XLVII. ON THE NATURE OF THE CELL WALL CONSTITUENTS OF *LAMINARIA* SPP. MANNURONIC ACID.

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The nature of the cell wall constituents of the red and brown algae has been the subject of study in this laboratory for some time past [Haas and Hill, 1921; Haas, 1921; Russell-Wells, 1922; Haas and Russell-Wells, 1923, 1929]. The present investigation, which is concerned primarily with *Laminaria* spp., was undertaken with the object of throwing some light on the mode of occurrence within the plant of some of the substances which have from time to time been described as being obtained from this genus. The search for the source of the methylpentose, fucose, in particular, has involved the study of the products of hydrolysis of two cell wall constituents known respectively as fucoidin and alginic acid and the results obtained are described herein.

Fucoidin.

The term fucoidin was first applied by Kylin [1913] to the highly mucilaginous material which exudes from freshly collected samples of *Laminaria* spp. and from *Fucus serratus*, *F. vesiculosus* and *Ascophyllum nodosum*. He found it to be a calcium salt and observed that when extracted from the weed with hot water the material was much less viscous; this fact he attributed to the conversion of the calcium salt into the alkali metal salt, though no evidence was offered for his assertion; he stated further that fucoidin contains pentoses and methylpentoses and established the presence of fucose among the products of hydrolysis of this substance.

Some years ago Haas and Russell-Wells [1923] obtained evidence of the existence of an ethereal sulphate grouping in *Laminaria*, and it has now been possible to show that this grouping is actually contained in fucoidin. The method employed for the isolation and purification of this substance was as follows.

The freshly gathered weed was soaked in distilled water whereby a very translucent and highly viscous extract was produced from which, by addition of an equal volume of absolute alcohol, a gelatinous precipitate was obtained. This substance when placed in water again swelled up and went into solution, but gradually lost its gelatinous character on keeping; it was purified by solution in water and re-precipitation by means of alcohol; but with the

gradual removal of salts, precipitation by alcohol became increasingly difficult and this method of purification was abandoned in favour of dialysis. When all dialysable salts had been removed the dialysed solution was poured into alcohol and the precipitated material was preserved in alcohol, since drying caused shrinkage to a tough horny material, which though still soluble in water had a greatly reduced swelling power.

On incineration the material yielded 30·93 % of ash, consisting for the most part of calcium sulphate. The calcium was shown to occur in the original material in the ionised condition, being precipitable by ammonium oxalate. That the substance was an ethereal sulphate was shown by the fact that the original material dissolved in water gave no reactions for the sulphate ion but that after hydrolysis it yielded precipitable sulphate in approximately double the quantity of that in the ash.

Sulphate in hydrolysed solution = 30·33 %,
ash = 15·10 %.

The presence of methylpentose, described by Kylin, was confirmed both by the colour reaction of Rosenthaler and by the isolation of an osazone crystallising in yellow needles, M.P. 170–173°, from the products of hydrolysis of the material with 3 % sulphuric acid in a boiling water-bath for 11 hours. The preparation from *Fucus* spp. of fucose was first described by Günther and Tollens [1890] and later by Votoček [1915] and by Clark [1922] who hydrolysed the entire weeds, after a preliminary soaking in dilute sulphuric acid. In view however of the evidence here presented for the occurrence of fucose in the water-soluble fucoidin of *Laminaria*, it appears that the preliminary soaking in dilute acid may remove some of the potential source of this sugar[1]. Heated with 12 % hydrochloric acid fucoidin produced furfuraldehyde which yielded a black phloroglucide soluble in alcohol; carbon dioxide was likewise given off, corresponding to about 7·3 % of a uronic acid complex, but, owing to the limited amount of material available, no attempt was made to ascertain its nature.

Algin and alginic acid.

The term algin was first applied by Stanford [1883, 1884, 1886] to a mucilaginous material occurring in *Laminaria* fronds; on extracting the weed with cold 2 % sodium carbonate and acidifying the resulting solution he obtained a precipitate which he described as alginic acid; of this acid he prepared a number of salts including an insoluble calcium salt containing 7·84 % of calcium.

Kylin [1915] applied the term algin to a supposed insoluble calcium salt which he assumed to occur in the cell wall; he stated that it was insoluble

[1] The completely exhausted residue from the extraction of 200 g. of *Laminaria* fronds with 2 % sodium carbonate, weighing 80 g., when hydrolysed under the conditions described by Clark [1922] for *Fucus vesiculosus*, yielded a very small quantity of an osazone which could not be definitely identified with that of fucose. From this fact it would appear that in the case of *Laminaria* at all events, the chief source of fucose would be the fucoidin.

or very sparingly soluble in water and suggested that hot water extracted from the weed an alkali metal salt of alginic acid produced from the calcium salt, but he did not support this by any experimental evidence. His explanation, moreover, of the action of sodium carbonate in extracting alginic acid was that it underwent double decomposition with the insoluble calcium salt yielding soluble sodium alginate.

In these circumstances it seemed worth while to examine the nature of the material extracted by hot water and to ascertain whether there were any limit to the extent to which the alginic acid contained in the cell wall could be extracted by this method. To this end 180 g. of *Laminaria* fronds were cut up, tied in a calico bag and extracted with boiling water. In view of the fact that water would also extract fucoidin, the first extracts were rejected until the absence of fucoidin could be established by hydrolysing with hydrochloric acid and showing that no sulphate was formed. The extraction was then continued until the extracts contained only negligible amounts of material; they were then evaporated to small bulk and precipitated with alcohol. The precipitated material was dissolved in a smaller volume of water and dialysed; after dialysis was complete, the material could no longer be precipitated by the addition of alcohol and was therefore recovered from solution by evaporation on a water-bath, when it weighed 2 g. Thus dried it would no longer dissolve in water. Analysis showed that it contained 3·46 % Ca and 0·45 % Mg, together with undetermined quantities of alkali metals. From these facts it was taken to be a water-soluble calcium, magnesium, sodium and potassium salt of alginic acid, since it gave, on acidification, a gelatinous precipitate in every way resembling alginic acid. That the extraction with hot water had not removed all the alginic acid from the cell wall was shown by the fact that the residue remaining, when treated with cold 2 % sodium carbonate, at once yielded an extract from which a copious precipitate of alginic acid could be obtained on acidification.

Direct evidence as to the correctness, or otherwise, of Kylin's assumption that alginic acid occurs in the cell wall in the form of an insoluble calcium salt is not easy to furnish, although the readiness with which cold dilute sodium carbonate extracts this acid from the weed is rather against this assumption. The following facts however provide indirect evidence which casts doubt on the occurrence of the calcium salt. From the loss in weight by 200 g. of the weed on exhaustive extraction with 2 % sodium carbonate it was estimated that *Laminaria* contains about 60 % of its weight of alginic acid. Such a proportion of this acid, if occurring as assumed by Kylin, in combination with calcium, would require a calcium content of the weed of 5·1 %, calculated on the basis of the figure given by Stanford for the percentage of calcium in the calcium salt. The calcium content of the whole weed was however found by analysis to be only 1·99 %.

From the above facts it is concluded that the cell wall of *Laminaria* contains alginic acid in two forms, one a soluble calcium, magnesium, alkali metal

salt, present only to the extent of about 1 %, and the other in the free state which appears to be the material extracted by cold dilute sodium carbonate; there does not thus appear to be any need to assume, as Kylin did, that the bulk of the alginic acid occurs in the form of an insoluble calcium salt.

Hydrolysis of alginic acid.

The first attempt to investigate systematically the hydrolysis of alginic acid was undertaken by Hoagland and Lieb [1915]. These authors obtaining their alginic acid from *Macrocystis pyrifera* subjected it to hydrolysis with hydrochloric acid and claimed to have obtained evidence that this substance yielded pentoses which were somewhat doubtfully identified by means of phenylhydrazine as xylose and arabinose. Some years later Schmidt and Vocke [1926] in an examination of alginic acid prepared from *Fucus serratus* described the hydrolysis of this material by successive treatments with anhydrous formic acid, 80 % sulphuric acid, and finally boiling 4·5 % sulphuric acid; they claimed to have established that alginic acid is a polymerised glycuronic acid by preparing a cinchonine salt having M.P. 204°, which is the M.P. given by Neuberg [1900] for the cinchonine salt of glycuronic acid prepared by the hydrolysis of euxanthone.

In view of these conflicting results we decided to reinvestigate the hydrolysis of alginic acid with a view to ascertaining whether it could be regarded as a potential source of pentoses or even of fucose.

For our experiments we chose sulphuric acid in preference to the hydrochloric acid employed by Hoagland and Lieb. The alginic acid[1] was heated under reflux with 8 times its weight of 2 % sulphuric acid in a boiling water-bath for 3 hours. At the end of this time the acid solution was filtered from the insoluble residue A (see below). On removing the sulphuric acid with barium carbonate it was found that the filtrate contained considerable quantities of barium in solution indicating that an acid forming a soluble barium salt had been produced.

In order to ascertain whether any hexose or pentose sugars had been produced as well, as had been suggested by Hoagland and Lieb's experiments on alginic acid from *Macrocystis pyrifera*, the barium salt in solution was precipitated by addition of alcohol. The aqueous alcoholic filtrate was filtered and evaporated to dryness under reduced pressure; the resulting light brown residue containing barium proved to be some of the barium salt which had escaped precipitation. In order however to ascertain whether it contained any sugars as well, it was repeatedly extracted with 80 % alcohol. Hardly any material was dissolved, but the combined extracts were evaporated and were found still to contain barium. Addition of phenylhydrazine acetate to the cold solution gave an immediate precipitate similar to the one described

[1] For much of the alginic acid used in these experiments we have to thank Messrs Nobel and Co., Explosives Ltd., Ardeer, Scotland.

on p. 409 and this proved to be insoluble in all organic solvents, showing that it contained no sugar hydrazones. The absence of sugars was thus established, and consequently alginic acid has to be excluded as a possible source of fucose.

Our results therefore failed to corroborate the observations of Hoagland and Lieb that alginic acid gave rise to pentoses on hydrolysis. In order to ascertain whether alginic acid from *Macrocystis pyrifera* was in any way different from that of *Laminaria* we procured some of the former weed from California through the good offices of the Californian Academy of Sciences, and on subjecting it to the same conditions of hydrolysis with sulphuric acid as described above we obtained substantially the same result, namely a solution containing a soluble barium salt. On treating this solution with phenylhydrazine as described by these authors we obtained in the cold a red-brown precipitate which contained barium but were unable to establish the presence of sugars.

In view of the statement by Schmidt and Vocke referred to above it seemed likely that the barium salt produced in our experiments might be that of glycuronic acid. On preparing the cinchonine salt by addition of the calculated amount of cinchonine sulphate to the barium salt we obtained a substance whose melting point, 195–197° (on one occasion 203°), seemed sufficiently close to that quoted by Schmidt and Vocke to suggest that it actually was cinchonine glycuronate; the specific rotation however, was $[\alpha]_D = 112{\cdot}8°$ as compared with $[\alpha]_D = 138{\cdot}6°$ as given by Neuberg. On preparing the quinine salt[1] an even greater discrepancy was found; the constants given by Neuberg for quinine glycuronate are M.P. 180° and $[\alpha]_D = -80{\cdot}1°$ and the analysis indicated the formula $C_{26}H_{34}O_9N_2$. Our salt on the other hand had M.P. 162–163° and $[\alpha]_D = -175{\cdot}3°$, while the analysis showed that the substance contained two molecules of water of crystallisation, as may be seen from the following figures[2]:

	C %	H %
Found	56·01	6·92
Calc. for $C_{26}H_{34}O_9N_2$, $2H_2O$	56·32	6·86

The residue A (p. 406) remaining after the hydrolysis of alginic acid for 3 hours with 2 % sulphuric acid was then subjected to further hydrolysis, with a view to obtaining an increased yield, by heating with 5 % sulphuric acid for 8 hours. After removal of the sulphuric acid a new cinchonine salt was obtained having M.P. 161° and $[\alpha]_D = 154°$; this substance gave the following results on analysis:

	C %	H %	N %
Found	59·21	6·87	5·4
Calc. for $C_{25}H_{32}O_8N_2$, H_2O	59·29	6·71	5·33

This salt therefore differed from that described by Schmidt and Vocke both in M.P. and in composition, since it contained a molecule of water of

[1] See Addendum.

[2] All the micro-analyses were carried out by Dr Ing. A. Schoeller of Berlin.

crystallisation. It was produced in much smaller yield than the cinchonine salt M.P. 195–197°. No explanation is at present offered for the existence of these two salts, more particularly since only one quinine salt has, so far, been obtained, but in any case neither of them agreed in physical constants with cinchonine glycuronate.

There seemed therefore no doubt that the acid produced by the hydrolysis of alginic acid from *Laminaria* was not glycuronic acid; as moreover the constants obtained did not agree for the cinchonine salt of galacturonic acid and furthermore alginic acid on oxidation yielded no trace of mucic acid, it seemed natural to conclude that the acid in question might be the hitherto unknown mannuronic acid, especially in view of the plentiful occurrence of mannitol in *Laminaria* and allied fucoids.

To settle this question experiments were undertaken[1] to ascertain the nature of the dicarboxylic acid produced on oxidation of the aldehydo-acid in question. To this end both alginic acid and its product of hydrolysis were oxidised and in each case mannosaccharic acid was identified as the diamide, thus proving conclusively that the aldehydo-acid was not glycuronic acid but the hitherto undescribed mannuronic acid.

Oxidation to mannosaccharic acid.

(*a*) *Alginic acid.* Earlier experiments on the oxidation of alginic acid by heating on a water-bath with nitric acid in search of the possible formation of mucic acid failed to produce anything but oxalic acid. By carefully regulating the oxidation however a different result was obtained.

5 g. of dried and finely ground alginic acid were covered with 7·5 g. of nitric acid (sp. gr. 1·2); the acid was at once absorbed leaving the alginic acid as a coarsely granulated mass; this was heated under a reflux condenser for 24 hours at 50°; about 50 cc. of water were now added and after thorough mixing the insoluble residue was filtered off on a Büchner funnel. The filtrate was then rapidly evaporated over a water-bath to about half its volume and then to dryness in a desiccator over sodium hydroxide, when a light lemon-yellow syrup resulted. On stirring this with concentrated ammonia it turned brown and deposited in the course of a few minutes a mass of crystals which on recrystallising from water separated in well formed rhombs melting at 188–189°.

Analysis gave the following results:

	C %	H %	N %
Found	34·39	5·84	13·21
Calc. for $C_6H_{12}O_6N_2$	34·61	5·77	13·46

[1] Experiments to this end were already in progress when we accidentally came across a paper by Cretcher and Nelson [1929], whose publication we had overlooked, in which these authors had already come to the same conclusion and had actually established the production of mannosaccharic acid on oxidation. Notwithstanding this we completed our investigation and now publish our results because they were arrived at independently and by slightly different methods.

(*b*) *Mannuronic acid.* 5·4 g. of barium mannuronate dissolved in 20 cc. of water were freed from barium by the addition of the requisite amount of dilute sulphuric acid and, after filtering, the solution was treated with 16 g. of bromine. The mixture was thoroughly shaken at intervals during 4½ days, after which the excess of bromine was removed by a rapid current of air until the liquid was of a light straw colour. The liquid was then diluted and treated with freshly precipitated silver oxide until neutral and then with hydrogen sulphide to precipitate any dissolved silver as sulphide. The filtrate evaporated to dryness in a desiccator left a colourless viscous residue which on addition of concentrated ammonia deposited rhombic prisms; these after crystallising twice from water melted at 188–189°.

Mannuronic acid.

The oxidation experiments described above showed undoubtedly that alginic acid gave rise on hydrolysis to mannuronic acid, since oxidation of the product led to mannosaccharic acid, identified by comparison of the M.P. of its diamide with that of an authentic specimen and the determination of a mixed melting point.

A sample of the mannuronic acid prepared from its barium salt left a light amber-coloured resin which did not crystallise. An attempt further to characterise the barium salt by treatment of the cold aqueous solution with phenylhydrazine acetate resulted in the production of an immediate red-brown precipitate containing barium. This material on extraction in a Soxhlet with chloroform lost its red colour and became orange-coloured. This substance was insoluble in organic solvents but dissolved sparingly in boiling water separating again rapidly on cooling in spherical aggregates. In spite of repeated attempts, no concordant analyses could be obtained indicating a homogeneous substance. This observation agrees with the experience recorded by Neuberg and Neimann [1905] of difficulty in obtaining a uniform product by the action of phenylhydrazine upon glycuronic acid.

Attempts to prepare a *p*-bromophenylhydrazone resulted in the production of brown resin. Similarly attempts to prepare a crystalline dinitrophenylhydrazone were unsuccessful.

Conclusion.

An attempt has been made to elucidate the nature of the cell wall constituents of the Laminariaceae known as fucoidin and alginic acid. The former has been shown to be the calcium salt of a sulphuric acid ester of a non-reducing polymerised uronic acid complex combined with a methylpentosan, since it yields on hydrolysis sulphate ions and the methylpentose fucose; the nature of the uronic acid has not been established.

Alginic acid has been shown to be a polymerised form of the hitherto undescribed mannuronic acid, and to contain no pentosan or methylpentosan groupings. These facts emphasise the distinctive metabolism of the brown

algae, which appears to be founded upon a mannose, rather than a glucose basis—as is shown by the known occurrence of mannitol and mannitan [Haas and Hill, 1929], and the apparent absence of glucose in any appreciable amounts.

SUMMARY.

1. Evidence is furnished that the substance described by Kylin as fucoidin is an ethereal sulphate. The substance contains also some uronic acid complex the nature of which has not been determined.

2. Alginic acid occurs mainly as such in the free state in the cell wall and to a limited extent only in the form of a water-soluble calcium magnesium alkali metal salt.

3. Alginic acid from *Laminaria* yields on hydrolysis mannuronic acid whose quinine salt is here described for the first time (see Addendum).

No evidence could be obtained of the formation of sugars by the hydrolysis of alginic acid and this substance cannot therefore be regarded as a source of any of the sugars which have been obtained by the hydrolysis of *Laminaria* weed as described by Müther and Tollens [1904], Manske [1930] and others.

4. Alginic acid on oxidation with nitric acid yields mannosaccharic acid.

5. Fucose obtained by the hydrolysis of the complete weed of *Laminaria* is derivable in part from fucoidin and in part possibly from the cell wall residue remaining after exhaustive extraction with sodium carbonate, but none is produced from alginic acid.

ADDENDUM (March 27th, 1931).

Since the above was written it was found that when the solution obtained by heating alginic acid with 2 % sulphuric acid was further heated for 8 hours with 5 % sulphuric acid the resulting liquid, on treatment with cinchonine sulphate, yielded the low-melting salt, M.P. 161° and $[\alpha]_D = 154°$. This shows definitely that the nature of the cinchonine salt obtained depends upon the strength of acid and the time employed in the hydrolysis, and that the cinchonine salt of low melting point is not obtained solely from the further hydrolysis of the residue remaining after initial treatment with 2 % sulphuric acid (as implied on p. 407), but may actually be obtained by further hydrolysis of the solution which previously yielded the salt of high melting point.

Attempts to prepare two different quinine salts from the two sources which yielded the two distinct cinchonine salts gave the following results: hydrolysis with 2 % acid yielded a quinine salt of M.P. 168° and $[\alpha]_D = -173{\cdot}5°$, while hydrolysis with 5 % acid yielded a quinine salt of M.P. 162–163° and $[\alpha]_D = -175{\cdot}3$. The difference between the physical constants in these two cases is hardly sufficient to justify the conclusion that the two salts are really distinct substances, but on the other hand the existence of two cinchonine salts requires the existence of two quinine salts.

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