CARBOHYDRATE SULPHATES

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It is not always realised how widespread is the occurrence of carbohydrate sulphates in Nature. In the plant kingdom many algal polysaccharides belong to this group: the red seaweeds *Chondrus crispus* and *Gigartina stellata* contain the carragheen polysaccharides, *Dilsea edulis* and *Irideæ laminarioides* contain galactan sulphates, and the important polysaccharide agar-agar which occurs in many species of *Rhodophyceæ* is either a member or a close relative. In the still more common brown seaweeds (*Phæophyceæ*) the polysaccharide sulphate fucoidin is found.

Still greater interest is attached to the animal carbohydrate sulphates, which include the chondroitin sulphate of cartilaginous tissue, the mucoitin sulphate of the gastric mucosa which is probably identical with the hyaluronic acid sulphate of the cornea, and the blood-anticoagulant heparin. The jelly coat of sea-urchin eggs contains polysaccharide sulphates,[1] and galactose sulphates have been reported in brain lipids.

The functions of the compounds under review are not always well understood; in the red algæ the polygalactose sulphates usually make up the major part of the plant, but this is not so with the fucoidin of the brown seaweeds where the principal polysaccharide is alginic acid and considerable amounts of laminarin and mannitol occur at the proper seasons.

In the animal group the main purpose of heparin is clear, and chondroitin sulphate is believed to act as a cementing material in connective tissue, probably as a gel-like network in association with protein.[2] The animal carbohydrate sulphates in loose combination as salts with proteins,[3] as in the mucins derived from mucoitin sulphate, form viscous, slimy layers with lubricating and protective properties; furthermore, by an inhibiting action on the action of trypsin and pepsin, autodigestion of the gastric mucosa is prevented.[2]

Quite apart from their complexity as carbohydrates, it is not surprising that our knowledge of the constitution of the natural carbohydrate sulphates is scanty when one considers their distinctive physical properties, such as solubility in water to form viscous solutions in some cases, and gels in others. They also occur as mixtures of salts of several different metals and, especially in the animal group, are difficult to separate from proteins. The only means of purification are usually dialysis or electrodialysis and fractional precipitation. Progress has been delayed too by an inadequate knowledge of the properties and reactions of simple monosaccharide sulphates.

[1] E. Vasseur, *Acta Chem. Scand.*, 1948, **2**, 900.
[2] G. Blix, *Biochem. J.*, 1946, **40**, vi.
[3] Karl Meyer, *Cold Springs Harbor Symp. Quant. Biol.*, 1938, **6**, 91.

Monosaccharide Sulphates

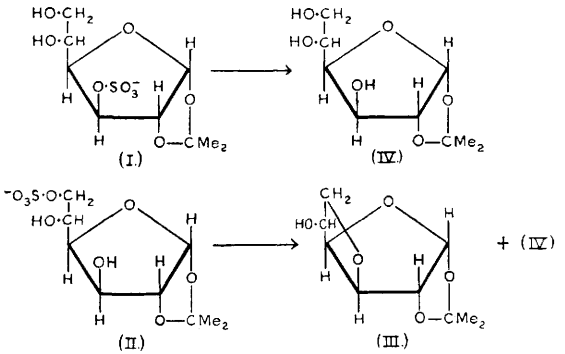
Monosaccharide sulphates are usually prepared by treating the sugar derivative dissolved in pyridine with chlorosulphonic acid,[4] sulphuryl chloride,[5] or, preferably,[6] with the pyridine–sulphur trioxide complex.[7] By choosing appropriate derivatives it is possible to prepare sulphates, usually as the barium or brucine salts, with this group in a definite position in a particular sugar molecule, and to compare the properties of such products with those of the sulphates prepared directly from the parent sugar. Thus H. Ohle [8] by the partial hydrolysis of 1 : 2-5 : 6-di*iso*propylidene glucose-3 sulphate obtained 1 : 2-*iso*propylidene glucofuranose-3 sulphate which differed from the monosulphate obtained by the direct sulphation of 1 : 2-*iso*propylidene glucofuranose. Removal of the *iso*propylidene group from the last-mentioned substance gave the glucose monosulphate which is obtained directly from glucose ; it is inferred, therefore, that the hydroxyl group on $C_{(6)}$ was sulphated in the last two preparations. On the other hand, galactose-6 sulphate prepared through 1 : 2-3 : 4-di*iso*propylidene galactopyranose was shown [9] to differ from the galactose sulphate prepared directly from galactose. Where more than one free hydroxyl group exists, owing to the vigorous nature of the sulphating agents generally used and the difficulties of isolating crystalline derivatives, the possibilities of the formation of mixtures of monosulphates and of polysulphates must be borne in mind so that it is necessary to use the direct method with caution. In any event the synthetic method of attack is only of limited application and other methods have been sought in attempts to answer the outstanding question of the location of the sulphate groups.

It has been claimed [10] that hydrolysis by acids may be used to distinguish poly- from mono-sulphates since the former are hydrolysed more rapidly, but differences in the rate of hydrolysis of selected monosaccharide sulphates with acid reagents [5, 9, 10] have been shown to be too small to act as a guide. More useful, although not by any means always decisive, is a study of the behaviour of the unknown sulphate under alkaline conditions.

The Hydrolysis of Carbohydrate Sulphates with Alkalis.—A salt such as sodium methyl sulphate is relatively stable to hot aqueous alkali, so that the observation [9] that di*iso*propylidene galactose 6-sulphate was not attacked by sodium hydroxide solution (2N.) during 6 hours at 100° is hardly surprising. On the other hand, the glucose and galactose sulphates prepared directly from the free sugars yielded all the sulphate in ionisable form within five minutes at 100° in N/10-sodium hydroxide, but since disruption of the monosaccharide residues occurred this observation is of no diagnostic value. When α-methylglucopyranoside sulphate was examined, heating

[4] C. Neuberg and L. Liebermann, *Biochem. Z.*, 1921, **121**, 326.
[5] P. A. Levene and G. M. Meyer, *J. Biol. Chem.*, 1922, **53**, 437.
[6] R. B. Duff, *J.*, 1949, 1597. [7] P. Baumgarten, *Ber.*, 1926, **59**, 1166, 1977.
[8] *Biochem. Z.*, 1922, **131**, 601 ; 1923, **136**, 428.
[9] E. G. V. Percival and T. H. Soutar, *J.*, 1940, 1475.
[10] T. Soda and W. Nagai, *J. Chem. Soc. Japan*, 1935, **56**, 1258.

with alkali caused rapid hydrolysis of the sulphate group with the production of an anhydromethylhexoside, and on further investigation [11] it was established that the sulphates of α- and β-methyl-glucopyranosides and -galactopyranosides and α-methylmannopyranoside gave the corresponding 3 : 6-anhydromethylhexosides. It was concluded, therefore, that unless the removal of the sulphate group can lead to the production of an anhydro-ring, for which a suitably placed hydroxyl group is necessary, hydrolysis of the sulphate group with alkali proceeds only slowly. Further evidence in support of this view was collected in the glucofuranose series.[12] The hydrolysis of 1 : 2-*iso*propylidene glucofuranose-3 sulphate (I) with alkali was found to be exceedingly slow—in fact just as slow as for 1 : 2-5 : 6-di*iso*propylidene glucose-3 sulphate (12% hydrolysis in 47 hours at 100° in 2·8N-sodium hydroxide). On the other hand, the corresponding 6-sulphate (II) was readily hydrolysed to give almost equal amounts of 1 : 2-*iso*propyl-

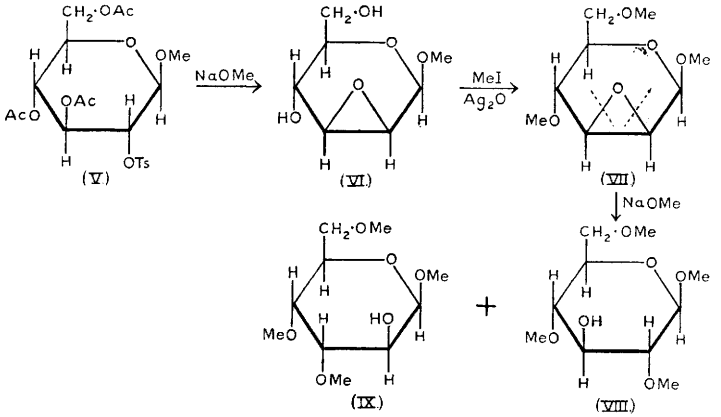


idene 3 : 6-anhydroglucofuranose (III) and 1 : 2-*iso*propylidene glucofuranose (IV).

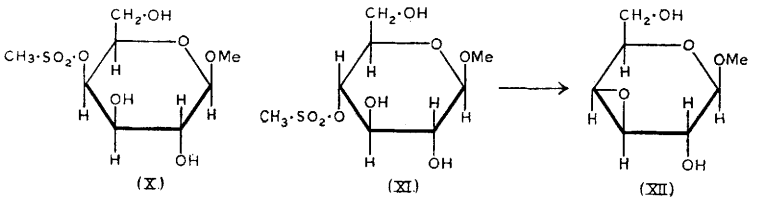
Leaving aside for the moment the fact that the primary alcoholic group in (I) is apparently suitably placed for 3 : 6-anhydride formation (3 : 6-anhydro-derivatives have been isolated from methylglucofuranoside-3 sulphates [12]), it is necessary here to draw attention to the close parallel between the behaviour of sulphates and of such sulphonic esters as toluene-*p*-sulphonates and methanesulphonates. Thus di*iso*propylidene galactopyranose 6-toluene-*p*-sulphonate, di*iso*propylidene glucofuranose 3-toluene-*p*-sulphonate,[13] and 1 : 2-*iso*propylidene glucofuranose 3-toluene-*p*-sulphonate [14] are hydrolysed with comparative difficulty by alkali and no 3 : 6-anhydride is produced from the last substance. On the other hand methylhexoside 6-toluene-*p*-sulphonates are readily converted into the 3 : 6-anhydromethylhexosides,[15] and a knowledge of the properties of these compounds was of the first importance in developing the subject under discussion.

[11] Duff and Percival, *J.*, 1941, 830. [12] Percival, *J.*, 1945, 119.
[13] J. W. H. Oldham and G. J. Robertson, *J.*, 1935, 685.
[14] Ohle and H. Wilcke, *Ber.*, 1938, **71**, 2316.
[15] See S. Peat, " Advances in Carbohydrate Chemistry ", Academic Press, New York, 1946, vol. 2, p. 37.

There is, however, a property of the sulphonic esters of even greater fundamental importance which is observed when the sulphonic ester group is adjacent to a free hydroxyl group and in *trans*-relationship to it. Treatment of such compounds with an alkaline reagent, usually sodium methoxide, causes an ethylene oxide ring to appear between the carbon atoms concerned with Walden inversion on the atom which carried the sulphonic ester residue. To give but one of many examples, 3 : 4 : 6-triacetyl β-methylglucoside 2-toluene-*p*-sulphonate (V) on treatment with sodium methoxide



gives 2 : 3-anhydro-β-methylmannopyranoside (VI).[16, 17] The dimethyl derivative (VII) of (VI), by the further action of sodium methoxide, gives 2 : 4 : 6-trimethyl β-methylglucoside (VIII) and 3 : 4 : 6-trimethyl β-methylaltroside (IX), in practically equal amounts.[17] At the point of entry of the new methyl groups Walden inversion takes place and the formation of two products shows that the ethylene oxide ring may be broken in either of the two ways indicated by the dotted line in (VII). It should

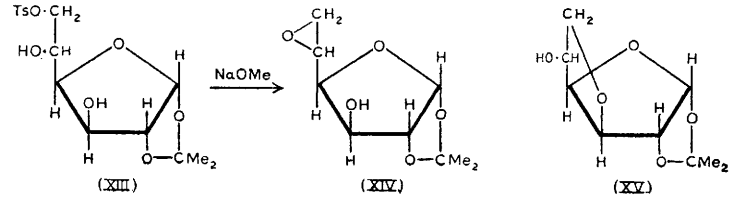


be pointed out, however, that two products cannot invariably be recognised in such a reaction, the chances of the ethylene oxide ring breaking in a particular way depending presumably on steric factors. If sodium hydroxide, instead of sodium methoxide, acts on (VII), a mixture of 4 : 6-dimethyl β-methyl-glucoside and -altroside is produced. When the sulphonic ester group is *cis*- to the neighbouring hydroxyl group no anhydride

[16] W. N. Haworth, E. L. Hirst, and L. Panizzon, *J.*, 1934, 154.
[17] W. H. G. Lake and Peat, *J.*, 1938, 1417.

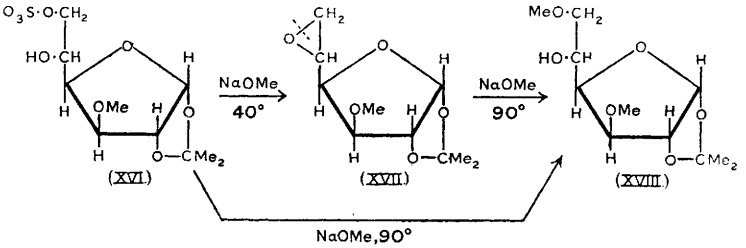
formation takes place and hydrolysis of the ester is comparatively difficult. Thus 4-methanesulphonyl β-methylgalactoside (X) is unaffected by sodium methoxide under conditions where the corresponding glucoside (XI) is readily converted into 3 : 4-anhydro-β-methylgalactoside (XII).[18]

With such examples in mind, it became of obvious interest to determine whether carbohydrate sulphates could also take part in transformations



involving the intermediate formation of ethylene oxide rings, especially as the interconversion of sugars in Nature might, in some cases, depend on such processes.

A superficial examination of the examples of the hydrolysis of sulphates already cited would appear to indicate that 3 : 6-anhydro-rings, but not ethylene oxide rings, were formed. There is evidence, however, that the formation of a 3 : 6-anhydride is in some cases preceded by the production

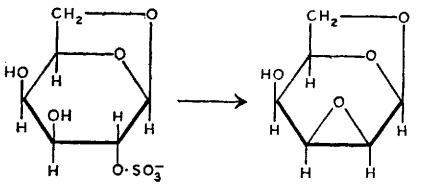


of an ethylene oxide derivative. Thus E. Seebeck, A. Meyer, and T. Reichstein [19] showed that 1 : 2-*iso*propylidene 5 : 6-anhydroglucofuranose (XIV), prepared from the corresponding 6-toluene-*p*-sulphonate (XIII), underwent a transformation into the more stable 3 : 6-anhydride (XV) even on storage in a desiccator and that, contrary to an earlier claim,[20] no L-idose derivatives could be obtained on the fission of (XIV) with alkaline reagents. Clearly, therefore, the observation that alkaline hydrolysis of the corresponding sulphate gave (XV) could be interpreted in a similar fashion. In order to arrive at a decision, barium 3-methyl 1 : 2-*iso*propylidene glucofuranose-6 sulphate (XVI) was prepared. The inclusion of a methyl residue at $C_{(3)}$ not only prevented the formation of a 3 : 6-anhydride but also conferred sufficient solubility in methanol to permit the use of

[18] A. Müller, M. Moricz, and G. Verner, *Ber.*, 1939, **72**, 745.
[19] *Helv. Chim. Acta*, 1944, **27**, 1142.
[20] Ohle and L. von Vargha, *Ber.*, 1929, **62**, 2435.

sodium methoxide as a hydrolytic agent; normally the barium salts of sulphate esters are soluble only in aqueous media. Treatment with sodium methoxide at 40° gave 3-methyl 1 : 2-*iso*propylidene 5 : 6-anhydroglucofuranose (XVII) (50% yield), and by more vigorous treatment 3 : 6-dimethyl 1 : 2-*iso*propylidene glucofuranose (XVIII) was obtained. The production of (XVIII) is explained by the fact that the entering methoxyl anion attaches itself apparently exclusively to the primary carbon atom in an ethylene oxide derivative of this type.[22]

Although the experiment outlined proves beyond doubt that the hydrolysis of a sulphate ester can give an ethylene oxide derivative, because the sulphate group was not attached to an asymmetric centre and the fission of the ethylene oxide ring takes place in only one direction, the Walden inversion so characteristic of the hydrolysis of sulphonic esters could not be observed. An attempt to settle this point by the hydrolysis of 6-methyl β-methylgalactopyranoside-2 sulphate [21] failed because of extensive decomposition with the production of reducing substances on treatment with sodium methoxide, the glycosidic methoxyl group apparently becoming labile to alkali when a sulphate group is adjacent. By taking advantage of the well-known stability to alkali of 1 : 6-anhydrides this difficulty has been overcome [6] and the analogy between the sulphate and sulphonic esters is now complete. 1 : 6-Anhydro-β-D-galactopyranose-2 sulphate (XIX) was converted by means of sodium methoxide into the known 1 : 6-2 : 3-dianhydro-β-D-talopyranose (XX).

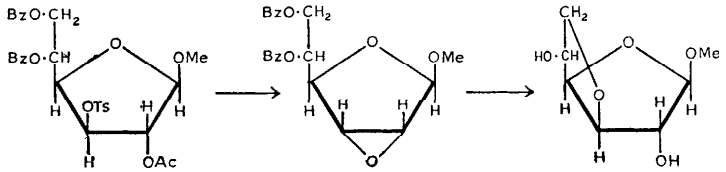


(XIX) (XX)

The failure to isolate an anhydro-derivative from 1 : 2-*iso*propylidene glucofuranose-3 sulphate (I) mentioned on page 370 can now be explained to some extent. There is no hydroxyl group available for the production of an ethylene oxide derivative, but one occurs in the most nearly comparable case of the methylglucofuranoside-3 sulphate (αβ-mixture), which was shown [12] to give 3 : 6-anhydromethylglucofuranosides together with methylglucofuranosides on hydrolysis with alkali. The toluene-*p*-sulphonates furnish a similar pair of examples,[14] for 3-toluene-*p*-sulphonyl 1 : 2-*iso*propylidene glucofuranose gives no anhydro-derivative with alkali whereas 5 : 6-dibenzoyl 3-toluene-*p*-sulphonyl 2-acetyl β-methylglucofuranoside (XXI) gives 5 : 6-dibenzoyl 2 : 3-anhydro-β-methylallofuranoside (XXII) on treatment with alkali in aqueous acetone and this with boiling sodium hydroxide (2N.) produces 3 : 6-anhydro-β-methylgalactofuranoside (XXIII).

[21] Duff and Percival, *J.*, 1947, 1675.
[22] Ohle and K. Tessmar, *Ber.*, 1938, **71**, 1843.

It will be clear from the above that the isolation of a particular 3 : 6-anhydro-derivative by the hydrolysis of an ethereal sulphate does not, of itself, establish at once the position of the original sulphate group, and it



is not always an easy task to identify small quantities of the isomers which may have been formed by the fission of any ethylene oxide rings produced at an intermediate stage. It is possible to say, however, that, if the sulphate group is readily hydrolysed with alkali, then an adjacent *trans*-hydroxyl group is present, or that the sulphate group is on $C_{(6)}$ (in a hexose) and that a free hydroxyl group is present at $C_{(5)}$ or $C_{(3)}$ or both. If the sulphate group is stable to alkali then none of these conditions applies, and, by taking into account also the results of methylation experiments, etc., it is sometimes possible to deduce the position of the sulphate group in a polysaccharide ethereal sulphate.

Natural Carbohydrate Sulphates

The representation of an unknown substance as a sulphuric ester may be deduced from the fact that, while no sulphate ions can be detected in solution until after hydrolysis, cations such as calcium are detectable, *e.g.*, by precipitation with ammonium oxalate. Another important check [23] is the fact that the amount of sulphate estimated in the whole polysaccharide either by hydrolysis or by fusion with sodium peroxide is double the amount found in the ash, *e.g.*, $2RO{\cdot}SO_3K \longrightarrow K_2SO_4 + SO_3 +$ products of combustion. In practice this 2 : 1 ratio is seldom realised because of the reduction of sulphate to sulphide during the ashing process.

Although the complete structure of a natural polysaccharide sulphate has in no case been worked out fully, some progress has been recorded in a few instances. Some of the difficulties involved in the isolation and purification of these sulphates have been mentioned already. For experimental purposes they are only stable as salts, since the free acids are relatively strong acids. Since, unlike alginic acid, the acids are soluble in water there is no simple method of isolating the free acid, apart from dialysis against mineral acids, or of converting them from the naturally-occurring mixtures of salts into the salts of a single cation. None of the salts is insoluble, so the facile double decomposition reactions like the conversion of sodium into calcium alginate cannot be applied as a means of isolation and purification. A further handicap in structural investigations lies in the difficulty of acetylation and of methylation—the former because of the presence of an inorganic ion attached to the sulphate group

[23] P. Haas, *Biochem. J.*, 1921, **15**, 469.

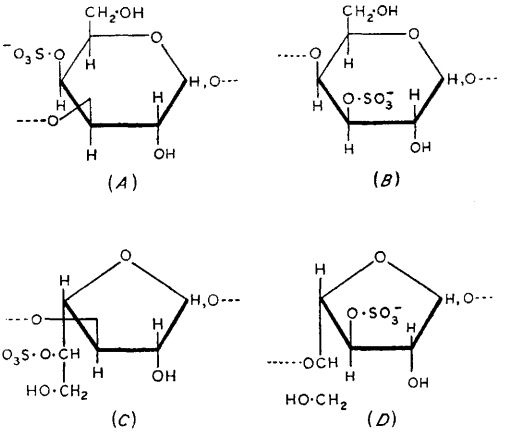
making the resulting salt difficult to disperse in pyridine, and the latter for two reasons: first, the methylated derivatives are soluble in water and insoluble in organic solvents so that dialysis is necessary for purification at each stage, and, secondly, the presence of the sulphate groups hinders the methylation process itself. There is also another difficulty. If it were possible to remove the sulphate groups without affecting the glycosidic links, an examination of the products of hydrolysis of the methylated desulphated polysaccharide, in comparison with those from the original methylated substance, would fix the positions of the sulphate groups. Unfortunately the sulphate groups cannot be removed by alkaline hydrolysis under ordinary conditions, and acid hydrolysis splits the polymeric links. The ideal process would be the use of enzymes, but apart from T. Soda's alleged glucosulphatase,[24] isolated from *Charonia lampas*, little work has been done in this field; preliminary experiments in Edinburgh with certain molluscs show, however, that some hydrolysis of sulphate groups can be induced.

Polysaccharide Sulphates of Marine Algæ.—**(a) Carragheenin.**—The material extracted by water from the red seaweeds *Chondrus crispus* and *Gigartina stellata* (Irish moss, carragheen), of some importance as a thickening and emulsifying agent and as an article of diet, has been the subject of chemical studies for at least eighty years. F. A. Flückiger [25] obtained mucic acid by oxidation with nitric acid and B. Tollens *et al.*[26] confirmed the presence of galactose. At various times the presence of fructose,[27] pentoses (or methylpentoses [28]), and 2-ketogluconic acid has been reported.[29] The work of Haas [30, 32] and of B. Russell-Wells [31] showed that two polysaccharides appeared to be present, the product extracted from *Chondrus crispus* by cold water containing less calcium, but more sodium and potassium, than the hot extract. The significant observation was also made that the sulphate residues were very stable to alkali. Nova Scotia *chondrus* extracts [33] were found to have a much higher potassium content and a 3 : 1 ratio of total to ash sulphate, attributed to the presence of ammonium salts, but by dialysis against appropriate solutions pure calcium and potassium salts were obtained which gave the correct 2 : 1 ratio.

In none of the above investigations was any progress made towards deciding the constitution of the polysaccharides concerned, but a step in this direction was made by T. Dillon and P. O'Colla [34] who, by treating carragheenin with acetic anhydride and sulphuryl chloride, isolated an acetylated galactan devoid of sulphate residues, although considerable

[24] Soda *et al.*, *Bull. Chem. Soc. Japan*, 1931, **6**, 258; 1933, **8**, 148; 1934, **9**, 83.
[25] "Repertorium of Pharmacie", 1868, p. 350.
[26] J. Hadecke, R. W. Bauer, and B. Tollens, *Annalen*, 1887, **238**, 302.
[27] F. Bente, *Ber.*, 1875, **8**, 416.
[28] A. Müther and B. Tollens, *ibid.*, 1904, **37**, 302.
[29] E. G. Young and F. A. H. Rice, *J. Biol. Chem.*, 1946, **164**, 35.
[30] *Biochem. J.*, 1921, **15**, 469.
[31] B. Russell-Wells, *ibid.*, 1922, **16**, 578.
[32] Haas and Russell-Wells, *ibid.*, 1929, **23**, 425.
[33] M. R. Butler, *ibid.*, 1934, **28**, 759.
[34] *Nature*, 1940, **145**, 749.

degradation occurred. Workers in Edinburgh [35] studying the hot- and cold-water extracts of *Chondrus crispus* demonstrated that the polysaccharides were essentially identical, although differing in their mineral constituents, and that the principal sugar unit was D-galactose.* Both extracts could be methylated with sodium hydroxide and methyl sulphate without this loss of sulphate residues, and 2 : 6-dimethyl and 2-methyl galactose were recognised as the principal products of hydrolysis of the methylated polysaccharides. The red alga *Gigartina stellata*, which closely resembles *Chondrus crispus*, was also investigated.[36] The material extracted with hot water (ash, 17·5 ; Ca, 3·7 ; Mg, 1·0 ; SO_4, 23·9%) gave on methylation a product (ash, 18·2 ; OMe, 18·6 ; Ca, 3·8 ; Mg, 0·9 ; SO_4, 24·7%) from which on hydrolysis a good yield of crystalline 2 : 6-dimethyl galactose was obtained. Assuming the sulphate groups to be linked directly to the galactose residues it is possible to decide how these are arranged in the polysaccharide, which is considered to be fundamentally the same from both sources. Clearly the hydroxyl groups on $C_{(2)}$ and $C_{(6)}$ are free, which leaves four possible structures :



Quite apart from the improbability that the units occur in the furanose form in the polysaccharide, a conclusion based on the rate of hydrolysis by acids, (*A*) is the only possible formulation, (*B*) on treatment with alkali would lose the sulphate group to form a 2 : 3- and probably also a 3 : 6-anhydride, (*C*) would give a 5 : 6- and (*D*) a 2 : 3- and 3 : 6-anhydride. In all these cases the sulphate group would be easily removed, in sharp distinction to the facts. On the other hand (*A*) could not give rise either to a 3 : 6-anhydride or to an ethylene oxide ring, and would be expected to resist alkaline hydrolysis. For these reasons it was concluded [35, 36] that

[35] J. Buchanan, E. E. Percival, and E. G. V. Percival, *J.*, 1943, 51.
[36] E. T. Dewar and Percival, *J.*, 1947, 1622.

* Recently, by chromatographic separation on a cellulose column, methylated L-galactose derivatives have been isolated from the hydrolysis products of certain methylated carragheenin fractions.[37]

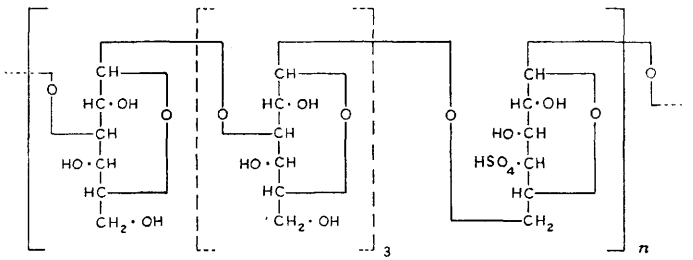
the galactose units in carragheenin are galactopyranose residues linked through the 1 and 3 positions and carrying the sulphate group on $C_{(4)}$. Confirmation of this view has been obtained [37] by the isolation of 2 : 4 : 6-trimethyl galactose from the hydrolysis of methylated partly-degraded specimens of carragheenin from which the sulphate residues had been removed, and Dillon [38] has also supported the allocation of the 1 : 3-linkages.

Much remains to be done before the full constitution of carragheenin is settled, for the yield of galactose obtained on hydrolysis represents only about two-thirds of the organic matter present. Fructose has been stated to be a constituent,[27, 28] and certainly *ca.* 20% of a constituent giving the colorimetric tests of a ketose can be detected.[35, 36] The isolation of a crystalline derivative of 2-keto-D-gluconic acid has been recorded,[29] but it is by no means certain that this product, estimated to make up about 3% of the polysaccharide, is present as such in the original material.

Two possibilities exist: one that carragheenin is a mixture of a polygalactan sulphate, and a labile polysaccharide or mixture of polysaccharides which has eluded characterisation so far, and the other that the unidentified residues are present with galactose in the same polymeric structure.

(b) **The galactan sulphate of** *Dilsea edulis*.—V. C. Barry and Dillon [39] isolated a galactan sulphuric ester from the red seaweed *Dilsea edulis* by extraction with dilute acids. The sulphate content is much lower than for carragheenin and corresponds to one sulphate group in four or five galactose residues.

A tentative formula (XXIV) has been advanced for the repeating unit, from oxidation experiments with periodic acid, only one galactose unit in five being attacked. The sulphate residue was found to be stable to alkali



(XXIV.)

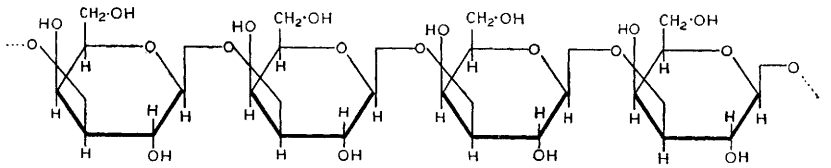
and is therefore assigned to $C_{(4)}$ instead of to $C_{(6)}$, and 1 : 3-linkages predominate in accordance with the evidence that most of the galactose residues are untouched by periodate.

(c) **Agar-agar.**—There is some doubt whether agar-agar, the important polysaccharide extracted from *Gelidium* spp. and related algæ, can be regarded strictly as a sulphuric ester. Various estimates of the sulphur

[37] R. Johnston and Percival, unpublished.
[38] *Proc. Chem. Soc.*, 1949, 34.
[39] *Proc. Roy. Irish Acad.*, 1945, **50**, 349.

content of commercial agar give values approaching 2%, but specimens [40] prepared from *Gracilaria confervoides* (S, 0·43%), *Gelidium crinale* (S, 0·47%), and *Gelidium latifolium* [41] (S, 0·36%) contain much less. There is a strong suspicion, however, that, because agar contains 3 : 6-anhydro-L-galactose units in its molecule, sulphate residues were present at an earlier stage.

The main structural feature of agar is a chain of β-D-galactopyranose units linked through the 1 : 3-positions (XXV) since the chief hydrolysis



(XXV.)

product of methylated agar was shown to be 2 : 4 : 6-trimethyl D-galactose.[42] Subsequent investigations [43] showed that 3 : 6-anhydro-L-galactose residues were present also and this was thought at the time to be related to the discovery that hepta-acetyl DL-galactose could be isolated on the acetolysis of agar.[44] It was shown subsequently,[45] however, that the acetolysis of 3 : 6-anhydro-β-methyl-D-galactopyranoside gave the same DL-galactose derivative, and that the racemisation was due to a rearrangement made possible by the special symmetry of the galactose series. W. G. M. Jones and S. Peat [46] isolated 2 : 5-dimethyl 3 : 6-anhydro-L-galactonic acid from methylated agar which had been dialysed in acid solution, remethylated, and hydrolysed, the production of the free acid being attributed to the formation of a free aldehyde by the opening of the pyranose ring in the 3 : 6-anhydro-L-galactose residue (a typical feature of 3 : 6-anhydrogalactopyranosides [47]), followed by atmospheric oxidation. 2 : 5-Dimethyl 3 : 6-anhydro-L-galactonic acid was also isolated [48] by the acetolysis of methylated agar followed by oxidation, remethylation, and hydrolysis of the mixture of disaccharide esters produced. It is certain therefore that the 3 : 6-anhydro-L-galactose residues are joined to the main chain through $C_{(4)}$.

Jones and Peat [46] interpreted their results as showing that agar is made up of repeating units which are composed of a chain of nine D-galactopyranose units linked through 1 : 3-positions, terminated by an L-galactopyranose residue linked through $C_{(4)}$ carrying a sulphate group on $C_{(6)}$

[40] Percival, *Nature*, 1944, **154**, 673.

[41] V. C. Barry and T. Dillon, *Chem. and Ind.*, 1944, **63**, 167.

[42] Percival and J. C. Somerville, *J.*, 1937, 1615.

[43] S. Hands and Peat, *Chem. and Ind.*, 1938, **57**, 937 ; *Nature*, 1938, **142**, 797 ; Percival, Somerville, and I. A. Forbes, *ibid.*, p. 797 ; Percival and Forbes, *J.*, 1939, 1844.

[44] N. W. Pirie, *Biochem. J.*, 1936, **30**, 369.

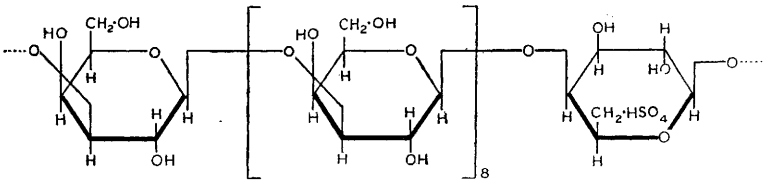
[45] T. L. Cottrell and Percival, *J.*, 1942, 749.

[46] *Ibid.*, p. 225.

[47] W. N. Haworth, Jackson, and F. Smith, *J.*, 1940, 625.

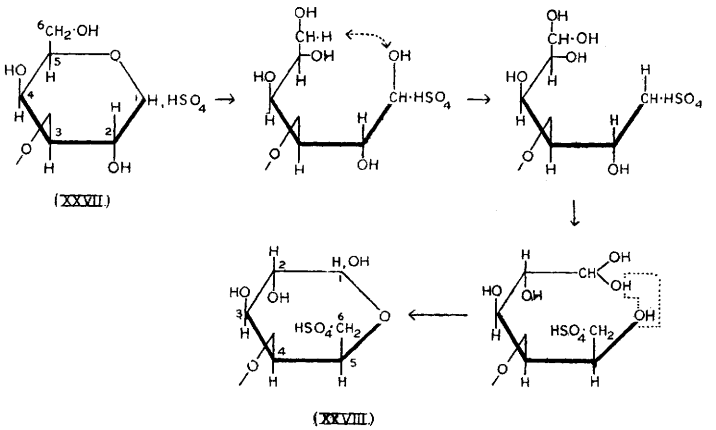
[48] Percival and T. G. H. Thomson, *J.*, 1942, 750.

(XXVI), from which the 3 : 6-anhydride is produced during methylation. This view has been contested [41, 42, 48] as an over-simplification of the problem and on the ground that the sulphur content of natural agar is too low to account for the yields of 3 : 6-anhydro-L-galactose derivatives actually isolated. It is possible though that the 3 : 6-anhydro-ring was formed at some stage in the elaboration of the polysaccharide by the alga.



(XXVI)

A speculation is made by Peat [46] as to the possible origin of the L-galactose residue linked through $C_{(4)}$. He points out that a D-galactopyranose residue (XXVII) linked through $C_{(3)}$ with a sulphate group on $C_{(1)}$ could, by following the sequence of events depicted below involving an oxidation–reduction process, become transformed into an L-galactose residue (XXVIII)



(XXVII)

(XXVIII)

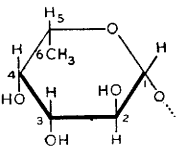
carrying the sulphate on $C_{(6)}$ and linked through $C_{(4)}$. It is also suggested that the sulphate group plays a part in the synthesis of agar in the same way as the phosphate group does for starch, but evidence in support of either suggestion is likely to be difficult to obtain.

(d) Fucoidin. H. Kylin [49] isolated, from various common brown seaweeds, a soluble polysaccharide called fucoidin which gave a methylpentose on hydrolysis. This substance was shown to be a sulphuric ester by M. Bird and P. Haas [50] since the sulphate contained in the ash (15·1%) was half the total sulphate (30·3%). A similar polysaccharide was isolated from

[49] *Z. physiol. Chem.*, 1913, **83**, 171. [50] *Biochem. J.*, 1931, **25**, 403.

Macrocystis pyrifera [51] and the principal building unit was identified as L-fucose.[52] G. Lunde and his co-workers [53] prepared fucoidin from *Laminaria digitata*, and estimated that 33—37% of the polysaccharide was L-fucose and that 35·5—37·7% of the sulphate was present in combination with sodium together with smaller quantities of calcium and magnesium. Since 20% of the molecule could not be accounted for, the formula $R,R'O{\cdot}SO_2{\cdot}ONa$ was suggested where R = L-fucose, R′ = unknown. Several different sources of fucoidin have now been examined, and it is now possible to account for 99% of the components of the polysaccharide.[54] One of the difficulties in the analysis of fucoidin is that the substance stubbornly retains water and ethanol, but a specimen from *Himanthalea lorea* after correction for these components gave: fucose, 56·7; galactose, 4·1; uronic acid, 3·3; xylose, 1·5; sulphate, 38·3; and metals (chiefly calcium), 8·2%. A calcium polyfucosan monosulphate ($C_6H_9O_7SCa_{0·5}$) would give: fucose, 66·9; sulphate, 39·2; and Ca, 8·2%. It is not possible to say at present whether the small quantities of carbohydrate material other than L-fucose are combined in a single polysaccharide, but the broad picture of fucoidin as a polyfucosan ethereal sulphate seems to be a good working hypothesis for future studies.

The sulphate residues in fucoidin are very stable to alkali; this excludes the possibility of a 1:4-linkage since, with a sulphate group on either $C_{(2)}$ or $C_{(3)}$ in a 1-substituted L-fucose (XXIX), there would be a *trans*-hydroxyl group in the reciprocal position. The polymeric link must, therefore, be on $C_{(2)}$ or $C_{(3)}$.



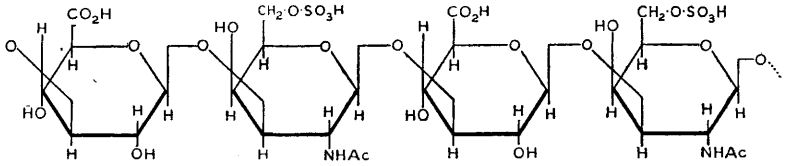
(XXIX.)

Animal Carbohydrate Sulphates.—(a) Chondroitin sulphate. Nasal septa, trachea, aorta, tendons, sclera, etc., contain chondroitin sulphate, which is the only member of the animal group in which progress has been made towards determination of structure. Said to comprise some 40% of dried cartilage, it is best extracted with calcium chloride,[55] a method which gives less-degraded products than that used for the original isolation in 1861.[56] P. A. Levene and F. B. LaForge [57] showed that equimolecular quantities of chondrosamine (2-amino-2-deoxy-D-galactose), acetic acid, D-glucuronic acid, and sulphuric acid were produced on hydrolysis and these ratios have been confirmed recently.[58] A degraded chondroitin sulphate of small molecular weight and devoid of sulphate has been examined by H. G. Bray, J. E. Gregory, and M. Stacey.[59] Evidence is presented, based on methylation and hydrolysis, that this product contains a terminal group of D-gluco-

[51] D. R. Hoagland and L. L. Lieb, *J. Biol. Chem.*, 1915, **23**, 287.
[52] W. L. Nelson and L. H. Cretcher, *ibid.*, 1931, **94**, 147.
[53] G. Lunde, E. Heen, and E. Öy, *Z. physiol. Chem.*, 1937, **247**, 189.
[54] A. G. Ross, Thesis, Edinburgh, 1949.
[55] Karl Meyer and E. M. Smyth, *J. Biol. Chem.*, 1937, **119**, 507.
[56] C. Fischer and C. Boedeker, *Annalen*, 1861, **117**, 111.
[57] *J. Biol. Chem.*, 1914, **18**, 237.
[58] M. L. Wolfrom, D. J. Weisblat, J. V. Karabinos, W. H. McNeely, and J. McLean, *J. Amer. Chem. Soc.*, 1943, **65**, 2077.
[59] *Biochem. J.*, 1944, **38**, 142.

pyruronic acid (isolated as the amide of 2 : 3 : 4-trimethyl α-methyl-D-glucuronoside), associated with doubly-linked glucuronic acid and acetyl chondrosamine residues (appearing as dimethyl derivatives). A branched-chain structure is suggested for chondroitin sulphate, but no conclusions are drawn as to the position of the sulphate groups or the linkages involved.

Kurt H. Meyer and his associates,[60] however, propound a straight-chain structure. The relation between viscosity and molecular weight ($27—33 \times 10^3$) as estimated by methods dependent on the presence of a free reducing group is held to indicate a straight-chain molecule containing about 120 monosaccharide residues, and the specific rotation ($[\alpha]_D^{20} - 31°$) is taken as evidence of β-linkages between them, as in (XXX).



(XXX)

The evidence for the 1 : 3-linkages and the allocation of the sulphate group to $C_{(6)}$ in the acetyl galactosamine residue is, briefly, as follows.

One macro-molecule (120 units) reduces only four periodic acid molecules so that large numbers of adjacent hydroxyl groups are excluded. By cautious hydrolysis it is claimed that half the sulphate groups can be removed without attacking more than 3% of the glycosidic links, and the product obtained still requires only four molecules of periodate for complete oxidation. The sulphate group is therefore not adjacent to a free hydroxyl group, and the possibility that a high proportion of the glucuronic acid residues are present as sulphated terminal groups is rendered remote. After methylation and hydrolysis, followed by periodate oxidation, ammonia is evolved almost quantitatively; therefore a free hydroxyl is adjacent to the amino-group in the partly methylated chondrosamine, which means that $C_{(3)}$ is either a main linking point or is blocked by a sulphate group in the nitrogenous unit. Furthermore, after the same treatment one molecule of formaldehyde is liberated per disaccharide period, which shows that $C_{(6)}$ is not methylated and that the methylated chondrosamine is the 4-methyl derivative. The consumption of periodate by the hydrolysed methylated chondroitin sulphate—three molecules per disaccharide period—is accounted for by the 4-methyl galactosamine, so that the dimethyl glucuronic acid fragment uses no periodate, from which it is concluded that it is 2 : 4-dimethyl glucuronic acid. The glycosides produced on methanolysis consumed one molecule of periodate per disaccharide period with a quantitative elimination of ammonia, in agreement with this result. (XXX) requires that the sulphate residue should be stable to alkali since there is no possibility of anhydride formation; this fits in with the observa-

[60] Kurt H. Meyer, M. E. Odier, and A. E. Siegrist, *Helv. Chim. Acta*, 1948, **31**, 1400.

tion that repeated methylations with sodium hydroxide and methyl sulphate do not eliminate the grouping.

(b) Mucoitin sulphate. Mucoitin sulphate occurs in gastric mucosa [61] and has also been isolated from ox cornea.[62] The constituents are *N*-acetylglucosamine, sulphuric acid, and D-glucuronic acid in molecular proportions,[58] although it was only recently [63] that D-saccharic acid was obtained by the oxidative degradation of mucoitin sulphate to confirm the latter constituent. The arrangements of the building stones of the molecule are quite unknown at present.

It is of interest that the substance hyaluronic acid,[64] isolated from vitreous humour and present in umbilical cord, synovial fluid, etc., is thought to be mucoitin sulphate devoid of the sulphate residues.

(c) Heparin. The blood-anticoagulant heparin, first isolated in 1918 by W. H. Howell and E. Holt [65] from dog liver, was early recognised to be carbohydrate in nature and to contain nitrogen, shown later [66] to reside in D-glucosamine. The presence of a uronic acid was inferred by the usual method and this was eventually proved to be D-glucuronic acid.[67] That heparin was a polysulphate was suggested by E. Jorpes [68] in 1935, and A. F. Charles and D. A. Scott's crystalline barium salt [69] was shown to be a salt of a sulphuric ester. Charles and A. R. Todd [70] submitted evidence that heparins from different sources, *e.g.*, lung and liver, appeared to be analytically identical and also that conversion into the ammonium salt, followed by reconversion into the barium derivative, did not impair the physiological activity. It was also observed that periodic acid did not attack heparin, and the substance was represented as a mucoitin sulphate containing five sulphuric ester residues in a tetrasaccharide unit $(C_{28}H_{38}O_{38}N_2S_5)_2Ba_5,24H_2O$, with two carboxyl and two acetamido-residues. Charles and Todd also observed a diminution of physiological activity with the progressive removal of sulphate groups. Jorpes,[71] on the other hand, considers heparin to be a mixture of polysaccharides containing variable amounts of sulphate residues, such as di- and tri-sulphuric esters of a glucuronic–glucosamine disaccharide unit, of which the crystalline component is a relatively small fragment. In connexion with this view, two components have been detected on electrophoresis.[72] According to M. L. Wolfrom [58] the crystalline barium acid heparinate is the same both analytically and biologically whether isolated from such varied sources as beef lung, or dog, pork, or beef liver. The sulphate residues are all combined with barium but the carboxyl groups

[61] Levene and J. López-Suarez, *J. Biol. Chem.*, 1916, **25**, 511.
[62] Karl Meyer and E. Chaffee, *ibid.*, 1941, **138**, 491.
[63] Wolfrom and Rice, *J. Amer. Chem. Soc.*, 1946, **68**, 532.
[64] Karl Meyer and J. W. Palmer, *Amer. J. Ophthalmol.*, 1936, **19**, 859.
[65] *Amer. J. Physiol.*, 1918, **47**, 328.
[66] E. Jorpes and S. Bergström, *Z. physiol. Chem.*, 1936, **244**, 253.
[67] Wolfrom and Rice, *J. Amer. Chem. Soc.*, 1946, **68**, 532.
[68] *Biochem. J.*, 1935, **29**, 1817.
[69] *Ibid.*, 1936, **30**, 1927.
[70] *Ibid.*, 1940, **34**, 112.
[71] *Ibid.*, 1942, **36**, 203.
[72] Wolfrom and Rice, *J. Amer. Chem. Soc.*, 1947, **69**, 2918.

are free. The claim is made that heparin contains no acetyl residues, and that the glucosamine residues are joined to the glucuronic acid units of the polymer by >CH·NH·CH< links to the potential reducing groups of the latter. There is no direct evidence to support this unique type of linkage or to indicate any other structural features of the heparin molecule.

The apparent connexion between "heparin activity" and sulphate content [70] has led to a search for more accessible materials for physiological studies. An extract from *Chondrus crispus* [73] has been shown to be about 40% as active as heparin, but fucoidin is inactive.[74] Polysaccharide sulphates have been synthesised and heparin activity demonstrated in many of them, although no product of practical value has been reported upon until recently, since most of the synthetic products have the disadvantage of high toxicity not possessed by heparin. Thus sulphated agar,[75] cellulose, starch, and glycogen sulphates [76] have been prepared.

A new clot-inhibitor, "Paritol," has been reported [77] which is presumably an alginic acid sulphate and resembles heparin in containing carboxyl and sulphate groups, but differs from it in the absence of glucosamine residues. This product is claimed to be no more toxic than heparin and, since its physiological action lasts twice as long, may be substituted for heparin for short-term administration to human beings, although clinical tests are not complete.

[73] A. R. Todd, private communication.

[74] A. F. Charles, private communication.

[75] C. Neuberg and C. H. Schweitzer, *Monatsh.*, 1937, **71**, 46.

[76] E. Chargaff, F. W. Bancroft, and M. Stanley-Brown, *J. Biol. Chem.*, 1936, **115**, 155; Bergström, *Z. physiol. Chem.*, 1936, **238**, 163; P. Karrer, H. Koenig, and E. Usteri, *Helv. Chim. Acta*, 1943; H. Gebauer-Fulnegg and O. Dingler, *J. Amer. Chem. Soc.*, 1930, **52**, 2849; W. Traube, B. Blaser, and E. Lindemann, *Ber.*, 1932, **65**, 603.

[77] *Chem. and Eng. News*, 1949, **27**, 2162.