modifications have been made, using different alkaline reagents, bleaches, etc.

Freundler and co-workers removed the alginic acid from the sample by means of sodium carbonate solution followed by water and hydrochloric acid washings. They obtained a cellulosic material, (in yields varying from 11-17%) which they could not free from nitrogen and sulphur and which was probably fairly impure.

Colin and Ricard(3) dissolved out the alginic acid in the same way, the residual material being boiled with dilute solutions of sulphuric acid and potassium hydroxide. The product was named "algulose" for reasons given later, and the yields from Laminaria flexicaulis were from 4.3-7.6% and from Laminaria saccharina from 2.8-10.9%.

Dillon and O'Tuama(4) varied the method slightly by treatment first with hydrochloric acid (1%), followed
by repeated extractions with dilute ammonia, till the extracts were free of alginate. The solid residue was again washed with acid and extracted with sodium hydroxide (5%), nine times, at boiling point for a few minutes. Finally the product was bleached with acid calcium hypochlorite, washed and dried. Naylor and Russell-Wells (5) used the same method as Kylin but showed that a much whiter product could be obtained by using a freshly gathered sample of seaweed as distinct from that from an old air-dried sample.

A considerable amount of work on algal cellulose has been done by Viel (6) who used the method of Cross and Bevan (7) modified and adapted to the particular case of algae. The seaweed was first extracted with cold acetone for several days until no more colour could be removed and then several times with water which removed soluble carbohydrates, some alginate and salts. The residue was boiled three times with sodium hydroxide (1%), washed and chlorinated during which process the sample turned yellow. Finally the yellow substance was washed with sodium sulphite solution (1%) and water. Several treatments with chlorine and sulphite were often necessary to obtain a white or cream-coloured product which was washed with alcohol and ether and dried. This author quotes the following figures.
Species | Percentage of cellulose
--- | ---
*Fucus vesiculosus* | 2.10
*Fucus serratus* | 2.81
*Laminaria saccharina* | 6.91
*Laminaria cloustoni* | 5.04

Hilpert, Becker and Rossèe\(^8\) carried out some extractions in an attempt to show the presence or absence of cellulose in marine algae, in which samples of *Fucus serratus* were extracted with various boiling reagents. Using sodium sulphite solutions of 15% and 30% the residues represented 15.1% and 13.4% of the dried seaweed. With sodium hydroxide (20%), in the case of fronds, less than 1% solid matter remained although the stipes yielded up to 12%. Treatment of the products with strong hydrochloric acid gave, depending on the strength of the acid, substances with high carbon content (48-59%) though with more dilute acid little reaction took place. It was suggested that an anhydro-polysaccharide is formed by this treatment, as opposed to hydrolysis. On this basis it was denied that the substance was a true cellulose.

Crude fibre determinations by Atsuki and Tomoda\(^9\) on samples of *Laminaria* gave yields of 6% which are stated to consist entirely of hemicelluloses but not of cellulose.
As can be seen from the above account all the methods are based on the removal of all other substances to leave the insoluble cellulose. Water or acid extraction removes all the soluble carbohydrates such as mannitol, laminarin and fucoidin, while converting insoluble calcium alginate into alginic acid in the case of acid extraction. The latter is then dissolved out with an alkaline reagent and the remaining impurities removed by bleaching and sulphite treatment. The products obtained appear to have been of varying degrees of purity and, in at least one case, the material was not regarded as cellulose at all.

Chemical Nature and Characteristics of Algal Cellulose.

Numerous authors have investigated the chemical properties of algal cellulose, especially with reference to its relation to land plant cellulose. Many attempts have been made to prove the identity or otherwise of the two substances and to date the results are conflicting and the conclusions indefinite. A point worth noting is the relatively small percentage of cellulose found in seaweeds as compared with land plants suggesting that this substance serves a different purpose in the structure of the plant in each case. A review of the evidence bearing on the chemical constitution of algal cellulose will show how the varied
views arose.

The product obtained by the above methods was usually a powder with a markedly fibrous structure if compared with, for example, esparto cellulose, but Dillon and O'Tuama (4) amongst others have noted its solubility in Schweitzer's reagent and blue coloration with iodine and sulphuric acid, both typical tests for cellulose. Viel (6) also observed that it gave a violet coloration with zinc chloride and potassium iodide which according to Haas is characteristic. Viel also quotes microanalytical figures which agree well with normal cellulose as shown below although any polysaccharide based on a hexose, e.g. a mannann, would give the same figures:

<table>
<thead>
<tr>
<th></th>
<th>Carbon</th>
<th>Hydrogen</th>
<th>Ash</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fucus vesiculosus</td>
<td>43.79</td>
<td>6.35</td>
<td>2.95</td>
</tr>
<tr>
<td>Fucus serratus</td>
<td>43.58</td>
<td>6.33</td>
<td>2.86</td>
</tr>
<tr>
<td>Laminaria saccharina</td>
<td>43.93</td>
<td>6.29</td>
<td>1.87</td>
</tr>
<tr>
<td>Laminaria cloustoni</td>
<td>44.32</td>
<td>6.41</td>
<td>1.45</td>
</tr>
<tr>
<td>((C_6H_{10}O_5)_n)</td>
<td>44.44</td>
<td>6.17</td>
<td></td>
</tr>
</tbody>
</table>

It is not stated whether these figures are on an ash-free basis.

Acetylation.

Acetylation experiments were carried out by Dillon and O'Tuama (4) using the method of Öst in which the
substance is added to a solution of zinc chloride in acetic acid and treated with acetic anhydride, followed by dilution with acetic acid and precipitation in water. They obtained an acetyl compound soluble in chloroform similar to that obtained from cellulose. Similar results were obtained by Naylor and Russell-Wells (5), and also by Miwa (10) who quotes yields of triacetate equivalent to a cellulose percentage of from 3.7-14.3% for Japanese seaweeds.

Methylation.

The only available reference to the methylation of algal cellulose occurs in the paper by Dillon and O'Tuama (4) who carried out methylation by treatment with sodium hydroxide solution (45%) in the presence of acetone. Dimethyl sulphate was then added with stirring over a period of ten hours with cooling. After three methylations in this manner the product was soluble in chloroform on standing. No figure for a methoxyl value is given.

Conversion to a Viscose.

The same authors (4) prepared a viscose from moist cellulose by soaking in sodium hydroxide solution (18.5%) followed by twelve hours treatment with carbon disulphide. After dissolving in dilute sodium hydroxide and precipitation in dilute hydrochloric acid,
a product resembling viscose was obtained.

**Hydrolysis to Free Sugars.**

Most of the foregoing information would appear to favour the possibility of the identity of marine and land plant cellulose, but no conclusive evidence has been brought forward. Hydrolysis experiments have, on the other hand, in at least one case, suggested the opposite view. Colin and Ricard\(^3\) hydrolysed their product with sulphuric acid and found only traces of reducing sugars in the hydrolysate. They therefore concluded that the material was not cellulose and hence gave it the name "algulose," but it is doubtful if their conditions of hydrolysis were sufficiently vigorous for complete breakdown to glucose.

Willstätter and Zechmeister\(^11\) showed that by treatment with fifty times its weight of saturated hydrochloric acid (density 1.21) for 24 hours at room temperature, cellulose is converted almost quantitatively into glucose, the yield being 96% of the theoretical. Following this method Viel\(^6\) carried out comparative experiments with algal and cotton celluloses. He found that the former took longer to dissolve but that the sugars formed gave an osazone resembling glucosazone. Estimations of glucose by Bertrand's method gave the following percentages based on the theoretical yield
of glucose.

| Cellulose            | %  
|----------------------|---
| Cotton cellulose     | 90.4  
| Cellulose ex F.vesiculosus | 94.4  
| " " F.serratus      | 90.5  
| " " L.saccharina    | 89.9  
| " " L.cloustoni     | 90.9  

Dillon and O'Tuama\(^4\) also investigated the hydrolysis of algal cellulose with sulphuric acid (72\%). After 48 hours standing the solution was diluted and heated in an autoclave at 120°C for one hour, neutralised, treated with phenylhydrazine acetate yielding an osazone which, after purification, had a melting point of 200-201°C, and which they concluded was glucosazone. Originally they thought the cellulose might be a polymer of mannose but could find no trace of mannose phenylhydrazone after hydrolysis as described above. Little further experimental work has been carried out on the hydrolysis of algal cellulose.

**Conclusions Derived from Chemical Evidence.**

The balance of evidence seems to be in favour of the existence of an algal cellulose similar in properties to land plant cellulose. The results of Viel and Dillon and O'Tuama show that the substance is in all probability built up from glucose residues but on the
other hand the structure of the polysaccharide has not been elucidated. In particular, no investigator has shown that the fundamental 1:4-β-linkages between the glucose units, known to exist in normal cellulose, are present in algal cellulose. In view of this fact a portion of the following research has been devoted to attempts to prepare cellobiase octa-acetate from the cellulose since this would give strong presumptive evidence in favour of 1:4-β-linkages. Other comparative physical and chemical properties have received attention.

Algal Cellulose from a Botanical Aspect.

Land plant cellulose normally forms the main constituent of the cell walls but it is clear that the percentage of cellulose in algae is insufficient for this purpose. The precise function of cellulose is not definitely known although several workers have investigated the matter, notably Naylor and Russell-Wells, Miwa, Wurdack and Moss. The general opinion is that alginic acid in some form constitutes most of the cell wall but it is not impossible that there is a fine network or internal scaffolding of cellulose round which other components such as alginates form the wall itself.

Industrial Application of Algal Cellulose.

Isolated instances are known of this cellulose
being used commercially. Deschiens\(^{(14)}\) recalls that in Germany the seaweeds have been used to prepare nitrocelluloses and also for paper-making. Herzog\(^{(15)}\) also describes a paper made from algal filaments although Hottenroth established that neither technically nor economically could the Sargasso Sea algae be used for paper-making owing to a lack of sufficiently fibrous structure in their cellulose.
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    147, (1933-8).


    No.3, 531.


    (1926).


Experimental

Preliminary Experiment on Preparation of Algal Cellulose.

The following attempted method of preparation is based on a combination of the methods previously quoted in the introduction to this section, with minor modifications.

A sample of *Fucus vesiculosus* (10 g.), air-dried and ground in a Christy and Norris mill to pass a 60-mesh sieve, was steeped for one and a half hours in hydrochloric acid solution (250 ml.; N). The insoluble matter was removed by centrifuging and treated with sodium carbonate solution (400 ml.; 3%) at 40°C. for three hours, allowed to stand overnight, centrifuged and the residue washed three times with distilled water. After suspending the impure cellulose in water, chlorine gas was passed into the solution for three hours after which time the solid had changed colour from its original brownish-green colour to yellow. Separation and washing at the centrifuge was followed by suspension in sodium sulphite solution (3%) and boiling. Sodium hydroxide was added to the boiling solution to give 1% solution and boiling continued for five minutes, during which process the substance reverted to its original brownish colour but not so dark as before. A second treatment with chlorine and
sodium sulphite solution improved the colour still further. Finally the product was washed with sodium hydroxide solution (1%), washed with water, alcohol and ether, collected on a 1 l sintered crucible and dried in vacuo over $\text{P}_2\text{O}_5$ yielding a hard, horny, brownish grey material. Yield, 0.21 g.

The product from this extraction was almost completely soluble in cuprammonium solution but with difficulty, presumably owing to its physical state.

**Larger Scale Extraction.**

In view of the moderate success with the preliminary experiment it was decided to carry out a larger scale extraction using a sample of *Laminaria digitata* as it was thought that this species might give a cleaner product. The ground fronds (3 Kg) were soaked overnight in sulphuric acid (15 l.; N/10), centrifuged, kept with sodium carbonate (20 l.; 3%) for three days, centrifuged and re-extracted with sodium carbonate (10 l.; 1%). The residue was suspended in water and bleached with free chlorine, followed by several washings with water. After treatment for two hours in a boiling water bath with sodium sulphite (3%), sodium hydroxide was added to give a concentration of 1% and heating continued for ten minutes followed by washing at the centrifuge. A further extraction with
sodium carbonate (3%) and two with sodium hydroxide (1%) were carried out. The product at this stage was greyish in colour.

**Bleaching with Sodium Chlorite.**

The greyish material was suspended in water and acetic acid added to give a pH value of 4, sodium chlorite solution (0.4%) added and the solution left overnight. Little change in colour could be detected in the separated material.

**Bleaching with Calcium Hypochlorite.**

Treatment with calcium hypochlorite solution (4.7%) in a similar manner, had likewise no effect on the colour.

**Final Separation of Cellulose.**

The product after treatment with the bleaching agents was dialysed against running water for fourteen days, separated off, washed with alcohol and dried in vacuo over phosphorus pentoxide.

An ash estimation showed the presence of 15% of inorganic matter, which was rich in calcium and contained 8% of siliceous matter, insoluble in hydrochloric acid (50%).

**Reduction of Ash Content.**

By shaking the cellulose in suspension with dilute hydrochloric acid overnight and dialysing free of acid,
the ash content was reduced to 9%. A second treatment gave a final figure of 7.22% ash containing 4.89% silica. The final product was dried in a vacuum dessicator over phosphorus pentoxide, yielding a lumpy greyish, horny material, extremely difficult to break up.

**Estimation of Glucose Produced on Hydrolysis.**

The method of Monier-Williams\(^1\) was adopted for this estimation as follows, a control experiment being carried out at the same time, using cotton cellulose:

Cellulose (1 g.) was treated with sulphuric acid (5 ml., 72%) at 15°C. for one week. After standing, the acid concentration was reduced by dilution with water to 500 ml., the solution boiled for 15 hours, followed by neutralisation with barium carbonate, filtration, and evaporation to dryness in vacuo. The resulting glass was extracted by refluxing with boiling methyl alcohol (100 ml.) for 3 hours, a little charcoal added, the solution filtered rapidly with suction, and finally evaporated to dryness in a current of air and the crystalline residue recrystallised from absolute alcohol and weighed.

Cotton cellulose gave a yield of 89.72% of the theoretical yield of glucose and the algal cellulose 88.12% residual matter based on the original weight corrected for ash content. Assuming the residue to
be glucose, a polarimetric determination in water solution gave a rotation equivalent to 82% glucose based on the cellulose.

**Indications of the Presence of Glucose in Hydrolysate and no other Sugars.**

A portion (20 mg.) of the above residue from hydrolysis was dissolved in water (2 c.c.) and to the solution added sodium acetate (0.3 g.) and phenylhydrazine hydrochloride (0.2 g.). After dissolution, the mixture was heated for 30 minutes in a boiling water bath and allowed to cool, yielding an osazone, M.Pt. 199-200°C. A mixed melting point with glucosazone gave a result of 201°C. and a microscopic examination confirmed glucosazone.²

An estimation of glucose in the product of hydrolysis was carried out by the method of Shäffer and Somogyi³ as applied to laminarin⁴ in which determinations of reducing power in solution are made before and after incubation at 37°C. with yeast⁵. By adsorption the glucose is removed from the solution and, by the use of a micro-copper reagent, the amount of glucose may be determined if a blank experiment with glucose itself is carried out simultaneously. When applied to the sugars in the hydrolysate above a figure of 78% glucose was obtained and the amount of other
sugars present, shown by the difference in reducing power before and after yeast treatment, was negligible.

**Attempted Isolation of Cellobiose Octa-acetate from Algal Cellulose.**

Haworth and Hirst's Method (6)

**Control Experiment.** Filter paper (1 g.) was disintegrated and stirred into a mixture of acetic anhydride (4 ml.) and sulphuric acid (0.6 ml.) and stirring continued till the paper was pulped (about ten minutes). After heating in a calcium chloride solution bath at 115°C. with stirring till the solution went dark red, it was poured into water and the separated solid washed by decantation over several hours, filtered off, washed and recrystallised from alcohol. M.Ft. 221-222°C. The quoted yield is 40%, but, in this case, no higher yields than 20% could be obtained in a series of five experiments.

**Using Algal Cellulose.** When algal cellulose (0.97 g.) was treated with the above reagents, no pulping occurred, so the solution was heated to 115°C. for ten minutes when the solution turned brown without passing through a red stage. After decanting into water, the undissolved residue was weighed (0.55 g.), and the material, precipitated in the water, separated as in the control experiment (0.09 g.), recrystallised from alcohol and examined. This was doubtfully crystalline,
had a melting point of 187°-189°C. and specific rotation \([\alpha]^{15}_{D} + 20.8^\circ\) in chloroform. By chromatographing the chloroform solution on an alumina column, using chloroform as eluent, only a small quantity of material (0.028 g.) passed through having no definite melting point. The alcohol mother liquor from the original recrystallisation was evaporated to give a buff coloured solid (0.086 g.) with a mean molecular weight by iodine number (Bergman and Machemer\(^7\)) of 902. The result suggests that the degradation of the cellulose had not proceeded as far as cellobiose, probably mainly due to the difficulty of reaction with the hard lumps of cellulose.

**Further Purification of Cellulose.**

In view of the difficulty in dealing with the hard material obtained to date, it was decided to investigate the further purification of the algal cellulose. A quantity of cellulose was dissolved in cuprammonium solution by shaking for two days, diluting the solution with water and reprecipitating the cellulose by just acidifying with sulphuric acid. After washing and dialysis against running water until free from copper and acid (1 week), the cellulose was separated and washed with water and alcohol. The slurry in alcohol was then vacuum distilled with the addition of benzene in the last stages to give a dry material of a
greyish colour which could be readily powdered.

**Conversion of Cellulose to Cellobiose Octa-acetate by the Method of Hibbert and Parana**

**Control Experiment.** Cotton cellulose (1 g.) was treated with acetic anhydride (4 ml.) and sulphuric acid (0.1 ml.) for 19 days at 50°C. in a test-tube with ground glass reflux condenser. At the end of this time glacial acetic acid (5 ml.) was added, mixed and the solution poured into water (250 ml.) and allowed to stand overnight. The precipitated octa-acetate is treated with a little charcoal and recrystallised from hot alcohol.

Yield, 0.24 g., $[\alpha]^{150}_D + 45.0$, M.Pt. 222°C.

The low yield was shown to be in part due to destruction of the octa-acetate during the 19 days, by treatment of cellobiose octa-acetate itself in the same manner.

**Application to Algal Cellulose.**

Samples of algal cellulose (0.240 g. ash free) and cotton wool (0.238 g.) were each treated in the above manner with appropriate quantities of reagents.

Cotton cellulose Yield 0.074 g. or 31%. M.Pt. 222-223°C.

Algal cellulose Yield 0.083 g. or 34%. M.Pt. 220-221°C.

After recrystallisation the yields went down to 26% and 24% respectively and the melting points both
went up to 223°C. A second recrystallisation gave:

- Cotton cellulose. M.Pt. 223°C.
- Algal cellulose. M.Pt. 224°C.
- Mixed Melting Point. 223°C.

The algal cellobiose octa-acetate had specific rotation $[\alpha]_{D}^{24} = 40$ (c. 0.34 in CHCl₃), and microanalysis (Weiler and Strauss) gave C, 49.77% H, 5.79% as compared with the calculated values of 49.56% and 5.65%.

**Method for Estimation of Cellulose.**

As the method outlined earlier to obtain algal cellulose was obviously rather tedious for estimation purposes, a more rapid method was sought.

Preliminary experiments involving, for example, the use of sintered crucibles in which the sample was placed along with sodium carbonate solution showed that the viscosity of the extract produced was much too high for filtration even through a crucible of the highest porosity.

The method eventually adopted was based on the estimation of "Residual Fibre" as used by the Association of Official Agricultural Chemists, Washington (9). The determination was carried out as follows:

Dried ground seaweed (2 g.) was boiled under reflux for half an hour with sulphuric acid (200 ml.; 1.25%), filtered through a hardened filter paper with suction
and washed free of acid with hot water. The residue was scraped from the filter without difficulty in the form of a cake and reboiled with sodium hydroxide (200 ml.; 1.25%), the frothing being kept under control by a gentle stream of compressed air introduced via the condenser down a glass tube to within an inch or two of the liquid. After half an hour the previous filtration and washing is repeated, again using a hardened filter, followed by washing with alcohol and ether rendering the removal of the residue from the filter easy. Bleaching is carried out by standing the cellulose in chlorine water (100 ml. saturated) overnight and the white product is filtered under suction through a weighed hardened filter and washed free of chlorine with water. Finally the cellulose is washed on the filter with hot sodium hydroxide solution (50 c.c., \( \frac{1}{10} \)) followed by water, till free of alkali, alcohol and ether, being dried at 50°C. for ten minutes and weighed.

When the above method was applied to the so-called rock weeds, e.g. *Fucus* spp., it was found necessary, owing to the sliminess of the residues, to centrifuge after the extractions with caustic soda and chlorine water as filtration was difficult.

Using this method, cellulose estimations on *Laminaria* fronds gave 3.27%, *Laminaria* stipes, 7.03%,
and *Fucus vesiculosus* mixed stipes and fronds, 1.70%.

It was also shown that the final treatments with chlorine and sodium hydroxide were necessary as the cellulose from the sample of *Laminaria* fronds was dried and weighed before each of these treatments giving:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage residue before treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorine</td>
<td>5.62%</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>3.79%</td>
</tr>
</tbody>
</table>

**Preparation of Cellulose by the above Method.**

By the above method 30 g. of *Laminaria* fronds yielded a practically white specimen of cellulose (1.3 g.) which gave all the usual tests for cellulose, namely (1) blue colour with iodine and sulphuric acid, (2) blue colour with zinc chloride and iodine provided the cellulose was allowed to swell overnight in zinc chloride solution, (3) solubility in cuprammonium solution.

The ash content of the sample was 3.2% and its fluidity, determined by the British Cotton Industry Research Association's standard method, was 46 reciprocal poises. The fluidity of cellulose solutions varies from 2 poises$^{-1}$ for raw cotton cellulose upwards compared with 72 poises$^{-1}$ for the solvent itself (cuprammonium solution).

Treatment of the above cellulose by solution in cuprammonium solution and reprecipitation with acid gave a poorer coloured product but with reduced ash
content. The colour may be due to traces of copper not completely removed by dialysis.

Future experiments on cellulose were carried out on samples prepared by the new method.

**Hydrolysis to Glucose.**

Determinations of the glucose formed from a sample prepared thus, on hydrolysis, gave, by the Monier-Williams method (see p. 22) a figure of 80·1% which was confirmed by the Shaffer-Somogyi method which also showed no trace of other reducing sugars.

A drop of a solution (1%) of the sugar formed in the hydrolysis was placed on a chromatogram fitted up as described by Partridge (11) using as solvent n-butanol (40 parts) water (50 parts) and ethanol (10 parts). Only one spot appeared on development with ammoniacal silver nitrate and this was shown to be glucose by comparison with a standard solution of glucose.

**Effect on Cotton Cellulose of Treatment as in Extraction.**

A sample of standard cotton prepared at the Shirley Institute (0·2521 g.) was treated with acid, alkali, chlorine, etc. exactly as in the preparation of cellulose from seaweed. The resulting material weighed 0·2015 g., i.e. a loss of 15%. Fluidity measurements were also made before and after the figures being 2 poises⁻¹ and 28 poises⁻¹ respectively. Chain length determinations by reaction with potassium...
periodate\(^{12}\) as described below, showed a decrease from 826 to 360 units after treatment.

These results indicate that a certain amount of degradation has taken place during the extraction processes and it must be assumed that a similar effect obtains in the preparation of algal cellulose accounting to some extent for the low fluidity (46 poises\(^{-1}\)) found.

**Uptake of Periodate by Algal Cellulose.**

Sodium periodate\(^{13}\) in neutral solution reacts with adjacent free hydroxyl groups, thus

\[
\text{-C(OH)-C(OH)} + \text{NaIO}_4 \rightarrow \text{-CHO} + \text{OH}^- + \text{IO}_3^-
\]

to give two aldehyde groups, one mole of periodate being required to react with each pair of hydroxyls. In the case of cellulose, each glucose residue has two adjacent free -OH groups on C-atoms 2 and 3 and therefore requires one mole of periodate for each glucose unit. Determinations of the uptake of periodate were therefore carried out on algal cellulose.

Algal cellulose (0.2916 g. containing moisture, 1.94% and ash, 5.03%) was mixed with \(\text{M/4} \text{ sodium periodate solution (20 ml.)}\) and shaken. At intervals, after allowing the solution to settle to a clear liquid, samples (1 ml.) were withdrawn and the periodate content determined with sodium arsenite solution (0.0926N) after addition of bicarbonate and potassium iodide and standing for five minutes. The first sample was taken after
one hour to determine the exact strength of periodate and thereafter at 1, 3, 5, 7 and 9 day intervals.

<table>
<thead>
<tr>
<th>Results</th>
<th>Time</th>
<th>Titration (ml. arsenite/ml. soln.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hour</td>
<td>5.17</td>
</tr>
<tr>
<td></td>
<td>1 day</td>
<td>3.88</td>
</tr>
<tr>
<td></td>
<td>3 days</td>
<td>3.42</td>
</tr>
<tr>
<td></td>
<td>5 &quot;</td>
<td>3.37</td>
</tr>
<tr>
<td></td>
<td>7 &quot;</td>
<td>3.32</td>
</tr>
<tr>
<td></td>
<td>9 &quot;</td>
<td>3.32</td>
</tr>
</tbody>
</table>

The total uptake of periodate is therefore equivalent to $1.85 \times 0.0926 \times 20$ ml. of normal arsenite which is found to be equivalent to an uptake of 1.02 moles of sodium periodate for every glucose residue (162 g.) in the original cellulose, no account being taken of the practically negligible amount of periodate used to oxidise end groups to formic acid.

**End Group Determination with Potassium Periodate.**

End groups in a chain of glucose units on reaction with periodate give rise to three molecules of formic acid, two from one end and one from the other.\(^{(12)}\) By using potassium periodate solution, over-oxidation of the products of reaction, to give further quantities of formic acid, is avoided owing to the low concentration of the relatively insoluble potassium periodate. By
estimation of the formic acid produced, a measure of the length of the chain may be obtained.

Algal cellulose (0.4853g. moisture and ash 6.97%) was mixed in a bottle with sodium periodate (M/4; 10 ml.) solid potassium chloride (2 g.) and water (30 ml.) and shaken mechanically for fourteen days. Samples of 5 ml. were withdrawn at intervals of 4, 7, 10 and 14 days and titrated in a micro-burette with sodium hydroxide (N/100) using methyl red as indicator, after addition of two drops of ethylene glycol and standing sixty minutes to destroy excess periodate. A blank determination without cellulose showed, after one hour, acidity equivalent to 0.01 ml. N/100 NaOH.

<table>
<thead>
<tr>
<th>Results</th>
<th>Time (days)</th>
<th>ml. N/100 NaOH used</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>0.72</td>
</tr>
</tbody>
</table>

By allowing for the amount of formic acid removed in each sampling and assuming three moles of formic acid to be produced for each chain, the above results give a chain length of 161 glucose units.

X-ray Examination of Cellulose.

X-ray powder photographs were kindly taken by Professor W.T. Astbury of Leeds University, of algal cellulose from Laminaria cloustoni and of the same regenerated from cuprammonium solution. Copies are
Professor Astbury reports that the sample of cellulose, containing some impurities, has the same X-ray diagram as that of cotton cellulose and that the regenerated material is very similar in pattern to mercerised or "hydrate" cellulose, normally obtained by swelling ordinary cellulose.

I. X-ray diagram of algal cellulose from *Laminaria cloustoni*.

II. X-ray diagram of cellulose regenerated from I after dissolution in cuprammonium hydroxide.
Methylation of Algal Cellulose.

Cellulose (1.4 g.) was methylated according to the method of Haworth and co-workers (14). The methylations were carried out in the cold in an atmosphere of nitrogen with fast stirring. To the cellulose was added sodium hydroxide (150 c.c.; 30%) and the mixture stirred until gelatinisation occurred (4 hrs.) when a mixture of dioxan (15 ml.) and dimethyl sulphate (15 ml.) was added slowly and stirring continued for nine hours. The insoluble material was centrifuged off and the liquid extracted with chloroform, which was removed under diminished pressure and the residue added to the insoluble material for further methylation. Five methylations were done in the same way. After the final methylation the semi-solid material separated on top and was removed diluted, neutralised with acetic acid (glacial) and dialysed till free of sulphate and acetate. The product was separated from the solution, washed with water and acetone at the centrifuge and dried in vacuo at 60°C. Yield 0.28 g., OCH₃, 25.0%. The chloroform-soluble portion was extracted with the hot solvent and the solvent removed yielding a glass (0.13 g., OCH₃, 35.1%) (cf. fully methylated cellulose ca 41%).
A method has been evolved for the routine determination of cellulose in seaweeds. Unfortunately the results of experiments on cotton cellulose indicate a certain degree of degradation by the employment of the reagents used, but owing to the extremely complex mixture of substances forming a seaweed it is necessary to use a number of fairly strong reagents to remove the other materials and leave the cellulose in a reasonable state of purity.

The algal cellulose so obtained has been investigated chemically and the results would seem to confirm the earlier opinions of Viel, Dillon and O'Tuama, etc., in so far as the presence of glucose units in the molecule is concerned. The foregoing work, however, also shows the presence of no other reducing sugar either by reducing tests or by paper chromatographic analysis indicating that the polysaccharide consists solely of chains of glucose units. Chain length determinations indicate a chain length of about 161 units but it is possible the actual lengths of the chains in the cellulose as it occurs in seaweed are somewhat greater since the chain length of cotton cellulose is more than halved by the extraction process.

The production of cellobiose octa-acetate known to
contain a 1:4-β-linkage between two glucose units shows that it is extremely probable that the same linkages occur in algal cellulose and the roughly similar percentage yield from algal and cotton cellulososes of octa-acetate indicates that 1:4-β-linked glucose units form the main proportion of the molecule. Assuming then that the building unit is glucose in which C-atoms 1 and 4 are used in links with other units the presence of a pyranose ring would allow C-atoms 2 and 3 to be free which is confirmed by the uptake of sodium periodate at the rate of one mole of periodate for each glucose unit.

These facts taken in conjunction with the conclusion drawn from X-ray examination make it highly probable that algal cellulose has the same general structure as land plant cellulose.

In seaweeds the percentage of cellulose present varies in the samples analysed from 1.7% in a sample of Fucus to 7.0% in a Laminaria stipe. The indications are that the stipe contains more cellulose than the frond.
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PART II

ALGINIC ACID
INTRODUCTION

Alginic acid occurs in most varieties of brown seaweeds (Phaeophyceae) as a major constituent and was one of the earliest investigated materials obtained from marine algae. In British seaweeds it occurs in percentages as high as thirty per cent of the dry weight and occasionally higher at certain periods of the year. Generally speaking, the seasonal variation in alginic acid content is considerable in the fronds of the plant, but small in the stipes as shown by the analytical results published by Lunde(1)

Preparation of Alginic Acid from Seaweed.

Alginic acid or algin, as it was originally called, is readily extracted from algae by treatment with sodium carbonate solution which gives a viscous mucilaginous solution of the polysaccharide in the form of a soluble salt from which the acid may be regenerated on acidification. This method was used by Stanford(2) as early as 1883, when he discovered alginic acid and investigated the properties of a wide range of its metallic salts. A more satisfactory product can be obtained if some of the other materials present are first removed before extraction with alkali. Thus, extraction with alcohol removes mannitol and pigments, followed by dilute hydrochloric acid, which removes
fucoidin and at the same time converts any calcium alginate present into alginic acid which can readily be extracted with alkali. The product so obtained is in the form of a fibrous, white to brownish substance, which shows promise of being of use industrially in the textile industry. The colour of the alginic acid is very variable depending on its source, that obtained from the genus *Laminaria* being moderately white while the genus *Fucus* generally gives a much browner product. Similar variations can be observed in the viscosities of the alkaline solutions, probably due to the state of aggregation of the molecule.

**Constitution of Alginic Acid.**

This thesis is not directly concerned with the constitution of alginic acid but only with its estimation in seaweed, however, some account may be given of the earlier work on the subject. About 1915 Hoagland and Lieb\(^3\) investigated alginic acid and claimed that on hydrolysis, one of the products was xylose while Schmidt and Vocke\(^4\) obtained what they believed to be D-glucuronic acid as a cinchonine salt. The presence of xylose in the polysaccharide was shown to be doubtful when Cretcher and Nelson\(^5\) showed that their samples of alginic acid contained up to 98% uronic acid. The presence of free carboxyl groups was readily
demonstrated by solution in sodium hydroxide and back titration with acid, giving a neutralisation equivalent of about 180. Decarboxylation of the free acid occurs very easily and is, in fact, the basis of one of the methods of estimation, and, therefore, suitable conditions for hydrolysis were difficult to find. Eventually Nelson and Cretcher (6) using 80% sulphuric acid for several days succeeded in obtaining partial hydrolysis and proved, for the first time, the presence of D-mannuronic acid in a natural product, the conclusion being that alginic acid was a polyuronide of mannuronic acid. This result was confirmed by the work of Bird and Haas (7), Miwa (8), and Niemann and Link (9).

It was found that full methylation of alginic acid was difficult to achieve. Hirst, Jones and Jones (10) however, obtained a degraded alginic acid by boiling the sample with 10% methanolic hydrogen chloride, which they succeeded in methylating by the thallium method. Vigorous hydrolysis of the methylated material with 4% methanolic hydrogen chloride at 150°C. for 24 hours yielded the methyl ester of 2:3 dimethyl methyl-D-mannuronoside (a). This was hydrolysed and oxidised to give 2:3 dimethyl-D-mannosaccharic acid (b) which on further oxidation with periodic acid gave glyoxylic acid (c) and the half aldehyde of meso-dimethoxysuccinic
acid (d) identified as the crystalline dimethyl ester of meso-dimethoxysuccinic acid (e).

Thus:

Methylated degraded alginic acid

\[ \text{hydrolysis} \]

(a)  

(b)  

(c)  

(d)  

(e)
Direct oxidation of alginic acid with periodic acid, followed by bromine confirmed these results. Periodic acid will only attack free adjacent hydroxyl groups so that if positions $C_2$ and $C_3$ were free as indicated above, each uronic acid residue would require one mole of periodic acid for reaction. This was in fact found to be the case. The product of the reaction would be a dialdehyde oxidisable with bromine to a tricarboxylic acid. Lucas and Stewart\(^{11}\) carried out the oxidation and hydrolysed the dialdehyde so formed with acid to give glyoxal and $D$-erythunronic acid. The acid formed by oxidation with bromine was also hydrolysed; yielding glyoxylic and mesotartaric acids. These results proved conclusively that neither $C_2$ nor $C_3$ were involved in either ring structure or bridge linkage.

Confirmation of the type of linkage in alginic acid was furnished by the X-ray studies of Astbury\(^{12}\) and Palmer and Hartzog\(^{13}\). They found the period along the fibre axis to be 8.7 Å as compared with 10.3 Å for cellulose. This is due to the fact that when hexopyranose rings are constructed in the "chair" form using standard bond lengths and angles, the formation of $\beta$-linkages between $C_1$ and $C_4$ in contiguous rings is possible in two ways. In the first case the rings are
inclined at an angle of 20° with one another, and the resultant chain is almost linear. This represents cellulose and agrees with the observed figure of 10.3 Å. In the second case where the rings are at an angle of 90°, the theoretical value would be 8.7 Å which agrees with that of alginic acid. Thus it appears that the structure of alginic acid is definitely non-linear and the molecule has a "puckered" appearance.

From the above experimental evidence, it was concluded that alginic acid was a polyuronide built up of D-mannuronic acid units containing a pyranose ring and linked between carbon atoms 1 and 4. The following formula was therefore proposed:

\[
\begin{align*}
&\text{COOH} \\
&\text{H} \quad \text{OH} \quad \text{OH} \\
&\text{H} \quad \text{H} \quad \text{O} \\
&\text{H} \quad \text{H} \quad \text{H} \\
&\text{H} \quad \text{H} \\
&\text{COOH} \\
&\text{H} \quad \text{OH} \\
&\text{H} \quad \text{H} \\
&\text{H} \\
&\text{H} \\
&\text{O} \\
\end{align*}
\]

The length of the uronic acid chain is a matter of some doubt as periodate oxidation with the potassium salt is inapplicable owing to the presence of the acid group at one end of the chain. The only other method in use at present is based on viscosity measurements. Heen\(^{14}\) determined the viscosity of solutions of sodium alginate in water and his results indicated a chain length of about 80 uronic acid units. This result
would appear to be on the low side when the extreme viscosity of the solutions is considered. Further investigations into the method are being carried out at present in this University. More recent X-ray work by Palmer\(^{(15)}\) indicates that the molecule consists of a long straight chain of high molecular weight, having, in the case of alginic acid, twofold, and sodium alginate, threefold symmetry.

**Other Polyuronides in Nature.**

Polyuronides occur abundantly in natural products notably in seed mucilages in the form of pectic materials, plant gums, hemicelluloses and certain bacterial polysaccharides. They are readily obtained by extraction with water and precipitation in alcohol, and are non-reducing to Fehling's solution.

**Industrial Applications of Alginic Acid.**

Industrially two main methods of extraction are used as follows.

(a) **Green's Method.**\(^{(16)}\) The seaweed is leached for some time with cold water, followed by treatment with sodium carbonate solution which extracts the alginic acid. The solution is diluted considerably and a mixture of aluminium alginate and silica added to cause the difficultly filterable cellulosic residue to settle, when the clear solution may be decanted and the
alginate precipitated with calcium chloride solution. The solid calcium alginate is then removed and reconverted to the acid with hydrochloric acid.

(b) Leghoanec and Herter's Method. In this case seaweed is treated with calcium or other alkaline earth metal salt solution which removes other polysaccharides and renders all the alginate present insoluble. Alkaline extraction and dilution follow as before, then air is blown into the solution in the form of a fine stream of bubbles, thereby carrying the insoluble material to the surface. At the same time an oxidising agent (hypochlorite) is added and after the scum has collected it is skimmed off, leaving the clear solution of alginate from which alginic acid is recovered as in the first method.

Within recent years alginic acid has become a commercial product of considerable importance. Transparent films may be formed by the evaporation of sodium alginate solutions or by precipitation of calcium alginate under suitable conditions. Partially owing to the viscosity of its solutions it is widely used as an emulsifier in the cosmetic and food industries, e.g. in face creams and ice-cream. For this purpose it is marketed as sodium alginate under the trade name of Manulol, in various grades, depending on the viscosities of the solutions, which may also be used in finishing and waterproofing of cloth and in
A considerable industry is also developing around the use of alginates for making yarns, by a method similar to that used in the viscose rayon process for silk yarn. Most of the alginate yarns are in the form of calcium alginate but other metals such as barium, chromium, and beryllium have been tried. The main industrial application of calcium alginate rayon, at present, is in its use in the "disappearing fibre" process. Here, use is made of the fact that if alginate yarn is used in conjunction with other fibres, materials may be woven and then treated with soap and soda to remove the alginate, giving various special effects in the finished product. Thus it may be used to strengthen twillless yarns until they are woven, to obtain missed thread effects by interweaving periodical threads of alginate and for various other effects in weaving not readily obtainable using insoluble yarns.

It is obviously necessary in view of these various applications to have a suitable method for the estimation of alginic acid in seaweeds in order to determine the optimum time of harvesting, etc.

**Estimation of Uronic Acids.**

The methods of estimation of uronic acids in general can be divided into groups:
(a) Methods depending on (1) the presence or reaction of the acidic carboxyl group, and (2) the production of furfural with strong hydrochloric acid.

(b) Methods involving the use of colour reactions.

(a) Methods based on Carboxyl Group or Furfural Production.

Free uronic acids are readily estimated by the slow direct titration with sodium hydroxide using phenolphthalein as indicator or by the addition of excess alkali and back titration with acid. In the case of alginic acid, the latter procedure is preferable owing to its insolubility in water but easy solubility in alkali. An improved method for alginic acid was published by McGee, Fowler and Kenyon(19) depending on the fact that if a solution of calcium acetate is added in excess to solid alginic acid, an exchange takes place, and an amount of acetic acid is liberated equivalent to the alginic acid present. The acetic acid released may be titrated with alkali after the reaction has proceeded for one hour.

A more general method, which is applicable not only to free uronic acids but also to their salts was developed by Tollens(20) and more recently modified by McCready, Swenson and Maclay(21) in which use is made of the ease with which uronic acids undergo decarboxylation. The uronic acid is heated to 120°C. with 19% hydrochloric acid and the carbon dioxide which is split
off is carried, by a stream of air, through a solution of sodium hydroxide in which it is absorbed. After precipitation of the carbonate formed with barium chloride, the excess alkali is titrated with acid to phenolphthalein and from the alkali used, the amount of carbon dioxide formed is calculated and converted, by a suitable factor, to uronic acid. This method can be applied to polysaccharides in which a uronic acid is only one of the building units possibly in quite small amount, but is not suitable for alginic acid estimations in seaweed until it is definitely known that no other uronic acids are present. It also requires a special apparatus, not suitable for routine estimations.

Using the same reagents as in the previous method, Lefèvre and Tollens\(^{(20)}\) determined uronic acids by distilling off the furfuraldehyde formed by decomposition and estimating it by converting it into its phloroglucide and weighing. This method is difficult to carry out and involves a number of corrections to attain the final result.

(b) Methods based on Colorimetric Reactions.

Various phenolic and nitrogenous compounds react with carbohydrates in the presence of sulphuric acid to give colourations in solution, the colours usually being reddish. Gurin and Hood\(^{(22)}\) investigated the
reaction of carbazole, in concentrated sulphuric acid, with various hexoses and found that, in general, the depth of colour was proportional to the concentration of hexose.

Egami (23) showed that the principle could be applied to other carbohydrates such as pentoses, uronic acids, etc. The colour developed varied from brown to purple and could, in the case of a single substance, be indicative of the group to which the substance belonged. Using a Pulfrich Photometer, Egami observed the variation in absorption \( \log \frac{I}{I_0} \) of the various groups, depending on the type of S-filter used and found that the uronic acid group had the highest absorption when filter \( S_{53} \) (green) was used. The colour was developed by adding the sugar solution (1 ml. containing 0.05-0.40 mg. of sugar) to sulphuric acid (10 ml. of a mixture by volume of 8:1 \( H_2SO_4/H_2O \)) cooled in iced water. After thorough cooling the carbazole reagent (0.3 ml.; 0.5\% in alcohol) is added and the solution heated in a water bath for 10 minutes, cooled and the colour allowed to develop.

Dische (24) who discovered the carbazole reaction in 1929 (25) has recently developed a method which he claims, under his experimental conditions, to be specific for hexuronic acids and is carried out as follows: 1 ml. of a solution containing 5 to 100 \( \gamma \) of uronic
Acid is added with cooling to pure concentrated sulphuric acid (6 ml.) and heated for 20 minutes in a boiling water bath. After cooling to room temperature, carbazole solution (0.2 ml., 0.1% in alcohol) is added with shaking. After a few minutes, a pink to violet-red colour appears, depending on the concentration, the intensity of colour increasing for 2 hours, and gradually diminishing again after a further hour.

According to Dische, at concentrations at which uronic acids give a deep violet colour, no visible reaction is given by true sugars. At higher concentrations hexoses give a brownish and pentoses a yellow colour. Dische suggests that the uronic acid reacts first with sulphuric acid and the reaction product then gives the coloured substance with carbazole. In the case of alginic acid he found that the intensity of colour produced was somewhat lower than that produced from glucuronic acid, and also showed that the maximum absorption was observed in photoelectric colorimetric measurements using a filter in the green region. By the use of a Klett-Summerson photoelectric colorimeter and an absorption-concentration calibration curve, determinations of uronic acids were made by Dische and the method applied to tissues and body fluids. The method has been applied to the sugar components of protein by Seibert and Atno(26) and of pregnant mare's
serum by Friedmann\(^{(27)}\) with reasonable success. All the above authors emphasise the importance of using pure sulphuric acid and carbazole as with impure materials side reactions occur which are liable to modify the colour produced.

The above method has been described in some detail as it has been used, as described in the experimental part, as a basis for the estimation of alginic acid in seaweed.

**Estimation of Alginic Acid in Seaweed.**

The present method used by the Scottish Seaweed Research Association for routine estimations of alginic acid in seaweed samples was developed by Cameron, Ross and Percival\(^{(28)}\). It is essentially an estimation of alginic acid by titration after its extraction from seaweed in a purified form and may be summarised as follows.

The ground sample is treated with sulphuric acid \((0.2N)\) in the cold overnight, and the residue removed and washed by means of a sintered glass crucible. Extraction of alginic acid from the residue is effected with sodium carbonate \((3\%)\) at \(50^\circ\text{C.}\) for two hours and standing overnight. The solution is then filtered by means of a Gooch crucible and the residue washed with sodium carbonate solution \((1.5\%)\) and water. The alginate is then precipitated as a calcium salt by
running the filtrate and washings into calcium chloride solution (10%) and allowing to stand for two hours. The calcium alginate is collected in a sintered crucible and converted to alginic acid by washing with hydrochloric acid (N) followed by water, and finally estimated by one of the two titration methods described earlier. The calcium acetate method is preferable particularly with specimens of *Fucus* which give a rather brown product which on solution in alkali yield dark solutions in which the colour of the indicator is somewhat difficult to see, whereas with calcium acetate the solution remains clear.

**Development of a More Rapid Method.**

The present research has been undertaken in order to try to adapt the carbazole colorimetric method to the estimation of alginic acid in seaweed samples with a view not only to routine estimations but also, owing to the extremely small concentrations required, to determinations on sections of living algae.
BIBLIOGRAPHY

Throughout the following experimental work, all light absorption readings and colour comparisons were made with a Spekker Photoelectric Absorptiometer, Type H 560. The principal reagents used were as follows:

**Sulphuric Acid.** Pure analytical reagent (A.R.).

**Carbazole.** B.D.H. Carbazole was purified by recrystallisation twice from glacial acetic acid. Two grades of carbazole were used, the first giving, on solution in alcohol, a faint blue fluorescence and the second, no fluorescence at all. Blank determinations, as described later, using the first carbazole solution tended to give slightly higher figures than the second.

**Preliminary Experiments.**

Preliminary experiments were carried out, based on the methods of Egami\(^1\) and Dische\(^2\), as described in the introduction to this section, of estimating alginic acid and other sugars. Solutions were made up as follows.

(a) **Alginic acid.** Commercial alginic acid (40 mg.) was dissolved in sodium hydroxide (15 ml.; N/10) and diluted to 100 ml., giving a concentration of 0.40 mg./ml.
(b) **Mannitol, Glucose and Rhamnose.** Solutions of the same concentrations (0.40 mg./ml.).

These sugars were selected as being the most probable interfering substances in the application of the method to seaweed, rhamnose being used instead of fucose owing to its greater availability.

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**Sugami's Method**

Sulphuric acid solution (8 parts by vol. \( H_2SO_4 \): 1 part water) was cooled and to it added 1 ml. of sugar solution with cooling. Carbazole solution (0.3 ml.: 0.5% in alcohol) was then added and the test-tube, containing the solution, placed in a boiling water-bath for 10 minutes, cooled again and the colour allowed to develop for 2 hours. In the case of mannitol and rhamnose only a very slight darkening of the solution was observed on treatment with the acid and no further change could be seen on addition of carbazole. Using glucose solution, a purplish colour appeared and with alginic acid, a magenta colour. A blank, using no sugar, yielded a yellowish solution. Measurements were therefore made of the absorption \( \left( \log \frac{I}{I_0} \right) \) ratio using as blanks, water and a carbazole blank for both the glucose and alginate samples and using a series of coloured filters supplied with the instrument. The maximum transmission of these filters occurs at the wavelengths given in brackets (in Angstrom units).
The results were as follows: the measurements being made in 1 cm. absorptiometer cells. In the following and all subsequent tables the decimal figures represent readings of log. $I/I_0$ in the Spekker Photoelectric Absorptiometer using samples and blanks as indicated.

**Spekker Readings.**

<table>
<thead>
<tr>
<th>Sample (0.4mg/cc)</th>
<th>Blank</th>
<th>Alginate Water</th>
<th>Glucose Water</th>
<th>Alginate Glucose Carb. Blank</th>
<th>Carb. Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Violet 601 (4300)</td>
<td>0.472</td>
<td>0.485</td>
<td>0.207</td>
<td>0.188</td>
<td></td>
</tr>
<tr>
<td>Blue 602 (4700)</td>
<td>0.524</td>
<td>0.390</td>
<td>0.365</td>
<td>0.204</td>
<td></td>
</tr>
<tr>
<td>Blue-Green 603 (4900)</td>
<td>0.659</td>
<td>0.381</td>
<td>0.534</td>
<td>0.238</td>
<td></td>
</tr>
<tr>
<td>Green 604 (5200)</td>
<td>0.660</td>
<td>0.420</td>
<td>0.545</td>
<td>0.287</td>
<td></td>
</tr>
<tr>
<td>Yellow-green 605 (5500)</td>
<td>0.535</td>
<td>0.413</td>
<td>0.404</td>
<td>0.280</td>
<td></td>
</tr>
<tr>
<td>Yellow 606 (5800)</td>
<td>0.384</td>
<td>0.343</td>
<td>0.272</td>
<td>0.212</td>
<td></td>
</tr>
<tr>
<td>Orange 607 (6000)</td>
<td>0.255</td>
<td>0.238</td>
<td>0.153</td>
<td>0.150</td>
<td></td>
</tr>
<tr>
<td>Red 608 (7000)</td>
<td>0.154</td>
<td>0.208</td>
<td>0.038</td>
<td>0.066</td>
<td></td>
</tr>
<tr>
<td>No filter</td>
<td>0.408</td>
<td>0.371</td>
<td>0.295</td>
<td>0.219</td>
<td></td>
</tr>
</tbody>
</table>

**Dische's Method.**

In this case the same sugar solutions were diluted to contain 0.1 mg./c.c. and this solution (1 ml.) was mixed with 6 c.c. pure concentrated sulphuric acid, the test-tube being cooled in a bath of iced water then heated in boiling water for 20 minutes, cooled to room temperature and carbazole solution (0.2 ml.; 0.1% alcoholic) added. The colour was allowed to develop for two hours according to Dische's directions. The previous results obtained with mannitol and rhamnose being confirmed it was decided that further consideration
of these compounds was unnecessary, as they would not interfere with the use of the method for alginic acid estimations. Glucose and alginic acid gave similar colours as in Egami's method and measurements of log $\frac{I}{I_0}$ were therefore made as before, using as blank the pure sulphuric acid treated with carbazole alone.

**Results:**

<table>
<thead>
<tr>
<th>Filter</th>
<th>Alginate/Carb. Blank</th>
<th>Glucose/Carb. Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Violet 601</td>
<td>0.057</td>
<td>0.069</td>
</tr>
<tr>
<td>Blue 602</td>
<td>0.059</td>
<td>0.062</td>
</tr>
<tr>
<td>Blue Green 603</td>
<td>0.068</td>
<td>0.057</td>
</tr>
<tr>
<td>Green 604</td>
<td>0.072</td>
<td>0.048</td>
</tr>
<tr>
<td>Yellow Green 605</td>
<td>0.067</td>
<td>0.042</td>
</tr>
<tr>
<td>Yellow 606</td>
<td>0.041</td>
<td>0.032</td>
</tr>
<tr>
<td>Orange 607</td>
<td>0.020</td>
<td>0.023</td>
</tr>
<tr>
<td>Red 608</td>
<td>Almost zero</td>
<td>0.006</td>
</tr>
<tr>
<td>No filter</td>
<td>0.107</td>
<td>0.047</td>
</tr>
</tbody>
</table>

**Choice of Method for Further Investigation.**

Dische's experimental method was eventually selected for further investigation as it was more convenient to apply owing to the use of undiluted sulphuric acid. It was also found in the preliminary experiments that, after heating with acid, the solution produced by the second method showed less darkening in colour due to decomposition. Owing to its giving the highest readings the green 604 filter was selected for future use.
Time of Development of Colour and Alginate Concentration Limits.

Following Dische's method, absorptiometer readings were taken of colours produced from varying concentrations of alginic acid. Measurements were made after two hours and after overnight development, and the results do not agree with Dische's claim of full development in two hours.

Results:

Measured against carbazole blank.

<table>
<thead>
<tr>
<th>Concentration of Alginic acid</th>
<th>Time of development</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 hours</td>
</tr>
<tr>
<td>0.05 mg./c.c.</td>
<td>0.026</td>
</tr>
<tr>
<td>0.10 mg./c.c.</td>
<td>0.078</td>
</tr>
<tr>
<td>0.20 mg./c.c.</td>
<td>0.105</td>
</tr>
<tr>
<td>0.50 mg./c.c.</td>
<td>0.175</td>
</tr>
<tr>
<td>1.00 mg./c.c.</td>
<td>0.213</td>
</tr>
</tbody>
</table>

These results indicate that, at a concentration of 0.05 mg./ml., the absorptiometer reading is rather low for accuracy and at a concentration of 1.00 mg./ml., there is a possibility of there being insufficient carbazole present to react with all the alginic acid. This contingency was investigated later but meanwhile, experiments were limited to solutions containing between 0.10 and 0.50 mg. of alginic acid in 1 ml. of solution. Duplicate experiments on samples within these limits confirmed that, under the experimental
conditions, standing overnight before taking readings was necessary for full development of the colour.

**Comparison of Alginate Blank and Carbazole Blank.**

It was observed that during earlier experiments there was a slight production of brown colour during the treatment of the alginic acid solution with concentrated sulphuric acid before the addition of carbazole. A series of readings were therefore made using as blank (1) alginate solutions of appropriate concentration treated with sulphuric acid and heated as in the determination, and (2) normal carbazole blanks.

**Results:**

<table>
<thead>
<tr>
<th>Conc(^n)</th>
<th>Alginate blank</th>
<th>Carbazole blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10 mg./ml.</td>
<td>0.144</td>
<td>0.151</td>
</tr>
<tr>
<td>0.20 mg./ml.</td>
<td>0.188</td>
<td>0.203</td>
</tr>
<tr>
<td>0.50 mg./ml.</td>
<td>0.434</td>
<td>0.475</td>
</tr>
</tbody>
</table>

The determinations in (1) were repeated to check the possibility of duplicating results giving:

<table>
<thead>
<tr>
<th>Conc(^n)</th>
<th>Reading.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10 mg./ml.</td>
<td>0.128</td>
</tr>
<tr>
<td>0.20 mg./ml.</td>
<td>0.188</td>
</tr>
<tr>
<td>0.50 mg./ml.</td>
<td>0.434</td>
</tr>
</tbody>
</table>

Readings were also taken using the various colour
filters and it was found that the green 604 filter definitely gave the highest figures. All future readings were made using this filter. The above comparison between (1) and (2) confirms the visual evidence that the brown colouration on treatment with acid increases slightly with increased concentration and it was, therefore, decided to use an alginate blank to allow for this discolouration. Experimentally it was found simplest to treat, instead of the original 1 ml. of alginate solution, 3 ml. with 18 ml. of sulphuric acid and after heating for 20 minutes, to divide the solution into two parts, to one of which carbazole is added, the other being used as a blank. This method has been used throughout the following experimental work.

**Effect of the Presence of Glucose in the Same Solution.**

Glucose solutions ranging in concentration from 0.10 - 0.30 mg./ml. of solution were made up and treated with sulphuric acid and carbazole as before, readings being made against glucose blanks with the following results.

<table>
<thead>
<tr>
<th>Conc.</th>
<th>Spekker Readings</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10 mg./ml.</td>
<td>0.063</td>
</tr>
<tr>
<td>0.15 &quot;</td>
<td>0.076</td>
</tr>
<tr>
<td>0.20 &quot;</td>
<td>0.078</td>
</tr>
<tr>
<td>0.25 &quot;</td>
<td>0.150</td>
</tr>
<tr>
<td>0.30 &quot;</td>
<td>0.098</td>
</tr>
</tbody>
</table>
The blank determination with sulphuric acid alone gave solutions considerably darker than those with similar concentrations of alginic acid. Determinations on mixed solutions of glucose and alginic acid also gave anomalous results with increasing concentration of glucose and the results could not be duplicated on repeating with the same solutions. Typical results are shown below:

<table>
<thead>
<tr>
<th>Conc(^n) mg.</th>
<th>Spekker Readings</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10 Alginic Acid + 0.10 Glucose</td>
<td>0.189</td>
</tr>
<tr>
<td>0.10 mg.</td>
<td>0.208</td>
</tr>
<tr>
<td>0.10 mg. + 0.15 mg.</td>
<td>0.185</td>
</tr>
<tr>
<td>0.10 mg. + 0.20 mg.</td>
<td>0.154</td>
</tr>
<tr>
<td>0.10 mg. + 0.25 mg.</td>
<td>0.167</td>
</tr>
</tbody>
</table>

In order to try to eliminate the effect of glucose and the darkening of the blank, the heating was omitted, the carbazole was added immediately after the addition of the acid and the solution left overnight before taking readings.

Results:

<table>
<thead>
<tr>
<th>Conc(^n)</th>
<th>Spekker Readings</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.20 Alginic acid/ml.</td>
<td>0.102</td>
</tr>
<tr>
<td>0.20 Glucose/ml.</td>
<td>0.043</td>
</tr>
<tr>
<td>0.20 mg. A.A. + 0.20 mg. Glucose/ml.</td>
<td>0.075</td>
</tr>
</tbody>
</table>

The depth of colour developed with pure alginic
acid was much weaker than previously at the same concentration, and the results show that the glucose interferes in this method also.

The results obtained in the presence of glucose solution therefore indicated that it would be necessary to eliminate glucose from any solution of alginic acid before attempting to estimate the latter by the use of carbazole by Dische's method. The method, on the other hand, showed promise of being applicable to the estimation of alginic acid in the absence of hexoses.

**Purification of Commercial Alginic Acid.**

A cleaner sample of alginic acid was obtained from the commercial product (Hopkin and Williams) by dissolving a quantity in sodium carbonate solution, reprecipitating with hydrochloric acid solution and dialysing free of salts. The product was filtered and dried in vacuo at 40°C. and gave, with the calcium acetate method, a figure of 70% of the theoretical yield of acetic acid. The low figure may have been in part due to strongly retained moisture, as much purer alginic acid was prepared from the same source as described later. Solutions of various concentrations were then made up, allowing for the purity of the sample, by dilution from a solution containing 0.357 g./250 ml. (i.e. 1 mg. pure alginic acid/ml. of solution),
made up by dissolving the solid in sodium hydroxide (N/10, 30 ml.) then diluting to the required volume.

**Application of the Modified Carbazole Method.**

The method modified as a result of the preliminary experiments was applied to a solution containing 0.2 mg. alginic acid/ml. of solution as follows:— To the solution (3 ml.) was added concentrated sulphuric acid (18 ml.) with cooling in water, the mixture then being heated in a boiling water bath for 20 minutes. The solution was then divided into two portions and to one portion carbazole solution (3 ml.: 0.1%) was added, the other portion being used as an absorptiometer blank. After standing overnight absorptiometer readings were taken. In three separate experiments the readings were (1) 0.434, (2) 0.466, (3) 0.600. As it was thought that more efficient cooling during the addition of the acid would be beneficial, three further determinations were made using cooling in iced water giving results of (1) 0.468, (2) 0.460, (3) 0.457. Intermediate readings taken before standing overnight showed that full development of the colour had not occurred in 3 hours.

**Improved Mixing during Addition of Acid.**

As it was apparent that local heating during addition of acid had an influence on the result, a standard method of stirring was devised. A Quickfit
chuck, driven by a high speed electric motor, was fitted with a tubular stirrer consisting of a glass tube (bore about 3 mm.) perforated with holes for about an inch from the bottom end. Compressed air was blown down the stirrer from the top and the combination of air and rotation gave satisfactory stirring during addition of the acid from a burette, the experiment being carried out in an 8" x 1" test-tube cooled in iced water. Using this method, the alginic acid solution used above gave concordant readings averaging 0·410.

Using other allied substances it was found that furfural and arabinose gave no colour and galacturonic acid at the same concentration gave a much higher reading of 1·260.

**Effect of Increased Time of Heating.**

By increasing the time of heating with acid to 3 hours a lower reading was obtained (0·314) for the same concentration (0·20 mg./c.c.).

**Variation in Intensity of Colour with Increasing Concentration of Alginic Acid.**

Following the modified method, a series of readings were made at various concentrations of alginic acid giving these results.
The low results at higher concentrations were shown to be due to insufficient carbazole since addition of 0.6 ml. instead of 0.3 ml. of 0.1% carbazole gave readings for 0.20 mg./c.c., 0.30 mg./c.c. and 0.40 mg./c.c. of 0.433, 0.470, and 0.722 respectively. Since increasing amount of carbazole involved adding more alcohol, it was decided rather to increase the concentration of carbazole to 0.2% in alcohol and add the original 0.3 ml. Using this increased carbazole concentration, the results were:

<table>
<thead>
<tr>
<th>Conc°</th>
<th>Spekker Reading</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.20  mg./ml.</td>
<td>0.335</td>
</tr>
<tr>
<td>0.30  mg./ml.</td>
<td>0.607</td>
</tr>
<tr>
<td>0.40  mg./ml.</td>
<td>0.880</td>
</tr>
<tr>
<td>0.50  mg./ml.</td>
<td>1.120</td>
</tr>
</tbody>
</table>

However a reading taken at 4 hours standing instead
of overnight for 0.20 mg./ml., was 0.437 thus showing that the increased carbazole concentration had decreased the time of development. It was also shown that heating after addition of carbazole produced a brown colouration which produced unsatisfactory results.

Rate of Development of Colour.

Using the standard method but with increased carbazole concentration, a solution of alginic acid (0.20 mg./ml.) gave the following results, readings being taken on the absorptiometer at the times stated.

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>Spekker Readings</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.491</td>
</tr>
<tr>
<td>0.75</td>
<td>0.489</td>
</tr>
<tr>
<td>1.0</td>
<td>0.491</td>
</tr>
<tr>
<td>1.5</td>
<td>0.483</td>
</tr>
<tr>
<td>2</td>
<td>0.477</td>
</tr>
<tr>
<td>3</td>
<td>0.457</td>
</tr>
<tr>
<td>4</td>
<td>0.448</td>
</tr>
<tr>
<td>5</td>
<td>0.443</td>
</tr>
<tr>
<td>6</td>
<td>0.428</td>
</tr>
<tr>
<td>22</td>
<td>0.337</td>
</tr>
</tbody>
</table>

In view of these results, three-quarters of an hour's standing before reading was selected as convenient for standardisation purposes. Using these conditions (¼ hour standing and 0.2% carbazole) the
following results were obtained for varying concentrations.

<table>
<thead>
<tr>
<th>Conc.</th>
<th>Spekker Readings</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05 mg./ml.</td>
<td>0.187</td>
</tr>
<tr>
<td>0.075 &quot;</td>
<td>0.219</td>
</tr>
<tr>
<td>0.10 &quot;</td>
<td>0.275</td>
</tr>
<tr>
<td>0.15 &quot;</td>
<td>0.390</td>
</tr>
<tr>
<td>0.20 &quot;</td>
<td>0.480</td>
</tr>
<tr>
<td>0.30 &quot;</td>
<td>0.653</td>
</tr>
<tr>
<td>0.40 &quot;</td>
<td>0.830</td>
</tr>
<tr>
<td>0.50 &quot;</td>
<td>1.020</td>
</tr>
</tbody>
</table>

These results when graphed gave a moderately straight line and therefore it was decided to prepare pure alginic acid for calibration purposes.

**Preparation of Pure Alginic Acid.**

Commercial alginic acid was dissolved in sodium carbonate solution (3%) and reprecipitated by the addition of a very slight excess of concentrated hydrochloric acid followed by immediate dilution with four times the volume of alcohol. The precipitate was readily filtered on a sintered glass crucible and washed with 25% alcohol till free from chloride, followed by pure alcohol. The product was dried at 40°C./0.1 mm., giving a pure white powder containing 94.42% alginic acid by both the excess sodium hydroxide
and the calcium acetate titration methods. The specific rotation as sodium alginate was \( [\alpha]_D^-139.4^\circ \) (c, 1.421 in water).

**Application of the Method to Pure Alginic Acid.**

A solution in sodium hydroxide (0.2592 g. alginic acid in 15 ml. \( \frac{N}{10} \) NaOH diluted to 250 ml.) was made up equivalent to 1 mg./ml. and appropriate dilutions made. Using 3 ml. of solution, 18 ml. conc. \( \text{H}_2\text{SO}_4 \), cooling in iced water and 0.2% carbazole the following results were obtained after standing \( \frac{1}{2} \) hour.

<table>
<thead>
<tr>
<th>Conc.</th>
<th>Spekker Readings</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>0.130</td>
</tr>
<tr>
<td>0.10</td>
<td>0.209</td>
</tr>
<tr>
<td>0.20</td>
<td>0.348</td>
</tr>
<tr>
<td>0.30</td>
<td>0.470</td>
</tr>
<tr>
<td>0.40</td>
<td>0.666</td>
</tr>
<tr>
<td>0.50</td>
<td>0.889</td>
</tr>
</tbody>
</table>

The solutions after treatment with acid were slightly brown and this was improved by cooling during addition of acid in a mixture of ice and salt (-8°C.) instead of iced water. It was also found convenient to add the alcoholic carbazole solution when the sulphuric acid solutions had cooled to 80°C. Using these modifications the results were as follows:-
<table>
<thead>
<tr>
<th>Conc^n</th>
<th>Spekker Readings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(1)</td>
</tr>
<tr>
<td>-</td>
<td>0.081</td>
</tr>
<tr>
<td>0.060</td>
<td>0.190</td>
</tr>
<tr>
<td>0.10</td>
<td>0.234</td>
</tr>
<tr>
<td>0.20</td>
<td>0.341</td>
</tr>
<tr>
<td>0.30</td>
<td>0.462</td>
</tr>
<tr>
<td>0.40</td>
<td>0.560</td>
</tr>
<tr>
<td>0.50</td>
<td>0.740</td>
</tr>
</tbody>
</table>

When the concentrations were plotted against absorptiometer readings on a graph a straight line resulted and this graph was used as a calibration curve for alginic acid determinations in seaweed samples.

**Effect of Purity of Concentrated Sulphuric Acid and Nitrogen for Air in Stirrer.**

A number of samples of concentrated sulphuric acid were obtained and absorptiometer readings made at timed intervals in the development of the colour with carbazole, using a solution of alginic acid obtained from a seaweed sample. In one case nitrogen was used instead of air in the stirrer.

The acids used were:

1. Griffin and Tatlock's Analytical Reagent with nitrogen.
2. Griffin and Tatlock's Analytical Reagent with air.
3. Macfarlane's P.B. acid with air.
4. Griffin and Tatlock's Pure Acid with air.
5. Jarvie's Bulk Acid with air.

The readings obtained in the absorptiometer were:

<table>
<thead>
<tr>
<th>Time</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.368</td>
<td>0.348</td>
<td>0.463</td>
<td>0.460</td>
<td>0.365</td>
</tr>
<tr>
<td>10</td>
<td>0.384</td>
<td>0.365</td>
<td>-</td>
<td>-</td>
<td>0.381</td>
</tr>
<tr>
<td>15</td>
<td>0.392</td>
<td>0.385</td>
<td>0.452</td>
<td>0.449</td>
<td>0.410</td>
</tr>
<tr>
<td>30</td>
<td>0.403</td>
<td>-</td>
<td>0.428</td>
<td>0.430</td>
<td>0.421</td>
</tr>
<tr>
<td>45</td>
<td>0.412</td>
<td>0.410</td>
<td>0.408</td>
<td>0.414</td>
<td>0.425</td>
</tr>
</tbody>
</table>

These figures indicate that the use of nitrogen does not affect the result but that the selection of a suitable acid is of prime importance and that each acid will require investigation into time of development of colour before use and also the preparation of a calibration curve.

Effect of Purity of Carbazole.

Using a purer sample of carbazole and new photocells in the absorptiometer, a lower blank and a slightly different graph were obtained but using the new figures and a control sample of alginic acid, the results were quite satisfactory.

Preliminary Experiments on Seaweed Samples.

A sample of Laminaria cloustoni containing 19.9% alginic acid (by the standard extraction method described
in the introduction) was used for these experiments. The sample (0.1 g.) was treated with sulphuric acid (N/5) as before to remove soluble carbohydrates and filtered through a 1x1 sintered glass crucible, the crucible and residue then being immersed in sodium carbonate solution (3%; 30 ml.) in a beaker overnight. The solution was then filtered through the crucible and made up to 100 ml. This solution (3 ml.) was then treated with sulphuric acid and carbazole as for pure alginic acid and the percentage of alginic acid obtained by comparison of the reading obtained with the graph obtained above. Filtration was found to be difficult and the result was low probably due to inefficient extraction.

By using, instead of a sintered crucible, a small filter paper and gravity filtration for removing the residue from the acid extraction, followed by immersion of the filter paper and residue in sodium carbonate solution, extraction was found to be more efficient if allowed to proceed over a week-end. By using this method followed by a second filtration and washing into a standard flask, an alginic acid percentage of 20.4% was obtained for the above sample.

Extraction with sodium carbonate by heating to 50°C. for two hours followed by standing overnight gave results, on the average, about 3% too low, but if the
paper was disintegrated with glass rods before heating to 50°C., better extraction was obtained and the results were satisfactory.

Method Adopted for Estimation of Alginic Acid in Seaweed (3)

The method finally adopted in view of the above experiments was as follows:-

Sample (0.1 g.) of dried ground seaweed of known moisture content is allowed to stand in sulphuric acid solution (10 ml.: N/5) in a small beaker overnight and then filtered off through a 5 cm. filter paper and washed. The filter paper and contents are transferred to a 100 ml. beaker containing sodium carbonate solution (20 ml.: 3%) and the paper disintegrated by means of two glass rods and the solution kept at 50°C. for two hours with occasional stirring and then overnight at room temperature. The solution is filtered through a 9 cm. filter paper into a 100 ml. graduated flask, the residue being washed with hot water and after cooling, the volume is made up. 3 ml. of solution are then added to an 8" by 1" test-tube and 18 ml. of sulphuric acid (pure conc.) added dropwise from a burette, the solution being cooled in ice and salt and stirred by the method described on page 65. After addition of the acid, the tube is heated in a boiling water bath for 20 minutes and then half of it
transferred to another tube. One tube is then cooled to 80°C. in a bath and to the solution, carbazole (0.3 ml.: 0.2% alcoholic) is added. After three quarters of an hour, the blank and sample are transferred to 1 cm. cells and, using the untreated half as blank, a reading is made on the photoelectric absorptiometer.

From this reading and using the calibration curve, the percentage of alginic acid may be obtained. The blanks using seaweed samples are slightly brown owing to the effect of the acid on pigments, etc. The following results were obtained for representative samples of seaweed. The figures obtained by the standard method are given for comparison.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Percentage of Alginic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>By carbazole</td>
</tr>
<tr>
<td>Laminaria digitata/10/46/L</td>
<td>17.4</td>
</tr>
<tr>
<td>&quot; cloustoni/9/46/L Frond</td>
<td>11.0</td>
</tr>
<tr>
<td>&quot; saccharina/10/46/L Stipe</td>
<td>21.5</td>
</tr>
<tr>
<td>&quot; &quot; Frond</td>
<td>10.8</td>
</tr>
<tr>
<td>Ascophyllum nodosum/1/47/L</td>
<td>29.5</td>
</tr>
<tr>
<td>&quot; /2/47/L</td>
<td>24.3</td>
</tr>
<tr>
<td>Fucus vesiculosus/10/46/O</td>
<td>14.1</td>
</tr>
<tr>
<td>&quot; serratus/10/46/O</td>
<td>16.3</td>
</tr>
<tr>
<td>&quot; spiralis/1/47/0</td>
<td>14.4</td>
</tr>
</tbody>
</table>

The figures following the species denote the month
and year of collection of the sample and L and O indicate whether the sample was collected in a sea-loch or in the open sea.

**Effect of Laminarin.**

Laminarin, a polysaccharide made up of glucose units would be expected to interfere in the estimation and this was found to be the case. A solution of laminarin (3%) was treated as in the estimation and found to give a colour equivalent to about 3% alginic acid, showing that the preliminary acid extraction, which removes most of the laminarin in the sample, cannot be omitted. This was also shown to be the case by carrying out (1) a direct extraction with sodium carbonate and (2) an acid extraction followed by sodium carbonate on a sample of seaweed containing 11·0% alginic acid. Estimations were carried out after both (1) and (2) and also on the acid extract giving the following results expressed as percentages of the sample, calculated as alginic acid.

1. Extraction with H₂SO₄, then Na₂CO₃ 11.4% Alginic Acid
2. " " H₂SO₄ 15.5% " "
3. Acid extract 5.7% " "

**Modification to the Micro Scale.**

The method has been found to be adaptable to the micro determination of alginic acid. A 10 mg. sample,
weighed on a semi-micro balance was treated with sulphuric acid (3 ml.: N/5) in a 10 ml. beaker overnight, filtered and washed through a 4.25 cm. filter paper, which was then returned to the beaker. The paper was broken up with two fine glass rods and extracted at 50°C. and overnight as before with sodium carbonate solution (3 ml.: 3%). The solution was then decanted through another small filter paper into a 10 ml. graduated flask, washed by decantation and the volume made up to the mark. Estimations were then made in the usual way.

Results:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Alginic Acid found</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminaria cloustoni/9/46/Frond</td>
<td>11.4%</td>
</tr>
<tr>
<td>(Alginic Acid 11.0%)</td>
<td></td>
</tr>
<tr>
<td>Ascophyllum nodosum/2/47/L</td>
<td>24.3%</td>
</tr>
<tr>
<td>(Alginic Acid 24.3%)</td>
<td></td>
</tr>
</tbody>
</table>

Estimation of Alginic Acid on Portions of Living Seaweed.

Freshly collected samples of Himanthalia lorea were drained and wiped free of water with filter paper and alginic acid estimations carried out on (a) 2" sections of the extreme tip of the frond and (b) 2" sections of the proximal portion. Two experiments were carried out:

A. About 1 g. was chopped up with a razor, weighed
accurately, treated with acid etc., and the alginic acid estimated.
B. About 1 g. was cut up and weighed as before but dried to constant weight in vacuo over P₂O₅, and ground in an agate mortar before estimation of alginic acid.

Results:-
1. Moisture determination from B.
   Tips  88.33%, Proximal portion 86.24%.
2. Alginic Acid Estimation from A.
   Tips  2.49%, Proximal portion 3.27%.
3. Alginic Acid Estimation from B.
   Tips  22.0%, Proximal portion 24.6%.
Allowing for moisture.
4. Tips  2.58%, Proximal portion 3.34%.

The results given in 2 and 4 show moderately good agreement, and that estimations can be carried out on wet seaweeds with reasonable accuracy.

Determination of Water Soluble Alginates in Seaweed.

Acid (N/5), cold water and hot water extractions were carried out on a sample (Laminaria digitata) and the alginic acid estimated in each case both on the extract and the residue by carbazole and by the standard precipitation and titration method with these results.
### Extraction Medium

<table>
<thead>
<tr>
<th></th>
<th>Acid N/5</th>
<th>Cold Water</th>
<th>Hot Water</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Extract</td>
<td>Residue</td>
<td>Ext.</td>
</tr>
<tr>
<td>Alginic Acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>by Carbazole</td>
<td>1.95</td>
<td>23.7</td>
<td>2.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5.52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>22.9</td>
</tr>
<tr>
<td></td>
<td>none</td>
<td></td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

These figures indicate that there is a small amount of water soluble alginate present which is mostly precipitated by the acid treatment and which is more soluble in hot water than in cold.
SUMMARY

1. A method has been evolved for the estimation of alginic acid in seaweeds based on the colour reaction between uronic acids, sulphuric acid, and carbazole.

2. The purity of the concentrated sulphuric acid used in this method has been shown to be of considerable importance.

3. The presence of laminarin in the sample interferes with the estimation to a small extent and this polysaccharide must be removed before analysis.

4. Application of the method shows good agreement with the earlier method of extraction and titration.

5. Determinations are normally made on samples of 0.1 g. but it is possible to reduce this quantity to 10 mg. with good results.

6. The method may be applied to wet fresh samples of seaweed for the determination of variations in alginic acid content in different parts of the plant.


PART III

FUCOIDIN
This section of the thesis, although headed fucoidin, actually consists of three parts, the first being concerned with the estimation of fucose; the second with the chemical composition of the polysaccharide fucoidin prepared from marine algae, and the third with determination of fucose in seaweeds. The investigations in the second part were carried out partly in an effort to determine a suitable factor for conversion of the estimated percentage of fucose to fucoidin in the original seaweed, and partly in an attempt to increase the present scanty knowledge of the latter polysaccharide.

Fucoidin belongs to the group of substances known as carbohydrate sulphuric esters which are of widespread occurrence in nature and particularly in seaweeds. Generally speaking these compounds can be extracted with water or dilute acids in the form of salts and purified by dialysis, although in the case of the Phaeophyceae, at least, owing to the presence of laminarin, soluble alginates etc., a more complex scheme of purification is necessary.

Polysaccharide ethereal sulphates, in the simplest form, may be represented by the formula:-

\[(R.O.SO_2. OM)_n\]
in which the portion within the brackets represents
the repeating unit in the polysaccharide.

\[ \text{R is the monosaccharide repeating unit.} \]

\[ \text{M is the metal, viz. Na,K, } \frac{\text{Ca}}{2} \text{ or } \frac{\text{Mg}}{2} \]

\[ n \text{ is the chain length and is unknown in most cases.} \]

In these compounds, it has been found that the
metallic portion of the molecule is ionised and, in the
case of calcium, may be quantitatively precipitated by
ammonium oxalate while the sulphate is unionised and a
solution of the polysaccharide will give no precipitate
with barium chloride solution until after hydrolysis
with hydrochloric acid. The monosaccharide repeating
unit \( \text{R} \), although indicated as a single sugar in the
formula, may consist of one or more simple sugars and
this is frequently found to be the case.

A prominent feature of these compounds is the
relation between the sulphate content of the ash
obtained on ignition and that found in the solution
after hydrolysis. On ignition there is insufficient
metal present to unite with the sulphate present and
some of the latter is lost as \( \text{SO}_3 \) thus:

\[ 2 \text{R.O.SO}_2\text{OM} \rightarrow \text{R}_2\text{SO}_4 + \text{SO}_3 + \text{products of combustion}. \]

On hydrolysis, however, free sugars are formed and
all the sulphate remains in solution as sulphuric acid.

\[ \text{R.O.SO}_2\text{OM} + \text{HCl} + \text{H}_2\text{O} \rightarrow \text{R.OH} + \text{M.Cl} + \text{H}_2\text{SO}_4 \]

Thus only half the sulphate obtained on hydrolysis
appears in the ash and this relation is characteristic of ethereal sulphates. It has been found essential when estimating the ash sulphate to treat the ash with concentrated sulphuric acid before weighing in order to convert all sulphur and metals present to sulphate, as, owing to the presence of carbonaceous matter, some of the sulphate may be reduced to sulphide, and sulphur will then be lost as hydrogen sulphide on solution in hydrochloric acid. Other losses occur due to the production of sulphur dioxide during ignition, but if the above precaution is observed, the 1:2 ratio is generally found to hold true.

The metallic portion of the molecule may be found to vary in the same polysaccharide if derived from different sources, but this fact does not normally have much effect on the characteristics of the polysaccharide. The presence of different metals in the same compound may also lead to different solubilities in water, hence cold water extraction of the polysaccharide may give chiefly the alkali metal salts whereas hot extraction will give chiefly the calcium salt.

**Fucoidin.**

This water-soluble ethereal sulphate occurs, along with the polyglucoside laminarin and the polyuronide alginic acid in various brown seaweeds. Kylin\(^1,2\)
working in Sweden where abundant seaweed deposits occur, was the first to isolate and name fucoidin obtained from various species of Laminaria and Fucus, by extraction of the weed with dilute acetic acid (0.5%). The extract was neutralised with sodium hydroxide, treated with lead acetate solution and the precipitate containing lead alginate, protein complexes, etc., was removed. The lead in the solution was removed by treatment with hydrogen sulphide and the filtered solution neutralized with sodium hydroxide after addition of more lead acetate when a precipitate was obtained consisting partly of lead hydroxide and partly of fucoidin, presumably as a lead complex. By treatment of the precipitate with sulphuric acid and neutralization with calcium carbonate, the lead was eliminated and from the solution on evaporation, fucoidin was obtained in admixture with some calcium sulphate. By hydrolysing the fucoidin for five hours at 100°C., Kylin was able to show the presence of fucose by the preparation of a characteristic osazone, and also claimed that pentose as well as methyl-pentose was present in the hydrolysate. Kylin also found that if hot extraction was used, a less viscous solution was obtained compared with that derived from cold extraction and this fact he attributed to the replacement of calcium by sodium in the polysaccharide.
Bird and Haas\(^3\) obtained fucoidin by soaking fresh samples of *Laminaria* in distilled water and precipitating fucoidin from the viscous extract by addition of alcohol. Purification was effected by repeated solution in water and reprecipitation in alcohol and finally by dialysis, evaporation and precipitation in alcohol.

Their product gave 30–93\% ash (chiefly calcium sulphate) and on hydrolysis yielded a total sulphate of 30–33\%, the sulphate in the ash being 15–10\%.

Haas and Russell-Wells\(^4\) had previously investigated the evidence of sulphate estimations in their relation to ethereal sulphates. Their findings coupled with the fact that the calcium in fucoidin could be precipitated with ammonium oxalate (hence Ca\(^+\)) led Bird and Haas to assume that fucoidin was a carbohydrate ethereal sulphate. The same authors confirmed the presence of methyl-pentose by the Rosenthaler colour reaction and preparation of an osazone, and, in addition, found evidence for the existence of 7–3\% of a uronic acid complex by distillation with 12\% hydrochloric acid and estimation of the carbon dioxide produced.

More recently Lunde, Heen and Oy\(^5\) have attempted to prepare as pure a sample of fucoidin as possible by collecting the viscous drops exuded from *Laminaria*.
**digitata** on standing, and purifying the product by boiling with alcohol. Since this is the only purification applied by these authors, it is difficult to see how the fucoidin did not contain any laminarin or soluble alginate although they investigated the possible presence of both these substances and found only 0.3% laminarin and also a small amount of protein (3.5%). Estimation of the ash gave figures, 26-30% containing 63-66% sulphate or 17-19% on the polysaccharide. On hydrolysis, the sulphate content was estimated to be 35.5-37.5% and the ash was found to be mainly sodium sulphate with small quantities of calcium, magnesium, and potassium sulphates. In neutral solution the addition of calcium or barium chlorides to fucoidin was said to produce a precipitate. By distillation with 13% hydrochloric acid, according to the method of Tollens,[6] an attempt was made to estimate the methylpentose quantitatively from the yield of methylfurfural weighed as a phloroglucide. By this means the fucose content of the polysaccharide was found to be 33-37% and the phloroglucide was completely soluble in alcohol indicating the absence of uronic acids which yield furfural phloroglucide under the same conditions, the latter substance being insoluble in alcohol. Under the circumstances, the uronic acid found by Bird and Haas was assumed to be
an impurity in their preparation, as was also the pentose found by Kylin since pentoses also yield furfural on distillation with hydrochloric acid. There remains the possibility of the presence of hexoses since, according to Kullgren and Tyden\(^7\), they give, under the above conditions, \(1-2\% \omega\)-hydroxy-methylfurfural the phloroglucide of which is soluble in alcohol.

The above analytical results of Lunde, et al., account for about 80% of the polysaccharide, i.e. the total of metal, sulphate, fucose and estimated impurities leaving 20% unaccounted for. In view of these results Lunde proposed the formula:

\[
(R \cdot R' \cdot O\text{SO}_3\text{OM})_n
\]

where \(R\) is fucose and represents about 60% of the total of \(R\) and \(R'\) and \(R'\) is another carbohydrate complex as yet unknown.

Conchie\(^8\) found a fucose content of 37.8% and sulphate 32.8% in a purified sample of fucoidin prepared by hot extraction of *Fucus vesiculosus*. On ignition the sulphated ash contained sulphate equivalent to 17.4% of the polysaccharide, the main metal present being calcium. By hydrolysis and treatment with methylphenylhydrazine the fucose was removed and a hypoiodite oxidation experiment indicated the presence of 49.5% aldose (calculated as hexose) in the "fucose-
free" syrup. Paper chromatographic analysis of the hydrolysed material according to the method of Partridge (9) suggested the presence of a hexose but the above author thinks that this may be due to an impurity. The same worker has carried out methylation experiments on fucoidin followed by hydrolysis of the methylated polysaccharide and separation of various fractions by (1) chromatographic adsorption and (2) solvent extraction. In no case was he able to detect methylated sugars other than fucose derivatives, except for traces of methylated hexoses and possibly a methylated uronic acid.

The main methylated sugars and their approximate proportions found by Coneirie in the sulphate free mixture of sugars, were as follows: 3-methyl methyl-L-fucosides (57%), 2:3 dimethyl methyl-L-fucosides (20%); methyl-L-fucosides (20%). On the basis of these results he proposes a tentative formula for the fucose-containing portion of the molecule, in which linkages through positions C1 and C2 and a sulphate group on C4 are the main features.

The foregoing survey of work carried out on fucoidin indicates that without doubt the substance is a carbohydrate ethereal sulphate containing a large percentage of fucose and sulphate but that there is still an unknown fraction amounting to between 20% and
30% of the polysaccharide. In the experimental work described later, an attempt has been made to find out the composition of this missing percentage and to purify fucoidin as highly as possible.

Polysaccharide from *Macrocystis pyrifera*.

This water soluble polysaccharide occurs in the brown seaweed *Macrocystis pyrifera* and is described owing to its resemblance to fucoidin. It was first isolated by Hoagland and Lieb\(^{10}\) who precipitated it from an aqueous extract followed by repeated precipitation with alcohol from acid solution to remove salts. The final product contained 35% calcium sulphate and was precipitated partially by various heavy metals and completely by ferric chloride and lead acetate. Using the method of Ellet and Tollens\(^{11}\) Hoagland and Lieb showed that the carbohydrate portion was mainly methylpentose along with a little pentose, the methylpentose being fucose as shown by the preparation of fucose phenylosazone. Nelson and Cretcher\(^{12}\) found fucose to the extent of 31.7% and showed that the polysaccharide had an ethereal sulphate grouping in the molecule. They showed also that 17.6% of titratable sulphate (as HSO\(_4\)) was present as compared with a total sulphate of 43.8% and proposed the formula \((R-O-SO_3^--O\text{H})_4\) for the ester where \(R\) is mainly fucose. A small quantity of uronic
acid (2.6%) was probably derived from alginic acid.

Fucose.

As this section is partially devoted to the determination of fucose, some description of the chemistry and methods of estimation of fucose will not be out of place at this point.

Fucose is found to occur naturally in both the D- and L- forms in the vegetable kingdom though not to any great extent, D-fucose or rhodeose as it was called by Votocek (13) being found in the glycosides convolvulin and jalapin whereas L-fucose occurs mainly in marine algae. L-fucose with which this thesis is concerned was first prepared from Fucus by Tollens and co-workers (14, 15) as a crystalline sugar although evidence for the presence of a methylpentose in seaweed had been obtained much earlier. Stenhouse (16) in 1872 had obtained from hydrolysed seaweed, by treatment with hydrochloric acid, a distillate which he called fucosol which was shown by Maquenne (17) to consist of a mixture of furfural and methylfurfural. L-fucose was also found in the hydrolysate from gum tragacanth (18). The preparation of fucose from seaweed is normally carried out by the method of Clark (19) who hydrolysed the weed with 3% sulphuric acid after soaking in dilute hydrochloric acid. It has been
suggested by later authors (3) that this soaking is wasteful as some of the fucoidin is lost by solution. After hydrolysis the solution is treated with alcohol to remove gummy substances, with basic lead acetate and filtered. From the resulting solution, the fucose is obtained as a phenyl-hydrazone which is decomposed with benzaldehyde to give free fucose. The constitution of L-fucose was determined as follows. Oxidation with nitric acid gives D-arabo-trihydroxyglutaric acid (20) (I) hence in fucose carbon atoms 2, 3 and 4 must have the configuration II or III. By cyanhydrin synthesis L-fucose gives two L-fuco-hexonic acids which on oxidation with nitric acid do not give mucic acid and therefore C2 and C3 of L-fucose cannot be in the cis-position, thus confirming formula II for carbon atoms 2 - 4. The preparation of IV by degradation (19) and the application of Hudson's lactone rule indicated the configuration of C5 and hence the conclusion that L-fucose was 6-desoxy-L-galactose (V). The structure in the case of the D-form has been confirmed by the synthesis of D-fucose from D-galactose by Freudenberg and Raschig (21)
Methylpentoses in general give Rosenthaler's colour reaction\(^{(22)}\) which consists of heating with 38% hydrochloric acid and a little pure acetone when a violet colour is obtained. On distilling with 12% hydrochloric acid, according to Widstoe and Tollens\(^{(23)}\), methylfurfural is obtained, whereas pentoses give furfural. Another test which serves to distinguish pentoses and methylpentoses is the reaction with \(\beta\)-naphthol-sulphonic acid in which pentoses give a blue colour and methylpentoses none.

**Estimation of Methylpentoses.**

The conversion of a methylpentose into methylfurfural by distillation with strong hydrochloric acid was the basis of all methods used for estimation of methylpentoses until quite recently. The reaction was first observed by Stone and Tollens\(^{(24)}\) in 1888, and developed into a method of estimation by Ellet and Tollens\(^{(25)}\) who determined the methylfurfural formed by precipitation as a phloroglucide. The method consists essentially of distilling the methylpentose with 12% hydrochloric acid at a standard rate, the quantity of acid in the flask being maintained by addition during the distillation. When complete, phloroglucinol in 12% hydrochloric acid solution is added to the distillate and after standing for several
hours, the precipitate is weighed in a Gooch crucible. Unfortunately the yield is not quantitative owing to the destruction of methylfurfural during distillation, the yields of precipitate being of the order of 50% or less of the theoretical. As a result Ellet and Tollens developed a purely empirical relation between the weight of precipitate and the weight of original methylpentose by means of control experiments. Later workers modified the method by using 13.15% hydrochloric acid along with a quantity of sodium chloride which maintained the concentration of acid during distillation (Kullgren and Tyden\(^{(26)}\)) and thiobarbituric acid was substituted for phloroglucinol. Powell and Whittaker\(^{(27)}\) used titration with potassium bromate to determine the methylfurfural present. Marshall and Norris\(^{(28)}\) investigated all the above methods and found phloroglucinol and thiobarbituric acid precipitation satisfactory when used with the distillation procedure of Kullgren and Tyden, but showed that a large number of factors influence the percentage yield compared with the theoretical, necessitating strict adherence to the conditions of whichever method is used and application of a number of correction factors to obtain an accurate result. Later Marshall and Norris\(^{(29)}\) also showed that using mixtures of methylpentose and uronic acid,
the method was satisfactory in some cases but in the presence of pentose numerous difficulties arose.

It is clear under the circumstances that the method would be extremely difficult to apply with accuracy to the complex mixture present in seaweed. Recently, however, Nicolet and Shinn\(^{30}\) have published an extremely neat method of estimation based on oxidation with periodic acid. Methylpentoses on treatment with periodic acid yield a mixture of formic acid and acetaldehyde thus,

\[
\begin{align*}
\text{CHO} & \quad \text{CHOH} \\
\text{CHOH} & \quad \text{CHOH} \\
\text{CHOH} & \quad \text{CHOH} \\
\text{CH}_3 & \quad \text{CH}_3
\end{align*}
\]

\[
\text{H}_2\text{O}_4 \rightarrow 4\text{H.COOH} + \text{CHO} + \text{CH}_3
\]

and by determining the quantity of acetaldehyde formed, a measure of the methylpentose present can be obtained. Under the same conditions hexoses and pentoses yield formic acid and formaldehyde. Nicolet and Shinn have shown that the yield of acetaldehyde is practically 100\% of the theoretical. The main possible interfering substances are protein hydrolytic products such as threonine, containing the group \(\text{CH}_3. \text{CHOH.CH.NH}_2\)\(^-\), which yield acetaldehyde with periodic acid but these
amino acids are not known to occur in seaweed. The method may be described in some detail as it is later used in the experimental section. The apparatus consists of three test-tubes fitted as a gas absorption train the first being fitted with a dropping funnel reaching to the bottom of the tube. The first tube contains the sugar (5-15 mg.) along with alanine (0.2 g.) which absorbs any formaldehyde formed, Nujol (1 drop) to prevent foaming, sodium bicarbonate (5 c.c.; N.), which reacts with formic acid and periodic acid, and finally sodium arsenite (10 c.c. N/10) to destroy excess periodate. The other two tubes contain sodium bisulphite (5 c.c. and 3 c.c. respectively, diluted to 25 c.c. of a solution containing 19 g. metabisulphite/litre). The solutions are mixed by the passage of carbon dioxide for a short time, then periodic acid (1-2 c.c., 0.5M) is added to the first tube via the tap funnel and carbon dioxide passed for a further hour at a rate of about 1 litre/min. The periodate requires to be in excess to the extent of twice that required for reaction with the sugar. The acetaldehyde formed is absorbed in the second and third tubes as a bisulphite compound. At the end of the hour the contents of these two tubes are washed out and mixed and titrated with iodine (N/10), using starch indicator, to near the end point and completed with N/50 iodine.
Saturated bicarbonate solution is then added to liberate the "bound bisulphite" and the titration continued to a new end-point. From the result of this latter titration, the amount of acetaldehyde formed and hence the original amount of methylpentose can be determined.

The above method has obvious advantages over that of Tollens, et al. insofar as no highly empirical calibration curves are necessary since the yield of acetaldehyde is almost theoretical (Nicolet and Shinn give figures of upwards of 98% for rhamnose in presence of tenfold excess of pentose and in presence of glucose and galacturonic acid). It is also applicable to small quantities of sugar and is not interfered with by hexoses, pentoses or uronic acids provided sufficient periodic acid is added.

In addition to the estimation of methylpentose, periodic acid oxidation may be used to estimate hexoses or pentoses by determining, by precipitation with dimedone, the formaldehyde produced. In the presence of methyl pentose, if the acetaldehyde is first aerated off, the method allows of the estimation of both hexose or pentose and methylpentose on the same solution.

Finally, recently, Dische and Shettles (31) have developed a colorimetric method for methylpentose estimation based on the reaction between the sugar and cysteine hydrochloride in strong sulphuric acid.
solution. The estimation is made by spectrophotometric measurements of the greenish yellow colour which is developed.
BIBLIOGRAPHY

PART I. Determination of Methylpentose.

The method used was that of Nicolet and Shinn(1) which has the many advantages described in the introduction. Standard solutions were prepared as follows.

1. 0.5 M Periodic acid containing 114 g. HIO4.2H2O./litre.

2. N/10 Sodium arsenite made up from pure As2O3 according to Cumming and Kay(2).

3. Sodium bisulphite solution prepared by passing SO2 into a cold saturated solution of sodium carbonate solution until the solution turned yellow. This solution was then diluted until 5 ml. of the diluted solution required 10 ml. of N/10 iodine by titration with starch indicator.

4. N/10 Iodine in potassium iodide solution.

5. N/100 Iodine prepared from 4 and accurately standardised with sodium thiosulphate solution.

Procedure following Nicolet and Shinn.

A solution (5 ml.) containing methylpentose (10-20 mg.) was pipetted into an 8" x 1" Pyrex test-tube and to it added alanine (0.2 g.), sodium bicarbonate (0.6 g.) and sodium arsenite solution (14 ml.; N/10)
and the test-tube fitted with a dropping funnel drawn out to reach the bottom of the tube and a short right-angled side arm. This side arm leads into a gas absorption train consisting of two similar test tubes containing respectively 4 ml. and 1 ml. of bisulphite solution diluted to 25 ml. Carbon dioxide was passed in via the dropping funnel for a few minutes in order to mix the solutions then the tap was closed and periodic acid (2 ml.; 0.5 M) was added to the funnel and run in under a low pressure of gas. When all the acid was added the rate of gas was increased to 1 litre/minute and aeration continued for one hour. At the end of this time the contents of the second and third tubes were mixed and titrated with N/10 iodine to near the end point and finished with N/100 iodine using starch indicator. Finally the acetaldehyde bisulphite compound was decomposed by the addition of saturated sodium bicarbonate solution (10 ml.) and the liberated bisulphite titrated with N/100 iodine. From this titration figure the amount of acetaldehyde formed and hence the original amount of methylpentose was calculated since 1 c.c. N/100 iodine = 0.82 mg. methylpentose.

Preliminary estimations were carried out using the above method on standard solutions of acetaldehyde in place of methylpentose and it was found necessary to aerate for 1½ hours, which time was later increased to
2½ hours in the case of seaweed samples to ensure of complete removal of the acetaldehyde. Under these circumstances the rate of gas flow could be reduced to 800 ml./min. thus avoiding a certain amount of splashing in the test-tubes.

Application to Methylpentose.

Rhamnose hydrate was used in control experiments owing to the limited quantity of fucose available. Estimations of rhamnose (10 mg. in 5 ml. sol.) as above gave results varying between 98-99%, but it was found that great care was necessary during the addition of the periodic acid to avoid frothing due to the production of carbon dioxide. To eliminate this, two drops of capryl alcohol were added to the first test-tube but owing to the carrying over of some of the alcohol, bad starch indicator end points were obtained in the titrations and the capryl alcohol was replaced by liquid paraffin which proved satisfactory, especially in the later experiments with seaweed samples. A second improvement was the replacement of carbon dioxide by nitrogen as aerating agent as, due to the high rate of gas, a Kipp's apparatus soon exhausted itself and when a cylinder of carbon dioxide was used difficulties arose due to freezing up in the nozzle of the reducing valve. The third modification applied was the replacement of the first test-tube by a
Quickfit 8" x 1" test-tube with side arm adaptor and ground in dropping funnel. In place of the other two test-tubes, Dreschel bottles (150 ml.) were used, the inlet tubes being drawn out slightly at the bottom and the bisulphite solutions being diluted to 75 ml. instead of 25 ml.

It was noted that the final titration with N/100 iodine must be carried out slowly as when the iodine is added the starch blue colour takes an appreciable time to disappear until the end point is reached when the blue colour must persist for at least two minutes. A little more bicarbonate solution may then be added to ensure complete decomposition of the "bound bisulphite" compound.

Estimation of Rhamnose in Presence of Hexose.

Estimations of rhamnose (10 mg.) were carried out in presence of glucose (10 mg. and 50 mg.) and gave results for rhamnose of between 99 and 100%.

Effect of Other Seaweed Constituents.

Experiments with mannitol (50 mg.) and alginic acid (125 mg.) showed that these substances gave no appreciable final titration with N/100 Iodine.

Variation of Yield of Acetaldehyde with Time of Aeration.

Samples of rhamnose were aerated for various periods of time giving acetaldehyde equivalent to
rhamnose as shown in the table below.

<table>
<thead>
<tr>
<th>Time of Aeration in hrs.</th>
<th>% Rhamnose found</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>51.4</td>
</tr>
<tr>
<td>1.0</td>
<td>76.6</td>
</tr>
<tr>
<td>1.25</td>
<td>83.5</td>
</tr>
<tr>
<td>1.5</td>
<td>100.6</td>
</tr>
</tbody>
</table>

Accuracy of the Determination at Various Levels of Methyl-Pentose.

Fucose in quantities varying from 20 mg. - 0.5 mg. in 5 ml. of solution were estimated with the following results.

<table>
<thead>
<tr>
<th>Fucose used</th>
<th>% Fucose found</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mg.</td>
<td>100.8</td>
</tr>
<tr>
<td>10 mg.</td>
<td>100.3</td>
</tr>
<tr>
<td>7 mg.</td>
<td>98.8</td>
</tr>
<tr>
<td>5 mg.</td>
<td>99.2</td>
</tr>
<tr>
<td>2 mg.</td>
<td>101.3</td>
</tr>
<tr>
<td>1 mg.</td>
<td>104.1</td>
</tr>
<tr>
<td>0.5 mg.</td>
<td>V. High.</td>
</tr>
</tbody>
</table>

It is thus clear that the method is reasonably accurate for quantities of fucose from 2-20 mg.

Effect of Time of Solution on Rhamnose Estimation.

Rhamnose estimations were carried out on (a) solutions immediately after dissolving rhamnose and (b) solutions allowed to stand overnight. In the
first case figures of 80-90% rhamnose were obtained whereas in the second the normal 98-100% results were obtained. This anomaly was assumed to be due to the time necessary for the setting up of an equilibrium between the ring and straight chain forms of rhamnose in solution since the presence of the ring prevents the production of acetaldehyde. It will be recalled that the periodic acid is rapidly destroyed by the sodium arsenite and the time of contact with the reagent is therefore limited and may be insufficient to permit the reaction to go to completion.

PART II. Investigation of Fucoidin.

In the following work all fucose determinations were carried out as described previously and sulphate and metallic estimations were done according to Cumming and Kay(2) the sulphates all being treated with concentrated sulphuric acid before weighing.

Preparation of Fucoidin.

Fucoidin was prepared from Fucus vesiculosus according to the method of Kylin(3) as modified by Conchie(4) as follows. The dried ground weed (1 Kg.) was extracted with hot water (3 l.) for 24 hours and filtered through muslin, and to the solution was added lead acetate solution until precipitation was complete.
After filtration, barium hydroxide solution was added until alkaline and the precipitate consisting of lead hydroxide and a fucoidin complex was removed and decomposed with dilute sulphuric acid (300 ml.; 4N in 1 l. water) by shaking overnight, dialysed till free from SO₄, filtered, evaporated under reduced pressure to small bulk, and the fucoidin precipitated by pouring into alcohol. After filtering, washing with alcohol and ether, the fucoidin was dried over P₂O₅ in vacuo, yielding a buff coloured solid. Hydrolysis with 2.5% sulphuric acid for 5 hours and methylpentose estimation gave a fucose content of 29.6% but the solution after hydrolysis was slightly brown and a small brown residue was present. Using 2.5% oxalic acid, the fucose percentage was 32.1%. The sulphate content was 27.3%.

**Hydrolysis followed Polarimetrically**

A sample of fucoidin was hydrolysed with (1) 2.5% oxalic acid and (2) 1% oxalic acid and the course of hydrolysis followed polarimetrically with these results.

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>1.0</th>
<th>1.5</th>
<th>2.5</th>
<th>3.0</th>
<th>3.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific Rot.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>2.5% Acid</strong></td>
<td>-140°</td>
<td>-60°</td>
<td>-38°</td>
<td>-36°</td>
<td>-36°</td>
</tr>
<tr>
<td><strong>1.0% Acid</strong></td>
<td>-140°</td>
<td>-60°</td>
<td>-43°</td>
<td>-38°</td>
<td>-37°</td>
</tr>
</tbody>
</table>
The fucose contents of the solutions after hydrolysis were respectively 31.5% and 31.9%. Since 2.5% acid gave more rapid hydrolysis it was adopted for later experiments.

**Purification by Reprecipitation in Alcohol.**

Reprecipitation in alcohol from water did not increase the fucose content but it was found that a slightly higher figure was obtained if the sample was dried overnight in vacuo at 40°C/0.1 mm and this procedure was adopted in future before making any determination.

**Purification with "Filter Cel."**

The sample of fucoidin was dissolved in water and to the solution added 2% Johns Manville "Filter Cel." After standing overnight, the solution was filtered twice through the "Filter Cel" and the polysaccharide recovered by evaporation and precipitation in alcohol. Fucose content - 35.4%. A second treatment with "Filter Cel" increased this to 37.4% and gave a product with SO₄, 31.7% and $\left[\alpha\right]_{D}^{150}-119^\circ$ (c, 1.0 in water).

**Fucoidin from Fucus spiralis**

A sample of fucoidin was obtained from this species in the usual way, except that the lead acetate precipitate was filtered off through "Filter Cel" thus
removing a considerable amount of the brown pigment in the solution. The resulting fucoidin had fucose, 35.8% which was not increased by further purification as before with "Filter Gel."

**Fucoidin from Laminaria cloustoni.**

A third sample of fucoidin (III) was obtained from *L. cloustoni* and purified as before to the highest fucose content obtainable.

For comparison the results of the three extractions are given in tabular form.

<table>
<thead>
<tr>
<th></th>
<th>( [\alpha]_{D}^{150} )</th>
<th>Sulphate</th>
<th>Fucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fucoidin I from F.v.</td>
<td>-119°</td>
<td>31.7</td>
<td>37.5</td>
</tr>
<tr>
<td>&quot; II from F.sp.</td>
<td>-118°</td>
<td>32.7</td>
<td>35.8</td>
</tr>
<tr>
<td>&quot; III from L.c.</td>
<td>-114°</td>
<td>33.5</td>
<td>36.5</td>
</tr>
</tbody>
</table>

**Ash Analyses of Fucoidin II and III.**

The sulphated ash contents of fucoidins II and III were respectively 22.9% and 24.2%, consisting of metals and sulphate as follows, the figures being given both as percentages of ash and of polysaccharide.

<table>
<thead>
<tr>
<th></th>
<th>II Ash</th>
<th>II Polys.</th>
<th>III Ash</th>
<th>III Polys.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulphate</td>
<td>71.80</td>
<td>16.4</td>
<td>73.2</td>
<td>17.8</td>
</tr>
<tr>
<td>Calcium</td>
<td>25.5</td>
<td>5.8</td>
<td>25.0</td>
<td>6.1</td>
</tr>
<tr>
<td>Magnesium</td>
<td>1.1</td>
<td>0.3</td>
<td>1.9</td>
<td>0.5</td>
</tr>
<tr>
<td>Sodium</td>
<td>1.4</td>
<td>0.3</td>
<td>1.1</td>
<td>0.3</td>
</tr>
<tr>
<td>Potassium</td>
<td>Trace</td>
<td>Trace</td>
<td>0.8</td>
<td>0.2</td>
</tr>
<tr>
<td>Total Metal</td>
<td>6.4</td>
<td>7.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Effect of Hydrolysis on Specific Rotation.

Samples of fucoidins II and III were dissolved in 2.5% oxalic acid and their rotations observed. Then hydrolysis was carried out for 4 hours and the rotations again observed. By allowing for the rotation of fucose ([α]_D^15° = -76.4) at a known percentage in the hydrolysate, a measure of the rotation of the remaining portion of the polysaccharide was obtained.

<table>
<thead>
<tr>
<th></th>
<th>Fucoidin II</th>
<th>Fucoidin III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sp. Rot° of hydrolysate</td>
<td>-23.6°</td>
<td>-24.6°</td>
</tr>
<tr>
<td>% of Fucose</td>
<td>35.8</td>
<td>36.5</td>
</tr>
<tr>
<td>Sp. Rot° of unknown portion</td>
<td>+10.6°</td>
<td>+9.5°</td>
</tr>
</tbody>
</table>

Hypoiodite Oxidation.

Measurements of the uptake of hypoiodite solution by fucose and by the two hydrolysates in the previous section indicated, after allowing for fucose, the presence of from 20-30% of other sugars calculated as hexose. These figures seem rather high and it is possible that some other material present was interfering.

Free Fucose in Fucoidin.

No evidence was found of any free reducing sugar in fucoidin either by methylpentose estimation or by hypoiodite.
Uronic Acids in Fucoidin.

By the method of MacCready, Swenson and Maclay by distillation with 19% hydrochloric acid, 6.6% uronic acid was found in fucoidin II.

Attempted Preparation of Fucose Phenylhydrazone and Osazone of Unknown Portion.

Fucoidin II (2 g.) was hydrolysed for 4 hours with oxalic acid (2.5%; 50 ml.), neutralized with barium carbonate, filtered, washed and the solution evaporated to give a glass. This glass was dissolved in water (10 ml.) and to the solution added 10 ml. of a solution containing phenylhydrazine (25 ml.), ethanol (100 ml.) and glacial acetic acid (3 ml.). The mixed solution was kept at 30°C. for half an hour then left overnight at 0°C. No phenylhydrazine crystallised until after dilution with an equal volume of alcohol and evaporation in a desiccator. The dilution and evaporation were repeated till no more precipitate was obtained, yielding in all 0.3 g. of fucose phenylhydrazone M.Pt. 167-8°C. after recrystallisation from alcohol. The solution after removal of the phenylhydrazone was evaporated and the residue dissolved in water and boiled with phenylhydrazine hydrochloride (0.5 g.) and sodium acetate (0.5 g.) for some hours giving a small quantity of osazone insufficient for identification.
Extraction of Laminaria cloustoni with Cold Water.

*Laminaria cloustoni,* dried and ground (400 g.) was shaken with cold water (4 l.) for several days. After filtration the fucoidin was prepared in the usual manner from the extract. Less than 0.1 g. was obtained, the fucose content being 22.3%.

Extraction of Sporing Tips of Fucus serratus.

The cold extraction was carried out as above to determine whether the slimy exudate from the sporing tips of seaweed fronds contained a large quantity of fucoidin but only a very small yield was obtained containing fucose 4.92%, uronic acid 6.93% and ash 82.80% consisting mainly of nitrates.

pH Value of Fucoidin Solution and Electrometric Titration with Sodium Hydroxide.

A freshly prepared fucoidin solution (0.2%) was found to have a pH value of 6.1, i.e. slightly acid, which on standing for a week was reduced to 4.0.

Fucoidin II (0.3536 g.) was dissolved in water (150 ml.) and titrated electrometrically with sodium hydroxide (0.0105) using a platinum-quinhydrone-N-calomel cell, the e.m.f. being read directly in millivolts on a Cambridge Portable Potentiometer, with the following results.
The calculated amount of oxalic acid was then added to precipitate the Ca\(^{++}\) and the titration repeated to obtain the neutralisation titration curve of the free sugar sulphuric acid, thus:

<table>
<thead>
<tr>
<th>ml. NaOH added</th>
<th>Galvanometer Reading (mV)</th>
<th>Calc. pH. value</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>100</td>
<td>5.54</td>
</tr>
<tr>
<td>0.5</td>
<td>86</td>
<td>5.77</td>
</tr>
<tr>
<td>0.8</td>
<td>74</td>
<td>5.98</td>
</tr>
<tr>
<td>1.0</td>
<td>68</td>
<td>6.09</td>
</tr>
<tr>
<td>1.3</td>
<td>53</td>
<td>6.35</td>
</tr>
<tr>
<td>1.5</td>
<td>43</td>
<td>6.52</td>
</tr>
<tr>
<td>2.0</td>
<td>22</td>
<td>6.88</td>
</tr>
<tr>
<td>2.3</td>
<td>12</td>
<td>7.05</td>
</tr>
<tr>
<td>2.5</td>
<td>8</td>
<td>7.12</td>
</tr>
<tr>
<td>3.0</td>
<td>&lt; 0</td>
<td>&gt; 7.26</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ml. NaOH (0.1045)</th>
<th>Galv. Reading (mV)</th>
<th>pH value</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>195</td>
<td>3.72</td>
</tr>
<tr>
<td>1.0</td>
<td>194</td>
<td>3.74</td>
</tr>
<tr>
<td>3.0</td>
<td>184</td>
<td>3.91</td>
</tr>
<tr>
<td>5.0</td>
<td>163</td>
<td>4.45</td>
</tr>
<tr>
<td>6.0</td>
<td>146</td>
<td>4.63</td>
</tr>
<tr>
<td>7.0</td>
<td>133</td>
<td>4.96</td>
</tr>
<tr>
<td>9.0</td>
<td>85</td>
<td>5.79</td>
</tr>
<tr>
<td>9.5</td>
<td>24</td>
<td>6.84</td>
</tr>
<tr>
<td>9.6</td>
<td>5</td>
<td>7.16</td>
</tr>
<tr>
<td>9.7</td>
<td>&lt; 0</td>
<td>&gt; 7.26</td>
</tr>
</tbody>
</table>
These results indicate that the free acid is quite a strong acid.

**Periodic Acid Uptake (6)**

Fucoidin II (0.2722 g.) was dissolved in water (10 ml.) and treated with 0.5 M. periodic acid (10 ml.). Samples (2 ml.) were withdrawn at intervals up to 14 days, diluted with water (10 ml.) and to the sample added N/10 sodium arsenite (20 ml.) and potassium iodide (1 ml.; 20%). After 15 minutes standing, the excess arsenite was titrated with N/10 iodine.

<table>
<thead>
<tr>
<th>Time</th>
<th>Vol. of N/10 Iodine (0.0932)</th>
<th>Difference 0.1N Iodine</th>
<th>H1O4 uptake per 0.2722 g. F.</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 mins.</td>
<td>10.66</td>
<td></td>
<td>0.0930</td>
</tr>
<tr>
<td>2 hrs.</td>
<td>11.70</td>
<td>0.97</td>
<td>0.1260</td>
</tr>
<tr>
<td>4 hrs.</td>
<td>12.07</td>
<td>1.32</td>
<td>0.1360</td>
</tr>
<tr>
<td>1 day</td>
<td>12.30</td>
<td>1.53</td>
<td>0.1466</td>
</tr>
<tr>
<td>3 days</td>
<td>12.54</td>
<td>1.75</td>
<td>0.1681</td>
</tr>
<tr>
<td>7 days</td>
<td>12.81</td>
<td>2.00</td>
<td>0.1922</td>
</tr>
<tr>
<td>14 days</td>
<td>13.01</td>
<td>2.19</td>
<td>0.2100</td>
</tr>
</tbody>
</table>

The oxidation did not appear to be complete after 14 days and was discontinued for lack of solution.

**Fucoidin from Large Scale Extraction of Fucus vesiculosus.**

A large scale extraction was carried out on behalf of the Scottish Seaweed Research Association at
the Ministry of Supply Research Establishment, Sutton Oak, St. Helens, following, in general, the procedure outlined earlier for the extraction of fucoidin except that a preliminary extraction with alcohol and benzene was carried out. The precipitate obtained on making the solution, containing lead acetate and fucoidin, alkaline with barium hydroxide was forwarded to the laboratory. This precipitate was treated with sulphuric acid, dialysed, filtered, evaporated and the fucoidin precipitated in alcohol as before. The fucose content of the pale brown fucoidin obtained was 30.0% but after two treatments with "Filter Cel", this was increased to 38.2%. (Fucoidin IV).

Hydrolysis of Fucoidin IV by Stages.

Samples of fucoidin IV (50 mg.) were hydrolysed with oxalic acid (2.5%) for varying periods of time and in each case the fucose content of the solutions was determined in the usual way and also the total reducing sugars, calculated as fucose, by hypoioidite oxidation.

<table>
<thead>
<tr>
<th>Time of Hydrolysis</th>
<th>Fucose</th>
<th>Total Sugars as Fucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 mins.</td>
<td>10.7</td>
<td>18.2</td>
</tr>
<tr>
<td>35 mins.</td>
<td>16.8</td>
<td>28.6</td>
</tr>
<tr>
<td>50 mins.</td>
<td>20.4</td>
<td>37.3</td>
</tr>
<tr>
<td>1.5 hrs.</td>
<td>29.6</td>
<td>42.3</td>
</tr>
<tr>
<td>2.5 hrs.</td>
<td>34.3</td>
<td>46.4</td>
</tr>
<tr>
<td>4.5 hrs.</td>
<td>37.2</td>
<td>57.9</td>
</tr>
</tbody>
</table>
Determination of Formaldehyde(7) and Formic Acid(8) produced by Periodate Oxidation.

Formaldehyde. On oxidation with periodic acid pentoses and hexoses each yield 1 molecule of formaldehyde along with formic acid thus:

\[
\text{CHO.}(\text{CHOH})_4.\text{CH}_2\text{OH} \xrightarrow{\text{HIO}_4} 5.\text{H.COOH} + \text{HCHO}. \\
\text{CHO.}(\text{CHOH})_3.\text{CH}_2\text{OH} \rightarrow 4.\text{H.COOH} + \text{H.CHO}. 
\]

The formaldehyde formed may be estimated quantitatively by precipitation with dimedone (Reeves(7) and Vorlänger(9)). Preliminary experiments were carried out with (1) 20 mg. glucose (2) 20 mg. glucose + 10 mg. rhamnose. The samples were dissolved in water (5 ml.) and treated as for the fucose estimation with the omission of the addition of liquid paraffin. After aeration to remove acetaldehyde formed in the second case, the solutions were just neutralised to methyl red with acetic acid, dimedone reagent (2 ml.; 80 mg/ml.) added and allowed to stand overnight. The precipitate is collected in a sintered crucible (0.3) washed with water, dried at 85-90°C. and weighed.

1 g. dimedone complex is equivalent to 0.10273 g. of formaldehyde.

Sample (1) gave 1.09 mols. of formaldehyde/mol. of glucose

" (2) " 0.96 " " "

Hence the presence of methylpentose does not
materially affect the yield of formaldehyde.

**Formic Acid.** Pentoses and hexoses yield formic acid as above with periodic acid and methylpentoses as below yield 4 molecules of formic acid

\[ \text{CHO} \cdot (\text{CHOH})_3\text{CHOH} \cdot \text{CH}_3 \rightarrow 4\text{H}_2\text{COOH} + \text{CH}_3 \cdot \text{CHO}. \]

Samples as (1) and (2) above in water (5 ml.) were treated overnight with periodic acid (2 ml.; 0.5 M), and a blank with the same periodic acid was also run. The excess periodate was converted to iodic acid with ethylene glycol and the increased acidity due to formic acid determined by titrating all three solutions with NaOH (N/10) after standing for 1 hour (methyl red).

(1) Glucose gave acidity equivalent to 4.90 mols. formic acid/mol. of glucose.

(2) Rhamnose gave acidity equivalent to 3.99 mols. formic acid/mol. of rhamnose after allowing for glucose present.

The above methods were applied to fucoidin IV (20 mg.), containing fucose 37.20%, after hydrolysis with oxalic acid. The solution after the determination of fucose was filtered to remove calcium oxalate, acidified and dimedone added as in the control experiment. The yield of formaldehyde was equivalent to 17.66% hexose (two determinations). The acidity as formic acid was determined by hydrolysis with oxalic acid, exact neutralisation with N/10 NaOH followed by periodate
oxidation overnight and titration as before. The yield of formic acid after allowing for 37.2% fucose was equivalent to 7.09% hexose.

As the two figures for hexose were at variance with each other it was decided to investigate the efficiency of the hydrolysis with oxalic acid by determining the sulphate set free on hydrolysis. A sample of fucoidin was hydrolysed with oxalic acid (2.5%) for 3/2 hours and to the solution barium chloride was added. The precipitate was filtered off, washed with hot dilute HCl to remove barium and calcium oxalates and weighed as barium sulphate. The solution was boiled for 3 hours after the addition of conc. HCl and the further precipitate of barium sulphate weighed. A total sulphate in fucoidin was also done by direct HCl hydrolysis.

Sulphate from oxalic acid hydrolysis 24.68%
" " filtrate from " 8.28%

32.96%

Total sulphate 33.55%

showing that incomplete hydrolysis occurs using oxalic acid.

Hydrolysis with Sulphuric Acid (2.5%).

The above experiments with periodic acid were carried out using (1) 2.5% oxalic acid and (2) 2.5%
sulphuric acid. In (2) the brown residue observed in earlier hydrolyses was absent and was presumed to be due to the impurities present.

Results.

\[
\begin{align*}
\text{Fucose} & \quad 37.3 & \quad 41.4 \\
\text{Hexose from formaldehyde} & \quad 17.9 & \quad 19.1 \\
\text{Hexose from formic acid} & \quad 12.7 & \quad 14.8
\end{align*}
\]

As a result of this work, later hydrolyses were carried out using 2.5% sulphuric acid.

Hydrolysis and Preparation of "Fucose-free" Material.

Fucoidin IV (9.68 g.) was hydrolysed with sulphuric acid (2.5%, 300 ml.) for 6 hours, the solution neutralised with barium carbonate, filtered and evaporated (40°C.; 15 mm.) to a syrup which was dried by re-evaporation with alcohol and benzene. Yield 7.3 g. The syrup gave the following analytical figures; \([\alpha]_D^{15} -28.1^\circ\); fucose 44.3%, hexose by (1) periodate and formaldehyde, 27.9% (2) periodate and formic acid, 27.8%.

The syrup was dissolved in water (40 ml.), diluted with alcohol (40 ml.) and to the solution added glacial acetic acid (0.5 ml.) and redistilled methylphenylhydrazine (10 ml.). After standing at 0°C. for 72 hours the methylphenylhydrazone was filtered off.
(G 1 crucible) washed with alcohol and ether and dried. Yield 4.94 g., M.Pt. 171°C. i.e., 94% recovery from the fucose present in syrup. The residual solution was freed from alcohol and water by evaporation to dryness at room temperature (15 mm.). On dissolution in alcohol a residue (0.2 g.) was obtained containing 25% ash. Both solution and residue were treated with alcohol (90 ml.), water (60 ml.) and benzaldehyde (10 ml.) and the solutions refluxed on a water bath for 4 hours, left overnight at 0°C. After filtration to remove benzalmethylphenylhydrazone the excess benzaldehyde was extracted with chloroform. The solutions were clarified with charcoal at 80°, extracted with ether to remove any benzoic acid and evaporated (40°C., 15 mm.) to give glassy products.

The small amount derived from the material insoluble in alcohol gave a naphthoresorcin test for uronic acids and a rough estimation by distillation with 19·0% HCl according to Tollens indicated the presence of about 30% uronic acid.

The main portion of glass had the following analytical figures; \([\alpha]_D^{150} +5.0\), fucose 5.38%, hexose from formic acid 51·90% and gave negative tests for pentose (Bial's Orcinol test) fructose (Bréderéck's and Seliwanoff's tests) and was reducing to Fehling's solution.
The treatment with methylphenylhydrazone, benzaldehyde, etc. was repeated in an attempt to reduce the residual fucose but after this second treatment, the fucose content of the syrup was still 4.5%. This syrup was used in the following experiments.

Attempted Preparation of a Lactone.

The syrup (0.4663 g.) was dissolved in water (10 ml.) and bromine (0.5 ml.) added to the solution which was left for 48 hours at room temperature. At the end of this time, the solution was still reducing so a further 0.5 ml. of bromine was added and the mixture left for 48 hours. The bromine was then blown off by aeration and the solution neutralized with silver carbonate, warmed to remove carbon dioxide, filtered and treated with hydrogen sulphide. After removal of silver sulphide, the solution was evaporated (40°C., 15 mm.) and the residue dried at 100°C. On titration with caustic soda only a negligible amount of acid was found to be present and the rotation of a solution was practically zero. It is doubtful if any sugar acid had been formed.

Attempted Preparation of an Osazone.

The syrup (0.16 g.) was treated with phenylhydrazine hydrochloride (0.4 g.) and sodium acetate (0.6 g.) in water (4 ml.) and the solution heated in boiling water
for 1/4 hour, cooled and left overnight. An osazone was obtained and filtered off, a second crop coming down on re-heating and cooling. These had melting points of 178°C and 186°C respectively and gave with galactosazone mixed M.Pts. of 174°C and 179-180°C. As the melting points were a little indefinite, the results are not conclusive. Microanalysis (Weiler and Strauss) gave the following results, the figures for pentosazone and hexosazone being given for comparison.

<table>
<thead>
<tr>
<th></th>
<th>Carbon</th>
<th>Hydrogen</th>
<th>Nitrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prepared osazone</td>
<td>60.29</td>
<td>6.56</td>
<td>15.60</td>
</tr>
<tr>
<td>Pentosazone</td>
<td>62.28</td>
<td>6.09</td>
<td>17.08</td>
</tr>
<tr>
<td>Hexosazone</td>
<td>60.34</td>
<td>6.21</td>
<td>15.65</td>
</tr>
</tbody>
</table>

These results seem to indicate that the unknown is a hexosazone.

Preparation of an Osotriazole: (10,11)

Phenylosazones when treated with copper sulphate solution as described below yield phenylosotriazoles thus:

\[
\begin{align*}
\text{HC} & = \text{NNHC}_6\text{H}_5 \\
\text{CuSO}_4 & \quad \rightarrow \\
\text{HC} & = \text{N} \\
\text{HC} & = \text{N} \\
\text{C}_6\text{H}_5 & \quad \rightarrow \\
\text{HC} & = \text{N}
\end{align*}
\]

These substances are crystalline with well defined melting points.
Control Experiment. Glucosazone (0.2 g.) was suspended in water (12 ml.) containing copper sulphate crystals (0.154 g.) in solution and the liquid refluxed for 1 hour with occasional shaking. The solution was then filtered hot and evaporated to 2 ml. in a stream of air and left overnight at 0°C. The precipitate was filtered off, dissolved in water (5 ml.), boiled with charcoal, filtered again hot and on cooling, needles of the osotriazole were deposited, M.Pt. 192-3°C. Yield 0.033 g.

The method was applied to the osazone obtained from the "fucose-free" syrup using the above quantities, giving an osotriazole (0.0138 g.), M.Pt. 196-7°C. and a mixed M.Pt. with phenyl-D-glucosotriazole of 193-4°C. Rotation in pyridine, -80°. cf. phenyl-D-glucosotriazole (8) M.Pt. 195-6°C, [α]D15°-81.6 (pyridine). These results indicate the presence of glucosazone and hence of glucose or mannose in the original syrup. The possibility of mannose being present was eliminated as it was found impossible to obtain mannose phenylhydrazone from the syrup under conditions in which a control mannose solution yielded a phenylhydrazone immediately.

Estimation of Glucose.

The amount of glucose present was determined by means of the Somogyi micro-copper reagent. The syrup (1.072 g., moisture 4.10%) was dissolved in 250 ml.
water and the solution diluted 5 times (10 ml. → 50 ml.),
at the same time a control solution of glucose was made
up containing 40 mg./100 ml. Measurements of reducing
power were carried out on the solutions as follows.
(1) 5 ml. distilled water, (2) 5 ml. glucose solution,
(3) 5 ml. diluted sample solution, (4) 5 ml. glucose
solution after incubation with yeast at 38°C. for ten
minutes, (5) 5 ml. sample incubated with yeast. The
incubation with yeast removes the glucose by adsorption
according to Harding and Selby.(23) This procedure
therefore, not only gives a measure of the glucose
present but indicates whether other reducing sugars are
present. In the present case the glucose content was
found to be 15.9% of the syrup and a similar amount of
another reducing sugar, not determinable accurately
owing to the unknown copper reducing power of the other
sugar.

Ash and Methoxyl Content of Syrup.

The ash content of the syrup was found to be 15.7%
and was shown to be mainly calcium sulphate. No
detectable methylated sugars were present.

Rate of Moisture Uptake of Fucoidin II.

A sample of fucoidin (0.0877 g.) was dried overnight
in vacuo (40°C.; 0.1 mm.) and the containing tube
suspended in a bottle in an atmosphere of 60% relative
humidity (saturated calcium nitrate at 20°C.). The tube was weighed at intervals with these results.

<table>
<thead>
<tr>
<th>Time</th>
<th>Weight of sample</th>
<th>Increase in Weight (% of dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0877</td>
<td>-</td>
</tr>
<tr>
<td>1 day</td>
<td>0.1026</td>
<td>16.99</td>
</tr>
<tr>
<td>2 days</td>
<td>0.1088</td>
<td>24.03</td>
</tr>
<tr>
<td>4 days</td>
<td>0.1104</td>
<td>25.86</td>
</tr>
<tr>
<td>6 days</td>
<td>0.1108</td>
<td>26.33</td>
</tr>
</tbody>
</table>

showing that the intensive drying normally carried out before analysis is absolutely necessary.

**Pentose Estimation on Fucoidin IV.**

Following the method of Marshall and Norris\(^\text{(13)}\) fucoidin IV (fucose 41.2%) was distilled with 13.16% hydrochloric acid and the furfural and methy1furfural obtained was precipitated and weighed as a thiobarbiturate. After allowance for the fucose present (from a factor determined in a separate experiment) the residual precipitate was found to represent 3.4% pentose.

**Attempt to Prepare Pure Fucoidin.**

As it was clear from the foregoing experimental work that the purification of fucoidin is a matter of extreme difficulty and considerable importance, it was decided to investigate the extraction and purification of fucoidin from freshly gathered seaweed. The species used was *Himanthalea lorea* which is readily
collected at low tide in a fairly clean condition. The samples used in the following experiments were collected at North Berwick.

**Preliminary Extraction.** 2 Kg. of fresh seaweed (May 24th, 1948) were extracted at 70°-75° with water (5-7 l.) for 17 hours. The solution, filtered through muslin was then treated as in the earlier extractions with lead acetate, barium hydroxide, sulphuric acid, etc. The fucoidin (1 g.) thus obtained was dissolved in water (200 ml.) and treated with "Filter Cel" (2 g.) and Merck's charcoal (1 g.) overnight, followed by heating to 90°C. for half an hour, a little calcium carbonate being added to correct any possible acidity during heating. This process was repeated on the filtered solution, followed by evaporation (40°C.; 15 mm.) and precipitation in alcohol. The fucose content after hydrolysis with sulphuric acid (2.5%; 3 hours) was 39.2% and \([\alpha]^{15}\_D\) -140°, and the product was pure white in colour. Paper chromatographic analysis showed only traces of sugars other than fucose.

The uronic acid content determined by distillation with hydrochloric acid was 6.2% and a determination of formic acid produced on periodate oxidation as described previously indicated an excess over that due to fucose, representing 3.4% sugar calculated as hexose.

In view of the apparent purity of this fucoidin a
large scale extraction of *Himanthalea lorea* was carried out.

**Large Scale Extraction.** Freshly gathered weed (1 cwt., June 24th, 1948) was minced and extracted in an indirectly heated metal boiler with 20 gallons of water for 24 hours at 70°C. At the end of this time the residues were removed in a hydraulic press and warm lead acetate solution added to the solution until complete precipitation resulted, when after settling overnight, the precipitate was removed in a filter press. The solution (25 gallons) was made alkaline with saturated baryta water until no more precipitate formed, the liquid being then removed first by decantation and finally filtration. After treatment of the solid by shaking overnight with dilute sulphuric acid (in excess) the lead and barium sulphates were removed at the centrifuge and the solution dialysed until free from acid (6 days). Finally the solution was evaporated (40°C.; 15 mm.) to 75 mls. and the polysaccharide precipitated in alcohol (6 gallons). Yield, 170 g. of a pale brown solid.

After purification three times with "Filter Cel" and charcoal as in the small scale, the product (fucoidin V) had the following analysis. Fucose, 42·1%, total sulphate, 31·8%, ash, 15·74%, sulphated ash, 23·00%, sulphate in ash, 16·5%, metals, 6·5%,
uronic acid, by 19% HCl distillation 2.83%, hexose by
formic acid, 4.00% (probably low in view of later
results), hexose by formaldehyde and dimedon, 3.83%.

Residue Obtained on Hydrolysis.

A small white residue appeared during hydrolysis
with sulphuric acid (2.5%). This was estimated on a
sample of fucoidin (0.3092 g.) which yielded 0.0100 g.
of residue, i.e. 3.23% containing ash 2.94%, sulphated
ash 3.16%. The residue was probably barium sulphate
since fucoidin solution gave a white precipitate with
cold H₂SO₄ but none with BaCl₂ and is probably due to
a slight exchange of barium for calcium in the
preparation of the polysaccharide.

Paper Chromatogram.

The hydrolysed solution on examination on a paper
chromatogram indicated, apart from fucose, a small spot
corresponding to galactose and a trace of uronic acid
which does not move from the starting line.

Further Purification of Fucoidin V.

Five further treatments with "Filter Cel" and
charcoal at 90°C. for half an hour resulted in a pure
white sample of fucoidin V containing:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Fucose</td>
<td>43.9% (Hydrolysed 3 hrs.; N H₂SO₄)</td>
</tr>
<tr>
<td></td>
<td>44.5% ( &quot; 8 &quot; &quot; )</td>
</tr>
<tr>
<td>Sulphate</td>
<td>32.4%</td>
</tr>
</tbody>
</table>
Ash 22.6%

Metals 6.9%

Hexose by formaldehyde 5.90%

Since N.H₂SO₄ was not found to have any deleterious effect in a control fucose determination it was used in future hydrolyses.

Quantitative Paper Chromatogram on Fucoidin V.

Following the method of Flood, Hirst and Jones (14) the reducing sugars present were estimated using rhamnose as the control sugar since the normal control used, namely, ribose, coincides with fucose on the chromatogram. Fucoidin (17.5 mg.) and rhamnose (9.8 mg.) were hydrolysed in a sealed tube with 0.5 N H₂SO₄ (0.5 ml.) and the solution neutralized and chromatographed. By extraction of the paper strips and comparison of reducing powers using Somogyi reagent, figures were obtained thus; fucose 49.6, 48.4%, galactose, 7.2, 6.8%.

Quantitative Hydrolysis.

Fucoidin (1.2466 g.) was hydrolysed with sulphuric acid (100 ml.; N), neutralized with barium carbonate, warmed to remove carbon dioxide, filtered and the residue washed with hot water. The filtrate and washings were evaporated (40°C.; 15 mm.), dried with alcohol and benzene to yield a glass (0.7551 g. or
60.7% having $[\alpha]^D_{150} = -54.8^\circ$ (c, 1.26 in water) fucose 75.0%, sulphate 7.80% as calcium sulphate and sulphated ash, 7.20%.

**Quantitative Chromatogram on Hydrolysed Glass.**

A mixture of the glass (8.2 mg.) and rhamnose (5.8 mg.) were quantitatively chromatographed as in the case of the polysaccharide, omitting the hydrolysis, giving figures for fucose of 79.1% and for galactose 5.2%. Calculated on the basis of 60.7% glass from the polysaccharide these results represent 48.01 and 3.15% respectively on the polysaccharide.

When a qualitative chromatogram was developed with aniline oxalate (sat.) instead of the usual ammoniacal silver nitrate, the galactose was definitely shown to be hexose (brown spot) and a second smaller spot was found to be a pentose (pink spot), the distance travelled by this latter sugar indicating xylose. (By comparison with control samples of galactose and xylose applied to the same paper).

A second quantitative chromatogram was carried out using a larger amount of material (21.5 mg.) in an attempt to estimate these two sugars more accurately, in this case the fucose being ignored. Found, galactose 5.8% on glass or 3.5% on polysaccharide, xylose 3.5% on glass or 1.3% on polysaccharide.
Calculation of Percentage of Unidentified Portion.

Using the analytical figures as obtained from quantitative chromatogram analysis, sulphate, metals and uronic acid a rough estimation of the missing percentage was obtained by calculating all the sugars as anhydrides and assuming one sulphate group for each fucose molecule. This last assumption is reasonably borne out by the analytical figures, and the fucose percentage was, therefore, reduced by the factor 129/164, i.e. fucose less one H_2O and one -OH group. The results are as follows:-

<table>
<thead>
<tr>
<th>Percentage from analysis</th>
<th>Percentages in polysaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fucose</td>
<td>48.0</td>
</tr>
<tr>
<td>Anhydrofucose</td>
<td>37.8</td>
</tr>
<tr>
<td>Galactose</td>
<td>3.5</td>
</tr>
<tr>
<td>Anhydrogalactose</td>
<td>3.0</td>
</tr>
<tr>
<td>Xylose</td>
<td>1.3</td>
</tr>
<tr>
<td>Anhydroxylose</td>
<td>1.2</td>
</tr>
<tr>
<td>Uronic acid</td>
<td>2.8</td>
</tr>
<tr>
<td>Uronic anhydride</td>
<td>2.5</td>
</tr>
<tr>
<td>Sulphate</td>
<td>32.4</td>
</tr>
<tr>
<td>Sulphate</td>
<td>32.4</td>
</tr>
<tr>
<td>Metals</td>
<td>6.9</td>
</tr>
<tr>
<td>Metals</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>83.8</td>
</tr>
</tbody>
</table>

The above figures show that about 15% of the polysaccharide remains unaccounted for. Since there was no evidence of sugars other than those identified being present, it was decided to investigate the possible presence of water or alcohol or both in the
samples after drying overnight at $40^\circ/0.1$ mm. since this procedure was followed before all analyses were carried out.

**Determination of Alcohol.**

A neat method for the determination of free alcohol in body fluids was published a few years ago by Newman (15). The method is based on the removal, by evacuation, of alcohol from its solution, the air and vapour as it is removed being passed through a solution of potassium dichromate in strong sulphuric acid which absorbs the alcohol. By titration of the solution of dichromate with thiosulphate after dilution and the addition of potassium iodide, a measure of the alcohol present can be obtained. Since no reagents other than sodium sulphate are added to the original solution, the only substances which might interfere in the determination are volatile organic materials, none of which are likely to be present in fucoidin after drying, other than alcohol. 1 c.c. N/40 Thiosulphate is equivalent to 0.2875 mg. alcohol.

The method was applied to samples containing 20-250 mg. alcohol/ml. of solution, as follows. Alcohol solution (1 ml.) was placed in a 100 ml. round bottomed Quickfit flask (A) connected by a
right-angled adaptor with vertical stop-cock (B) to a
delivery tube passing via an adaptor also with side arm, to the bottom of a quickfit 8" x 1" test-tube.

To the sample is added anhydrous sodium sulphate
(3 g., dried before use) to form a layer on the bottom
of the flask which is immediately connected to the apparatus. The receiver contains 10 ml. dichromate solution (5 ml. N/10 K$_2$CrO$_4$: 5 ml. conc. H$_2$SO$_4$).

Suction by water pump is applied to the receiver side arm (D) after closing the stop-cock (B) and the flask kept in a water bath at 50°C. for 15 minutes, when the pump is shut off by the stop-cock (D) and the stop-cock (B) opened to allow air to enter. The dichromate solution is then washed out of the receiver and diluted to 100 ml., potassium iodide (10 ml.; 20%) is added
and the iodine corresponding to the residual dichromate titrated with N/40 thiosulphate. A blank is also carried out. Using known alcohol solutions, results within 1% of the calculated amount were obtained.

The method was applied to a solution of fucoidin V and gave a result of 6.0% (average of three determinations, 5.9, 6.0, 6.1%).

**Determination of Water as Moisture in Fucoidin.**

The method adopted for this estimation was based on the method for determination of active hydrogen as described by Pregl-Grant (Zerewitinoff reaction) between substances containing active hydrogen and alkyl magnesium halides thus:

\[ R.H + CH_3MgI \rightarrow R MgI + CH_4 \]

By measurement of the methane produced, the percentage of active hydrogen can be determined. Flaschenträger adapted the method to the micro-scale. In pyridine, as solvent, at room temperature it has been found that in water there are two active hydrogen atoms and it was hoped that any water present would dissolve out into the pyridine although the polysaccharide itself is insoluble. The apparatus was built and the reagents magnesium, methyl iodide and iso-amyl ether purified and from them the Grignard reagent was prepared and purified according to Pregl-Grant. The experimental procedure described by the above author was followed in
The pyridine used was shaken with barium oxide overnight and then distilled over potassium hydroxide, but on carrying out a blank determination with this pyridine, it was found to yield methane equivalent to about 0.08% water. A negligible blank was only obtained after distilling the pyridine five times over phosphorus pentoxide. The samples were weighed into the apparatus in a small cup fitted with a long handle, and the nitrogen used to clear the apparatus was passed through wash bottles of alkaline pyrogallol and conc. sulphuric acid. All volumes of methane obtained were corrected to N.T.P. before calculation. Samples were then estimated as follows.

1. Glucose (2.88 mg.) gave 1.63 c.c. methane at N.T.P. representing 2.58% active hydrogen (Cal'd 2.78%).

   Solution of the glucose in pyridine was effected by warming to 50°C. and cooling before adding the Grignard reagent.

2. Starch (moisture 12.1%) was treated with pyridine at 50°C. for 2 minutes before reaction as for glucose but in this case the starch did not dissolve but on reaction yielded methane equivalent to 1.41% active hydrogen or 12.7% water. This favourable comparison with a known moisture content in a substance insoluble in pyridine indicated the feasibility of the application
of the method to fucoidin.

3. Fucoidin was treated with pyridine in three ways with these results.

(a) Heated at 50°C. for three minutes gave active hydrogen 1.18%.

(b) Heated at boiling point for three minutes gave active hydrogen 1.10%.

(c) Kept for 1½ hours at room temperature gave active hydrogen 0.98%.

The most satisfactory result was from (a) and was readily duplicated. As fucoidin contains 6.0% alcohol, allowance must be made for its active hydrogen of 6.0 / 46 or 0.13%, leaving 1.18 - 0.13 = 1.05% active hydrogen from fucoidin representing 9.45% water.

A determination carried out after leaving fucoidin over calcium nitrate solution (sat.) for 14 hours gave a figure of 22.0% water.

In view of the result obtained using starch it seems improbable that any other reaction takes place with an insoluble polysaccharide.

Conclusion.

These results for the determination of alcohol (6.0%) and water (9.4%) along with the previously calculated total of 83.8% give a final total analytical figure of 99.2%.
PART III. Determination of Combined Fucose in Seaweeds.

An attempt to determine the fucose content of hydrolysed seaweed samples was carried out as follows, using the method for methylpentoses as described on page 101 et seq.

A sample of dried, ground seaweed (0.5 g.) of known moisture content was weighed into a dry 8" x 1" quickfit test-tube and hydrolysis effected by heating in boiling water for 3 hours with sulphuric acid (5 ml.; N/2). At the end of this time the acid was neutralised with sodium bicarbonate and then excess sodium bicarbonate (0.6 g.), alanine (0.2 g.) and sodium arsenite solution (14 ml.; N/10) added and the tube fitted to the apparatus as described previously. (p.101). Periodic acid (3.5 ml., N/2) was added slowly, aeration with nitrogen carried out for two and a half hours and the acetaldehyde liberated determined in the usual way. From this result the percentage of fucose was obtained. Various samples gave the following figures;

<table>
<thead>
<tr>
<th>Species</th>
<th>Percentage of Fucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fucus vesiculosus/4/45</td>
<td>3.65</td>
</tr>
<tr>
<td>&quot; spiralis/4/45</td>
<td>3.65</td>
</tr>
<tr>
<td>&quot; serratus/4/45</td>
<td>2.15</td>
</tr>
<tr>
<td>Pelvetia caniculata/4/45</td>
<td>3.78</td>
</tr>
<tr>
<td>Laminaria digitata/4/45 Stipe</td>
<td>1.02</td>
</tr>
<tr>
<td>&quot; &quot; /4/45 Frond</td>
<td>1.14</td>
</tr>
</tbody>
</table>
It was then discovered that after the aeration was complete, in the absence of sodium arsenite solution, no excess periodic acid could be detected and it was therefore decided to carry out a further series of estimations using a smaller amount of sample and the same amount of periodic acid. It was found that using 0.1 g. samples, 3.5 ml. of periodic acid (M/2) was sufficient. Using the same conditions as before, the following results were obtained:

<table>
<thead>
<tr>
<th>Species</th>
<th>Percentage of Fucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fucus vesiculosus/12/46</td>
<td>11.21</td>
</tr>
<tr>
<td>&quot; spiralis/1/47</td>
<td>11.40</td>
</tr>
<tr>
<td>Pelvetia caniculata/3/47</td>
<td>12.20</td>
</tr>
<tr>
<td>Laminaria digitata/1/48</td>
<td>4.16</td>
</tr>
<tr>
<td>&quot; &quot; 11/44/0/Stripe</td>
<td>2.67</td>
</tr>
</tbody>
</table>

Although not strictly comparable with the previous results, the above figures were clearly much higher and it was also found that if the sample of *Fucus vesiculosus*, used above, was allowed to stand for 5 minutes with water instead of hydrolysing then an estimation with periodic acid gave a titration figure with iodine (N/100) equivalent to 1.90% of fucose. This was shown to be a false figure by paper chromatographic analysis of the water extract. No free reducing sugar could be detected.

Since there appeared to be a possibility that
Acetaldehyde was being produced from some source other than fucose or (2) another volatile aldehyde or ketone which reacted with bisulphite was present, a method of estimating acetaldehyde, which was specific, was sought.

**Estimation of Acetaldehyde.**

Fromageot and Heitz\(^{(19)}\) have published a colorimetric method for the estimation of amino-acids yielding acetaldehyde by permanganate oxidation, after conversion to the hydroxy acids, in the presence of other amino acids. They claim that the method is specific for acetaldehyde. From the point of view of the present work it was convenient since the estimation is carried out on the solution of acetaldehyde obtained as in the described estimation of fucose, i.e. after liberation of the aldehyde by bicarbonate and titration with iodine (\(\text{n/100}\)). The basis of the method is the formation of a blue coloration with sodium nitroprusside in the presence of piperazine.

**Application to Acetaldehyde Solutions.**

A bulk solution of acetaldehyde was made up containing about 0.15 ml. of aldehyde in 250 ml. of solution and an accurate sample (5 ml.) was added to excess bisulphite solution (5 ml.) in a graduated flask (100 ml.). The acetaldehyde was estimated as in the fucose estimation by destruction of excess bisulphite,
addition of saturated sodium bicarbonate and titration with N/100 iodine. The blue starch colour was discharged by the addition of one drop of sodium thiosulphate solution (N/10). A blank solution was prepared in the same way with no acetaldehyde.

The blue coloration was developed thus; acetaldehyde solution (from 2 to 6 ml.) was run from a burette into a ground glass stoppered test-tube and the blank solution added in appropriate amounts to give in each case a total volume of 6 ml. A blank solution (6 ml.) with no acetaldehyde is also measured out. To each sample and to the blank, sodium nitroprusside solution (0.5 ml.; 4% freshly prepared) and piperazine solution (1.5 ml.; 33%) were added, the tubes stoppered and shaken for a minute. The solutions were then transferred to 1 cm. Spekker Photoelectric Absorptiometer cells and readings of log I/Io taken against the blank solution, using a yellow filter, until a maximum reading was obtained (about 5 minutes). The results obtained using commercial acetaldehyde were not satisfactory and it was found that aeration of the acetaldehyde solution was necessary before estimation. This was carried out as in the fucose estimation except that the Breschel receiving bottles were replaced by 8" x 1" test-tubes and the volume of bisulphite solution in each reduced to 25 ml. in order to complete the
estimation in a 100 ml. flask. Using aeration and transferring the bisulphite solution to a 100 ml. flask before titration the following results were obtained; Acetaldehyde estimated by iodine titration, 0.025 mg/ml. of solution.

<table>
<thead>
<tr>
<th>Vol. of sol</th>
<th>Weight of acetaldehyde in 6 ml. (mg.)</th>
<th>Spekker readings (duplicated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.050</td>
<td>0.175, 0.177</td>
</tr>
<tr>
<td>3</td>
<td>0.075</td>
<td>0.261, 0.258</td>
</tr>
<tr>
<td>4</td>
<td>0.100</td>
<td>0.342, 0.343</td>
</tr>
<tr>
<td>5</td>
<td>0.125</td>
<td>0.429, 0.431</td>
</tr>
<tr>
<td>6</td>
<td>0.150</td>
<td>0.536, 0.531</td>
</tr>
</tbody>
</table>

A duplicate experiment on a new acetaldehyde solution gave:

Acetaldehyde by titration 0.032 mg/ml.

<table>
<thead>
<tr>
<th>Vol. of sol</th>
<th>Weight of acetaldehyde in 6 ml. (mg.)</th>
<th>Spekker readings</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.160</td>
<td>0.575</td>
</tr>
<tr>
<td>4</td>
<td>0.128</td>
<td>0.452</td>
</tr>
<tr>
<td>3</td>
<td>0.096</td>
<td>0.332</td>
</tr>
</tbody>
</table>

These results are in good agreement with the previous ones and all the figures when graphed give a moderately straight line (opposite).

Application to Rhamnose.

Rhamnose solution (5 ml., containing 15 mg.) was treated as in fucose estimation in the modified apparatus and estimated by titration and by the
colorimetric method with these results:

- Rhamnose by titration: 93.0%
- Rhamnose by colorimetric method (from graph): 92.4%

**Effect of Paraldehyde.**

Paraldehyde solutions were shown to give a coloration with the reagents but did not react with sodium bisulphite solution and this is thought to account for the results obtained before aeration was introduced.

**Application to Seaweed Samples.**

All estimations of fucose on seaweed samples by the liberation of acetaldehyde were carried out, up to the final titration, by the original method as modified above, using the higher ratio of periodic acid to samples (p.138). The weight of sample used was, however, increased to 0.2 g. and the periodic acid to 7.0 ml. for convenience. The solutions were made up to 100 ml. in every case and volumes used for estimation as indicated. The titration results were noted as a matter of interest and were found to be in agreement with those previously obtained (p.138).
Results:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Vol. of sol(^n) (allowing for moisture)</th>
<th>Spekker readings with blank</th>
<th>Acetaldehyde mg./6 ml. from graph</th>
<th>Percentage of fucose</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fucus vesiculosus</em></td>
<td>3 ml.</td>
<td>0.528, 0.530</td>
<td>0.149</td>
<td>8.1</td>
</tr>
<tr>
<td>12/46 (0.218 g.)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td><em>Pelvetia caniculata</em></td>
<td>3 ml.</td>
<td>0.510, 0.514</td>
<td>0.144</td>
<td>8.4</td>
</tr>
<tr>
<td>3/47 (0.203 g.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>Laminaria digitata</em></td>
<td>3 ml.</td>
<td>0.525, 0.528</td>
<td>0.150</td>
<td>8.5</td>
</tr>
<tr>
<td>6/48/L (0.253 g.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Estimation of Fucose. The method of Nicolet and Shinn for methylpentoses has been found to be satisfactory for the estimation of methylpentoses but when applied to various species of brown seaweeds (Phaeophyceae) somewhat high results were obtained, as shown by the determination, using the method of Fromageot and Heitz, of the acetaldehyde produced. The lower results by the second method show presumably, that the seaweeds contain other substances giving rise to volatile compounds which react with sodium bisulphite but the investigation has not proceeded sufficiently far to indicate what these substances might be.

The results indicate that fucose is most abundant in Fucus spp. and least abundant in the Laminariales, less fucose being found in the stipe than in the frond of the latter species.

For purposes of determination of seasonal variation in fucoidin content of seaweed, a factor for conversion from fucose to fucoidin will be necessary. Referring to the fucoidin obtained from Himanthalea lorea the percentage of fucose found was 48.0 in the sample purified as far as possible. If allowance is made for the water and alcohol (total 15.4%), the fucose content is increased to 56.7% and the factor would be 100/56.7
= 1.76. If on the other hand, the galactose, xylose and uronic acid found are assumed to be impurities, the factor would be slightly lower, in the region of 1.6.

**Fucoidin.** Fucoidin has been prepared from samples of *Fucus vesiculosus*, *Fucus spiralis*, *Laminaria cloustoni*, and *Himanthalea lorea*. Analytical figures show that the general composition of each sample was the same, allowing for the fact that the fucoidin from the last named seaweed was subjected to more intensive purification, the latter being a matter of considerable difficulty. The presence of an ethereal sulphate is confirmed by the 2:1 ratio of sulphate in polysaccharide to sulphate in ash and in every case, metallic analysis shows that the main metal present is calcium, amounting to about 86% of the total metals, as compared with Lunin (20) who found sodium to be in the highest proportion in his samples.

Alginic acid is known to occur in the form of soluble salts in seaweed and it seems probable that the small percentage of uronic acid found in every case may be due to the incomplete removal of extracted alginic acid though this would be difficult to prove. Glucose was shown to be present in the earlier samples of fucoidin, but here again, this may be due to laminarin, a polyglucosan, as an impurity. The percentage of
glucose found in the "fucose free" glass in fucoidin IV was 15·9 representing 4-5% of the polysaccharide. Galactose and xylose occur in fucoidin V but here again it would be a matter of difficulty to show whether these are impurities or not.

An alcohol content of 6·0% and a moisture content of 9·4% have been found in fucoidin V dried for 16 hours (40°C.; 0·1 mm.). The presence of alcohol in a polysaccharide prepared by precipitation in alcohol even after intensive drying is not an unknown phenomenon, since Berner(21) found similar results with inulin. A parallel example, in the case of strongly retained moisture, is furnished by chondroitin sulphuric acid (22) which requires three days or more at 60°C. in vacuo to remove the last traces of water.

The polysaccharide has a specific rotation, \([\alpha]^{15\circ}_D-140^\circ\) and its solution is slightly acid. The curve of neutralisation of the free acid indicates the presence of a strong acid, which would be expected of a substituted sulphuric acid. The presence of moisture in the dried sample is not so surprising when the polysaccharide has been shown to absorb about 20% moisture in one day in an atmosphere of 60% relative humidity.

The analysis of fucoidin V after allowance for water and alcohol gives the following figures;
Fucose 56.7%, galactose, 4.1%, xylose 1.5%, uronic acid 3.3%, sulphate 38.3% and metals 8.2%. The calculated composition of the calcium salt of a fucose mono-sulphuric ester is fucose 62.3%, sulphate (SO₄) 36.5% and calcium 7.6% indicating that approximately one sulphate group is present in fucoidin for each fucose unit.

Assuming (1) one sulphate group per sugar residue (2) calcium salt, (3) galactose and xylose also contain a sulphate group, the following calculation may be made. The sugars are reduced to anhydro sugars and also by one -OH group for the sulphate group, giving fucose 44.6%, galactose 3.3%, xylose 1.2%, uronic anhydride 3.0% and allowance made on the sulphate and calcium for galactose (2.2%, SO₄, 0.5% Ca) and xylose (0.8% SO₄, 0.2% Ca) leaving sulphate 35.3% and calcium 0.7%. The percentage composition of the fucose portion then becomes:

fucose 44.6, sulphate 35.3 calcium 7.5 = 87.4.

or as % 51.0 40.4 8.1%

The calculated percentage composition of a unit in a polysaccharide as linked to two other units is:

fucose 52.6%, sulphate 39.2%, calcium 8.2%

which is in good agreement with the observed figures. If on the other hand, the galactose and xylose are assumed to have no sulphate and metal, the composition
of the fucose portion becomes:

fucose 49.0\%, sulphate 42.1\%, calcium 9.0\%

which is still in reasonable agreement with the theoretical.

It must be emphasised that this calculation is based on possibly unwarranted assumptions and is merely made to show roughly what the analytical figures indicate. The results are all based on average figures since the polysaccharide may contain any structure from a straight chain to a highly complicated branched network. All the fucose units do not necessarily have sulphate groups attached and finally the polysaccharide with which this thesis deals may be a mixture of two or more polysaccharides of different constitution.
BIBLIOGRAPHY


Halsall, Hirst and Jones.


16. Pregl-Grant. Quantitative Organic Micro-
In conclusion the author wishes to express his most sincere thanks to Dr. E.G.V. Percival for his advice and encouragement throughout the period of the research, and to the Scottish Seaweed Research Association, under whose auspices the work has been carried out.