LVII. ON CARRAGEEN (*CHONDRUS CRISPUS*). III. THE CONSTITUTION OF THE CELL WALL¹.

By BARBARA RUSSELL-WELLS.

(Received June 21st, 1922.)

HISTORICAL.

MANY workers have from time to time investigated *Chondrus crispus*, but of recent years the most exhaustive work has been done by Sebor. The presence of galactose had been established by Flückiger and Obermayer [1868], and Bente [1875, 1876] had found among the products of the action of acid upon carrageen levulinic acid and a sugar, which showed little or no optical activity. Haedicke, Bauer and Tollens [1867] established in carrageen mucilage the presence of fructose and calculated that it contained 28 % of galactose. They suggested that these sugars might be present under the form of raffinose. Sebor [1900] determined to identify the remaining sugars. He confirmed the presence of galactose and fructose, found small quantities of pentosan and showed that raffinose is not present. He also stated that he had found evidence of glucose. He oxidised the carrageen mucilage, but, after filtering off the mucic acid, failed to obtain any acid potassium saccharate on treating with potassium carbonate and acetic acid. As an analysis of the silver salt gives the same result for both mucate and saccharate he tested the potassium solution for optical activity, but found it inactive, whereas the saccharate should be active. He therefore abandoned the oxidation products and tested for glucose in a hydrolysed solution of mucilage. He hydrolysed with 6N HCl to destroy the fructose, and when the solution no longer gave a Selivanoff reaction, added benzylphenylhydrazine. He separated off the benzylphenylhydrazone of galactose and found the remaining compound had a melting point of 163°, that of glucose benzylphenylhydrazone being 165°. He declared that the hydrazone was a mixture and his paper gives the impression that he was not satisfied with the agreement of melting points referred to above. This appears to be all the evidence he had in favour of the presence of glucose among the constituents of carrageen. Müther and Tollens [1904] state that glucose is among the constituents of carrageen, but all the evidence they offer is an estimation of the amount of silver present in the silver salt of an oxidation product; this is obviously useless since the amount of silver is the same in the salts of both saccharic and mucic acids.

Abderhalden's *Biochemisches Handlexikon* [1911] contains the statement that the pure mucilage of carrageen corresponds to the formula $C_6H_{10}O_5$, but

¹ Thesis approved for the Degree of Doctor of Philosophy in the University of London.

it is not stated on what evidence this formula is based; it must be borne in mind that the mucilage is known to contain both nitrogen and considerable quantities of ash. Stocks and White [1903], on the other hand, have shown by analysis that the mucilage does not possess this formula.

	Found for ash free substance	Calculated for $C_6H_{10}O_5$
Carbon	37.94 %	44.44 %
Hydrogen	5.92	6.17
Oxygen	54.95	49.39
Nitrogen	1.19	—

These figures conclusively demonstrate that the formula $C_6H_{10}O_5$ does not agree with the results of analysis.

Since, as will appear later, it has now been proved that carrageen mucilage contains at least two substances which are carbohydrate esters of sulphuric acid, and various metallic radicles, any calculations of the above nature are futile.

Previous investigators have commented on the high ash content of carrageen and reported that it is largely composed of calcium sulphate, but most of them have ignored the significance of this and proceeded to examine the organic radicles obtained by various means from the plant. They also appear to have worked either on the seaweed itself or on a direct hot water extract. In 1921 Haas and T. G. Hill [1921] showed that this extract consists in reality of two distinct fractions, and in the same year Haas [1921] published a method for separating the two, together with a systematic investigation of the inorganic constituents of one of them. He found that while one was readily soluble in both hot and cold water, the other was readily soluble in hot, but only sparingly in cold water. The method of extraction and separation was based on this difference of solubility, and was, with slight modification, the method adopted in the preparation of larger quantities of the two substances required for the present investigation.

The distinctions between the hot extract (H.E.) and the cold extract (C.E.) described by Haas and Hill were confined to physical characteristics such as different solubility in cold water and different gelatinising properties.

It was also shown by Haas that the H.E. contained 17.6 % of ash which could not be reduced by dialysis and was found to be due to the presence of the calcium salt of an ethereal sulphate, in which the calcium is fully ionised and can be precipitated by the ordinary reagents, while the sulphate radicle is not ionised and can only be precipitated by barium chloride after hydrolysis of the compound.

Haas and Hill also state that they found nitrogen in both fractions. They also got positive reactions for protein in the seaweed itself and in the water-soluble product obtained from it.

From the foregoing it will be seen that carrageen can be separated into three constituent fractions, the cold water and hot water soluble portions and the residue. The physical characteristics of the two extracts have been described

by these previous workers, and the presence of an ethereal sulphate complex definitely established in the H.E.

PRESENT INVESTIGATION.

The present investigation was undertaken with a view to instituting a chemical comparison between the two extracts of carrageen, both as to their inorganic and organic constituents, to examine the residue of the weed, remaining after the extraction of both fractions, for cellulose and to discover if other examples could be found among the seaweeds of ethereal sulphate grouping.

The hand picked carrageen was first washed free from dust by holding it for a few seconds in running water, and then soaked for about an hour in distilled water. The aqueous extract thus obtained was poured off, filtered and evaporated, the resulting scales constituting the "cold extract." This process was then repeated and thus a further quantity of C.E. obtained. After only two hours' soaking much C.E. still remained in the weed but it was decided to sacrifice this quantity lest a more prolonged extraction should result in contamination with some of the H.E. which is also slightly soluble in cold water. The weed was therefore washed for several days by soaking in water until it was no longer slimy, all the wash water being rejected. The weed thus deprived of its C.E. and some of its H.E. was then washed several times in distilled water and air-dried. In order to obtain the H.E. the dried carrageen was heated on a water-bath with fresh distilled water and the liquid from this yielded, on filtration and evaporation, the "hot extract."

EXAMINATION OF THE COLD EXTRACT.

The crude C.E., just as it is taken from the evaporating pans, has a distinctly saline taste and on incineration was found to contain about 32 % of ash which showed a marked tendency to fuse. After dialysis the salt taste had disappeared and the ash content had fallen to 21.86 %. No amount of dialysis would reduce the ash further. The salts which dialysed out were, no doubt, mainly impurities arising from contact of the seaweed with seawater. During dialysis it was observed that the water outside the dialyser turned slightly yellow as though some organic substance had come through the parchment. This water was therefore collected and evaporated down over a water-bath on tin-lined copper trays. A small quantity of a brown, sticky, amorphous material was obtained; this did not dry in scales like the hot and cold extracts, and although composed very largely of the salts which had dialysed out from the C.E., gave a strong reaction with Molisch's reagent, thus showing the presence of a diffusible carbohydrate; as it did not reduce Fehling's solution to any appreciable extent it was thought possible that it might be trehalose, which Kylin [1915] claims to have found in carra-

geen. Accordingly a dilute solution was tested in a polarimeter, but not the slightest trace of rotation could be observed.

A qualitative analysis of the ash of the C.E. showed the presence of sulphate, calcium, magnesium and small quantities of sodium and potassium with traces of iron. These were all found in the ash of the H.E. as well, but here the amount of alkali metals was so small that the ash showed no tendency to fuse during the process of incineration.

The calcium in the C.E. as in the H.E. is fully ionised and can be precipitated quantitatively from a 0.35 % solution by means of ammonium oxalate. The actual figures obtained were as follow:

1. Calcium by incineration 3.98 %.
2. Calcium by direct precipitation 4.03 %.

The sulphate however is not ionised, no precipitate being formed by the addition of barium chloride. But, after hydrolysis with hydrochloric acid, barium chloride gives a copious precipitate of barium sulphate, showing that hydrolysis liberates the sulphate radicle in an ionised condition, while previously it was bound to some organic radicle. The method of hydrolysis employed was as follows:

About 0.5 g. of C.E. was dissolved in 200 cc. of water and 5 cc. of concentrated hydrochloric acid added. This solution was boiled over a Bunsen flame for at least six hours to ensure complete hydrolysis. On estimation the percentage of sulphate obtained after hydrolysis was found to be rather more than twice that obtained on incineration, the actual figures being:

	I	II	Mean
1. SO ₄ by incineration	13.75 %	14.16 %	13.95 %
2. SO ₄ by hydrolysis	30.14	30.32	30.23

As in the case of the H.E. it is extremely difficult to get rid of all moisture, the substance tending to char when heated in a steam oven owing to the liberation of sulphuric acid.

If the C.E. contained only a calcium salt of the ethereal sulphate corresponding to the formula which has been given for H.E., viz.

the sulphate ratio would be exactly two to one. Actually, however, it is more than two to one. This discrepancy could be accounted for on the assumption that some of the ethereal sulphate was combined with ammonium instead of with calcium. Such a combination might be present in a mixed calcium and ammonium salt, or there might be slight traces of the pure ammonium salt mixed with the calcium salt. There is not, however, enough evidence to show which combination is the more probable. It is obvious that in a salt of this

nature the sulphate moiety combined with (NH_4) would leave no ash on incineration, although such sulphate could be estimated after hydrolysis.

In order to discover if any ammonium radicle were in fact present, some of the C.E. was distilled with magnesium oxide, when ammonia was given off. This ammonia was estimated and the results, calculated as N, were as follow:

	I	II	Mean
N by magnesium oxide	0.24 %	0.22 %	0.23 %

The quantity of nitrogen found would, in the form of ammonium, satisfy 0.80 % of sulphate calculated as SO_4 . Thus assuming the nitrogen actually present is contained in such an ammonium salt as suggested above, the experimental value for incinerated sulphate is raised by 0.80 % from 13.95 % to 14.75 %, which is only 0.36 % below the value required by theory, a difference well within the limits of experimental error. In addition to the nitrogen which can be driven off by distillation with magnesium oxide, C.E. contains other nitrogen not liberated in this manner.

	I	II
N by Kjeldahl	0.55 %	0.57 %

As shown above, nearly half this nitrogen can be liberated by boiling with magnesium oxide and is therefore probably present in an ammonium radicle. The rest is almost certainly there as protein nitrogen. That protein is present in both H.E. and C.E. is indicated by the fact that both give a positive reaction with Millon's reagent and with nitric acid. Whether this protein is combined or not is at present impossible to say, but it is constantly present though it may be removed by prolonged heating with alkali.

Ammonium salt of extracts.

An attempt was made to prepare the ammonium salt of the C.E. in the hope of removing all the calcium and magnesium and so obtaining an ash free material. For this purpose some C.E. was dissolved in water, ammonia and ammonium chloride were added and then enough ammonium phosphate to precipitate all the calcium and magnesium. The solution was left to cool and settle over night and was filtered next morning. The liquid was filtered with the aid of the filter pump first through a Chardin and then through a quantitative filter-paper on a Buchner funnel. The filtrate, which was much clearer than a solution of ordinary C.E., was dialysed for five days to remove excess of ammonium phosphate and other impurities. Since it charred when evaporated to dryness over a water-bath, the filtrate was evaporated in a vacuum-desiccator and clear, almost colourless scales were obtained. These were dried to constant weight in the desiccator, and it was then found that the ash content had fallen from 21.26 % to 5.87 %. A qualitative analysis of the ash showed it to consist very largely of magnesia, from which it was concluded that the magnesium present in the C.E. is not in an ionised condition, or else it would have been precipitated by the phosphate.

In addition to magnesium the ash of the ammonium salt of the C.E. contained potassium and sodium but no calcium.

The same procedure was adopted with the H.E. The filtrate, after precipitation with ammonium phosphate, was dialysed for six days, and then evaporated over a water-bath in a platinum basin. Like the C.E. it showed a tendency to char, and when evaporated to small bulk turned acid and reduced Fehling's solution. It was therefore kept alkaline with ammonia all the time it was being evaporated, and when reduced to a small volume it was taken from the water-bath and set aside to cool. After cooling it set to a stiff jelly, proving that the loss of calcium does not destroy the gelatinising properties of the H.E. On complete evaporation in a vacuum the substance was found to be very like the ammonium salt of the C.E. in appearance, and consisted of clear, nearly colourless scales which readily absorbed moisture from the atmosphere. On incineration a considerable amount of ash was left, which like that of the C.E. contained magnesium. So here again the magnesium is present in an un-ionised condition.

Another sample of the ammonium salt of the H.E. was prepared by precipitating with ammonium oxalate instead of with ammonium phosphate and the ash in this was estimated and found to have been reduced from the original 17.6 % to 4.38 %.

COMPARISON OF ORGANIC SUBSTANCES IN HOT AND COLD EXTRACT.

The organic complexes in the two extracts of carrageen were also investigated with a view to discovering further differences of composition between the two fractions.

Both H.E. and C.E. consist largely of carbohydrates. Neither of them will reduce Fehling's solution without previous hydrolysis, but both will do so after such treatment.

Since galactose has been found in carrageen mucilage by Flückiger and Obermayer [1868] and in *Chondrus elatus* by Takahashi [1920] it was decided to test for it in the two extracts. They were therefore oxidised with nitric acid according to the Creydt modification of the Kent and Tollens method. Mucic acid was obtained from both, thus proving galactose to be present in both fractions. The amount of mucic acid obtained was then estimated, according to the method of van der Haar [1920], the average values obtained being 21.12 % from the H.E. and 24.82 % from the C.E. These figures correspond to 29.47 % and 33.72 % of galactose respectively in the two extracts.

The mother liquors from the mucic acid were examined qualitatively for saccharic acid, but in no case was any found; on the other hand, both tartaric and oxalic acid were shown to be present, the latter in larger quantity in the case of the H.E.

ESTIMATION OF PENTOSSES.

Sebor [1900] expressed the view that carrageen mucilage contained a small quantity of pentosan or methyl-pentosan. The H.E. and C.E. were accordingly tested by the Kröber-Tollens method to discover whether pentoses could be discovered in either or both of these fractions. The actual results obtained were: H.E. 1.89 % of pentosan (mean of two estimations); C.E. 1.38 % of pentosan.

Since concentrated solutions of carrageen mucilage will set to a jelly on cooling it was thought that this mucilage might contain pectic bodies. Accordingly a direct hot water extract of *Chondrus* was tested according to the method of von Fellenberg [1914], which consists in steam distilling an alkaline solution of the substance under consideration, and testing the distillate for methyl alcohol and acetone [Tutin, 1921]. Two grams of the extract of carrageen were therefore gently boiled for $6\frac{3}{4}$ hours under a reflux condenser with 100 cc. of $N/10$ NaOH, and the alkaline solution, which had darkened slightly, was then steam distilled. The distillate was practically neutral and slightly yellow in colour. It was tested for alcohol by the acid and bichromate test and for acetone by the iodoform and nitroprusside tests, but the result was negative in each case. This was therefore taken to prove the absence of pectic bodies from the mucilage of carrageen.

RESIDUE LEFT AFTER EXTRACTION BY COLD AND HOT WATER.

The residue of the *Chondrus* left after the removal of the C.E. and H.E. showed a great reluctance to part with its last traces of water-soluble material. It was boiled for several hours on a water-bath, the water being changed periodically, but each time this water on being filtered off and tested with Molisch's reagent gave a strong positive reaction. A fresh portion of residue was therefore heated over a Bunsen burner with three successive changes of water, but this treatment also failed to remove the last traces of water-soluble material. Recourse was then had first to a steriliser and finally to an autoclave. After several hours in each of these, however, water-soluble material was still coming out, and was found to contain calcium.

Kylin [1915] claims to have found an insoluble calcium compound in *Chondrus crispus*. He apparently cut sections of fresh weed, boiled them in water and then left them to soak over night, concluding that this treatment removed all water-soluble substances present. He then found calcium to be present in the sections and assumed it to be in an insoluble form. In view of the extreme difficulty experienced during the present investigation in getting rid of the last traces of water-soluble material from the residue of carrageen which had already been soaked for several days in water, it seems unlikely that all was removed by Kylin's treatment. Hence the calcium-containing substance he found was in all probability merely the last traces of the H.E.

Since it was found so difficult to extract the residue completely merely by heating with water, a portion was boiled under a reflux condenser with

3 % NaOH. After two days of this treatment the contents of the flask were filtered, and the residue on the filter-paper washed free from alkali. It was then soaked in dilute hydrochloric acid to remove the carbonate found to have been formed during the alkali decomposition. When washed free from acid the residue was found to be protein- and ash-free, and completely soluble in cuprammonia, showing it to be pure cellulose. Cellulose was also obtained from the residue by acid decomposition. This was effected by boiling for some hours with hydrochloric acid. The solution was filtered and the solid matter left on the filter-paper was washed and tested with cuprammonia. The total amount of cellulose in carrageen, estimated by alkali decomposition, was found to be 1.3 %.

CERAMIUM RUBRUM.

After it had been definitely established that the sulphate in both the C.E. and H.E. of carrageen is present as an ethereal sulphate linked to an organic radicle it seemed probable that this grouping might be found to occur in other seaweeds. Accordingly it was decided to examine another alga which was known to yield an ash with a high sulphate content, and for this purpose *Ceramium rubrum* was selected.

Three grams of dried *Ceramium* were heated with distilled water on the water-bath for an hour. The seaweed was then removed and the liquid filtered and evaporated. Scales of a transparent, horny material were obtained. Some of this material was dissolved in distilled water and the solution was divided into two portions *A* and *B*, barium chloride being added to each. To solution *A* a small quantity of concentrated hydrochloric acid was added while solution *B* was left neutral; both were then boiled for one and a half hours. At the end of this time a precipitate of barium sulphate had been formed in the acid solution *A* only, thus demonstrating the presence of the ethereal sulphate grouping in *Ceramium rubrum*. The investigation is being continued.

GENERAL.

Microscopic examination of sections of the thallus of *Chondrus* show that the cell walls become very thick and swollen in distilled water. This is what one would expect if they were largely composed of H.E. and C.E., since on being placed in water dried scales of these materials absorb much moisture and swell up enormously. While only 1.3 % of the dry weight of carrageen consists of cellulose the cell walls appear under the microscope to constitute the greater part of the section, and over 80 % of its weight consists of H.E. and C.E. Since these contain complex polysaccharides they are allied to cellulose and are possibly degradation products of it.

The exact nature of the organic radicle or radicles present in the ethereal sulphates has not been determined. There may be one bi-valent or two uni-valent groups in each molecule (see formula on p. 581).

The present investigation has shown that the two extracts, though differing chemically as well as physically, are nevertheless closely allied. It may be that each extract contains a mixture of various closely related bodies and that different ones predominate in each extract. The similarity of the oxidation products points to both extracts consisting of substances belonging to the same group of compounds, but there is not enough evidence to show whether the differences are due to different bodies or mixtures in different proportions of the same substances. On the whole the former of these alternatives appears the more likely.

SUMMARY.

1. Dialysable organic matter can be separated from the C.E. of carrageen.
2. The C.E. contains calcium and ammonium ethereal sulphates and its ash contains, besides sulphate and calcium, magnesium, sodium, potassium and traces of iron. The ash of H.E. also contains these radicles, but it has less sodium and potassium and more calcium than that of C.E.
3. The ammonium salts of both extracts have been prepared by replacing the ionised calcium by the ammonium radicle.
4. Un-ionised magnesium is present in both extracts.
5. The main oxidation products of both extracts consist of mucic, oxalic and tartaric acids. More mucic acid, but less oxalic acid, is obtained from C.E. than from H.E.
6. Pentose radicles are present in both extracts, but more in cold than in hot.
7. Pectic bodies are absent from both extracts.
8. Cellulose is present in the residue of carrageen left after extraction with both cold and hot water.
9. There are indications that the ethereal sulphate grouping is present in *Ceramium rubrum*.

In conclusion the writer wishes to express her thanks to Dr Haas for his help and advice during the progress of the work, and his kind interest throughout.

REFERENCES.

- Abderhalden (1911). *Biochemisches Handlexikon*, 2, 75.
Bente (1875). *Ber.* 8, 417.
— (1876). *Ber.* 9, 1158.
von Fellenberg (1914). *Chem. Zentr.* 2, 942.
Flückiger and Obermayer (1868). *Repert. Pharm.* 380.
van der Haar (1920). Monosaccharide u. Aldehydsäuren
Haas (1921). *Biochem. J.* 15, 469.
Haas and Hill (1921). *Ann. Appl. Biol.* 7, 352.
Haedicke, Bauer and Tollens (1867). *Annalen*, 238, 302.
Kylin (1915). *Zeitsch. physiol. Chem.* 94, 337.
Müther and Tollens (1904). *Ber.* 37, 298.
Sebor (1900). *Oesterr. Chem. Zeit.* 3, 441.
Stocks and White (1903). *J. Soc. Chem. Ind.* 22, 4.
Takahashi (1920). *J. Coll. Agr. Hokkaido Imp. Univ. Japan*, 8, 183.
Tutin (1921). *Biochem. J.* 15, 494.