

LXXXVII. ON THE SIGNIFICANCE OF THE ASH CONTENT OF CERTAIN MARINE ALGAE.

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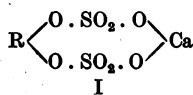
INTRODUCTORY.

COMPARATIVELY little attention has, in the past, been paid to the question whether certain ash constituents occurred as such in the plant or whether they had been produced during the process of incineration; thus while the sulphate or phosphate in an ash may be due to the accumulation within the plant of salts containing these radicles, they may, on the other hand, have been produced during incineration by the oxidation of organically combined sulphur or phosphorus, more especially if the ash has a strongly alkaline reaction, for in such cases the incineration amounts almost to alkali fusion.

It has long been known that many sea-weeds on incineration give ashes containing a relatively high percentage of calcium sulphate, but no particular significance was attached to this fact by earlier workers. By means of micro-chemical tests it should of course be easy to recognise the presence of certain ash constituents and so to establish their pre-existence within the plant before incineration. Such reactions, however, as can be carried out are due to dissolved ions and indicate the presence of salts which it should be possible to remove by dialysis. If such be the case no great significance attaches to their presence as they may merely be mobile products of metabolism or may be part of the plant's saline nutrients. If then a plant, or its aqueous extract still retains a considerable quantity of ash constituents even after exhaustive dialysis there is reason to suspect that such constituents are held in some form of chemical combination which precludes their free movement and consequently renders them incapable of dialysis.

It was found some time ago [Haas, 1921] that *Chondrus crispus* presents such a case, in which the ash is rich in calcium sulphate even after the material has been subjected to prolonged dialysis. Both chemical and micro-chemical analysis revealed the presence of ionised calcium but failed entirely to disclose any sulphate. To account for these facts it became necessary to

assume the existence in the plant of some form of masked sulphate such as an ethereal sulphate of the type represented by the formula



Chemical evidence for the correctness of this assumption was furnished by the fact that the masked sulphate could be converted into ionised sulphate by hydrolysis.

One of the consequences of this observation has been the recognition within the plant of colloidal electrolytes, a class of substances whose existence had not hitherto been suspected in this connection. The establishment of the presence of ionised calcium attached to a complex colloidal aggregate suggested the possibility for the manifestation of a variety of interesting physical chemical phenomena, of which osmotic pressure and conductivity are the most obvious. By means of the very simplest form of parchment osmometer it has been possible to demonstrate the existence of sufficient osmotic pressure to produce a steady rise in the stem of the thistle funnel. Conductivity measurements were kindly carried out for us by Mr F. C. Harwood in the physical chemistry laboratory of this college, and a preliminary account of some of his results has already appeared elsewhere [1923].

What may be the significance to the plant of the presence of osmotically active colloidal electrolytes in the cell wall is at present a matter of speculation. Whether these substances represent transitory phases in which the plant retains calcium sulphate in chemical combination, to be hydrolysed when occasion arises, it is not as yet possible to say; further evidence will have to be accumulated before it can be stated whether there is any seasonal variation in the occurrence of such compounds.

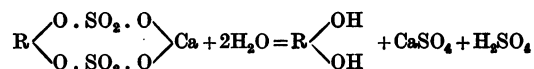
That the occurrence of ethereal sulphates is a fairly widely distributed phenomenon is shown by the present results in which the occurrence of similar compounds is described in the following Rhodophyceae and Phaeophyceae: *Ceramium rubrum*, *Delesseria sanguinea*, *Delesseria alata*, *Polysiphonia fastigiata*, *Plumaria elegans*, *Ascophyllum nodosum* and *Laminaria digitata*.

Since we commenced work on this subject Neuberg and Ohle [1921] have been able to establish by methods similar to our own the existence of the ethereal sulphate grouping in agar, the extract of a species of *Gelidium*. The physical chemistry of this substance has likewise since received attention at the hands of various workers, notably Samec and his collaborators [1921, 1922], and more recently Fairbrother and Mastin [1923].

In view of these facts it would appear necessary to urge the desirability of a more careful examination of the ash constituents of plant material in general with the object of elucidating, if possible, the nature of the combination of these substances in the plant.

METHODS.

In the case of *Chondrus* the proof of the existence of an ethereal sulphate depended on the fact that the percentage of sulphate contained in the hydrolysed solution of the hot extract (H.E.) was just twice that contained in the ash, in accordance with the equation:



from which it is clear that of the two molecular proportions of sulphate here given only the one combined with the calcium would be found in the ash, the other being lost during incineration.

In endeavouring to establish the existence of ethereal sulphate groupings in other material by similar means it has however not always been found possible to establish the exact relation of 2 : 1. The reasons for this discrepancy may be due either to experimental difficulties connected with the incineration or the hydrolysis, or to the inherent properties of the particular type of ethereal sulphate concerned.

A. With regard to difficulties encountered in the hydrolysis it has been found that the reaction is somewhat slowly effected by boiling with hydrochloric acid, due possibly to a tendency towards a reversal of the reaction as noted by Cunningham [1918]. The use of potassium chlorate or other oxidising agent during hydrolysis to assist in the destruction of the organic complex is of course precluded in this connection, since it is essential to ensure that all the sulphate found is due to hydrolysis and that none shall have been produced by the oxidation of organically combined sulphur. After a number of trials however the following conditions were found to effect the complete hydrolysis of such ethereal sulphates, the accuracy being confirmed by simultaneous estimation of total sulphur by Benedict's method.

About 0.75 g. of the substance is placed in a tall form beaker with 60 cc. of water to which 40 cc. of concentrated hydrochloric acid are added. The beaker is then covered with a clock-glass and heated over a sand-bath for six hours, water being added from time to time to replace that lost by evaporation. The resulting dark coloured solution is then filtered from any brown insoluble matter, which is nearly always present, and the filtrate is precipitated with barium chloride.

With regard to the incineration it has been found that the results obtained are very often rather more than half those obtained by hydrolysis. This is notably the case where the ash is strongly alkaline, e.g. *Laminaria* (p. 703), whereas on the other hand in the case of *Chondrus* hot extract, in which the ash is neutral, the ratio comes out exactly 2 : 1.

B. As regards the effect of the type of ethereal sulphate combination upon the ratio it must be borne in mind that the occurrence of an ammonium salt of the type $R \cdot O \cdot \text{SO}_2 \cdot \text{ONH}_4$ will at once throw out the ratio, since the

whole of its sulphate will be lost on incineration; such a case has already been described [Russell-Wells, 1922]. Similar remarks apply to the case of a true ethereal sulphate of the type $R_1 \cdot O \cdot SO_2 \cdot O \cdot R_2$ in which R_1 and R_2 are both organic complexes; in the case of such a compound of course the whole of the sulphate radicle should likewise be lost on incineration.

The general method of procedure may now be given, as it is essentially the same for all the cases investigated.

The carefully hand-picked material is extracted with hot water over a water-bath or in an autoclave at 105° ; the extract is then filtered over a Buchner funnel, first through calico and then through Chardin filter paper. The filtrate is thereupon placed in a parchment dialyser with a little thymol and the water is replaced morning and evening until it no longer gives any reaction for either sulphate or chloride; the dialysed liquid is evaporated to dryness on a platinum dish and the resulting scales are dried for analysis.

The necessity for thorough dialysis must be emphasised inasmuch as no great significance attaches to sulphate found in a hydrolysed solution unless precautions have been taken to ensure removal of any free sulphate, a consideration which is particularly important in the case of sea-weeds which are normally surrounded by a medium containing sulphates.

With the object of saving time a few analyses were carried out directly upon the weed, without preparing an extract, on the assumption that while adhering sulphate would undoubtedly throw out the ratio, indications of a distinctly higher proportion of sulphate on hydrolysis would be reliable evidence of the presence of ethereal sulphate. Although the method gave valuable indications in some cases it cannot be recommended owing largely to the fact that many weeds give a strongly alkaline ash, which tends to retain some of the sulphuric acid that should be lost on incineration; this is particularly the case with *Laminaria* (p. 703).

The weeds which have been examined for the presence of ethereal sulphate were all selected for their relatively high ash content and the high proportion of sulphate in their ash and it may be safely assumed that this is a reliable criterion for judging of the likelihood of such combinations occurring in any new plant material.

In deciding from the results of an analysis whether a substance contains ethereal sulphate, undue importance should not be attached to the establishment of the exact ratio 2 : 1; the deciding factor should rather be that the dialysed product yields on hydrolysis a relatively high proportion of sulphate and that this proportion exceeds that found in the ash.

Evidence of the occurrence of phosphoric acid in organic combination has also been obtained in the case of *Chondrus*, but here it is of course not possible to rely upon the difference in the amounts found on hydrolysis and incineration since phosphoric acid is not volatile; the evidence is therefore based entirely upon the establishment of phosphate in a dialysed solution after hydrolysis.

EXPERIMENTAL.

Chondrus crispus.

During the dialysis of the hot and cold extracts (H.E. and C.E.) of carrageen it was noticed that these substances, even when free from dialysable salts, drew a very considerable volume of water into the dialyser indicating that they must exert a definite osmotic pressure. To verify this, solutions of pure C.E. and H.E. of the same strength were placed in simple osmometers consisting of thistle funnels with pig's bladder stretched across them. In a short time a rise was observed in each case which was maintained for as long as the tube was left in position, a matter of about a week. A very much greater rise was observed in the case of the C.E. than in that of the H.E.

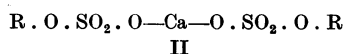
To obtain some measure of the osmotic pressure exerted by the C.E. in aqueous solution a freezing point determination was made with the following results:

A 1.5 % solution gave $\Delta = 0.045^\circ$ which corresponds to an osmotic pressure of 0.54 atmosphere.

Furthermore measurements kindly carried out for us by Mr F. C. Harwood showed the molecular conductivity at the same concentration to be 65.1 and that at infinite dilution to be 110, giving a value for the degree of ionisation of $\alpha = 0.59$.

A calculation of the minimum molecular weight of the C.E. from its calcium content of 4 % gives the value 1000, while from the depression of 0.045° the value 620 is obtained, which is in fairly close agreement with value 590 obtained by multiplying the theoretical molecular weight of 1000 by the degree of ionisation 0.59.

The evidence obtained from the conductivity measurements moreover points to a basicity of 2 for the acid radicle combined with the calcium, which lends support to the formula I (p. 697) previously ascribed to this substance in preference to the possible alternative formula II



With regard to the question of the occurrence of ethereal phosphate estimations were made on two occasions of the amount of phosphate in solutions of carefully dialysed H.E. when it was found that the amount contained in this material was 0.11 % calculated as P_2O_5 . We have however some hesitation in putting forward this figure as the correct value for the amount of phosphoric acid in the material, since we have experienced great difficulty in attempting to hydrolyse quantitatively samples of pure sodium glycerophosphate, a substance of relatively simple constitution when compared with H.E. Similar difficulty in hydrolysing ethereal phosphate has been experienced by Robison [1922].

Ceramium rubrum.

For this weed, as for all others, the ash content varies according to the amount of washing it has received previous to analysis, as may be seen from the following figures obtained from different samples: 11.58, 12.32, 13.05, 13.88, 25.41; it must however be borne in mind that excessive washing is to be avoided owing to the danger of losing some water-soluble constituents other than salts.

In the first instance a direct analysis of the weed was made without extraction; the sample selected was the fourth one quoted above which had been collected in the neighbourhood of Plymouth during the month of May. The following figures were obtained:

	%
Ash in the crude weed	13.88
SO ₄ in above ash	15.63
SO ₄ in weed by hydrolysis	4.13
SO ₄ " " incineration	2.17

It was then decided to examine an extract of the weed and for this purpose a sample obtained from Port Erin during the month of April was heated with water in an autoclave for one hour at 110°; after filtering, the residue was heated once more with a fresh quantity of water and the combined extracts were dialysed and evaporated; the dried material, weighing about 10 % of the air dry weed, forms clear light yellow gelatinous scales which swell up considerably in cold water and dissolve completely on standing without the application of heat.

Samples of the dialysed material from different sources gave the following figures on analysis:

	%	%	%
Ash in the extract	9.2	8.35	7.88
SO ₄ in the above ash	36.03	37.62	42.76
SO ₄ in the extract (by hydrolysis)	8.18	—	8.85
SO ₄ " " (by incineration)	3.31	3.14	3.37

It will be seen that in this case the ratios of the two sulphate estimations in the first and third cases are 2.47 : 1 and 2.62 : 1 respectively; as explained above this may be due either to the presence of ammonium groups or to the occurrence of a true organic ester; the matter is still under investigation.

To determine whether the substance has any osmotic properties a solution was prepared from 1.2 g. of the extract in 70 cc. of water. Placed in an osmometer there was a rise of 9.3 cm. in seven days thus showing that the substance exerts a distinct osmotic pressure.

Delesseria sanguinea.

In this case the weed, collected near Plymouth in May, was rapidly washed in two or three changes of water and then dried. Analysis of the air dry material gave the following figures:

	%
Ash in the washed weed	16.83
SO ₄ in above ash	44.15
SO ₄ in the weed (by hydrolysis)	14.47
SO ₄ " " (by incineration)	7.43

An extract of the above was prepared by heating for two hours in an autoclave at 110°; the mucilaginous solution on evaporation yielded clear gelatinous scales which are readily soluble in water. Analysis of the dialysed material gave the following results:

	%
Ash in the extract	16.01
SO ₄ in the above ash	68.39
SO ₄ in the extract (by hydrolysis)	23.84
SO ₄ „ „ (by incineration)	10.68

Osmotic properties were established by means of the osmometer in which a solution containing 1.5 g. of dialysed extract in 70 cc. of water registered a rise of 3 cm. in 40 hours. These facts together with the ratio of 2.23 : 1 for the two sulphates confirm the presence of ethereal sulphate.

Delesseria alata.

In this case only the weed itself was analysed. The material was kindly collected for us at Port Erin in July by Miss Knight, of Liverpool University. Analysis gave the following results:

	%
Ash in the washed weed	12.24
SO ₄ in the above ash	33.84
SO ₄ in the weed (by hydrolysis)	9.77
SO ₄ „ „ (by incineration)	4.17

Here again the excess of sulphate obtained by hydrolysis over that found on incineration proves the presence of ethereal sulphate.

Polysiphonia fastigiata.

The ash content of this weed again depends very much upon the amount of washing, as is shown by the following figures obtained from the same sample before and after washing:

	%	%
Ash before washing	24.88	28.19
Ash after washing	9.34	9.99

Analysis of an unwashed sample gave the following figures:

	%
Ash in the unwashed weed	24.88
SO ₄ „ above ash	24.84
SO ₄ „ unwashed weed (by hydrolysis)	11.80
SO ₄ „ „ „ (by incineration)	6.18

An extract was made of a sample of weed kindly collected for us near Folkestone in the month of March by Mr Chater of this Department. Heated at 105° for one hour the weed yielded to water about 20 % of its weight, giving a mucilaginous solution which on evaporation left light brown shiny transparent scales.

The substance is somewhat sparingly soluble in cold water but dissolves readily on warming.

An analysis of this material after dialysis gave the following figures:

	%
Ash in the extract	13.95
SO ₄ " above ash	61.89
SO ₄ " extract (by hydrolysis)	21.65
SO ₄ " " (by incineration)	8.63

The remarkably high percentage of sulphate in the ash is noticeable and also the fact that the ratio of sulphates is 2.5 : 1. It is intended to investigate this matter further.

Plumaria elegans.

This weed, of which we have not seen any previously published analyses, is characterised by an ash containing a high proportion of silica as is shown by the following:

	%	%
Ash	17.5	17.09
Silica in ash	49.76	49.61

Extracted in an autoclave at 110° the weed yields about 10 % of its weight to water. The extract evaporated to dryness over a water-bath leaves dark very brittle scales which are only partially soluble again in water even on boiling; if however the extract is evaporated in a vacuum at room temperature the resulting scales behave quite differently in water, swelling up and going into solution on standing to form a mucilaginous liquid.

Analysis of the dialysed extract of a sample obtained from Port Erin in the summer gave the following:

	%
Ash in the extract	15.94
SO ₄ in the above ash	41.41
SO ₄ in the extract (by hydrolysis)	9.07
SO ₄ " " (by incineration)	6.6

Ascophyllum nodosum.

This weed was selected from among the Phaeophyceae on account of the high proportion of sulphate in its ash. The material here examined was kindly collected for us by Mr Chater near Folkestone during January. Extraction with water in an autoclave at 110° yielded a brown mucilaginous solution which was dialysed until free from adherent salts. On evaporating over a water-bath there remained opaque horny scales of a light chocolate brown colour which were insoluble in water; when however the liquid was evaporated in a vacuum at the temperature of the room the resulting solid was found to be completely soluble in cold water. The substance exhibits in a very marked degree the phenomenon of adhesion, and if dried on a glass or silica dish the scales in cracking off remove large flakes from the surface. The residual weed after exhaustive extraction with water in the autoclave still yields a quantity of substance to 5 % sodium carbonate from which it is precipitated on the addition of acid; this substance, which is presumably alginic acid, would appear to be distinct from the water-soluble

extract here described. An analysis of this substance gave the following figures:

	%
Ash in the extract	16.98
SO ₄ „ above ash	55.34
SO ₄ „ extract (by hydrolysis)	11.41
SO ₄ „ „ (by incineration)	9.40

The presence of ethereal sulphate may be deduced from these figures in spite of the fact that the ratio of the two sulphates is only 1.2 : 1, since the known high alkalinity of the ash of this weed readily accounts for the large amount of sulphate obtained on incineration.

Corroborative evidence for the existence of an ionised salt, *i.e.* of ethereal sulphate, was furnished by placing a 1.7 % solution of the dialysed material in an osmometer when a rise of 17.5 cm. was produced in three days.

Laminaria digitata.

The following analyses were obtained from a number of samples of the weed collected at different times and places:

	I	II	III	IV ¹
	%	%	%	%
Ash in the weed	32.36	48.99	13.53	13.9
SO ₄ „ above ash	9.97	5.78	25.63	24.71
SO ₄ „ weed (by hydrolysis)	3.12	2.11	2.69	2.15
SO ₄ „ „ (by incineration)	3.22	2.83	3.46	3.43

Attention may be drawn to the striking difference in ash content between the samples, due no doubt to a difference in the amount of preliminary washing; furthermore in consequence of the large amount of saline impurities, presumably other than sulphate, in samples I and II the percentage of sulphate calculated upon the ash is in these cases comparatively low.

It is worthy of note moreover that in all cases the percentage of sulphate on hydrolysis is actually rather less than that on incineration; nevertheless the presence of an appreciable amount on hydrolysis suggests the possibility of ethereal sulphate. It seemed probable therefore that the ratio might have been thrown out by the incineration sulphate being too high, owing to the alkalinity of the ash. That this explanation is probably correct was shown by a comparison of the alkalinity of the ash of the weed with that of the ash of the extract. For this purpose samples of both weed and extract were incinerated and aqueous extracts of equal weights of the two ashes were titrated against *N*/50 hydrochloric acid with the following results:

	<i>N</i> /50 acid required
Alkali from 0.0388 g. of ash of weed	5.85 cc.
„ „ „ extract	0.24 cc.

Further confirmation was obtained by the analysis of a dialysed autoclave extract of the weed whose analysis is quoted above under column IV. This sample gave the following results:

	%
Ash in the extract	17.67
SO ₄ „ above ash	50.19
SO ₄ „ extract (by hydrolysis)	15.17
SO ₄ „ „ (by incineration)	8.87

¹ This sample was kindly collected for us at Menai Bridge in May by Miss Purvis of University College, Bangor.

It will be seen how different a result is obtained from an analysis of the weed itself and from an extract thereof; the ratio of the two sulphates is now 1.71 : 1 owing no doubt mainly to the lower alkalinity of the ash permitting a much larger proportion of the sulphate to escape on incineration; furthermore the removal of the extraneous salts by dialysis has doubled the percentage of sulphate in the ash as compared with that of the weed. The presence of ethereal sulphate is therefore established in the water-soluble extract of *Laminaria*.

Cetraria islandica.

This lichen was selected for comparison with the above mentioned algae not because it was suspected of containing ethereal sulphate but owing to the general similarity in the physical properties of its aqueous extract with those obtained from these algae.

The following figures were obtained by analysis of a commercial sample of "Iceland Moss"; the material was air dried and not washed.

	%
Ash in the crude lichen	3.58
SO ₄ " above ash	4.53
SO ₄ " lichen (by hydrolysis)	0.02
SO ₄ " " (by incineration)	0.16

From the above figures it is clear that this material contains no ethereal sulphate grouping and incidentally it is shown that the gelatinising properties of the substance are in no way dependent upon the existence of such a grouping.

In order to ascertain whether the mucilaginous substance extracted from Iceland moss exerted any osmotic pressure an extract was prepared and dialysed; it was then evaporated and a 1.5 % solution was placed in an osmometer but no rise whatever was recorded after three days.

SUMMARY.

1. From the evidence furnished it appears that there exist among the marine algae a number in which calcium, and probably sodium and potassium, sulphate occur in chemical combination with an organic complex in the form of an ethereal sulphate.

2. The presence of such ethereal sulphates is established by a comparison between the amounts of sulphate contained in the ash and in the hydrolysed solution of the material concerned.

3. For the purpose of such a comparison it is best to employ a dialysed aqueous extract, although in some cases conclusive evidence is furnished by an analysis of the weed itself.

4. The ethereal sulphates above referred to have their metallic constituent freely ionised but the sulphate is masked by being in combination with a complex colloidal aggregate; they accordingly belong to the group of colloidal

electrolytes and as such have a measurable conductivity and exhibit osmotic phenomena.

5. Attention is drawn to the occurrence in the cell wall of a mucilaginous material having osmotic properties, and the question of its significance to the plant is thereby raised.

In conclusion the authors wish to express their indebtedness to the Directors of the Marine Biological Stations at Plymouth and Port Erin for kindly obtaining for them supplies of much of the material required for this investigation.

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