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by

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Title

# Investigation of the Polysaccharide Content of Irish Moss (Chondrus crispus).

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# INTRODUCTION.

The occurrence of ethereal sulphates among products of animal origin is not uncommon, but it would appear that Haas and co-workers (1), working on Carrageen or Irish Moss (Chondrus crispus), were the first to prove the existence of carbohydrate esters of sulphuric acid among plant products.

Carrageen or Irish moss is a dark purple, branching, cartilaginous seaweed, which is found on the coasts of Northern Europe and North America. When dried and bleached, it forms the Irish Moss of commerce, which is being used extensively as a thickener of jams and jellies. It is also claimed that it has value as a food-stuff in diseases of the lungs and general weakness. Carrageen is used in many patent medicines and is also applied as a size and for the stiffening of silk.

Various views have been put forward from time to time concerning the composition of Carrageen mucilage. The discordant results obtained in the early days, were mainly due to the fact that, while some investigators worked on the weed as such, others examined the aqueous extract. In all cases the early workers contented themselves with hydrolysing the material with mineral acids or oxidising with nitric/

nitric acid. Bente (2) found that, on heating the moss with acids, laevulinic acid was prepared. Flückiger and Obermayer (3) showed that oxidation with nitric acid gave mucic acid. Haedicke, Bauer, and Tollens (4) examined the effect of hydrolysis with dilute acids and succeeded in isolating 2 g. of a crystalline hexose from 500 g. of the weed. This hexose gave a good yield of mucic acid and it was therefore concluded to be galactose. These workers also stated that fructose was present on the basis of a positive Seliwanoff reaction and the fact that laevulinic acid could be obtained on hydrolysis of the moss with acid. On further examination of the hydrolysis products of Carrageen, Müther and Tollens, (5) obtained a slight "furfural reaction" with aniline acetate after hydrolysis with hydrochloric acid, which indicated the presence of a small quantity of pentose or methyl pentose. More evidence was given for the presence of fructose by the isolation and identification of a phenylhydrazone of methyl hydroxy furfural, which according to Lintner, Düll and Kiermayer (6), indicated the presence of fructose. Glucose also appeared to be present.

Working along these lines, Tollens and his pupils/

pupils have shown that the Carrageen polysaccharide contains galactose and fructose residues, the latter in such quantity as to induce Tollens (7) to place Carrageen among the fructosans, without, however, assigning to it any definite formula. Sebor (8), on the other hand, went a little further in expressing the opinion that Carrageen mucilage is a complex carbohydrate produced by the combination of galactose, dextrose, and laevulose residues, together with a small quantity of pentose as an impurity.

It has long been known that Carrageen on incineration yields a considerable amount of ash consisting largely of calcium sulphate. Although it was found impossible to reduce the ash content by dialysis, the possible significance of this fact escaped all investigators until comparatively recently. In 1921, Haas and Hill (1) showed that two distinct fractions could be obtained by the aqueous extraction of the moss, and in the same year Haas (9) published a method of separating the two, together with a systematic investigation of one of them. He found that, while one extract was readily soluble in both cold and hot water, the other was readily soluble in hot water, but only sparingly soluble/

soluble in cold water. The method of extraction was based on this difference and was on the following lines. The hand-picked weed was washed once or twice with cold water and then soaked in distilled water for 1 hour. The aqueous extract was then filtered, and, on evaporation to dryness, the filtrate gave a residue, which was referred to as the Cold Extract or C.E. The remaining weed was washed continuously for several days and, after squeezing out and drying, it was warmed on the water-bath with water. The resulting solution, after filtration and evaporation, yielded the so-called Hot Extract or H.E. The distinctions between the two extracts were confined to physical characteristics, such as the different solubilities and different gelatinizing powers.

Haas (9) also showed that the two extracts had high ash contents, the C.E. having 21.6% and the H.E. averaging 17.6%. These figures could not be reduced, even on prolonged dialysis. A systematic investigation of H.E. was then made and the following facts were discovered:-

1) The ash was found to consist largely of calcium sulphate.

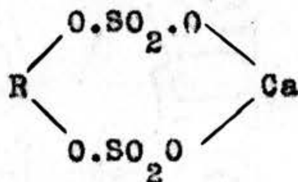
2) The calcium could be quantitatively precipitated from an aqueous solution of the H.E. using ammonium oxalate/

oxalate and the estimations, so carried out, agreed well with those made on the ash.

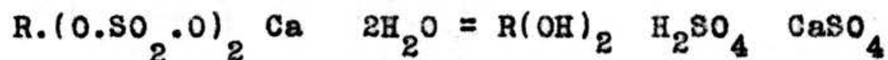
3) No precipitate of barium sulphate could be obtained on adding barium chloride to an aqueous solution of the H.E. until hydrochloric acid was added and the solution boiled for a few minutes. A copious precipitate was then obtained.

4) The sulphate in the ash of H.E. was half that obtained after hydrolysis of the H.E.

This showed that it had the general properties of an ethereal sulphate and Haas assigned to it the following general formula.




It can be seen that, on ignition, half of the sulphate would be lost as sulphuric acid and this accounts for the difference obtained in the sulphate content of the ash and that obtained after hydrolysis of the H.E.



$$R.(O.SO_2.O)_2\ Ca \quad 2H_2O = R(OH)_2 \quad H_2SO_4 \quad CaSO_4$$

Haas and Hill also noted that both the C.E. and H.E. contained nitrogen, but the form this took in the compounds was not specified.

Russell-Wells (10) made a comparison of the two extracts/

extracts both as to their organic and inorganic constituents. Using the Cold Extract (C.E.), a similar series of results to those obtained by Haas working on the H.E. was obtained and thus the same general formula for an ethereal sulphate was assigned to the C.E. Russell-Wells found that the ratio of sulphate after hydrolysis to the sulphate in the ash was slightly more than the 2:1 ratio found in the case of H.E. This could be explained by the assumption that some of the sulphate was combined with ammonium instead of calcium. She found that the C.E. contained calcium and ammonium radicals and that the ash contained besides calcium and sulphate, sodium, potassium and traces of iron. The ash of H.E. also contained these radicals, but less sodium and potassium and more calcium than that of the C.E. Unionised magnesium was found in both extracts. A comparison was made between the organic constituents of the two extracts and it was found that more mucic acid and less oxalic acid was obtained from C.E. than from the H.E. Russell-Wells also found that pentose units were present in both extracts but to a greater extent in C.E.

Harwood (11) subjected the C.E. to a physico-chemical examination with a view to throwing some light/

light on its constitution. This author showed that it was strongly ionised in solution and its conductivity at infinite dilution was found to be of the same order as that of calcium sulphate and from this he concluded that the colloidal ion of the C.E. must possess a mobility similar to that of the sulphate ion. His results were in favour of Haas's view that the extract was a calcium salt of a sulphuric ester but as to the basicity of the acid, the physical evidence was contradictory.

In a further paper Haas and Russell-Wells (12) investigated the hydrolysis of Carrageen mucilage. This was the outcome of an attempt to isolate the carbohydrate complex free from sulphate. It was found that conditions which favoured the separation of the sulphate from the H.E., involved the complete breakdown of the carbohydrate complex. For a long while, it had been known that the mucilage lost its gelatinizing power when heated with dilute acids such as lemon juice or vinegar. Haas and Russell-Wells found that if a 2% solution of H.E. was heated at 80° for 45 minutes with 0.15 N sulphuric acid the resulting liquid had acquired reducing properties, but contrary to expectations, this was not due to the production of free sugar, but to the formation of a/

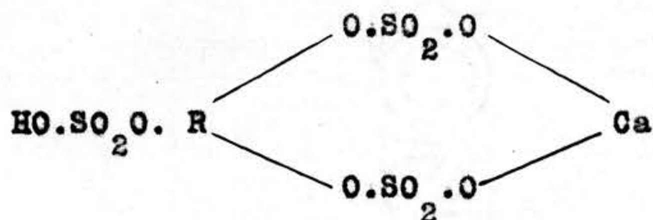
a non-dialysable product, which proved to be an ethereal sulphate. The solution, obtained after hydrolysis, was found to contain two ethereal sulphates, which could be separated by dialysis. The residue left over after dialysis was shown to contain galactose, fructose and pentose. The dialysate gave colour reactions for pentose and fructose but, on oxidation with nitric acid, no mucic acid was produced showing the absence of galactose. It did not reduce Fehling's solution till after hydrolysis and was optically inactive.

The presence of glucose in Carrageen had been postulated by Sebor (8) and subsequently by Muther and Tollens (5), on the basis of the analysis of the silver salt of a presumed saccharic acid. This was an inconclusive piece of evidence as the silver content of silver saccharate is clearly the same as that of silver mucate and this result provides no real evidence for the presence of glucose. As both the dialysate and the residue gave the Seliwanoff test for ketose (evidence on which presence of fructose was based), Haas and Russell-Wells (12), destroyed the fructose by hydrolysing with 5% hydrochloric acid both the dialysate and the residue until a positive Seliwanoff test was no longer obtained. The /

The resulting solutions were treated with phenylhydrazine and the residue alone gave glucosazone. This could only have been obtained from glucose (or mannose). Potassium hydrogen saccharate was also isolated from the potassium salt of H.E. after the mucic acid, formed by the oxidation of the H.E. with nitric acid, had been removed. A further attempt was made with various strengths of sodium, potassium and barium hydroxides to hydrolyse the sulphate from the carbohydrate complex without degrading the latter. As in the case of the acid hydrolysis, it was found that any attempt to remove the sulphate resulted in a complete breakdown of the carbohydrate portion to simple sugars. In the case of the alkali hydrolysis, however, it was found that a quantitative separation of sulphate could not be effected even on boiling directly with 5% sodium hydroxide for 6 hours.

On the ground that the fractionation of the extract, as suggested by Haas, appeared to be artificial as neither of the fractions C.E. or H.E. appeared to be pure chemical compounds, a Canadian investigator Miss M.R. Butler (13) adopted a different procedure of extraction and obtained a so-called standard extract. This was obtained by heating the moss, washed free from chloride, with distilled water/

water on the water-bath and the filtered extract was concentrated and precipitated in alcohol. A white, amorphous powder was obtained with an ash content averaging 18.6%. The ash was found to contain varying quantities of potassium, up to 12%. Butler found that the ratio of the sulphate after hydrolysis, to the sulphate in the ash was nearer 3 : 1 than the ratio found by Haas in his investigation of H.E. (9), viz. 28.3% for the sulphate after hydrolysis and 11.5% for the sulphate in the ash. This suggested to her the possibility of there being three sulphate groups in the molecule, only one of which was retained in the ash, and the following possible formula was suggested.



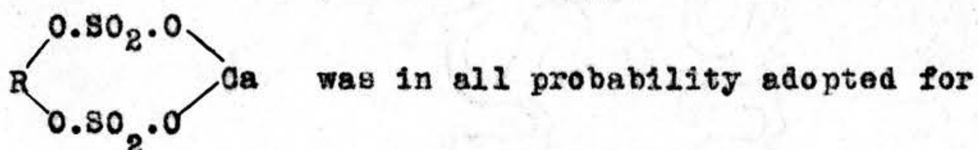

By dialysing a 1% solution of the extract against a frequently changed solution of potassium chloride until the dialysate gave a negative test for calcium, Butler prepared a potassium compound which, on incinerating, gave an ash of practically pure potassium sulphate. In the same way the ammonium salt was prepared. The potassium compound, dialysed/

dialysed against a frequently changed solution of calcium chloride, gave a pure calcium compound. The analytical figures for this preparation differed very greatly from those of the original extract and, from this, she concluded that Haas's assumption, that the original extract was wholly a calcium salt, or even chiefly so, was false. By examining the pure potassium and calcium compounds, she found that the ratio 2:1 of the sulphate after hydrolysis to sulphate in ash was re-established. The fact that this ratio was nearer 3:1 in the case of the original extract was explained by saying that the extract probably consisted of a mixture of calcium, potassium, and ammonium ethereal sulphates.

The sulphate content of the pure potassium salt was 28%. On the basis of the formula $R(O.SO_2.O.K)_2$, the molecular weight (presumably of the simplest unit) would be 685 or that of the carbohydrate portion 415. Based on the formula $R(O.SO_2.O.K)_3$ the molecular weight would be 1030 or that of the carbohydrate portion 625. Butler stated that the nature of the compound indicated that it was more complex and this led her to suggest the following alternative formulae as representing the constitution.

$$\left[R(O.SO_2.OK)_2\right]_n \qquad \left[R(O.SO_2.OK)_3\right]_n$$

It is clear, however, that the formulation $R.O.SO_2.O.K.$ would on the above basis give rise to a 'molecular weight' for R of ca. 200 which would be more closely in agreement with R as a monosaccharide residue. Nelson and Cretcher (14) pointed out for the polysaccharide isolated from Macrocystis pyrifera, that the results for such ethereal sulphates could equally well be explained by a formula representing a chain of hexose units each carrying one sulphate residue. In any case the original idea of Haas




was in all probability adopted for convenience on account of the divalency of calcium and the objections to it raised by Nelson and Cretcher seem to rest on a misunderstanding as to the nature of R.

In a later paper Miss Butler (15) has investigated the nitrogen content of Carrogeen and it was found that the amount of nitrogen present in the polysaccharide complex was dependent on the amount originally present in the plants, viz. 13% of the total. Repeated precipitation failed to reduce the nitrogen content of the extract. The mode of occurrence of the nitrogen was unknown.

Since/

Since the discovery that the polysaccharide of Carrageen was an ethereal sulphate, several of the marine algae have been shown to contain ethereal sulphates of polysaccharides. Haas and Russell-Wells (16) obtained evidence of the existence of an ethereal sulphate grouping in Laminaria spp. and Bird and Haas (17) showed that this grouping also exists in fucoidin, which is a water soluble carbohydrate, first named and isolated by Kylin (18) from various species of Laminaria and Fucus. A corresponding water soluble carbohydrate was isolated from Macrocystis pyrifera by Hoagland and Lieb (19) and later this was shown to contain an ethereal sulphate grouping by Nelson and Cretcher (14).

Bird and Haas (17) described the extraction and purification of fucoidin from Laminaria spp. They extracted the weed by soaking in distilled water and, at first, Kylin's method of purification by precipitation and re-precipitation with alcohol was used (18). It was found, however, that, with the gradual removal of the salts, precipitation became more difficult and therefore, this method was abandoned in favour of purification by dialysis. The completely dialysed substance was precipitated in alcohol. The product, on incineration, was found to/

to have an ash content of 30.93% which was found to be mostly calcium sulphate. The calcium was shown to exist in the original substance in the ionised condition, as it could be quantitatively precipitated by the addition of ammonium oxalate to the aqueous solution of the extract. On the other hand, this aqueous extract gave no reaction for sulphate ion. Further evidence for the presence of an ethereal sulphate grouping was obtained by comparing the figures for sulphate after hydrolysis and the amount of sulphate contained in the ash.

Amount of sulphate obtained after hydrolysis
= 30.33%

Amount of sulphate contained in the ash
= 15.10%

Of the carbohydrate portion of fucoidin, very little is known. Kylin (18) described the presence of methyl pentose and this was confirmed by Bird and Haas (17) by the colour reaction of Rosenthaler, and by the isolation of an osazone m.p. 170° - 173°, from the products of hydrolysis of the material with 3% sulphuric acid. The preparation from Fucus spp. of the methyl pentose fucose, was first described by Gunther and Tollens (20) and by Votoček (21), and/

and by Clark (22) who hydrolysed the entire weeds after a preliminary soaking in dilute acid. Bird and Haas (17) also obtained evidence of the presence of a uronic acid to the extent of 7.3%.

Fucoidin has also been prepared from the droplets exuded from Laminaria digitata in air, by Lunde, Heen and Öy (23). Analytical data on the material indicated that it had the general formula $R(O.SO_2.OM)$, in which R was a carbohydrate radical containing 60% of fucose. Neither uronic acid nor pentose was found in this case. M is chiefly sodium, but some potassium and very small amounts of calcium and magnesium were found to be present.

The only recorded instance in which an attempt has been made to determine the structure as distinct from the composition of the carbohydrate portion of one of these ethereal sulphates, is given by Hassid's work on Irideae laminarioides (24) and (25). The plants, after thorough washing with alcohol, were extracted with water for several hours on the steam bath and then, after filtration, the filtrate was evaporated to small volume and precipitated in alcohol. The precipitate was obtained in the form of a snow white powder, after filtration and drying. Proof was obtained of the ~~the~~ presence of a sodium ethereal sulphate grouping from the following experimental/

experimental data:-

1) The sulphur in the ash (5.8%), was half the sulphur obtained after hydrolysis (11.5%).
2) The aqueous solution of the extract gave no positive test for sulphate with barium chloride.
3) The substance can be dialysed free from sodium by the electrodialysis method of Greenberg and Greenberg (26).
4) A quantitative analysis on 50 mgm of the ash obtained on incineration gave the following figures:- 34.8 mgms sulphate, 13.5 mgm sodium, and 1 mgm each of calcium and magnesium. These figures are in close agreement with the assumption that the ash is mainly sodium sulphate.

In order to find the units making up the organic portion of the molecule, Hassid hydrolysed the material using 2% sulphuric acid (24). The hydrolysis product, a syrup, was inoculated with a crystal of galactose and, on standing for several days, crystals separated out, which formed a phenyl-osazone melting at 194-196°, corresponding to galactose phenyl osazone. A quantitative determination showed that galactose was present to the extent of 54.2%. Pentose and uronic acid were shown/

shown to be absent and from this it was concluded that the carbohydrate portion of the ester, was made up solely of galactose units.

In the second paper (25), Hassid describes the acetylation of the sodium sulphuric ester of galactan (the name given to the extract) using pyridine and acetic anhydride. He obtained a product of acetyl content corresponding to a diacetyl compound. It was also found that the ethereal sulphate grouping could be removed, without destroying the carbohydrate complex, by 0.5 N sulphuric acid. Alkaline hydrolysis, using 5% baryta solution, gave a product which appeared to have undergone some decomposition and the yield of free galactan was not so good.

Hassid then succeeded in methylating the sodium sulphuric ester of galactan by the method of Haworth and Learner (27) and obtained, after two further methylations with the Purdie reagents, a product described as the dimethyl ether of the original sodium sulphuric ester. The hydrolysis of the fully methylated substance was effected with 2% sulphuric acid at 105-110$^{o}$ for 7 hours. The product of hydrolysis was reducing and did not yield a precipitate of barium sulphate on boiling with/

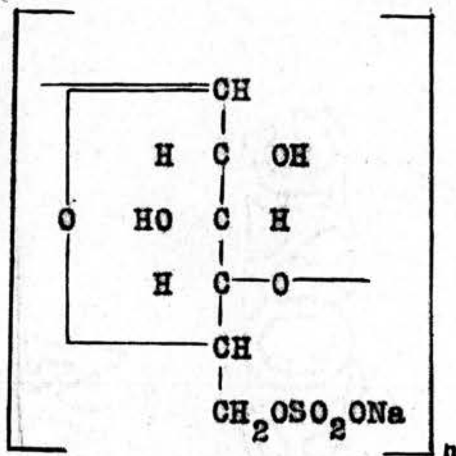
with hydrochloric acid and adding barium chloride. In this way a methylated monosaccharide was isolated containing no sulphate residue. The methylated monosaccharide was then converted into the glycoside, and a crystalline dimethyl-methyl-galactoside of unknown constitution was isolated. By methylating the galactan, prepared by acid hydrolysis of the original sulphuric ester, he obtained a methylated galactan. The methoxyl content of this corresponded with that of a tri-methyl galactan. On hydrolysing this compound, Hassid obtained on distillation an uncrystallizable syrup corresponding to a trimethyl galactose. No osazone could be prepared from this and it was therefore concluded that position 2 was occupied by a methoxyl group. On oxidation of this trimethyl compound with bromine water and then with nitric acid, a substance described as an arabodimethoxydi-methyl-glutarate was obtained. The fact that he obtained this dimethoxy compound proved, provided a pyranose ring structure was assumed, that one of the methoxyl groups was on the primary alcohol group in the trimethyl galactan. If a 1:6 linkage between the galactose units was present, then Hassid argued that a trimethyl galactose, with no methyl group/

group on position 6, should have been obtained on hydrolysis of the fully methylated galactan. On subsequent oxidation of the trimethyl galactose with bromine and then nitric acid a trimethoxy glutaric ester should have been obtained instead of the dimethoxy glutaric ester actually obtained. On this argument he rejected the idea of the linkage between adjacent galactose residues involving position 6 in the anhydro-galactose unit. The linkage between both of the first carbon atoms of the individual galactose units, whatever that may imply, was excluded on the grounds that a trimethyl compound would have been prepared on methylating the sodium sulphuric ester of galactan, instead of the dimethyl compound prepared. The 1:2-linkage between the galactose units was removed as a possibility by the fact that no osazone could be prepared from the trimethyl galactose, prepared by the hydrolysis of the fully methylated, sulphate free galactan showing that one of the methoxyl groups occupied position 2. This is, to some extent, a dangerous argument since an osazone may have been formed which was soluble in the reaction mixture and in any case a dimethyl galactosazone should have resulted (cf. the isolation of 4:6-dimethylgalactosazone/

zone from 2:4:6-trimethyl galactose (28)). Assuming the usual pyranose structure for galactose, the idea of a 1:5-linkage was rejected. This left only two possibilities, the 1:3-linkage and the 1:4-linkage. Hassid discarded the former on the ground that it had never been discovered in a naturally occurring substance, whereas the latter is common in many naturally occurring polysaccharides. Since that time, however, the 1:3-linkage between galactose units has been found in agar by Somerville and Percival (28), in damson gum by Hirst and Jones (29) and in gum arabic by Smith (30).

The position of the ethereal sulphate grouping was left in doubt but Hassid gave the probable position as that of the sixth carbon atom in the galactose unit. His argument was that the sixth carbon atom would protrude from the ring and the steric position of groups attached to this atom would tend to give them greater activity than groups attached to other atoms. Hassid determined the molecular weight of the so-called sodium sulphuric ester of galactan by the micro-chemical method of Rieche (31) and from this, he deduced that the chain length must be approximately equal to six galactose sulphate units and he gave a tentative structural formula but it is clear that it must be accepted with/

with reserve.




where n = 6 (approx.).

It can be seen from the foregoing that very little is known as to the structure of the carbohydrate portion of these ethereal sulphates and, with the exception of Hassid's work on Irideae laminarioides, no attempt has been made to determine the linkages involved in making up the complex molecules. An attempt has been made in the present investigation to help to remedy this deficiency by a study of the polysaccharides obtained from Carrageen.

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# EXPERIMENTAL

## Preparation of Carrageen Extracts.

A modification of the method described by Haas (1) was used. Instead of two extracts, however, three were prepared as follows. The moss (300 g.) purchased in 5 lb. lots from a well-known firm, was washed twice with cold water and then, after draining, steeped in water (5 l.) for 1 hour. The extract was then filtered through muslin and the filtrate concentrated at 50°/20 mm. to 300 c.c. It was then added drop by drop to ethyl alcohol (1 l.) with mechanical stirring, to yield a fibrous, greyish-white precipitate. This was dehydrated in fresh alcohol, filtered, washed with ether and finally dried in a vacuum over calcium chloride. This was the Cold Extract I. or C.E.I. (25 g.). The remainder of the moss was again washed twice with water, and soaked in water (5 l.) for a further 24 hours. The extract so obtained was concentrated and precipitated as described above to yield Cold Extract II (C.E.II 40 g.). After washing in running water for 7 days the moss was transferred to muslin bags, and the extraction was then continued in water (10 l.) on the steam bath. After 6 hours the aqueous solution was removed and fresh water was/

was added. This process was repeated twice. The combined extracts were concentrated to 300 c.c. as already described for C.E.I., to yield the Hot Extract (H.E.) (90 g.).

Investigation of the H.E.

The H.E. was non-reducing to Fehling's solution and after solution in hot water set to a rigid gel on cooling. In dilute solution, however, the optical rotation could be obtained.

$[\alpha]_D^{18^\circ} + 63^\circ$ (c, 0.3 in water).

Ash Determination.

After drying over phosphoric oxide in a vacuum at 98-100$^\circ$, about 1 g. of the H.E. was weighed accurately into a tared silica crucible and incinerated. After the last traces of carbon had been removed with nitric acid and further ignition, a few drops of sulphuric acid were added. The crucible was then heated first gradually, finally strongly to remove the excess sulphuric acid. The treatment with sulphuric acid was repeated to constant weight. The ash content so obtained averaged over six experiments 18.7%.

It is noteworthy that the ash content of the H.E. was not reduced after 6 days' dialysis against running water.

Analysis/

Analysis of the Ash.

A quantitative analysis for sulphate, calcium, potassium and sodium was made. The ash (ca. 0.2 g.) was dissolved in dilute hydrochloric acid (2N). The solution was filtered free from a slight amount of insoluble residue and made up to a 100 c.c. in a standard flask. This solution was used for all the following determinations. The determination of potassium and sulphate was made on 40 c.c. of the solution. Since potassium was to be determined as perchlorate (inaccurate in the presence of sulphate) the sulphate was first removed by precipitation as barium sulphate and determined in this way (2). The potassium could then be estimated without interference (3). Calcium was determined volumetrically (on 10 c.c.) by precipitation as calcium oxalate, filtering and after redissolving the precipitate in dilute sulphuric acid, the solution was titrated with standard potassium permanganate (4). The sodium was determined on 5 c.c. of the original solution, as sodium zinc uranyl acetate (5). The results are tabulated below:-

| | Ash | H.E. (calc. from Ash). |
|---|---|---|
| $SO_4$ | 66.6% | 12.5% |
| K | 2.5% | 0.48% |
| Ca | 29.9% | 5.6% |
| Na | 1.0% | 0.19% |

Determination of sulphate in H.E.

The H.E. was oxidised by fusion with a mixture of sodium peroxide and sodium carbonate. The fused mass was dissolved in water and the sulphate determined gravimetrically as barium sulphate (6). ($SO_4$, 23.8%).

Complete Hydrolysis of the H.E.

A method was sought whereby the H.E. could be completely hydrolysed with the removal of the sulphate groups and degradation of the carbohydrate portion to simple sugars. The H.E. (3 g.) was hydrolysed on a boiling water bath with 2N-sulphuric acid (100 c.c.) for 3 hours. The hot solution was filtered to remove inorganic salts and, after cooling, neutralised with barium carbonate in the presence of charcoal. The solution was filtered and evaporated at 50°/16 mm. to give a syrup (2 g.). $[\alpha]_D^{18°}$ +32.4°, (c, 0.5 in water). A portion of this syrup (1 g.) was treated with nitric acid (5 c.c., d 1.15) and the solution evaporated almost to dryness on the steam bath. On dilution with water and leaving to stand overnight, crystals separated m.p. 213°. A mixed melting point with an authentic specimen of mucic acid (m.p. 213-214°) showed no depression, indicating the/

the presence of galactose in the syrup. The amount of galactose present was determined by the formation of galactose methylphenylhydrazone. The syrup (0.90 g.) was dissolved in water (30 c.c.) and an equal volume of alcohol was added. To this solution methylphenylhydrazine (1.5 g.) and glacial acetic acid (0.3 c.c.) were added. The solution was left to stand for 24 hours at -3°. Crystalline galactose methylphenylhydrazone (0.33g.) was deposited, and was recrystallised from alcohol (m.p. 187°). In a similar experiment 1.553 g. galactose methylphenylhydrazone (m.p. 188°) was obtained from 1.004 g. galactose. Calculated amount of free galactose in the syrup 23.3%. In these estimations the galactose methylphenylhydrazone was dried over phosphoric oxide in a vacuum for several days.

In an attempt to raise this value, another method of hydrolysis was carried out. The H.E. (3.093 g.) which was dried over phosphoric oxide in a vacuum at 98-100°, was hydrolysed with 3% aqueous oxalic acid for 20 hours at 98-100°. The solution, after cooling, was filtered free from residue (0.53 g.) and, after neutralising with calcium carbonate, was filtered and evaporated to a syrup at 50°/16 mm. (2.46 g.). This syrup was dissolved in water (30 c.c.) and alcohol (30 c.c.)/

(30 c.c.), methylphenylhydrazine (2 c.c.) and glacial acetic acid (1 c.c.) were added. After 48 hours at $-3^{\circ}$ galactose methylphenylhydrazone (1.44g.) was obtained, corresponding to a galactose content of 46.4% in the syrup, or 36.9% in the H.E. Several of these estimations were carried out with similar results.

Typical Preparation of the "Galactose free Syrup".

After washing the galactose methylphenylhydrazone thoroughly with water the remaining sugars in the filtrate and washings were recovered according to the method of Lüdtke (7). The combined filtrate and washings were evaporated to 20 c.c. Alcohol (30 c.c.) and benzaldehyde (3.5 c.c.) were added and the solution boiled under a reflux condenser for 5 hours. The solution was then cooled in ice for 15 hours, filtered, the precipitate thoroughly washed with water, and the alcohol removed from the filtrate and washings by evaporation. The aqueous solution, so obtained, was extracted with ether, decolourised with charcoal and evaporated to dryness at 50°/16 mm. to give a syrup (1 g.) i.e. 32.1% of the original H.E. $[\alpha]_D^{15^{\circ}} + 9.7^{\circ}$ (c, 1.0 in water). All the specimens of "galactose free syrup" prepared in this way had similar properties. The Seliwanoff test for ketose was positive but the Bredereck test resulted in the development/

development of a green coloration; whereas with fructose a dark blue coloration developed quite easily. The syrup was reducing to Barfoed's reagent, appearing to show the absence of a disaccharide. Attempts to form mannose phenylhydrazone (which separates out quite easily in the cold) failed. The pine splint test for digitoxose and other 2-desoxy sugars was negative. The syrup was also tested for pentose by observation of its action on aniline acetate after hydrolysis with 12% hydrochloric acid. Slight evidence of the presence of furfural in the hydrolysis mixture was obtained. The amount of pentose present was estimated according to the method of Marshall and Norris (8). According to this estimation there was a small proportion of pentose (2.4%) and methyl pentose (1.2%) present in the "galactose free syrup".

Osazone Formation.

The "galactose free syrup" (1 g.) was dissolved in water (25 c.c.) and pure phenylhydrazine (1.5 g.) mixed with glacial acetic acid (3 c.c.) was added. Sodium bisulphite (0.1 g.) was added according to the modification of Hamilton (9). After 75 minutes at 95-100$^{\circ}$, an impure osazone was obtained (0.25 g.). On further heating a second crop was obtained (0.05 g.); very pure crystals (m.p. 205$^{\circ}$) resembling glucosazone in/

in crystalline form could be obtained from the first crop by washing with pyridine and alcohol. Mixed m.p. with an authentic specimen of glucosazone (m.p. 207-208°) gave m.p. 203-204°. The main body of the osazone was recrystallised from alcohol, pyridine and water to yield a product m.p. 206-208°. Mixed m.p. (with glucosazone) 204-206°. Mixed m.p. (with galactosazone, m.p. 195-196°) 190-195°. From this it was concluded that the osazone consisted mainly of glucosazone.

Methylation of the H.E.

An attempt was made to acetylate the H.E. using pyridine and acetic anhydride with a view to subsequent simultaneous deacetylation and methylation. The H.E. (5 g.) was shaken with pyridine (100 c.c.) for 1 hour at 80°, but showed no sign of dissolving. After cooling, a mixture of acetic anhydride (50 c.c.) was added gradually with stirring. After 3 days at 80° the H.E. remained apparently completely undissolved and on filtration, and pouring into water no precipitate was obtained. Many attempts to acetylate the H.E. varying the conditions, were abortive.

As acetylation had failed, it was necessary to turn to direct methylation. Several methods were attempted, but a modified version of that used by Baldwin and Bell in the methylation of galactogen (9) was found to give the best results. The H.E. was dissolved in water (60 c.c.) and mechanically stirred at 60°. Dimethyl sulphate (120 c.c.) and 40% potassium hydroxide (360 c.c.) were added in ⅛th portions at intervals of 2 minutes. Subsequently dimethyl sulphate (80 c.c.) and 40% potassium hydroxide/

hydroxide (160 c.c.) were added in ⅛th portions at intervals of 10 minutes. The temperature was raised to 80° for 60 minutes and the solution cooled, neutralised with acetic acid and dialysed in parchment bags against a rapid stream of running water, until free from sulphate (8 days). The dialysed solution was evaporated at 50°/16 mm. to yield a scaly solid (7.7 g.) OMe, 9.2%. This was dissolved in 40% potassium hydroxide (30 c.c.), stirred at 55° in the presence of acetone (50 c.c.), and dimethyl sulphate (60 c.c.) and 40% potassium hydroxide (150 c.c.) were added in 1/10th portions at intervals of 10 minutes. The solution was heated at 75° for 1 hour, freed from sulphate by dialysis and worked up as described above to yield a solid (7.6 g.) OMe 12.1%. Two further methylations failed to increase the methoxyl content beyond 14.2%.

Methylated H.E. was obtained as a hygroscopic solid, which was dried over phosphoric oxide in a vacuum at 98-100° before analysis. The ash )17.7%) was found to cohtain calcium (19.9%). Sulphate was detefmined by oxidation as already described for the H.E. (p. ). ($SO_4$ in methylated H.E. 27.0%).

<u>Attempted acetylation of the methylated H.E.</u>

The/

The methylated H.E. (3 g. OMe 14.1%) was dissolved in water (20 c.c.) and pyridine (100 c.c.) was added. To the homogeneous solution obtained a mixture of acetic anhydride (50 c.c.) and pyridine (50 c.c.) was added, the solution was heated at 70° for 6 hours and allowed to stand for 24 hours at 18°. The gelatinous precipitate obtained, was filtered and dried (1 g.). ($CH_3CO$ nil; OMe 14.8%).

Hydrolysis of the methylated H.E.

The methylated H.E. (7 g.) was heated with 3% aqueous oxalic acid (100 c.c.) at 98-100° for 20 hours. The solution so obtained was neutralised, after filtration and cooling, with calcium carbonate, filtered and evaporated at 50°/20 mm. to yield a syrup (5 g.) OMe 16.8%; $[\alpha]_D^{18°}$ +32° (c, 0.3 in water).

Acetylation of the hydrolysis product.

The syrup was dissolved in pyridine (50 c.c.) by warming at 60°. To the cooled solution, acetic anhydride (25 c.c.) was added and the solution heated at 98°-100° for 30 minutes. After standing for 72 hours at 16° the solution was poured into water (500c.c.) and the aqueous solution, together with/

with the syrupy solid which separated, was extracted four times with chloroform (1 l.) and the extract washed with dilute sulphuric acid (250 c.c.), then with a saturated solution of sodium bicarbonate (100 c.c.) and finally with water. After drying over anhydrous sodium sulphate, the chloroform extract was evaporated at $50^{o}$/16 mm. to give a syrup (5.6 g.).

Fractional distillation of the acetylated syrup.

The acetylated syrup was introduced into a small distilling flask and distilled in a high vacuum, the operation being carried out as quickly as possible, in order that the syrup might be exposed to a high temperature for a minimum period. The following illustrates the separation effected.

| | Bath Temp. | OMe % | Yield |
|---|---|---|---|
| 1. | $132-140^{o}$/0.03 mm. | 13.0 | 0.39 g. |
| 2. | $165-180^{o}$/0.03 mm. | 15.2 | 3.46 g. |
| 3. | $190-200^{o}$/0.03 mm. | 9.3 | 1.01 g. |

The second fraction was submitted to a further distillation with the following result.

| | Bath Temp. | OMe % | Yield |
|---|---|---|---|
| 2a. | $165-170^{o}$/0.03 mm. | 18.5 | 2.75 g. |
| 2b. | $185-195^{o}$/0.03 mm. | 9.6 | 0.47 g. |

Complete/

Complete methylation of Fraction 3.

To a portion of the syrup (0.54 g.; OMe, 9.3%) dissolved in acetone (10 c.c.) and water (5 c.c.), dimethyl sulphate (15 c.c.) and 30% sodium hydroxide (35 c.c.) were added in small portions at intervals of 10 minutes. During the first three additions at 35° the dimethyl sulphate was kept in excess. The remainder of the reagents were added at 56° after which the solution was heated at 75° for 30 minutes before extracting the reaction mixture with chloroform (1 l.). After washing with water, the extracts were dried over anhydrous sodium sulphate, filtered, and evaporated to yield a syrup, which was given three further methylations after Purdie's method. The syrup was dissolved in methyl iodide (15 c.c.) and four additions of silver oxide (4 g. in all) were made at intervals of 60 minutes, the solution being kept at 50° for 7 hours. The mixture was then filtered and the residue extracted with acetone. After the third methylation the solvent was removed to yield a syrup (0.15 g.).

The glycosidic methoxyl residue was removed by dissolving the syrup in 7% hydrochloric acid (5 c.c.) and heating at 98-100° for 2 hours. The solution was neutralised with barium carbonate, alcohol (50 c.c.) was/

was added to precipitate barium salts, which were filtered off and the solution evaporated to dryness at 50°/16 mm. The residue was extracted three times with ether and from the filtered extracts a syrup (0.11 g.) was obtained on evaporation.

Preparation of 2:3:4:6-Tetramethyl Galactose Anilide.

The anilide was prepared from the above syrup (0.11 g.) by heating with aniline (0.05 g.) in alcohol (10 c.c.) at 90° for 1 hour. On cooling white needles separated which were filtered and recrystallised from alcohol (0.05 g.; m.p. 192-193°). A mixed melting point with an authentic specimen of 2:3:4:6-tetramethyl galactose anilide (m.p. 192°) showed no depression.

Osazone formation.

A specimen of the monomethyl hexose acetate (0.42 g. OMe 9.3%) was deacetylated by Zemplen's method (10). The syrup was dissolved in chloroform (2 c.c.) and added to a solution of sodium methoxide (0.1 g. sodium in 1 c.c. methyl alcohol). The mixture was kept at 12° for 3 hours. After neutralisation with acetic acid (2N), the solution was extracted with water and the aqueous layer extracted with chloroform. The aqueous solution (20 c.c.) after addition of phenylhydrazine (0.35 g.), glacial acetic acid (0.7 c.c.), and/

and sodium bisulphite (0.1 g.) was heated at 98-100°. After 45 minutes followed by cooling, an osazone separated which was filtered and the heating continued. A second crop was obtained in this way after 3 hours.

The crude first crop (0.08 g.) had a negligible methoxyl content (m.p. 170-175°). After two recrystallisations from alcohol, pyridine, and water, the melting point rose to 189-191°, not depressed on admixture with an authentic specimen of galactosazone (m.p. 192-194°). The second crop was similar to the first, giving galactosazone on recrystallisation from alcohol, pyridine, and water.

A portion of the monomethyl hexose tetraacetate (0.5 g.) was dissolved in nitric acid (3 c.c. ; d 1.42) and the solution evaporated almost to dryness at 70°. No trace of mucic acid was found after dilution of the solution and standing for 24 hours, although under identical conditions galactose pentaacetate gave a good yield of mucic acid.

Examination of Fraction 2a.

The specimen was a pale yellow viscous syrup, reducing to Fehling's solution. $n_D^{12°}$ 1.4598; Found OMe 18.5; Calc. for $C_{14}H_{22}O_9$ OMe 18.6%.

A portion (0.5 g.) was subjected to complete methylation as previously described for Fraction 3. One/

One methylation with dimethyl sulphate was carried out, followed by three treatments with methyl iodide and silver oxide, and the product was hydrolysed with 7% hydrochloric acid to remove the glycosidic methyl group. The resulting syrup (0.20 g.) on treatment with aniline (0.10 g.) and alcohol (5 c.c.) gave an anilide (0.20 g.) m.p. 191-192°. Mixed melting point with 2:3:4:6-tetramethyl galactose anilide (m.p. 192°), 190-191°.

Osazone formation.

A portion (0.51 g.) of the dimethyl hexose triacetate was deacetylated by Zemplen's method as already described for the mono-methyl hexose tetraacetate. The first crop (0.06 g.; OMe 7.4%) was obtained after heating at 98-100° for 1 hour. After filtration, the heating was continued for another 1½ hours and a second crop was obtained (0.03 g.; OMe 7.6%). On prolonged heating (8hours in all) a third (0.01 g.) and a fourth crop (0.01 g.) was obtained. The first crop, on recrystallisation from alcohol gave a pure crystalline osazone m.p. 199-201°. Mixed melting point with an authentic specimen of 6-methyl galactosazone (m.p. 200-201°) 200-201°. Mixed melting point with galactosazone (m.p. 194-195°), 165-175°.

Analysis; Found OMe 8.0; Calc. for monomethyl galactosazone; $C_{19}H_{24}O_4N_4$ OMe, 8.3%.

This crystalline osazone was also obtained on recrystallising/

recrystallising the other three crops from alcohol.

The above result was confirmed by another similar preparation. The dimethyl hexose triacetate (0.5 g.) gave four crops of osazone (total yield 0.1 g.), which all gave 6-methylgalactosazone on recrystallisation.

The Changes in rotation during Glycoside Formation.

A portion of the dimethyl hexose acetate (0.25 g.) was deacetylated using Zemplen's method. The aqueous solution was evaporated to dryness and the residue dissolved in dry methyl alcohol. Sufficient dry methyl-alcoholic hydrogen chloride was added to bring the concentration of hydrochloric acid in the solution up to 1%, allowance being made for the sodium present.

$[\alpha]_D^{13^\circ}$ in 1% methyl-alcoholic hydrogen chloride (C, 1.1) $+43^\circ$ (5 minutes) $+32^\circ$ (19 hours), $+23^\circ$ (25 hours), $+9^\circ$(41 hours), $+2^\circ$(3 days), $-9^\circ$ (4 days), $-21^\circ$(6 days), $-27^\circ$(7 days), constant value;

Glycoside Formation on Authentic 6-Methyl Galactose.

$[\alpha]_D^{17^\circ}$ in 1% methyl-alcoholic hydrogen chloride (C, 1.0) $+54.0^\circ$ (5 minutes), $+50^\circ$(30 minutes), $+34^\circ$ (90 minutes), $+18^\circ$(2 hours), $-6^\circ$($3\frac{1}{2}$ hours), $-28^\circ$ (5 hours), $-40^\circ$ ($6\frac{1}{2}$ hours),

(6½ hours), -51°(8½ hours), -57°(21 hours), constant value;

Glycoside Formation on Galactose.

$[\alpha]_D^{16°}$ in 1% methyl-alcoholic hydrogen chloride (c, 1.1), +55°(10 minutes), +45°(20 minutes), +30° (45 minutes), +15°(2 hours), 0°(5 hours), -50°(24 hours), constant value;

Glycoside Formation on Pentaacetyl Galactose.

The pentacetyl galactose (0.50 g.) was treated as described for the dimethyl hexose acetate. $[\alpha]_D^{15°}$ in 1% methyl-alcoholic hydrogen chloride (c, 1.8) +100°(5 minutes), +91°(1 hour), +4°(19 hours), -88°(42 hours), constant value.

Attempted Lactone Formation.

A portion of the dimethyl hexose acetate (0.39 g.) was deacetylated by Zemplen's method, and the aqueous solution evaporated at 50°/16 mm. to dryness. The residue was dissolved in water (5 c.c.) and bromine (2 c.c.) was added, the solution being maintained at 35°. Tests were made at frequent intervals with Fehling's solution but even after 8 days the solution was still reducing. This unexpected result was twice confirmed.

Attempts to degrade the dimethyl hexose with concentrated/

concentrated nitric acid according to the method of Haworth, Hirst, and Stacey (10), with a view to isolating identifiable products such as *d*-or *i*-dimethoxy succinamide proved unsuccessful.

Methylation of the "Galactose Free Syrup."

The syrup (4 g.) prepared as already described was heated at 80° with 3% dry methyl-alcoholic hydrogen chloride (150 c.c.) until it was non-reducing to Fehling's solution (6 hours). Considerable decomposition took place. The solution was neutralised with silver carbonate, filtered, the residue extracted thoroughly with water and evaporated at 50°/16 mm. to yield a syrup (1.64 g.). This syrup failed to crystallise even after inoculation with α- and β-methylglucosides. The glycoside was dissolved in acetone (30 c.c.) and methylated at 50° using dimethyl sulphate (25 c.c.) and 30% sodium hydroxide (55 c.c.) in the usual way. The solution was heated at 75° for 1 hour, cooled, extracted four times with chloroform (500 c.c.). The extract was washed with water (100 c.c.), dried over anhydrous sodium sulphate and evaporated to yield a syrup (1.0 g.). This was subjected to a further methylation using methyl iodide (25 c.c.) and silver oxide (6 g.) and heating at 50° for 7 hours. After filtering, the residue was extracted 4 times with chloroform (100 c.c.) and the combined filtrates were evaporated to dryness. The syrup obtained (1.1 g.) was/

was subjected to 3 further methylations with methyl iodide and silver oxide to yield a syrup (1.1 g.).

Distillation of the Methylated "Galactose Free Syrup".

The syrup was distilled in a high vacuum as follows:-

| | Bath Temp. | OMe % | $n_D^{18^\circ}$ | Yield |
|---|---|---|---|---|
| 1. | 100-110°/0.03 mm. | 56.8 | 1.4452 | 0.27 g. |
| 2. | 110-113°/0.03 mm. | 49.6 | 1.4502 | 0.13 g. |

After the removal of these fractions the bath temperature was raised to 160°/0.05 mm. but no further distillation took place. The operation was therefore discontinued and the residue, a viscous syrup (0.60 g.), was remethylated using methyl iodide and silver oxide (6 g.). The product gave OMe 23.3%. A small portion of this syrup on boiling with 2N hydrochloric acid failed to reduce Fehling's solution. The further investigation of this fraction was discontinued owing to lack of material.

Examination of Fraction 1.

To the colourless syrup (0.23 g.), 5% hydrochloric acid (5 c.c.) was added and the solution heated at 98-100° for 3 hours. After neutralisation with barium carbonate and addition of alcohol to precipitate barium salts, the solution was filtered and/

and evaporated at $50^{o}$/16 mm. The residue was extracted four times with ether and then evaporated to give a syrup (0.2 g.), which failed to crystallise. A portion was dissolved in light petroleum bp. 60-80° and allowed to evaporate in air. The syrup, obtained, failed to crystallise even after inoculation with a crystal of tetramethylgluoppyranose.

A portion of the reducing syrup was subjected to anilide formation in the usual way. No crystalline anilide could be isolated.

Examination of Fraction 2.

Fraction 2 was evidently incompletely methylated so that the colourless mobile syrup (0.12 g.) was subjected to a further methylation with methyl iodide (10 c.c.) and silver oxide (2 g.). The product obtained (0.11 g. $n_D^{18°}$ 1.4484) was hydrolysed with 7% hydrochloric acid for 5 hours at 98-100$^{o}$ (stronger treatment than that accorded to fraction 1). All attempts, to crystallise the reducing syrup obtained (0.09 g.), failed.

A second attempt to methylate the "galactose free syrup" and isolate identifiable methylated free sugars was carried out without success.

Hydrolyses of the H.E. with Dilute Acids.

Attempts were made to hydrolyse the H.E. with varying strengths of mineral and organic acid. Using very dilute acid it was found that a great change could be brought about in the nature of the solution. From being gelatinous and non-reducing, the solution became mobile and strongly reducing. The specific rotation decreased and became constant. The H.E. (0.3 g.) was dissolved in an aqueous solution of the acid (50 c.c.) and heated at 98-100° the rotation being observed at intervals. It was found that $[\alpha]_D^{18^\circ}$ +50° (initial value) fell to $[\alpha]_D^{18^\circ}$ +15° (constant value). Using N/10 aqueous oxalic acid this change was brought about in 75 minutes, with N/20 sulphuric acid in 45 minutes, with N/75 sulphuric acid in 3 hours, and with N/100 sulphuric acid in 5 hours.

Hydrolysis of the H.E. with N/75 sulphuric acid.

The H.E. (2.43 g.) was dissolved in N/75 sulphuric acid (100 c.c.) and heated for 3 hours on the water bath at 98-100°. The solution, after filtration and cooling, was neutralised with barium carbonate. The filtrate was evaporated to 20 c.c. and added drop by drop to alcohol (500 c.c.). A white precipitate was obtained, which was filtered, dehydrated in fresh alcohol/

alcohol, washed with ether and dried in a vacuum (1.45 g.). Henceforth this will be called fraction A. The filtrate and washings were combined and evaporated at $50^{\circ}/16$ mm. to a glass, (0.45 g.). (Fraction B).

Examination of Fraction A.

Fraction A was purified by re-precipitation from aqueous solution in alcohol and was finally obtained as a hygroscopic, white powder, which was reducing to Fehling's solution and gave $[\alpha]_D$ + 32.4 (c, 0.4 in water). On boiling with hydrochloric acid and adding barium chloride a copious precipitate of barium sulphate was obtained. The ash content was determined in the same manner as that used for the H.E. and was found to average 18.9% over 6 determinations. The sulphate content of fraction A was determined by oxidation as already described for the H.E. ($SO_4$, 27.9%).

Hydrolysis of Fraction A.

Fraction A(thoroughly dried over phosphoric oxide in a vacuum at 98-100°, 2.68 g.) was dissolved in 3% aqueous oxalic acid (100 c.c.) and heated on a boiling water bath for 20 hours. The solution was filtered, cooled, neutralised with calcium carbonate in the presence/

presence of charcoal, filtered, and evaporated at 50°/ 20 mm. to yield a syrup(1.88 g.). The galactose content was determined by formation of galactose methylphenylhydrazone as described previously (p. ). The syrup (1.88 g.) gave galactose methylphenylhydrazone (1.77 g.) corresponding to a galactose content of 60.1% in the syrup or 42.7% in fraction A.

Preparation of "Galactose Free Syrup."

The filtrate and washings obtained after the preparation of the galactose methylphenylhydrazone were evaporated to 20 c.c. and treated according to the method of Lüdtke (7) to recover the galactose free syrup as already described for the H.E. (p.29). Yield 0.48 g.; 25.8% of the syrup or 17.8% of fraction A.

Attempted methylation of Fraction A.

Fraction A (12 g.) was dissolved in water and methylated as previously described for the H.E. The product after the first methylation (7 g.) gave OMe, 14.1% and that obtained after the second methylation (3 g.) gave OMe, 14.9%. This method was abandoned owing to the poor yields obtained for which no reason could be assigned.

Hydrolysis/

Hydrolysis of Fraction B.

Fraction B was reducing to Fehling's solution and gave $[\alpha]_D^{18^\circ}$ +21.9. A trace of galactose was found in the syrup but no evidence for the presence of other free sugars was obtained. No precipitate of barium sulphate was obtained on addition of barium chloride until after boiling with 2N hydrochloric acid. Fraction B (0.4 g.) was dissolved in 3% aqueous oxalic acid (25 c.c.) and heated at 98-100° for 20 hours. The solution was filtered, cooled, and neutralised with calcium carbonate. After filtration, the solution was evaporated at 50°/16 mm. to a glass (0.32 g.). The galactose content of this product was 33.3% (determined as galactose methylphenylhydrazone).

Acetylation of Fraction B.

Fraction B(2 g.) was acetylated by solution in pyridine (20 c.c.) and addition of acetic anhydride (10 c.c.), then heating at 98-100° for 30 minutes. After 48 hours at 18°, the solution was poured into water (500 c.c.), and extracted with chloroform (1 l.). The extract was washed with dilute sulphuric acid (100 c.c.) a saturated solution of sodium bicarbonate and finally with water. After drying over anhydrous sodium sulphate the extract was evaporated to yield a glass (2.1 g.).

Deacetylation/

Deactylation and attempted osazone formation on this product failed to produce any osazone.

Methylation of the Acetylated Fraction B.

The acetylated syrup (1 g.) was dissolved in acetone and methylated according to the method of Haworth and Leitch (11). To the solution, dimethyl sulphate (3 c.c.) and 30% sodium hydroxide (3 c.c.) were added in the cold, gradually and with stirring. The solution was left overnight and the remaining methylation carried out in the usual way, dimethyl sulphate (15 c.c.) and 30% sodium hydroxide (40 c.c.) being added in 1/10th portions at 10 minute intervals at 55$^{\circ}$. The temperature was raised to 80$^{\circ}$ for 30 minutes, the solution cooled, extracted four times with chloroform (500 c.c.), the extract dried and evaporated to dryness to yield a syrup (0.55 g.). This was given a further three methylations using silver oxide and methyl iodide to yield a syrup (0.48 g.). This was distilled as follows:-

Fraction 1.

0.12 G.; b.p. 100-115$^{\circ}$(bath temp.) / 0.03 mm.; $n_D^{14^{\circ}}$ 1.4458; OMe, 55.5%; $[\alpha]_D^{14^{\circ}}$ +55.5$^{\circ}$ (c, 0.5 in water).

Fraction 2.

0.05 G.; b.p. 120-130$^{\circ}$ (bath temp.) / 0.03 mm.; $n_D^{14^{\circ}}$ 1.4498;

Fraction 3/

Fraction 3.

0.07 G. b.p. 170-180$^{\circ}$ (bath temp.) / 0.03 mm.; $n_D^{14^{\circ}}$ 1.4812;

Residue, 0.40 G.

Examination of Fraction 1.

The syrup (0.10 g.) was hydrolysed with 5% hydrochloric acid (5 c.c.) for 5 hours at 98$^{\circ}$-100$^{\circ}$. The cooled solution was neutralised with barium carbonate and worked up in the usual way to give a syrup (0.09 g.), which failed to crystallise, even after extraction with light petroleum (b.p. 60-80$^{\circ}$) and allowing the solution to evaporate in air. This syrup (0.07 g.) was subjected to anilide formation in the usual way. A crystalline anilide was obtained (0.03 g.) m.p. 190-191$^{\circ}$. No depression was obtained on admixture with an authentic specimen of 2:3:4:6-tetramethyl galactose anilide (m.p. 192$^{\circ}$).

Typical Hydrolysis of the Methylated H.E. with N/75 Sulphuric Acid.

The methylated H.E. (7 g.) was hydrolysed with N/75 sulphuric acid, as already described for the H.E., to yield two fractions, one insoluble in alcohol (2 g.) (fraction C) and the other soluble in alcohol (3 g.) (fraction D).

Fraction C.

OMe, 9.4%, Ash 19.6% ; $[\alpha]_D^{17°}$ (C, 0.3 in water) nil.

Fraction D.

OMe 15.4%; Ash 15.5% ; $[\alpha]_D^{17°}$ (C, 0.6 in water) 14.4°.

Fraction C (3 g.) was remethylated using 40% potassium hydroxide (260 c.c.) and dimethyl sulphate (140 c.c.) as previously described for H.E. After dialysis the solution was evaporated to yield a glass (2.5 g.) OMe 9.4%.

This product was completely hydrolysed with 3% oxalic acid and worked up in the usual way. The syrup obtained (1.7 g.) gave OMe, 10.2%. A portion of this syrup (0.3 g.) was dissolved in water (10 c.c.) and alcohol (10 c.c.), methyl phenylhydrazine (0.5 g.) and glacial acetic acid (0.1 c.c.) were added, the solution being kept at -3° for 24 hours. A precipitate (0.05 g.) was obtained which crystallised from alcohol in white needles m.p. 187°, and showed no depression on admixture with an authentic specimen of galactose methyl-phenylhydrazone (m.p. 188°).

## Discussion.

From the literature it was found that two methods have been employed to extract the polysaccharides of Carrageen. Haas (1), taking advantage of the fact that extraction with cold water removed one constituent leaving behind a polysaccharide which could then be extracted with hot water. Butler (12) on the other hand, chose to make only one extraction with hot water, on the ground that the separation effected by Haas did not appear to give definite chemical entities. In the present work, however, a modified version of the method of Haas was used, since the physical characteristics of the cold and the hot extracts differed so widely, that the possibility clearly existed of definite differences in the chemical constitution of the polysaccharides. Three extracts were therefore prepared C.E. I, C.E. II, and H.E.

It soon became apparent that the problem presented special experimental difficulties so that it was decided to concentrate on a study of the hot extract (H.E.). Haas's conclusion (12) that the H.E. was a polysaccharide ethereal sulphate was confirmed by the following experimental evidence similar to that on which his conclusion was based:-

1)/

1) The ash content of the H.E. (18.9%) could not be reduced by dialysis so that the inorganic residues were obviously firmly bound to the rest of the molecule.

2) No precipitate of barium sulphate was obtained on adding barium chloride to an aqueous solution of the H.E. until after hydrolysis. This, combined with the fact that it was not removed by dialysis showed that the sulphate group was not ionised.

3) On the addition of ammonium oxalate to an aqueous solution of the H.E. an immediate precipitate of calcium oxalate was obtained. The ash obtained on ignition of the H.E. in the presence of sulphuric acid contained calcium (29.9%), potassium (2.5%), sodium (1.0%) and sulphate ($SO_4''$, 66.6%) showing it to consist chiefly of calcium sulphate (calc. Ca, 29.5%; $SO_4$, 70.6%).

4) The sulphate content of the H.E. ($SO_4$, 23.8%) was approximately double that of the ash ($SO_4$, 12.5%).

An attempt was then made to identify the sugars comprising the carbohydrate portion of the molecule. On complete hydrolysis with 3% aqueous oxalic acid a syrup was obtained which was found to contain galactose/

galactose to the extent of 46.4% (36.9% of the H.E.). The syrup was freed from galactose by the method of Lüdtke (7) to yield a "galactose free syrup" the yield of which was 32.1% based on the weight of the original polysaccharide. Several investigators claimed to find fructose, glucose and a pentose present (see pp. 2-3). It was found that the "galactose free syrup" gave glucosazone but this could have been obtained from either glucose, fructose or mannose. The Seliwanoff test for ketose was positive but the Bredereck test (13) was negative, so that the presence of fructose in any considerable quantity was doubtful. Mannose also appeared to be absent since no mannose phenylhydrazone could be isolated on treatment with phenylhydrazine at room temperature. A quantitative determination of the pentose content of the "galactose free syrup" showed that pentose (2.4%) and methyl pentose (1.2%) were present only to a small extent. These results appeared to agree with those of Haas and Russell-Wells (14) as indicating that d-glucose was present in the products of hydrolysis. Since tetramethyl glucopyranose is readily obtained crystalline it was hoped to/

to confirm this conclusion by methylation, separation by fractional distillation in a high vacuum of the methylglycosides obtained, followed by hydrolysis, but complete failure to secure any additional evidence by this method must be admitted. For some unexplained reason the yields obtained on methylating the "galactose free syrup" were poor and in no case, in spite of repeated methylation, could the required constants for a fully methylated methylhexoside be reached, the methoxyl content being invariably low. Hydrolysis to the free sugar failed to yield any identifiable products. It may be that the "galactose free syrup" is a complex mixture of sugars but the importance of deciding upon its composition in view of the fact that it comprises such a high proportion of the products of the hydrolysis of the H.E. cannot be denied.

Methylation of the H.E. proved to be very difficult. Acetylation having been found impracticable by the usual methods, direct methylation was attempted. Since the completion of this work, however, Dillon and O'Colla (15) have apparently succeeded in acetylating the polysaccharides obtained from Carrageen, using acetic anhydride and acetic acid, sulphur dioxide and chlorine being used as catalysts. On deacetylation of/

of the products, these workers claim to have removed the ethereal sulphate groups and to have isolated two galactans, one soluble in hot water and the other soluble in cold water. The exact details of this work have not yet been published but from the method of preparation and the fact that these substances appear to yield galactose exclusively on hydrolysis, it would seem that some degradation of the original mucilage must have taken place. After several methods of methylation had been tried, the method used by Baldwin and Bell (9) for the methylation of galactogen was finally adopted and after four methylations the product gave OMe 14.2%.

The methylated H.E. was shown to have retained the sulphate grouping by determinations of the sulphate content ($SO_4''$ in the methylated H.E. 27%) and the ash content (17.7%). The possibility of the presence of potassium sulphate or potassium methyl sulphate incorporated during the methylation process can be excluded in view of the long process of dialysis employed during the isolation of the methylated polysaccharide sulphate, which still retained a high proportion of calcium (19.9% in the ash).

On complete hydrolysis of the methylated H.E. followed/

followed by acetylation and distillation, the products obtained consisted of what appeared to be a monomethyl hexose tetraacetate and a dimethyl hexose triacetate in the proportions of <u>ca</u> 1 : 2.

The monomethyl hexose tetraacetate on complete methylation and anilide formation gave 2:3:4:6-tetramethyl galactose anilide. On deacetylation and osazone formation, the monomethyl fraction (0.5 g.) gave galactosazone (0.12 g.) 1 g. galactose gives 1.22 g. galactosazone (16) . That this galactosazone was not derived from galactose pentaacetate seemed clear since no mucic acid could be obtained on oxidation of the monomethyl tetraacetate.

Although the yield of osazone was by no means quantitative, in other cases, cf. Percival and Munro (17), Percival and Ritchie (18) when the hydroxyl group on position 2 was replaced by a methoxyl residue the yields were invariably poor.

The dimethyl hexose acetate fraction on complete methylation and anilide formation also gave 2:3:4:6-tetramethyl galactose anilide. On deacetylation and osazone formation a monomethyl osazone was obtained showing that one of the methoxyl groups occupied position 2. On recrystallization the monomethyl osazone gave 6-methyl galactosazone proving the/

the presence of 2 :6-dimethyl galactose. This result is naturally open to criticism on the ground that the starting material was a syrup and 6-methyl galactose might have been present as the tetraacetate, together with more completely methylated galactose acetates. The yield of osazone was such as to make this possibility remote because as has already been explained, the yield of osazone from sugars in which the hydroxyl group on position 2 is substituted by a methoxyl group, is always poor. No other identifiable methyl galactosazones could be detected and a search for dimethyl galactosazones proved abortive.

The possible presence of 2:4-dimethyl galactose was rendered improbable by the fact that the specific rotation changed from $[\alpha]_D^{13^\circ} +43^\circ$ to $[\alpha]_D^{13^\circ} -27^\circ$ (constant value) on standing at room temperature in methyl-alcoholic hydrogen chloride (1%). Percival and Ritchie (18) showed that the specific rotation of 2:4-dimethyl galactose $[\alpha]_D^{18^\circ} +49^\circ$ reached equilibrium $[\alpha]_D^{18^\circ} +33^\circ$ in 1 day in methyl-alcoholic hydrogen chloride (2%) although galactose and its derivatives with a free hydroxyl group on the fourth carbon atom yield a mixture of galactofuranosides with a negative rotation e.g. the 2:3-methyl galactose isolated by Robertson and Lamb (19) under the same conditions fell from $[\alpha]_D^{15^\circ} +38^\circ$ to $[\alpha]_D^{15^\circ} -24^\circ$ in 7 days. Oxidation with nitric acid followed by esterification, distillation and amide formation/

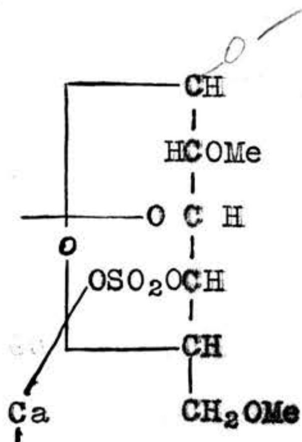
formation failed to produce either d- or l-dimethoxy succinamides just as in the case of the oxidation of 2:4:6-trimethyl galactose isolated from agar (20) so that both 2:3- and 3:4-dimethyl galactose derivatives appear to be absent.

An attempt was made to remove the ethereal sulphate from the H.E. by hydrolysis without destroying the polysaccharide complex. Hassid had found it possible to obtain a free galactan from the sodium sulphuric ester of galactan obtained from Irideae laminarioides (21) by hydrolysis with 5% sulphuric acid. In the case of the H.E., however, it was found that conditions involving the removal of the sulphate residue also involved the complete breakdown of the polysaccharide portion of the molecule as already noted by Haas and Russell-Wells (15). As an outcome of this investigation it was found that after hydrolysis with N/75 sulphuric acid (cf. Haas and Russell-Wells, p. 9), the product could be divided into two fractions, one insoluble in alcohol and the other soluble in alcohol. The fraction insoluble in alcohol formed the main proportion of the product. It was reducing but still retained the general properties of an ethereal sulphate. On methylation of this fraction the methoxyl content (OMe 14.8%) after two methylations was similar to that obtained for the methylated H.E. (OMe 14.2%). Because of the poor yield obtained after methylation/

methylation the investigation of this portion was discontinued. The fraction soluble in alcohol was obviously a mixture since it was shown to contain some free galactose (2.3%) although qualititative tests indicated that it also contained an ethereal sulphate residue.

It is only possible to draw conclusions about the galactose portion of the polysaccharide. The galactose units may be linked directly or through other anhydro-sugar units e.g. glucose, so it is difficult to advance even tentative theories as to the structure. It is clear from the non-reducing character of the H.E. that the units, as would be expected, are linked by the hydroxyl residue on position 1.

Although methylation was difficult and acetylation apparently impossible and a methoxyl content corresponding to that required for the calcium salt of a polymerised dimethyl anhydro-galactose monosulphate I was never reached, a substance having such a structure as II cannot be assumed since this would demand $SO_4$ 53.3% whereas the value found for the H.E. was $SO_4$ 23.8%.

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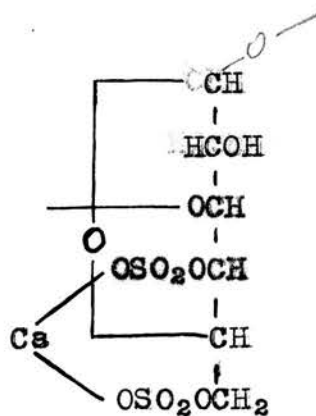
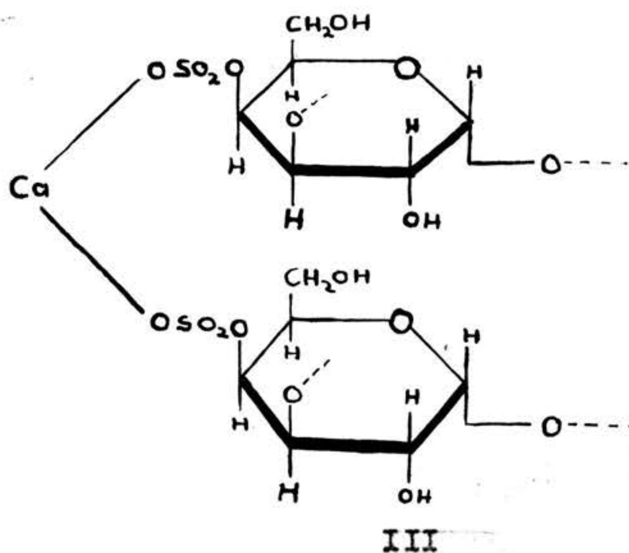



II

It is difficult to understand why with less calcium (found Ca. 5.6%) and sulphate in the H.E. than is demanded for I a methoxyl content no greater than 14.2% can be introduced (calc. for monomethyl galactose monosulphate OMe 11.3; for dimethyl galactose monosulphate OMe 21.5% - as the calcium salt). It would appear that the primary alcohol residue in some of the galactose units is difficult to methylate since 2-methyl galactose and not 6-methyl galactose is found in the monomethyl fraction. It is possible that a shielding effect on the primary alcohol group at position 6, by a sulphate residue in its neighbourhood may account for this.




III

Assuming the pyranose ring to be present and if, as appears to be the case the 2:6-dimethyl galactose is the "key" degradation product, the sulphate residue and the second link between the units must be on position 3 or position 4. The 1:3 linkage between galactose units has already been found in agar (20) in damson gum (22) in gum arabic (23) and in galactogen (9). At present this point cannot be decided and it may even be that the sulphate residues occupy different positions in different hexose units.

It would seem clear that as calcium is divalent, if I is taken as a model two anhydro-galactose sulphate residues must be linked together as shown in III. This structure may also persist in concentrated solution and may account for the gel-forming properties of the polysaccharide as well as for the difficulties experienced in acetylation and in methylation.

## Summary.

1. An investigation of the polysaccharide (H.E.) obtained by extracting Carrageen with hot water has been carried out and the presence of the ethereal sulphate groups has been confirmed.

2. Complete hydrolysis of the H.E. gave galactose (36.9%) and a reducing syrup (32.1%) from which glucosazone was isolated, but the source of the glucosazone is uncertain.

3. Unsuccessful attempts were made to acetylate the H.E. but exhaustive methylation yielded a product (OMe 14.2%) which also resisted acetylation.

4. Hydrolysis and acetylation of the methylated product gave a monomethyl and a dimethyl hexose acetate. Both these partially methylated acetates gave 2:3:4:6-tetramethyl galactose anilides on methylation, hydrolysis and treatment with aniline. Also on suitable treatment the acetates gave respectively galactosazone and 6-methyl galactosazone indicating the original acetates to be 2-methyl galactose tetraacetate and 2:6-dimethyl galactose triacetate.

5. Support for this view of the structure of the latter/

latter was found as follows. No other identifiable methyl galactosazones could be isolated, the free sugar appeared to form a galactofuranoside with cold methyl-alcoholic hydrogen chloride indicating a free hydroxyl group on position 4 and oxidation with nitric acid failed to produce dimethoxy succinamides so that adjacent hydroxyl groups were not methylated.

6. Assuming that 2:6-dimethyl galactose is the "key" degradation product of the methylated H.E., tentative suggestions are made as to the possible structure of that portion of the polysaccharide which is composed of anhydro-galactose units.

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