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THE MOLECULAR STRUCTURE OF SOME

POLYSACCHARIDE SULPHATES

by

Alistair Penman, B.Sc.

---o0o---

Thesis presented for the degree of Doctor of Philosophy.

To my Wife and Parents.
I should like to express my gratitude to Professor Sir Edmund Hirst, Professor N. Campbell and Professor J.I.G. Cadogan for the provision of laboratory facilities.

My sincere thanks are due to my Research Supervisor, Dr. D.A. Rees, who has been a constant source of help and encouragement throughout my course of study.

I should like to thank my colleagues, past and present, in the Chemistry Department for their help and stimulating discussion, and Marine Colloids Inc. for financial support.

Finally, I wish to thank my wife for typing this manuscript.

Part of this work has been published in the form of two papers, reprints of which are inserted at the end of this Thesis.
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**GENERAL INTRODUCTION**

Polysaccharides linked to sulphate half ester groups are widespread in animal and human tissue and also in marine algae but have never been isolated from higher plants or bacteria. This seems quite remarkable in view of the wide biological difference between algae and multicellular animals. Unlike carbohydrate phosphates, which play such an important role in cell metabolism, polysaccharide sulphates seem to be present in cell walls and intercellular regions more for their physical properties than for their chemical reactions. Two very striking properties of these polymers are their polyelectrolyte character, due to the presence of ionised sulphate ester groups, and their ability to form gels and highly viscous solutions. It is rather remarkable that many polysaccharide sulphates, from algae and animal tissue, have a strictly alternating structure of two sugar units, an arrangement that is rare in other types of polysaccharides, hyaluronic acid being a notable exception.

The presence of ester sulphate groups has a considerable effect on the structure determination of a polysaccharide and gives rise to certain problems. It has been suggested in the literature\(^1\) that sulphate ester groups, like acetyl groups, migrate under certain reaction conditions, but to date there is no good evidence to substantiate this claim. Methylation followed by hydrolysis is by itself not sufficient to characterise the positions of the glycoside linkages in sulphated polysaccharides as this does not distinguish between the site of sulphation and of glycosidic substitution. Several other methods have been used in conjunction with methylation to unambiguously characterise the position of ester sulphate and a section of the research done/..
done for this Thesis is concerned with extending existing methods and
developing new techniques for the location of sulphate half ester
groupings on polysaccharides.

Additional evidence for the positioning of sulphate can be obtained
after desulphation of the polysaccharide, which is usually achieved with
minimum glycosidic bond cleavage using methanolic hydrogen chloride, a heterogeneous reaction. By comparing the hydrolysis products of the
methylated polysaccharide with those of the methylated desulphated
polysaccharide the positions of the sulphate ester groupings can be
deduced. Further information can be obtained by periodate oxidation
before and after desulphation.

Infrared spectroscopy has proved useful in locating sulphate ester
groupings in some polysaccharides. All sulphate esters show a general
absorption band around 1240 cm⁻¹. Sulphates on pyranose sugars have
been further classified according to whether the ester is attached to
a secondary axial, a secondary equatorial, or a primary hydroxy group:
characteristic bands are supposed to appear at 850, 830 and 820 cm⁻¹,
respectively, due to the C-O-S vibration. It now appears doubtful
whether infrared spectra of carbohydrate sulphates can be interpreted
in such simple stereochemical terms. More details are given in Section B
Part Two.

It has been shown from experiments on model monosaccharide
sulphates that ester sulphate groups are exceedingly stable to alkali,
with two important exceptions. When there is a free hydroxyl group
adjacent and trans to the sulphate (Fig. 1. (1)), the alkali catalytically
cleaves the sulphate by an $S_N$ mechanism to form an epoxide ring (2),
which, on hydrolysis can open on either side to yield the desulphated
parent sugar (3) and new sugar (4). Cleavage of the epoxide ring with
sodium methoxide likewise may give two mono-O-methyl sugars (5, 6).
If/..
Fig. 1.

Fig. 2.
If the sulphate is linked to C3 or C6 (Fig. 2 (7)) then treatment with alkali can result in the formation of a 3,6-anhydro ring (6). These facts have often enabled deductions regarding the site of ester sulphate to be made for polysaccharides.

Aqueous acid hydrolysis of sulphate esters usually occurs at about the same rate as glycosidic hydrolysis, and a complicated mixture of products is therefore to be expected in structure analysis of polysaccharide sulphates by straightforward partial acid hydrolysis. Occasionally advantage can be taken of the high acid lability of certain glycosidic linkages to hydrolyse a polysaccharide to a relatively simple mixture of sulphated oligosaccharides, (see Section B, Part One for further details).

Polysaccharide Sulphates from Seaweeds

The sea has a vegetation consisting almost entirely of algae, which show a great diversity of form ranging from unicellular organisms to the giant seaweed *Macrocystis pyrifera*. The seaweeds are probably the best known members of the algae, and are found mostly attached to rocks in the continental shelf areas. The seaweeds are divided into three main classes, depending on the nature of the pigments present: Phaeophyceae (brown), Rhodophyceae (red) and Chlorophyceae (green). All three classes contain chlorophyll a, but the colour is masked in the red and brown seaweeds by biliproteins and the xanthophyll, fucoxanthin, respectively. As well as having characteristic pigments these classes can be distinguished by the types of polysaccharides which are found in their cell walls and intercellular regions. A common factor in all three classes is the presence of at least one sulphated polysaccharide, the structure of which varies widely from class to class.

Red Seaweeds/...
Red Seaweeds

The main polysaccharide of most red seaweeds seems to be a sulphated galactan which often shows a well-defined structural pattern namely a chain of units with the galacto configuration linked $\alpha 1,3 \beta 1,4$. Different species of red seaweed take this template and modify it to suit their own needs giving rise to a whole spectrum of different polysaccharides with a common structural base. Two of the most widely studied families of polysaccharides found in the red seaweeds are the agars and carrageenans, and part of the research reported in Section I has been concerned with a systematic structural examination of polysaccharides from various species in an attempt to find new types of polysaccharides belonging to these two classes. A review of the present knowledge of the structure of these sulphated galactans is given below with an attempt to show the structural relationships between the various types. The results are summarised in Table 1.

Carrageenans. Most of the early work done on carrageenans was performed on polysaccharides extracted from Chondrus crispus and Gigartina stellata, but similar polysaccharides have also been extracted from plants of different genera. The hot water extract from Chondrus crispus can be fractionated by addition of dilute potassium chloride solution into a soluble and insoluble fraction.

\(\kappa\)-Carrageenan. This is the fraction of carrageenan precipitated with potassium chloride and has been shown to be essentially a polymer of alternately arranged $\beta-D$-galactose 4-sulphate and 3,6-anhydro-\(\alpha-D\)-galactose units linked $1,3$ and $1,4$. The molar ratio of galactose : 3,6/..
### Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>1,3 linked unit</th>
<th>1,4 linked unit</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose</td>
<td>D-galactose</td>
<td>3,6-anhydro L-galactose</td>
<td>Gelidium amansii</td>
</tr>
<tr>
<td>Porphyrn</td>
<td>D-galactose, 6-O-methyl D-galactose</td>
<td>(L-galactose 6-sulphate)</td>
<td>umbilicalis</td>
</tr>
<tr>
<td>K-Carrageenan</td>
<td>D-galactose 4-sulphate</td>
<td>3,6-anhydro D-galactose, D-galactose 6-sulphate, 3,6-anhydro D-galactose</td>
<td>Chondrus crispus</td>
</tr>
<tr>
<td>L-Carrageenan</td>
<td>D-galactose 4-sulphate</td>
<td>3,6-anhydro D-galactose</td>
<td>D-fucoides spinosum</td>
</tr>
<tr>
<td>A-Carrageenan</td>
<td>D-galactose 2,6 disulphate</td>
<td>D-galactose 2,6 disulphate</td>
<td>Chondrus crispus</td>
</tr>
<tr>
<td>L-Carrageenan</td>
<td>D-galactose 4-sulphate</td>
<td>3,6-anhydro D-galactose</td>
<td>Chondrus crispus</td>
</tr>
</tbody>
</table>

* Structures may contain different proportions of one type of 1,3 linked unit relative to another, and similarly within the 1,4 linked series. Examples only are given here and classification is according to the predominant type of 1,3 and 1,4 linked unit.
3,6-anhydrogalactose sulphate, shows that there is more galactose and sulphate than this perfect repeating structure could accommodate and various experiments have shown that the basic regularity is overlaid by substitution and modification to produce what is known as a 'masked repeating structure'. Treatment of the polysaccharide with alkali produces an increase in 3,6-anhydrogalactose which was presumed to arise by sulphate elimination, indicating the presence of a 1,4 linked galactose 6-sulphate residue. Further evidence for this was provided by mild methanolysis of the polysaccharide before and after alkali treatment and measurement of the yields of 3,6-anhydro-(4-0-2-galactopyranosyl)-2-galactose dimethyl acetal (carrabiose dimethyl acetal) produced. Parallel experiments on carrabiose dimethyl acetal itself gave a measure of the loss due to side reactions. These reactions showed that on alkali treatment the corrected 'carrabiose content' of \( \kappa \)-carrageenan rose from 88 to 99%. That some of these 6-sulphated residues were also sulphated on position 2 was shown by their resistance to periodate oxidation. By oxidatively hydrolysing the methylated polysaccharide with bromine-sulphuric acid and identifying the products so formed, it has been shown that a small proportion of the 3,6-anhydrogalactose units are sulphated on position 2. This has been confirmed by infrared studies (see Section B, Part Two).

\( \kappa \)-Carrageenan. This polysaccharide is extracted from *Eucheuma spinosum* and by using methods similar to those described for \( \kappa \)-carrageenan, it has been shown that \( \kappa \)-carrageenan has a structure in which the 1,3 linked unit is galactose 4-sulphate and the 1,4 linked unit is mainly 3,6-anhydrogalactose 2-sulphate, with about 10% galactose 2,6-disulphate units.

\( \kappa \)-Carrageenan/..
\(\lambda\)-Carrageenan. The polysaccharide material remaining in the supernatant solution after potassium chloride fractionation of carrageenan consists of two distinct polysaccharides namely \(\lambda\) and \(\mu\) (sometimes known as third component) carrageenans. As yet no method has been developed for fractionating these two polymers in the native state, but if the mixture is treated with alkaline borohydride then the modified \(\mu\) component may be precipitated with potassium chloride. Structural studies have shown that alkali modified \(\mu\) is very similar to \(\kappa\)-carrageenan\(^{14,11}\) thus, if it is assumed that treatment with alkali converts 1,4 linked 6-sulphated galactose units into 3,6-anhydrogalactose then native \(\mu\) carrageenan must have a structure in which the 1,3 linked unit is galactose 4-sulphate and the 1,4 linked unit is galactose 6-sulphate, with varying amounts of 3,6-anhydrogalactose depending on source.\(^{13}\)

On examination of the structures of \(\lambda\), \(\kappa\) and \(\mu\) carrageenans, it is seen that they do not vary in the types of structural units which they possess but only in the extent to which they possess them. Thus \(\lambda\) and \(\mu\) carrageenans can be thought of as representing extremes of a structural spectrum with \(\kappa\) somewhere in the middle. By progressively adding potassium chloride to solutions of certain carrageenans, subfractions can be obtained having decreasing 3,6-anhydrogalactose contents which represent a transition from \(\kappa\) to \(\mu\) like carrageenans.\(^{14}\)

\(\lambda\)-Carrageenan. This polysaccharide, together with \(\mu\) carrageenan, is left in solution when carrageenan is fractionated with potassium chloride. As stated earlier \(\lambda\) and \(\mu\) carrageenans have not been fractionated in the native state, but occasionally \(\lambda\) is the sole component left in solution and thus can be examined in the uncontaminated state. A measure of the degree of contamination by \(\mu\)\(^{13}\) can be obtained by analysis for 3,6-anhydrogalactose\(^{15}\) or galactose 4-sulphate, as these residues do not occur in \(\lambda\).

First/..
First evidence that the 1,4 linked unit in \(\lambda\)-carrageenan was probably 6-sulphated, was the large increase in 3,6-anhydrogalactose content on treatment with alkaline borohydride; that this unit was also 2-sulphated was supported by the high sulphate ester content and the resistance of the molecule to oxidation by periodate. Better evidence for the structure was given by methylation analysis before and after desulphation and by the almost quantitative yield of carrabiose dimethyl acetal from the alkali treated polysaccharide on methanalysis. These experiments indicated that the 1,4 linked unit occurred as galactose 2,6-disulphate and that the 1,3 linked unit was galactose and its 2-sulphate. The configuration of the 1,3 linked unit was shown to be \(\alpha\) by isolation of the disaccharide 3-O-\(\alpha\)-galactopyranosyl-\(\alpha\)-galactose on acetylation followed by deacetylation.

\(\lambda\)-Carrageenan differs from the polysaccharides of the \(\kappa, \lambda, \kappa\) range in that it possesses galactose or its 2-sulphate as the 1,3 linked unit, rather than galactose 4-sulphate, and also in that it is not believed to contain 3,6-anhydrogalactose or its derivatives.

**Agar.** The agars, like the carrageenans, are a family of polysaccharides which occur in the red seaweeds and which have the same basic structural template namely a chain of units with the galacto configuration linked alternately \(\alpha\) 1,3 \(\beta\) 1,4. The distinguishing feature of the agar family is that the 1,4 linked unit is the \(\lambda\) sugar and not the \(\kappa\) as in carrageenans. Structural investigations have been carried out on several members of the agar family and a brief account of current knowledge is given below.

Agarose/...
**Agarose.** This polysaccharide is isolated from *Gracilaria* and *Gelidium* genera and unlike the other polysaccharides mentioned so far in this Thesis, agarose has not been shown to contain covalently linked sulphate half ester groups. It has a structure in which the 1,3 linked unit is \(\beta-D\)-galactopyranose and the 1,4 linked unit is 3,6-anhydro-\(\alpha\)-galactopyranose.\(^{16,19}\) Although agarose is not a sulphonated polysaccharide it is mentioned as it is thought to be the prototype for all gelling systems and increasing deviation from the agarose structure seems to result in decreasing gel strength. It is presumably 6-sulphated at some stage as this residue is the biological precursor of 3,6-anhydrogalactose.\(^{20}\)

**Porphyran.** This sulphonated polysaccharide is extracted from *Porphyra* species and it has been shown to have a structure of the masked repeating type. Its structure can be derived from that of agarose by formal replacement of some of the 1,4 linked units by \(\alpha\)-galactose 6-sulphate and by partial 6-\(\alpha\)-methylation of some of the 1,3 linked \(\beta\)-galactose units. This masked repeating structure for porphyran was proved by alkaline borohydride treatment of the polysaccharide followed by methylation to yield a product which was identical to methylated agarose.\(^{21}\)

The water soluble sulphonated polysaccharide from *Laurencia pinnatifida* has been shown to have a structure that is similar to but more complex than porphyran.\(^{22}\) The 1,3 linked unit can occur as \(\beta\)-galactose and its 6-\(\alpha\)-methyl ether whereas the 1,4 linked unit may be \(\alpha\)-galactose, its 2-\(\alpha\)-methyl ether, 3,6-anhydro-\(\beta\)-galactose and its 2-\(\alpha\)-methyl ether. Sulphate ester groups occur on C-6 of both \(\alpha\)-galactose and 2-\(\alpha\)-methyl-\(\beta\)-galactose, and on C-2 of \(\beta\)-galactose units. There is some evidence for branching in the molecule.

Other /...
Other Polysaccharides. Sulphated galactans similar to those in the agar and carrageenan families have been isolated from Glociopeltis furcata and Furcellaria fastigiata. Their structures are discussed in detail in Section A, Part One.

The sulphated polysaccharide from Aeodes orbitosa has been shown to be similar to \( \lambda \)-carrageenan in that it contains a 1,3 linked galactose 2-sulphate unit. It differs however, in that the 1,4 linked unit is unsulphated D-galactose. There seems to be evidence that the 1,3 linked units occur in greater concentration.

There are some red seaweed polysaccharides which do not seem to fit the pattern of strict alternation of 1,3 and 1,4 linkages. The mucilage from Dilsea edulis is thought to consist of two structurally dissimilar regions which may be in the same or different molecules. The major component is a chain of 1,3 linked galactose units, with the possibility of some branching, sulphate possibly occurring on position 4 of some of these. A second component may be an alternating chain of 1,3 and 1,4 linked galactose units in which some of the 1,3 linked units are 4-sulphated and some of the 1,4 linked units occur as the 6-sulphate or 3,6-anhydride.

Physical Studies on Red Seaweed Polysaccharides. The primary structure of a polysaccharide can be elucidated using chemical techniques but in order to obtain information about secondary or tertiary structure, physical methods have to be employed. The most powerful of all physical methods for elucidation of molecular shape and structure is X-ray diffraction. The red seaweeds provide many polysaccharides which have primary structures regular enough to be examined by diffraction techniques and information has been obtained about the solid state conformations of \( \kappa \)-, \( \lambda \)- and \( \lambda \)-carrageenan by X-ray examination of oriented fibres of these polysaccharides.
X-ray fibre photographs of \( \kappa \) - and \( \lambda \) -carrageenans show that these polysaccharides exist as double helices with three disaccharide residues in a complete turn of each single chain. That each of the chains has a right handed screw sense has been shown by model building to fit the parameters deduced from X-ray photographs. Similar experiments have shown that \( \lambda \) -carrageenan exists as a left handed three-fold helix.

It has been inferred from indirect evidence that the junction zones in the gel consist of aggregated double helices. This theory fits the experimental observation that the replacement of 3,6-anhydrogalactose by galactose 6-sulphate results in a marked decrease in gel strength. Computer calculations have shown that the result of this substitution is to produce a 'kink' in the regular helix. This kink will diminish the tendency towards double helix formation thus reducing the number of junction zones and weakening the gel.

**Green Seaweeds**

Many of the Chlorophyceae synthesise sulphated polysaccharides containing a wide variety of sugar residues and although partial fractionation has been achieved in some instances, in no case has a well characterised and structurally distinct polysaccharide sulphate been isolated. The water soluble polysaccharide isolated from Cladophora rupestris appears to be highly branched with xylose and galactose units at the periphery of the molecule and galactose, arabinose and rhamnose in the inner core. Sulphate ester groups are attached both to residues on outer branches and inner core, but there is not a great deal of evidence for the detailed primary structure of the molecule. The polysaccharide isolated from Codium fragile appears to be a highly branched sulphated polymer consisting mainly of galactose and arabinose residues which is similar in some respects to the Cladophora polysaccharide.
**Brown Seaweeds**

The sulphated polysaccharides isolated from the Phaeophyceae are usually of the fucoidan type. Although fucoidans isolated from different genera of brown seaweed appear to have somewhat different compositions, they all, as far as is known, comprise mainly L-fucose and ester sulphate. The fucoidan sample isolated from *F. vesiculosus* is thought to have a main chain of $\alpha 1,2$ linked fucose 4-sulphate units with about $10\%$ of $\alpha 1,3$ linked fucose 4-sulphate branch points.  

**Polysaccharide Sulphates from Animal Tissues**

In animal and human connective tissue the constituent cells are embedded with protein fibres, usually collagen, in an expanse of extracellular material known as matrix. This matrix consists of carbohydrate polymers many of which carry sulphate ester groups, and the structure of which vary from one tissue to another. Recent work has shown that these sulphated polysaccharides from connective tissue are conformationally very similar to polysaccharides of the agar–carrageenan type and it has been suggested that a common stereochemical theme underlies some of the properties and functions of all these polysaccharides. A brief discussion of the structures of these connective tissue polysaccharides is given below and the results are summarised in Table 2.

In connective tissue most of these polysaccharides are found linked glycosidically to a protein backbone and the polysaccharide chains, or fragments of them, can be released using enzymes or by base catalysed elimination at the serine residues. One fraction from bovine nasal cartilage consists of a polypeptide backbone with about twenty polysaccharide chains branching from it, mostly chondroitin 4-sulphate but with some other types. There are usually about one hundred and twenty sugar/..
### TABLE 2

**Alternating Co-polymers from connective tissue**

<table>
<thead>
<tr>
<th>Name</th>
<th>1,3 linked unit</th>
<th>1,4 linked unit</th>
<th>Typical Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyaluronic acid</td>
<td>N-acetyl-(\beta)-glucosamine</td>
<td>(\beta)-glucuronic acid</td>
<td>Vitreous humour in the eye.</td>
</tr>
<tr>
<td>Chondroitin 4-sulphate</td>
<td>4-sulphate</td>
<td>4-sulphate</td>
<td>Cartilage.</td>
</tr>
<tr>
<td>Chondroitin 6-sulphate</td>
<td>6-sulphate</td>
<td>6-sulphate</td>
<td>Cartilage.</td>
</tr>
<tr>
<td>Dermatan sulphate</td>
<td>N-acetyl (\beta)-galactosamine</td>
<td>(\beta)-iduronic acid</td>
<td>Skin.</td>
</tr>
<tr>
<td>Keratan sulphate</td>
<td>galactose, galactose</td>
<td>N-acetyl-(\beta)-glucosamine</td>
<td>Cornea of the eye.</td>
</tr>
</tbody>
</table>
sugar units in each chain with the polypeptide making up about one quarter of the weight of the complex.

Like the agars and carrageenans the polysaccharide chains are made up from sugar residues linked alternately 1,3 and 1,4, the 1,3 linked unit being a 2-acetamido-2-deoxy D-hexose and the 1,4 normally being a uronic acid residue. This sequence of units changes at the point where the polysaccharide is bonded to the serine residue of the protein chain; this structurally deviant portion being known as the 'linkage region'. In chondroitin 4-sulphate the linkage region is of the form:

\[
\beta 1 \rightarrow 3 \text{ gal} \beta 1 \rightarrow 3 \text{ gal} \beta 1 \rightarrow 4 \text{ x} \rightarrow \text{ serine-polypeptide}
\]

where gal = D-galactopyranose, x = D-xylopyranose.

Apart from this linkage region there is no convincing evidence for deviation from this alternating arrangement of 1,3 and 1,4 linkages. As in the agar-carrageenan type of polysaccharides, variation in the ratio of certain monomer units can take place to give structures of the masked repeating type.

These structures have been elucidated by a wide variety of methods, several of which are mentioned at the beginning of this Introduction. Advantage can be taken of the stability of uronosyl linkages to acid hydrolysis to hydrolyse these polysaccharides to sulphate-free disaccharide units. Chondroitin 4-sulphate on hydrolysis with oxalic acid gives the disaccharide 3-2-β-D-glucuronosyl-2-D-galactosamine (chondrosine), the structure of which has been proved by synthesis:

![Chemical structure of chondrosine]

Fig. 3. chondrosine

There/..
There are two mammalian polysaccharide sulphates which do not fit the pattern of strict alternation of 1,3 and 1,4 linkages, namely heparin and heparitin sulphate. Heparin occurs in circulatory tissue and possesses important biological properties, notably as an anticoagulant. For many years it was thought to have a structure in which 2-deoxy-2(sulphoamino)-\(\alpha-D\)-glucopyranosyl 6-sulphate and \(\alpha-D\)-glucopyranosyluronic acid 2-sulphate residues were joined via 1,4 glycosidic bonds in alternating sequences.\(^{33}\) Recent research has shown however, that \(L\)-idosyluronic acid residues\(^{34,35}\) occur in heparin. The structure of heparitin sulphate is related to that of heparin but differs in the degree of sulphation and that some of the glucosamine units are N-acetylated.

**Comparison of Seaweed and Animal Polysaccharide Sulphates**

Connective tissue glycosaminoglycans and the agar-carrageenan family of seaweed polysaccharides differ from polysaccharides from most other sources in having a strictly alternating structure of two sugar units with the glycosidic linkages being 1,3 and 1,4. The full extent of structural similarity is not immediately obvious because in the animal series the 1,4 linked residue is commonly \(\text{gluco}\), Reeves C1 and \(\beta\), whereas in seaweeds it is commonly \(\text{galacto}\), Reeves 1C and \(\alpha\). But by comparison of suitably drawn conformational formulae, such as \(\zeta\)-carrageenan and chondroitin 4-sulphate (Fig. 4), there is a possibility that the chain conformations are similar.

The entire group of polysaccharides can be considered to be derived from the same polyether skeleton composed of cyclohexane-1,4-diol and cyclohexane-1,3-diol residues, (I), Fig. 5. There is an example in each series which could be derived from the other conformation (II), Fig. 6., \(\lambda\)-carrageenan in which the 1,4 linked unit occurs in the C1 conformation, and dermatan sulphate in which the 1,4 linked \(L\)-iduronic acid residue could perhaps exist in the 1C conformation (normal chair for L-series). By taking the polymer skeleton (I) and using/..
Fig. 4. 

-κ-carrageenan.

Fig. 5. (I)

chondroitin 4-sulphate.

Fig. 6. (II)
using a computer to analyse the conformational effects of steric interactions between atoms on it for the polysaccharide structures given in Tables 1 and 2, it has been shown that over 99% of the conformations are excluded and virtually all of the remainder for each polysaccharide lie close together. On the basis of this similarity between the conformations, common properties might be expected related to this common element in stereochemistry. An example of this is that calculation has shown that sterically feasible double helices can be built for chondroitin sulphates, keratan sulphate, dermatan sulphate and agarose as well as for carrageenans for which they are experimentally established.

Biological Function

As stated earlier in this Introduction, polysaccharide sulphates seem to exist in nature more for their physical properties than for their chemical reactions. Their ability to form gels and hydrated networks and their polyanionic nature have been used as a basis for predicting their biological functions in both marine algae and animal connective tissue.

In the seaweeds these polysaccharides, due to their remarkable gelling properties, help to build up flexible structures which can withstand buffeting by the waves without damage. There is some evidence that in some species there has been some adaption so that stiffer gels are present in plants exposed to more severe wave action. This modification in gel strength seems to be due to variations in the sulphate ester content of the polysaccharides. Sulphated polysaccharides are anionic polyelectrolytes with marked cation exchange properties and this must be of some significance for plants living in a saline medium, for example, porphyran is probably responsible for the selective absorption of potassium ions/..
ions and exclusion of sodium ions in F. perforata. A further role of these polysaccharides may be the prevention of dessication in plants exposed by the tides; fucoidan appears to be particularly efficient in this respect as among the Fucales these growing highest on the shores which are exposed to the atmosphere for the longest periods, have on the average, considerably more fucoidan than those which are exposed for only short periods.

In connective tissue, the protein-polysaccharide networks act like molecular springs in that due to electrostatic repulsion between chains they tend to resist compression and when pressure is removed the chains expand and move as far apart as possible. Due to their polyanionic nature, it is thought that they have a role in calcification and bone formation and also control of electrolytes and water in extracellular fluids.
SECTION A

STRUCTURAL SURVEY OF CARRAGEENANS FROM VARYING SOURCES

PART ONE

Structural Units Present in the Polysaccharides

INTRODUCTION

In the General Introduction it was stated that the sulphated galactans from red seaweeds exist in nature more for their physical properties than for their chemical reactions. In order to understand this more fully, the physical properties of these polysaccharides must be explained in terms of their primary structures. One way in which this could be done would be to determine the primary structures of polysaccharides extracted from many different species of red seaweed and to attempt to correlate their primary structure with their observed properties. Any new structural type found in this way would then be examined conformationally in the attempt to account for any distinctive properties. It would be particularly useful if new types of regular structure could be found as these structures lend themselves particularly well to examination by diffraction techniques, an example being $\xi$-carrageenan from *Fucalespinosum* which gives exceptionally good X-ray fibre photographs.\(^2\)\(^4\) From a purely biological viewpoint, a survey of this type enables comparisons to be drawn between differing seaweed species as to the types of polysaccharide structure which they synthesise.

In this Section, details are given of a methylation study on fourteen polysaccharides extracted from red seaweeds collected in various parts of the world and supplied by Marine Colloids Inc. As stated earlier, methylation followed by hydrolysis does not distinguish between the/...
the site of sulphation and of glycosidic substitution but if the results are interpreted in terms of structures in which 3-linked β-residues and 4-linked α-residues are present in equal proportions, then the sulphation pattern can be deduced. Recent evidence has shown, for most of the polysaccharides in the survey, that 1,4 linkages occur alternately along the chain. 39 The polysaccharides have all been methylated under alkaline conditions and there is thus a distinct possibility of some base catalysed elimination of sulphate which must be taken into account in the interpretation of results.
EXPERIMENTAL

Methylation and Examination of Samples

The polysaccharides (1 g. of each) were separately dissolved in water (100 ml.), and each solution was cooled in ice. Dimethyl sulphate (10 ml.) and sodium hydroxide (30% w/v, 30 ml.), were added over 6 hr. with vigorous stirring under nitrogen for 16 hr. The addition was repeated four times at room temperature. The solution was dialysed, and concentrated under diminished pressure to 100 ml., and the entire cycle was repeated twice. Methoxyl analysis is not used as a direct criterion of the extent of methylation of these polysaccharides because the following factors render it unreliable: (a) cation exchange could occur to an indefinite extent during methylation; (b) impurities from the 30% sodium hydroxide cannot be removed by extraction of the polysaccharides into chloroform; (c) the hygroscopic nature of freeze-dried carbohydrate sulphates. The polysaccharides were taken to be fully methylated if, on hydrolysis, no galactose appeared in the hydrolysate. Most of the polysaccharides reached this stage after three methylation cycles: a few polysaccharides required a further two cycles, and some of the polysaccharides were suspected of being under-methylated even after repeated methylation cycles. As a further check on the extent of methylation, samples of the polysaccharides (20 mg. of each) were converted to their ammonium salt forms using Amberlite IR 120 (NH₄⁺ form) resin and analysed for OCH₃ and S. The OCH₃/S ratio could thus be evaluated and compared with the OH/S ratio calculated for the original polysaccharide.

The methylated polysaccharides were isolated by dialysis and freeze drying and examined by the following techniques:

(1)/..
(1) **Hydrolysis in 45% formic acid for 16 hr. at 100°**
followed by paper chromatography in solvents (a),
(b), (c) and (d), spray (1).

(2) **Oxidative hydrolysis (selected polysaccharides only).**

Each methylated polysaccharide (60 mg.) was dissolved in water
(3 ml.) and sulphuric acid (1.0N, 3 ml.) was added, followed by bromine
(5 drops). The solution was heated at 60° for 24 hr. after which time
the negative resorcinol-hydrochloric acid reaction on an aerated aliquot
showed that all 3,6-anhydrogalactoside linkages had been broken. The
bromine was removed from the main solution by aeration and sulphuric acid
(6.0N) was added to give a solution which was 1.0N with respect to acid,
and the mixture was heated at 100° for 15 hr. to hydrolyse the galactosyl
bonds and sulphate groups. Complete hydrolysis was confirmed by the
negative phenol-sulphuric acid reaction given by an aliquot sample after
neutralisation with sodium carbonate and reduction with potassium
borohydride. After neutralisation with barium carbonate, silver carbonate
was added to the suspension which was left in the dark with occasional
shaking for 72 hr., and then filtered and treated with Amberlite resin
IR 120 (H\(^+\) form). The solutions were examined by paper chromatography
in solvent (i) spray (4); comparison of the spot intensities at a
number of different loadings gave an approximate estimate of the relative
amounts of 3,6-anhydrogalactonic acids. Under these conditions the
relative mobilities were:

| 3,6-anhydrogalactonic acid     | 0.69 |
| 2,6-di-\(\beta\)-methylgalactonic acid | 0.82 |
| 3,6-anhydro-2-\(\alpha\)-methylgalactonic acid | 1.00 |
| 3,6-anhydro-2,5-di-\(\beta\)-methylgalactonic acid | 1.40 |
| 3,6-anhydro-2,4-di-\(\beta\)-methylgalactonic acid | 1.56 |
Solvent (i) was found to deteriorate on standing for more than a week, adversely affecting separations.

(3) Methanalysis (2.3% methanolic hydrogen chloride in a sealed tube for 16 hr. at 100°C, followed by neutralisation with silver carbonate). All polysaccharides were examined on column (a), with the exception of Furcellaria fastigiata (lambda fraction) which was examined on column(b).

RESULTS AND DISCUSSION OF INDIVIDUAL POLYSACCHARIDE STRUCTURES

(A) POLYSACCHARIDES OF THE K-6,4 RANGE

(I) Furcellaria fastigiata (K fraction)

Source - Furcellaria fastigiata collected in Northumberland Straits, Nova Scotia, summer 1965.

Molar ratio, galactose : 3,6-anhydride : sulphate 1.00 : 0.09 : 0.74.

The infra-red spectrum showed a peak at 845 cm⁻¹.

Hydrolysis followed by paper chromatography in solvent (f), spray (1), showed galactose with faint traces of xylose.

Analysis of the methylated polysaccharide gave C₁₁₂₂₂₂, H₂₂₂₂, O₂₆₆₂; 36.07%. Thus, C₁₁₂₂₂₂/S calcd. 4.26, Found 3.88.

Hydrolysis followed by paper chromatography of the methylated polysaccharide gave:

Degradation products of 3,6-anhydrogalactose derivatives

<table>
<thead>
<tr>
<th>Derivative</th>
<th>Relative Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4,6-tri-0-methylgalactose</td>
<td>(+++)</td>
</tr>
<tr>
<td>2,6-di-0-methylgalactose</td>
<td>(+++)</td>
</tr>
<tr>
<td>2,4-di-0-methylgalactose</td>
<td>(+)</td>
</tr>
<tr>
<td>mono-0-methylgalactose(s)</td>
<td>trace</td>
</tr>
</tbody>
</table>

Oxidative hydrolysis showed 3,6-anhydro-2-0-methylgalactonic acid as the major product, with no trace of 3,6-anhydrogalactonic acid. An additional product was detected having R = 1.19 in about 10% concentration. Methanalysis/...
**Methanolysis and gas chromatography.** (Fig. 7).

<table>
<thead>
<tr>
<th>Component</th>
<th>Retention Times</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>tetra-O-methylgalactosides</td>
<td>1.67</td>
<td>Trace</td>
</tr>
<tr>
<td>2,3,6-tri-O-methylgalactosides</td>
<td>2.76</td>
<td>Trace</td>
</tr>
<tr>
<td>2,4,6-tri-O-methylgalactosides</td>
<td>3.66, 4.17</td>
<td>Strong</td>
</tr>
<tr>
<td>3,6-anhydro-2-O-methylgalactose</td>
<td>6.15, 7.35, 16.45</td>
<td>Strong</td>
</tr>
<tr>
<td>derivatives</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,6-di-O-methylgalactosides</td>
<td>8.43, 10.29, 11.09, 14.40</td>
<td>Medium</td>
</tr>
<tr>
<td>2,4-di-O-methylgalactosides</td>
<td>13.61, (15.52)</td>
<td>Weak</td>
</tr>
</tbody>
</table>

The identification of 2,4,6-tri-O-methylgalactose and 2,6-di-O-methylgalactose shows that the 1,3 linked residues in the polysaccharide occur both as galactose and galactose 4-sulphate, in the approximate ratio of 3:2. The 1,4 linked unit is almost entirely non-sulphated 3,6-anhydrogalactose. This is borne out by the infra-red spectrum which shows strong absorption at 845 cm⁻¹ indicative of a galactose 4-sulphate residue; the absence of any absorption at 805 cm⁻¹ indicates that there are no 3,6-anhydrogalactose 2-sulphate units in the molecule. Paper chromatography shows the presence of 2,4-di-O-methylgalactose in quite substantial amounts, but this identification is not confirmed by gas chromatography although there is a distinct possibility of masking by the 3,6-anhydro-2-O-methylgalactoside peak with Rₜ 16.45. The most likely significance of the 2,4-di-O-methylgalactose is either (a) branch points through position 6, or (b) 1,3 linked galactose 6-sulphate units, i.e.

![Chemical structures](image)

**Fig. 8.**

The/...
GAS CHROMATOGRAPHY OF METHANOLYSATES OF METHYLATED

F. fastigiata (K).

G. canaliculata (K)

Fig. 7.

For conditions, see text.

Key to peak numbers:

1. Solvent and degradation products.
2. 2,3,4,6-tetra-0-methylgalactosides.
3. 2,3,6-tri-0-methylgalactoside (1 of 3 peaks).
4. 2,4,6-tri-0-methylgalactosides.
5. 3,6-anhydro-2-0-methylgalactose derivatives.
6. 2,6-di-0-methylgalactosides.
7. 2,4-di-0-methylgalactosides.
The identification of 2,4-di-\(\beta\)-methylgalactose after hydrolysis of methylated desulphated furcellaran, and the absence of any absorption at 820 cm\(^{-1}\) in the infra-red spectrum would be consistent with the former possibility. In view of this uncertainty only a tentative structure can be put forward for Furcellaria fastigiata (kappa):

1,3 linked component
\[\beta-D\text{-}galactopyranosyl 4\text{-}sulphate (2)*\]

1,4 linked component
\[\beta-D\text{-}galactopyranosyl\]

mainly 3,6-anhydro-\(\alpha\)-D-
\[\beta-D\text{-}galactopyranosyl (3) galactopyranosyl\]

Together with some relatively minor features such as 3-linked galactosyl 6-sulphate or 3,6-linked galactosyl, and 4-linked galactosyl residues.

*The figures are the proportion of one type of 1,3 linked unit relative to the other; similarly for the 1,4 linked units.

(II) Gigartina canaliculata (\(\kappa\) fraction)

Source - Gigartina canaliculata collected from the Pacific Coast of Baja, California in 1966.

Molar ratio of galactose : 3,6-anhydride : sulphate, 1.00:0.64:1.20.

The infra-red spectrum showed peaks at 850 cm\(^{-1}\) (strong) and 805 cm\(^{-1}\) (weak).

Hydrolysis and paper chromatography gave galactose as the only spot.

Analysis of the methylated polysaccharide gave CMe 14.80; 
S 8.17. Thus, OCH\(_3\)/S calc. 2.03 Found 1.87.

Hydrolysis followed by paper chromatography of the methylated polysaccharide gave

Degradation products of 3,6-anhydrogalactose derivatives in large amounts

- 2,4,6-tri-\(\beta\)-methylgalactose: trace
- 2,6-di-\(\beta\)-methylgalactose: (+++)
- mixed monomethylgalactosides: small amounts
Oxidative hydrolysis showed 3,6-anhydrogalactonic acid and its 2-0-methyl ether in the approximate ratio 1:4.

Methanolysis and gas chromatography. (Fig. 7.)

<table>
<thead>
<tr>
<th>Component</th>
<th>Retention Times</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>tetra-0-methylgalactosides</td>
<td>1.75</td>
<td>trace</td>
</tr>
<tr>
<td>2,3,6-tri-0-methylgalactosides</td>
<td>2.91</td>
<td>trace</td>
</tr>
<tr>
<td>2,4,6-tri-0-methylgalactosides</td>
<td>3.66, 4.18</td>
<td>medium</td>
</tr>
<tr>
<td>3,6-anhydro-2-0-methylgalactose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>derivatives</td>
<td>6.17, 7.54, 16.72</td>
<td>strong</td>
</tr>
<tr>
<td>2,6-di-0-methylgalactosides</td>
<td>8.43, 10.25, 11.60, 14.90</td>
<td>strong</td>
</tr>
</tbody>
</table>

The identification of 2,4,6-tri-0-methylgalactose and 2,6-di-0-methylgalactose indicates that the 3-linked residues in the polysaccharide occur both as galactose and its 4-sulphate, with the latter present in greater amount. Oxidative hydrolysis shows that only a minor proportion of the 3,6-anhydride units are 2-sulphated. This sulphation pattern is confirmed by the infra-red spectrum. The overall picture is therefore:

**1,3 linked component**

\[
\beta-D-galactopyranosyl 4-sulphate (4) \\
\beta-D-galactopyranosyl (1)
\]

**1,4 linked component**

\[
3,6-anhydro-\alpha-D-galactopyranosyl (4) \\
3,6-anhydro-\alpha-D-galactopyranosyl 2-sulphate (1)
\]

Other structural units, such as 4-linked galactosyl sulphate esters could also be present.

(III) *Gigartina Chamissoi* (∞ fraction)

**Source** - *Gigartina chamissoi var. lessonii* collected in Peru, 1966.

Molar ratio of galactose : 3,6-anhydride : sulphate, 1.00:0.82:1.31.

The...
The infra-red spectrum showed peaks at 650 cm\(^{-1}\) (strong) and 805 cm\(^{-1}\) (weak).

Hydrolysis and paper chromatography gave galactose and a faint trace of xylose.

Analysis of the methylated polysaccharide gave: OMe 13.64%; S 8.50%. Thus, OCH\(_3\)/S calc. 1.92 Found 1.66.

Hydrolysis followed by paper chromatography of the methylated polysaccharide gave:

Degradation products of 3,6-anhydrogalactose derivatives in large amounts:

- 2,4,6-tri-O-methylgalactose: barest traces only
- 2,6-di-O-methylgalactose: (++++)
- Mono-methylgalactose(s): barest traces only

Oxidative hydrolysis showed 3,6-anhydrogalactonic acid and its 2-O-methyl ether in the approximate ratio 1:4.

Methanolysis and gas chromatography:

<table>
<thead>
<tr>
<th>Component</th>
<th>Retention Times</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>tetra-O-methylgalactosides</td>
<td>1.79</td>
<td>trace</td>
</tr>
<tr>
<td>2,3,6-tri-O-methylgalactosides</td>
<td>2.85</td>
<td>trace</td>
</tr>
<tr>
<td>2,4,6-tri-O-methylgalactosides</td>
<td>3.61, 4.12</td>
<td>weak</td>
</tr>
<tr>
<td>3,6-anhydro-2-O-methylgalactose</td>
<td>6.16, 7.46, 16.49</td>
<td>strong</td>
</tr>
<tr>
<td>derivatives</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,6-di-O-methylgalactosides</td>
<td>8.43, 10.22, 11.68, 14.81</td>
<td>strong</td>
</tr>
</tbody>
</table>

The identification of 2,6-di-O-methylgalactose and the presence of 2,4,6-tri-O-methylgalactose in only trace amounts, suggests that the 3-linked residues are almost entirely sulphated on position 4. Oxidative hydrolysis shows that only a minor proportion of the 3,6-anhydride residues are sulphated. The overall picture is therefore:
Minor structural features are likely to include 4-linked galactose sulphates, and perhaps a trace of 3-linked galactose.

(IV) Ahnfeltia

Source - Ahnfeltia durvillaei collected in Peru, 1966.

Molar ratio of galactose : 3,6-anhydride : sulphate, 1.00:0.59:1.25.

The infrared spectrum showed peaks at 850 cm\(^{-1}\) (strong) and 805 cm\(^{-1}\) (weak).

Hydrolysis and paper chromatography gave galactose and a faint trace of xylose.

Analysis of the methylated polysaccharide gave %Me 15.04; S 7.97. Thus, OCH\(_3\)/S calc. 1.67 Found 1.95.

Examination of the methylated polysaccharide showed it to be similar to Gigartina chamaesoi (kappa) but having more of the 1,4 linked 3,6-anhydride units sulphated on position 2 and with a few of the 1,3 linked galactose units non-sulphated at position 4. Small amounts of mono-O-methylgalactoses were identified by paper chromatography indicating that the polysaccharide contains some 1,4 linked galactose 2,6 disulphate units.

The overall picture is therefore:

<table>
<thead>
<tr>
<th>1,3 linked component</th>
<th>1,4 linked component</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\beta)-D-galactopyranosyl 4-sulphate</td>
<td>(3,6)-anhydro-(\alpha)-D-galactopyranosyl (4)</td>
</tr>
<tr>
<td>(\beta)-D-galactopyranosyl</td>
<td>(3,6)-anhydro-(\alpha)-D-galactopyranosyl</td>
</tr>
<tr>
<td>2-sulphate</td>
<td>(1)</td>
</tr>
</tbody>
</table>

Minor/...
The infra-red spectrum shows peaks at 850 cm\(^{-1}\) (strong) and 805 cm\(^{-1}\) (medium).

Hydrolysis and paper chromatography gave galactose and faint traces of xylose.

Analysis of the methylated polysaccharide gave OMe 12.24%; S 6.43%. Thus, OCH\(_3\)/S calc. 1.46 Found 1.49.

Examination of the methylated polysaccharide showed it to be similar to the previous three polysaccharides but having an even greater proportion of the 1,4 linked 3,6-anhydride units 2-sulphated.

The overall picture is:

<table>
<thead>
<tr>
<th>1,3 linked component</th>
<th>1,4 linked component</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\beta-D)-galactopyranosyl 4-sulphate</td>
<td>3,6-anhydro-(\alpha-D)-galactopyranosyl (1)</td>
</tr>
<tr>
<td>3,6-anhydro-(\alpha-D)-galactopyranosyl</td>
<td>2-sulphate (4)</td>
</tr>
</tbody>
</table>

Other structural features presumably include 4-linked galactose sulphates; 3-linked \(\beta-D\)-galactopyranosyl is present in trace amounts.

(VII) *Eucheuma uncinatum*

Source - *Eucheuma uncinatum* collected from the Gulf of California in Spring 1966.

Molar ratio of galactose : 3,6-anhydride : sulphate, 1.00:0.45:1.44.

The infra-red spectrum shows peaks at 850 cm\(^{-1}\) (strong) and 805 cm\(^{-1}\) (medium).

Hydrolysis and paper chromatography gave galactose as the only spot.

Analysis of the methylated polysaccharide gave: OMe 11.71%; S 9.21%. Thus OCH\(_3\)/S calc. 1.39 Found 1.31.

Examination of the methylated polysaccharide showed it to be of the iota type, i.e. virtually all the 1,4 linked 3,6-anhydride being sulphated on position 2.
The overall picture is:

<table>
<thead>
<tr>
<th>1,3 linked component</th>
<th>1,4 linked component</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-D-galactopyranosyl 4-sulphate (9)</td>
<td>3,6-anhydro-α-D-galactopyranosyl</td>
</tr>
<tr>
<td>β-D-galactopyranosyl (1)</td>
<td>2-sulphate</td>
</tr>
</tbody>
</table>

Other structural features could include 4-linked galactosyl sulphate esters.

(VIII) *Aphardhiella tenera*

Source - *Aphardhiella tenera* collected at Hungars Creek, Virginia, November 1964.

Molar ratio of galactose : 3,6-anhydride : sulphate, 1.00:0.47:1.35.

The infra-red spectrum shows peaks at 850 cm⁻¹ (strong) and 805 cm⁻¹ (medium).

Hydrolysis and paper chromatography showed galactose and traces of xylose.

Analysis of the methylated polysaccharide gave CMe 12.44% ;
S 8.59%. Thus OCH₃/S calc. 1.57 Found 1.50.

Examination of the methylated polysaccharide showed it to have a structure almost identical to that of *Bucheuma uncinatum*, i.e. it is an iota carrageenan.

The overall picture is:

<table>
<thead>
<tr>
<th>1,3 linked component</th>
<th>1,4 linked component</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-D-galactopyranosyl 4-sulphate</td>
<td>3,6-anhydro-α-D-galactopyranosyl</td>
</tr>
<tr>
<td>2-sulphate</td>
<td></td>
</tr>
</tbody>
</table>

Other structural features presumably include 4-linked galactose sulphates, and there are very small amounts of non-sulphated 3-linked galactose and 4-linked 3,6-anhydride.
B. **LAMBDA TYPE POLYSACCHARIDES**

(IX) **Furcellaria fastigiata** (λ fraction)


Molar ratio of galactose : 3,6-anhydride : sulphate, 1.00:0.07:0.53.

The infra-red spectrum shows a peak at 845 cm⁻¹ (weak).

Hydrolysis and paper chromatography gave galactose with traces of xylose and fucose.

Analysis of the methylated polysaccharide gave OMe 25.31%; S 6.48%. Thus, OCH₃/S calc. 4.79 Found 4.17.

Hydrolysis followed by paper chromatography of the methylated polysaccharide gave

Degradation products of 3,6-anhydrogalactose derivatives

- 2,4,6-tri-O-methylgalactose
- 2,6-di-O-methylgalactose
- 2,3-di-O-methylgalactose
- 2,4-di-O-methylgalactose
- mono-O-methylgalactose(s)

Oxidative hydrolysis showed that a component having R 0.27, represented 80% of the detectable products. Small amounts of 3,6-anhydro-2-O-methylgalactonic acid, and a component having R 1.19 c.f. kappa fraction, were also observed.

Methanolysis/..
Methanolysis and gas chromatography.

<table>
<thead>
<tr>
<th>Component</th>
<th>Retention Times</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>tetra-O-methylgalactosides</td>
<td>1.33</td>
<td>trace</td>
</tr>
<tr>
<td>2,3,6-tri-O-methylgalactosides</td>
<td>2.48</td>
<td>very weak</td>
</tr>
<tr>
<td>2,4,6-tri-O-methylgalactosides</td>
<td>2.60, 3.14</td>
<td>strong</td>
</tr>
<tr>
<td>3,6-anhydro-2-O-methylgalactose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>derivatives</td>
<td>4.27, 4.83, 10.47</td>
<td>weak</td>
</tr>
<tr>
<td>2,6-di-O-methylgalactosides</td>
<td>5.52, 6.05, 6.88, 9.15</td>
<td>medium</td>
</tr>
<tr>
<td>2,3-di-O-methylgalactosides</td>
<td>5.52, 6.05, 7.64</td>
<td>weak</td>
</tr>
<tr>
<td>2,4-di-O-methylgalactosides</td>
<td>3.19, 9.15</td>
<td>trace</td>
</tr>
</tbody>
</table>

The interpretation of the data for this polysaccharide is complicated due to the difficulty in completely separating 2,3 and 2,6-di-O-methylgalactoses both by paper chromatography and g.l.c. When these two sugars are present in a mixture the presence of both can be detected but it is difficult to assess their relative proportions. A tentative attempt has been made to gauge the relative proportions of these two sugars and the structure proposed below is based on this assessment. Gas chromatography shows the presence of quite substantial amounts of 3,6-anhydro-2-O-methylgalactose, whereas the analytical figures for the native polysaccharide show only small amounts of 3,6-anhydrogalactose. Thus it seems likely that the 2-O-methyl 3,6-anhydrogalactose could have come from galactose 6-sulphate which underwent elimination during methylation. There is evidence that galactose 6-sulphate is more susceptible to elimination than 2,6-disulphate and this could account for the failure to identify 2,3-di-O-methylgalactose in the hydrolysates of this and other polysaccharides where it might be expected.

The/..
The identification of 2,4,6-tri-O-p-methylgalactose and 2,6-di-O-methylgalactose shows that the 1,3 linked units in the polysaccharide occur both as galactose and its 4-sulphate in the approximate ratio 2 : 1. The 1,4 linked unit appears to be a mixture of galactose, galactose 6-sulphate, and 3,6-anhydrogalactose. As with the kappa fraction, traces of 2,4-di-O-methylgalactose were observed and again the most likely significance of this sugar is either branch points through position 6 or 1,3 linked galactose 6-sulphate units.

The overall picture is:

<table>
<thead>
<tr>
<th>1,3 linked component</th>
<th>1,4 linked component</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-D-galactopyranosyl 4-sulphate (1)</td>
<td>α-D-galactopyranosyl 6-sulphate</td>
</tr>
<tr>
<td>β-D-galactopyranosyl (2)</td>
<td>3,6-anhydro-α-D-galactopyranosyl</td>
</tr>
<tr>
<td>α-D-galactopyranosyl</td>
<td></td>
</tr>
</tbody>
</table>

Together with some relatively minor structural features such as 3-linked galactosyl 6-sulphate or 3,6-linked galactosyl.

(X) *Gigartina canaliculata* (λ fraction)

Source - *Gigartina canaliculata* collected from the Pacific Coast of Baja, California in 1966.

Molar ratio of galactose : 3,6-anhydride : sulphate, 1.00:0.11:1.27.

The infra-red spectrum shows a peak at 830 cm⁻¹ (strong).

Hydrolysis and paper chromatography shows galactose as the only spot.

Analysis of the methylated polysaccharide gave OMe 11.97%;

S 10.07%. Thus, OCH₃/S calc. 1.45 Found 1.23.

This polysaccharide appeared to be slightly undermethylated even after repeated treatment with sodium hydroxide and dimethyl sulphate.

Hydrolysis/
Hydrolysis followed by paper chromatography of the methylated polysaccharide gave:

Degradation products of 3,6-anhydrogalactose derivatives

4,6 and/or 3,6-di-O-methylgalactose (++++)
mono-O-methylgalactose(s) (+)
galactose (+)

Methanolysis and gas chromatography. (Fig. 9).

<table>
<thead>
<tr>
<th>Component</th>
<th>Retention Times</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>tetra-O-methylgalactosides</td>
<td>1.65</td>
<td>trace</td>
</tr>
<tr>
<td>2,3,6-tri-O-methylgalactosides</td>
<td>2.84</td>
<td>trace</td>
</tr>
<tr>
<td>2,4,6-tri-O-methylgalactosides</td>
<td>3.68, 4.16</td>
<td>trace</td>
</tr>
<tr>
<td>3,6-di-O-methylgalactosides</td>
<td>5.83, 9.26, 13.40</td>
<td>medium</td>
</tr>
<tr>
<td>4,6-di-O-methylgalactosides</td>
<td>7.45, 13.40</td>
<td>strong</td>
</tr>
</tbody>
</table>

The mixture of 3,6- and 4,6-di-O-methylgalactoses occurring in the hydrolysate of this polysaccharide cannot be separated by paper chromatography. The glycosides are quite easily separable on g.l.c. however.

The only methyl ether which can be attributed to 3-linked residues is 4,6-di-O-methylgalactose, showing that these residues are completely 2-sulphated. The 3,6-di-O-methylgalactose must arise from 1,4 linked galactose 2-sulphate units. There is significantly less 3,6- than 4,6-di-O-methylgalactose and this, together with the presence of substantial amounts of monomethylgalactose, indicates the presence of 1,4 linked galactose 2,6 disulphate units.

The overall picture is:

1,3 linked component
β-D-galactopyranosyl 2-sulphate
1,4 linked component
α-D-galactopyranosyl 2-sulphate
α-D-galactopyranosyl 2,6-disulphate (assumed).
GAS CHROMATOGRAPHY OF METHANOLYSATE OF METHYLATED

G. canaliculata (λ)

Fig. 9.

On NPGA column at 175⁰.

Key to peak numbers:

1. Solvent and degradation products.
2. 2,3,6-tri-O-methylgalactoside. (1 of 3 peaks).
3. Mixture of 2,3,6- and 2,4,6-tri-O-methylgalactosides.
4. 3,6-di-O-methylgalactosides.
6. 4,6-di-O-methylgalactosides.
The failure to detect 3,6-anhydro-2-0-methylgalactose derivatives after methanolysis would seem to indicate that the 3,6-anhydride occurs mainly as the 2-sulphate.

(XI) *Gigartina chlamissoi* (λ fraction)

Source - *Gigartina chlamissoi* var. lessonii collected in Peru, 1966.

Molar ratio of galactose : 3,6-anhydride : sulphate, 1 : 0.16 : 1.17.

The infra-red spectrum shows a peak at 820-830 cm⁻¹ (strong).

Hydrolysis and paper chromatography shows galactose and a trace of xylose.

Analysis of the methylated polysaccharide gave OMe 13.10/;

S 9.51/. Thus, OCH₃/S calcd. 1.70 Found 1.42.

This polysaccharide appeared to be slightly undermethylated even after repeated cycles of sodium hydroxide and dimethyl sulphate.

The evidence for this polysaccharide is very similar to that for *G. canaliculata* (λ fraction), except that traces of 3,6-anhydro-2-0-methylgalactose derivatives were detected after methanolysis.

The overall picture is therefore:

1,3 linked component

β-D-galactopyranosyl 2-sulphate

1,4 linked component

α-D-galactopyranosyl 2-sulphate

α-D-galactopyranosyl 2,6-disulphate (assumed).

Minor structural features include 4-linked 3,6-anhydrogalactose, and probably its 2-sulphate.

(XII) *Gigartina atropurpurea* (λ fraction)

Source - *Gigartina atropurpurea* collected in New Zealand in 1965.

Molar ratio of galactose : 3,6-anhydride : sulphate, 1 : 0.14 : 1.37.

The/..
The infra-red spectrum shows a strong broad peak at 820-830 cm$^{-1}$.

Hydrolysis and paper chromatography show galactose and traces of xylose.

Analysis of the methylated polysaccharide gave OMe 9.32%; S 11.20%. Thus, OMe/S calcd. 1.29 Found 0.86.

This polysaccharide was definitely undermethylated by the criterion that there should be no galactose in the hydrolysate of the methylated polysaccharide, and the conclusions must therefore be qualified.

Hydrolysis followed by paper chromatography of the methylated polysaccharide gave:

Degradation products of 3,6-anhydrogalactose derivatives

- $2,3,6$-tri-$\Omega$-methylgalactose: barest traces
- $2,4,6$-tri-$\Omega$-methylgalactose: barest traces
- $3,6$ and/or $4,6$-di-$\Omega$-methylgalactose: (+++)
- $2,6$-di-$\Omega$-methylgalactose: (+)
- mono-$\Omega$-methylgalactoses: (++)
- galactose: (+)

Methanolysis and gas chromatography.

<table>
<thead>
<tr>
<th>Component</th>
<th>Retention Times</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>tetra-$\Omega$-methylgalactosides</td>
<td>1.73</td>
<td>trace</td>
</tr>
<tr>
<td>$2,3,6$-tri-$\Omega$-methylgalactosides</td>
<td>2.92</td>
<td>trace</td>
</tr>
<tr>
<td>$2,4,6$-tri-$\Omega$-methylgalactosides</td>
<td>3.84, 4.19</td>
<td>trace</td>
</tr>
<tr>
<td>$3,6$-di-$\Omega$-methylgalactosides</td>
<td>5.85, 9.15, 13.29</td>
<td>medium</td>
</tr>
<tr>
<td>$3,6$-anhydro-2-$\Omega$-methylgalactose derivatives</td>
<td>6.17, (7.54), 16.60</td>
<td>medium</td>
</tr>
<tr>
<td>$4,6$-di-$\Omega$-methylgalactosides</td>
<td>7.44, 13.29</td>
<td>strong</td>
</tr>
<tr>
<td>$2,6$-di-$\Omega$-methylgalactosides</td>
<td>8.43, (10.29), 11.69, 14.40</td>
<td>weak</td>
</tr>
</tbody>
</table>

The/..
The identification of 4,6-di-O-methylgalactose as a major product shows that the 3-linked residues in the polymer are almost entirely 2-sulphated, although the small amounts of 2,6-di-O-methylgalactose indicate some 1,3 linked galactose 4-sulphate. The 3,6-di-O-methylgalactose indicates the presence of 1,4 linked galactose 2-sulphate. The detection of 3,6-anhydro-2-O-methylgalactose derivatives after methanolysis indicates 1,4 linked 3,6-anhydrogalactose units whereas the analytical figures show only small amounts of 3,6-anhydrogalactose to be present in the native polysaccharide. Thus, it seems likely that there has been some elimination of 1,4 linked galactose 6-sulphate units during methylation. Paper chromatography shows substantial amounts of mono-O-methylgalactose indicating 1,4-linked galactose 2,6 disulphate units.

More recent research has shown that, in the alkali modified polysaccharide, all the 3,6-anhydrogalactose residues occur combined in contiguous carrabiose units. This raises two distinct possibilities: (a) the material designated G. atrojurpurea (lambda) consists of two polysaccharides, one of which is a polymer of galactose 2-sulphate units linked alternately α1,3 and β1,4 and the other a polymer in which the 1,3-linked unit is galactose 2-sulphate and the 1,4-linked unit a mixture of 3,6-anhydrogalactose, galactose 6-sulphate and galactose 2,6-disulphate; (b) the material consists of a single polymer made up of blocks of the two polymer types listed in (a).

C. OTHER TYPES OF POLYSACCHARIDE

(XIII) Pachymenia himantophora

Source - Pachymenia himantophora collected in New Zealand in 1965.
Molar ratio of galactose : 3,6-anhydride : sulphate, 1.00:0.06:0.77.

The infra-red spectrum showed a broad peak at 820-830 cm⁻¹.

Hydrolysis and paper chromatography showed galactose and a trace of xylose.

Analysis of the methylated polysaccharide gave OCH₃ 16.43%; S 8.90%. Thus OCH₃/S calc. 2.97 Found 1.91.

This polysaccharide was definitely undermethylated by the criterion that there should be no galactose in the hydrolysate of the methylated polysaccharide, and the conclusions must therefore be qualified.

Hydrolysis followed by paper chromatography of the methylated polysaccharide gave:

Degradation products of 3,6-anhydrogalactose derivatives

<table>
<thead>
<tr>
<th>Component</th>
<th>Retention Times</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>tetra-0-methylgalactose</td>
<td>trace</td>
<td></td>
</tr>
<tr>
<td>2,3,6-tri-0-methylgalactose</td>
<td>(++++)</td>
<td></td>
</tr>
<tr>
<td>2,4,6-tri-0-methylgalactose</td>
<td>trace</td>
<td></td>
</tr>
<tr>
<td>2,6-di-0-methylgalactose</td>
<td>(++)</td>
<td></td>
</tr>
<tr>
<td>4,6 and/or 3,6-di-0-methylgalactose</td>
<td>(++)</td>
<td></td>
</tr>
<tr>
<td>mono-0-methylgalactose(s)</td>
<td>(++)</td>
<td></td>
</tr>
<tr>
<td>galactose</td>
<td>(++)</td>
<td></td>
</tr>
</tbody>
</table>

Methanolysis and gas chromatography. (Fig. 10).

<table>
<thead>
<tr>
<th>Component</th>
<th>Retention Times</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>tetra-0-methylgalactosides</td>
<td>1.72</td>
<td>trace</td>
</tr>
<tr>
<td>2,3,6-tri-0-methylgalactosides</td>
<td>2.90, 3.50, 3.82, 4.23</td>
<td>strong</td>
</tr>
<tr>
<td>3,6-anhydro-2-0-methylgalactose</td>
<td>6.16, 7.52, 16.66</td>
<td>weak</td>
</tr>
<tr>
<td>derivatives</td>
<td>7.45, 12.98</td>
<td>medium</td>
</tr>
<tr>
<td>4,6-di-0-methylgalactosides</td>
<td>8.43, 10.25, 11.77, 14.91</td>
<td>medium</td>
</tr>
<tr>
<td>2,6-di-0-methylgalactosides</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
GAS CHROMATOGRAPHY OF METHANOLYSATE OF METHYLATED Pachymenia himantophora

Fig. 10.

For conditions, see text.

Key to peak numbers:

1. Solvent and Degradation products.
2. 2,3,4,6-tetra-\text{O}-methylgalactosides.
3. 2,3,6-tri-\text{O}-methylgalactosides.
4. 3,6-anhydro-2-\text{O}-methylgalactose derivatives.
5. 4,6-di-\text{O}-methylgalactosides.
6. 2,6-di-\text{O}-methylgalactosides.
The 4-linked unit in this polysaccharide would seem to be mainly galactose due to the presence of 2,3,6-tri-O-methylgalactose as a major product of hydrolysis and methanolysis. The 3,6-anhydro-2-O-methylgalactose probably arises from 1,4-linked galactose 6-sulphate units eliminated during methylation. The identification of 4,6-di-O-methylgalactose suggests the presence of 1,3 linked units sulphated at position 2, and the traces of 2,4,6-tri-O-methylgalactose detected by paper chromatography indicates that some of these residues are sulphate free. The 2,6-di-O-methylgalactose could have arisen from 3-linked units sulphated at position 4, although the spectroscopic evidence, no band at 850 cm⁻¹ for axial secondary sulphate, is against this. This sugar could also have arisen from a 4-linked 3-sulphate or from undermethylation. 2,6-Di-O-methylgalactose has been isolated after methylation and hydrolysis of the polysaccharide from Aeodes orbitosa, which, like Pachymenia himantophora is a member of the Grateloupiaceae, and in this case also there was no spectroscopic evidence for galactose 4-sulphate units. There seems to be a possibility that in polysaccharides extracted from seaweeds in the Grateloupiaceae family the 3- and 4-linked residues do not occur in equal proportions.

(XIV) Gloiopeltis furcata

Source - Gloiopeltis furcata collected in South Korea in 1967.

Molar ratio of galactose : 3,6-anhydride : sulphate, 1.00:0.52:0.94.

The infra-red spectrum shows a broad peak at 820 cm⁻¹.

Hydrolysis and paper chromatography showed galactose with about 10% xylose and a trace of fucose.

Analysis of the methylated polysaccharide gave OMe 19.69%; OMe/S calc. 2.74 Found 2.87.

Hydrolysis/...
Hydrolysis followed by paper chromatography of the methylated polysaccharide:

Degradation products of 3,6-anhydrogalactose derivatives

- 2,4,6-tri-O-methylgalactose: trace
- 2,4-di-O-methylgalactose: (++++)
- 2,6-di-O-methylgalactose: trace
- Mixed mono-O-methylgalactoses: (+)

On oxidative hydrolysis followed by paper chromatography the main product was 3,6-anhydro-2-O-methylgalactonic acid, together with 3,6-anhydrogalactonic acid and unidentified components having R 0.55 and 0.27. The approximate concentrations were in the ratio 10 : 2 : 1 : trace, respectively.

Methanolysis and gas chromatography. (Fig. 11).

<table>
<thead>
<tr>
<th>Component</th>
<th>Retention Times</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetra-O-methylgalactosides</td>
<td>1.71</td>
<td>trace</td>
</tr>
<tr>
<td>2,3,6-tri-O-methylgalactosides</td>
<td>2.89</td>
<td>weak</td>
</tr>
<tr>
<td>2,4,6-tri-O-methylgalactosides</td>
<td>3.56, 4.12</td>
<td>medium</td>
</tr>
<tr>
<td>3,6-anhydro-2-O-methylgalactose derivatives</td>
<td>6.10, 7.35, 16.00</td>
<td>strong</td>
</tr>
<tr>
<td>2,6-di-O-methylgalactosides</td>
<td>8.43, (10.00), 11.12, (14.50)</td>
<td>trace</td>
</tr>
<tr>
<td>2,4-di-O-methylgalactosides</td>
<td>13.45, 15.45</td>
<td>strong</td>
</tr>
</tbody>
</table>

The identification of 2,4-di-O-methylgalactose and the presence of only small amounts of 2,4,6-tri-O-methyl and 2,6-di-O-methylgalactose suggests that virtually all the 3-linked units are sulphated on position 6. Oxidative hydrolysis shows that most of the 3,6-anhydride is non-sulphated.
GAS CHROMATOGRAPHY OF METHANOLYSATE OF METHYLATED
Gloioeltis furcata

Fig. 11.

For conditions, see text.

Key to peak numbers:
1. Solvent and Degradation products.
2. 2,3,4,6-tetra-\(\text{O}\)-methylgalactosides.
3. 2,3,6-tri-\(\text{O}\)-methylgalactoside. (1 of 3 peaks).
4. Mixture of 2,3,6- and 2,4,6-tri-\(\text{O}\)-methylgalactosides.
5. 3,6-anhydro-2-\(\text{O}\)-methylgalactose derivatives.
6. 2,6-di-\(\text{O}\)-methylgalactosides.
7. 2,4-di-\(\text{O}\)-methylgalactosides.
The configuration of the 3,6-anhydrogalactose residues in this polysaccharide has been shown to be L by the isolation of agarobiose dimethyl acetal in high yield on partial methanolysis of the polysaccharide.\(^{41}\)

The overall picture is therefore:

\[ \begin{array}{ll}
\text{1,3 linked component} & \text{1,4 linked component} \\
\beta-D-galactopyranosyl 6-sulphate (9) & 3,6-anhydro-\alpha-L-galactopyranosyl (5) \\
\beta-D-galactopyranosyl (1) & 3,6-anhydro-\alpha-L-galactopyranosyl \\
& 2-sulphate (1) \\
\end{array} \]

Other structural features presumably include 4-linked galactose sulphates, and there might be minor amounts of 3-linked galactopyranosyl 4-sulphate.

The native polysaccharide on hydrolysis gave galactose and xylose in the approximate ratio 9 : 1, and thus it would be expected that some methylated xylose derivatives would be observed on hydrolysis and methanolysis of the methylated polysaccharide. This was not the case however, and a possible explanation could be that the xylose occurred in a contaminating polysaccharide of low molecular weight which was lost in the dialyses between methylation cycles.
In the Introduction to this Section it was stated that it would be very useful if new types of regular structure could be found so that they could be examined by diffraction techniques. From this viewpoint the survey has proved rather disappointing. It had been hoped that a regular lambda carrageenan would be found, i.e. one in which the 1,3 linked unit is entirely galactose 2-sulphate and the 1,4 linked unit is galactose 2,6-disulphate. The lambda fractions from *Chondrus crispus* and *Gigartina stellata* show about 30% undersulphation on the 1,3 linked units and partly as a result of this they do not give good X-ray diffraction diagrams. The lambda fractions from *G. atropurpurea*, *G. chamesai* and *G. canaliculata* are completely 2-sulphated on the 3-linked residues, but are undersulphated at position 6 of the 4-linked residues. There is a possibility that the material designated *G. atropurpurea* (lambda fraction) is in fact two polysaccharides, one of which is a polymer consisting of galactose 2-sulphate units linked alternately α1,3 β1,4. If this component could be isolated in the pure state, it would be expected, because of its regular structure, to give better X-ray fibre photographs than have been previously obtained from other lambda type polysaccharides. This polysaccharide is not in fact a lambda carrageenan but represents a new type of structure which could be designated β-carrageenan.

The kappa fraction from *G. chamesai* is similar to that from *C. crispus*, but the kappa fractions from *F. fastigiata* and *G. canaliculata* are novel in showing undersulphation on position 4 of the 1,3 linked unit. The λ-fraction from *F. fastigiata* seems to be related to μ-carrageenan isolated from *C. crispus* but differs in showing undersulphation on position 4 of the 1,3 linked unit. *Gymnoclonium* and *Ahnfeltia* polysaccharides seem to belong to the μ-carrageenan group whereas the polysaccharides...
polysaccharides from *E. isoforme*, *E. uncinatum* and *Aparrhidiella tenera* are of the \(\alpha\)-type although they are not as 'ideal' as \(\lambda\)-carrageenan from *E. spinosum* and thus do not give such good X-ray fibre photographs. The \(\lambda\)-carrageenan from *Aparrhidiella tenera* can be converted into short regular segments by splitting the polysaccharide by Smith degradation followed by 6-sulphate elimination with alkaline borohydride. These segments have proved useful in studying conformations in solution as their chain lengths appear to be too short to form the networks which cause complications in work of this type.

*Pachy menia* polysaccharide is a very interesting new type of carrageenan which shows some features of \(\kappa\) - and \(\lambda\)-carrageenans but in which most of the 4-linked residues are neither 3,6-anhydride nor sulphate esters, but simply galactose. The polysaccharide from *Gloiopeltis furcata* is a member of the agar and not the carrageenan family, but differs from both agarose, which is unsulphated, and porphyran, which has some of the 4-linked components 6-sulphated, in that the 3-linked residues occur almost entirely as the 6-sulphate.
PART TWO

The Synthesis of 3,6-Di-O-Methylgalactose

INTRODUCTION

In Part One it was reported that 3,6-di-O-methylgalactosides were identified in the methanolysates of methylated *G. canaliculata*, *G. chemissoi* and *G. atropurpurea* ( fractions). Authentic 3,6-di-O-methylgalactose has never before been synthesised although its isolation, from the water soluble polysaccharide from coconut kernel, has been claimed. In 1965, Dr. B.A. Rees isolated a small amount of material which had the same paper chromatographic mobility as 4,6-di-O-methylgalactose, solvent (c), but which, on methanolysis, gave a different pattern of g.l.c. peaks from the other known di-O-methylgalactoses. It was thus assumed that this material was 3,6-di-O-methylgalactose although it was never characterised and no intermediates in the preparation were isolated and characterised. This material was initially used as a reference standard in the methylation survey but it was later thought necessary to establish its identity more fully and thus the synthesis of 3,6-di-O-methylgalactose described in the Experimental Section was undertaken.

EXPERIMENTAL

The reaction scheme for the synthesis is shown in Fig. 12.

Stage 1: 1,3,4,6-tetra-O-acetyl-α-D-galactose. 45

D-galactose monohydrate (264 g.) was added with stirring to acetic/.
Fig. 12. The synthetic route to 3,6-di-O-methylgalactose.
Fig. 12 continued.

\[
\begin{align*}
\text{PhNHCOCOO} & \quad \text{CH}_3\text{OTr} \quad \text{OCH}_3 \\
\text{CH}_3 & \quad \text{MsO} \quad \text{OCH}_3 \\
\text{PhNHCOCOO} & \quad \text{CH}_2\text{CH} \quad \text{OCH}_3 \\
\text{CH}_3 & \quad \text{MsO} \quad \text{OCH}_3 \\
\text{PhNHCOCOO} & \quad \text{HO} \quad \text{CH}_2\text{OCH}_3 \\
\text{CH}_3 & \quad \text{HO} \quad \text{H}_2\text{OH} \\
\end{align*}
\]
acetic anhydride (1 l.) and perchloric acid (4 ml.) over a period of 45 minutes, the temperature being kept between 40° and 45° by cooling in iced water. After stirring for one hour at room temperature, the solution was cooled further to 15° before adding phosphorous tribromide (172 ml.) followed slowly by water (92 ml.). After a further hour at room temperature, the solution was cooled to 10° before addition of aqueous sodium acetate (200 g. in 250 ml. water, precooled to 5°), cautiously so that the temperature was kept in the range 45-50°. A further amount of sodium acetate (600 g.) in water (750 ml.) was then added before allowing the solution to stand for 30 minutes at 35-40°. The product was extracted into chloroform, washed with iced water followed by cold saturated aqueous sodium bicarbonate, then again with iced water, and dried over calcium chloride and treated with charcoal. Evaporation to dryness gave a syrupy residue which was crystallised from ether.

Yield 151.7 g. (29.6%), m.p. 146° (lit. 151°).

Stage 2: 1,3,4,6-tetra-O-acetyl-2-O-methanesulphonyl-α-D-galactose.

The product from the previous stage (150 g.) was slowly added to a stirred mixture of dry pyridine (200 ml.) and methanesulphonyl chloride (147 g.) in an ice-salt bath at -10°. After stirring for one hour at this temperature, the mixture was left at 0° overnight then poured into water. The solid which separated was removed on a filter and crystallised from ethanol.

Yield 149.3 g. (61.2%), m.p. 97-97° (lit. 96-98°);

\[ \alpha \] D + 86.4° (c 1.7 in chloroform).

Calculated for \( \text{C}_{15}\text{H}_{22}\text{O}_{12} \text{S} \) C 42.19, H 5.21, S 7.52.

Found C 43.48, H 5.53, S 7.30.

Stage 3/
Stage 3: Methyl 3,4,6-tri-α-acetyl-2-α-methanesulphonyl-β-D-galactopyranoside.

The product from the previous stage (141.3 g.) was dissolved in hydrogen bromide in acetic acid (45%, 400 ml.), and left at room temperature (3 h.) before dilution with chloroform (1500 ml.), followed by washing with iced water (thrice). After drying over sodium sulphate, evaporation gave a syrup which was dissolved in dry methanol (1500 ml.). Calcium sulphate (20 g.) and silver carbonate (140 g.) were added, and the mixture was stirred in the dark (16 h.), then filtered and evaporated. The syrupy residue was crystallised from ethanol.

Yield 73.0 g. (55.4%), m.p. 137-138° (lit. 139.5-140.5°; [α]D -10.3° (c 3.0 in chloroform).

Calculated for C14H22O11S C 42.15, H 5.53, S 8.04
Found C 41.95, H 5.54, S 7.60

Stage 4: Methyl 2-α-methanesulphonyl-β-D-galactopyranoside.

The product from the previous stage (73 g.) was refluxed with methanolic hydrogen chloride (0.3%, 1000 ml.) for 16 hours. After neutralisation with sodium bicarbonate, filtration, and evaporation, the syrupy product was crystallised from ethanol.

Yield 45.8 g. (91.9%), m.p. 145-146°. [α]D -19.9° (c 1.6 in pyridine).

Calculated for C8H16O6S C 35.30, H 5.88, S 11.76.
Found C 35.47, H 5.94, S 11.60.

To check that anomerisation had not occurred during deacetylation, part of the product (5 mg.) was heated in a sealed tube at 100° with pyridine-acetic anhydride (1:1 v/v, 1 ml.) for 1 hour. The solution was cooled, diluted with water, and extracted with chloroform. A sample of/...
of the chloroform layer was withdrawn for analysis by g.l.c. using an SE52 column (3% on Gas Chrom P) at 200°. This showed a single sharp peak, identical to that given by the product of Stage 3.

The Konigs-Knorr synthesis does not necessarily lead to the β anomer when there is a non-participating neighbouring group on C-2, and it was therefore necessary to check the configuration of the product. A small sample (10 mg.) was dissolved in sodium hydroxide (2N, 0.1 ml.) and left at room temperature (16 h.). After the addition of solid carbon dioxide then ethanol (5 ml.), the solution was filtered and analysed by paper chromatography, solvent (a), spray (5). The controls were the methyl 2,3-anhydro-α- and β-talopyranosides, kindly supplied by Professor J.G. Buchanan.

![Figure 13](image)

The reaction product was identical with the β-anomer, and well separated from the α, proving that the 2-O-methanesulphonyl glycoside had the β configuration.

**Stage 5**: Methyl 4,6-benzylidene-2-O-methanesulphonyl-β-D-galactopyranoside.

The product from the previous stage (45 g.) in redistilled tetrahydrofuran (1000 ml.), was mixed with toluene-p-sulphonic acid (80 mg.) and benzaldehyde dimethyl acetal (80 ml.). The solution was slowly/..
slowly concentrated by distillation at atmospheric pressure, until the volume was 100 ml. (30 minutes). It was then neutralised with sodium bicarbonate, filtered, and evaporated to dryness. The syrupy product was crystallised from ethanol.

Yield 43.2 g. (72.7%), m.p. 183-185°; [α]D + 20.2° (c 1.2 in pyridine).

Calculated for C15H20O8S C 50.26, H 5.88, S 8.50, OCH3 8.60.
Found C 50.26, H 5.88, S 8.50, OCH3 8.40.

**Stage 6:** Methyl 4,6-benzylidene-2-O-methanesulphonyl-3-O-methyl-β-D-galactopyranoside.

The product from the previous stage (42 g.), in N,N-dimethylformamide (390 ml.), was methylated with methyl iodide (130 ml.) and silver oxide (75 g.) by stirring in ice for 2 hours then at room temperature for 4 days, anhydrous conditions being maintained throughout. The mixture was diluted with chloroform, filtered and washed with sodium cyanide solution (1% in water). The cyanide solution was back-extracted with chloroform (five times), then the combined chloroform layers were washed with water and dried over sodium sulphate. The product was obtained by evaporation to dryness and crystallisation from ethanol.

Yield 28.4 g. (63.5%), m.p. 182-184°; [α]D + 30.2° (c 1.26 in chloroform).

Calculated for C16H22O8S C 51.3, H 5.88, S 8.55, OCH3 16.57.
Found C 51.19, H 6.17, S 8.60, OCH3 16.80.

**Stage 7:** Methyl 2-O-methanesulphonyl-3-O-methyl-β-D-galactopyranoside.

The product from the previous stage (27.5 g.), in aqueous formic acid/..
acid (40% v/v; 675 ml.), was heated on a boiling water bath for 30 minutes then cooled and benzaldehyde was removed by extraction (thrice) with benzene. The aqueous layer was evaporated to dryness and water was distilled from the residue repeatedly to remove formic acid, and the product was finally obtained by crystallisation from ethanol.

Yield 15.5 g. (71.1%), m.p. 161-163°, α D -5.14° (c 1.44 in acetone).

Calculated for C_{18}H_{18}O_{5} S  C 37.76, H 6.29, S 11.19, OCH_{3} 21.68.

Found  C 37.81, H 6.22, S 11.36, OCH_{3} 21.30.

A sample of the product (100 mg.) was converted to the acetate, which was crystalline, for examination by n.m.r. at 100 MHz in deuteriochloroform with tetramethylsilane as the internal reference. The spectrum was in accordance with the structure proposed and showed τ 6.40, 6.53 (3-proton singlets corresponding to two methoxy groups), 6.88 (3-proton singlet corresponding to the methyl of the sulphonyl ester), 7.03, 7.90 (3-proton singlets corresponding to two acetoxyl groups).

Stage 8: Methyl 2-O-methanesulphonyl-3-O-methyl-6-O-triphenylmethyl-β-D-galactopyranoside.

The pyridine used in this experiment was redistilled and kept over potassium hydroxide; immediately before use it was redistilled again from phosphorous pentoxide.

The product from the preceding stage (14.96 g.) in pyridine (200 ml.) was mixed with chlorotriphenylmethane (24.4 g.) and kept at room temperature for 16 hours before pouring into a large volume of iced water. The precipitate was removed by filtration and washed thoroughly with water, then with ether. The residue was crystallised from ethanol.

Yield 8.18 g. (29.8%), m.p. 92-94°; [α] D -18.92° (c 1.96 in chloroform).

Calculated/..
Calculated for $\text{C}_{28}\text{H}_{32}\text{O}_6\text{S}$: $\text{C} 63.63$, $\text{H} 6.06$, $\text{S} 6.06$, $\text{OCH}_3 11.74$.

Found: $\text{C} 63.88$, $\text{H} 6.24$, $\text{S} 6.46$, $\text{OCH}_3 11.69$.

N.m.r. data (conditions as above except that the solvent was carbon tetrachloride) were in agreement with the structure proposed: $\delta 2.5-2.9$ (15-proton multiplet corresponding to the trityl group), $6.53$, $6.59$ (two 3-proton singlets, corresponding to two methoxy groups), $7.09$ (3-proton singlet corresponding to the methyl of the sulphonyl ester).

**Stage 9:** Methyl-2-$\alpha$-methanesulphonyl-3-$\alpha$-methyl-4-$\alpha$-phenylcarbamoyl-6-$\eta$-triphenylmethyl-$\beta$-$\eta$-galactopyranoside.

The product from the previous stage (7.1 g.), in pyridine (80 ml., prepared as above), was heated at 100° for 2 hours with phenyl isocyanate (3.3 ml.). The product separated on pouring into water and was removed by filtration and recrystallised from ethanol-acetone.

Yield 6.90 g. (78.0%), m.p. 214-215°; $[\alpha]_D^2 -13.4^0$ (g 2.18 in chloroform).

Calculated for $\text{C}_{39}\text{H}_{37}\text{O}_9\text{NS}$: $\text{C} 64.91$, $\text{H} 5.72$, $\text{S} 4.95$, $\text{N} 2.16$, $\text{OCH}_3 9.57$.

Found: $\text{C} 64.77$, $\text{H} 5.87$, $\text{S} 5.13$, $\text{N} 2.31$, $\text{OCH}_3 9.76$.

N.m.r. data (conditions as above, in deuteriochloroform) were in agreement with the structure proposed: $\delta 2.42-2.83$ (20-proton multiplet corresponding to the trityl and phenylcarbamoyl protons), $6.44$, $6.50$ (two 3-proton singlets corresponding to two methoxy groups), $6.95$ (3-proton singlet corresponding to the methyl of the sulphonyl ester).

**Stage 10:** Methyl 2-$\alpha$-methanesulphonyl-3-$\alpha$-methyl-4-$\alpha$-phenylcarbamoyl-$\beta$-$\eta$-galactopyranoside.

The product from the previous stage (6.22 g.), in chloroform (60 ml.) was mixed with methanol (100 ml.) and acetyl chloride (1.2 ml.). The solution/...
solution was heated on a boiling water bath for 5 minutes, cooled, then neutralised with silver carbonate, filtered and evaporated to dryness. The residue was taken up in benzene and applied to a 'Florisil' (synthetic silica gel) column (40 x 3 cm. diameter), and eluted with benzene to remove trityl chloride. Carbohydrate material was eluted with benzene/methanol 9:1 and the resulting solution evaporated to dryness. The syrupy product was crystallised from ethanol.

Yield 3.71 g. (61.9%), m.p. 142-143°C; [α]D + 45.6° (c 1.96 in chloroform).

Calculated for C_{16}H_{23}O_9 NS; C 47.41, H 5.68, S 7.90, N 3.46, OCH$_3$ 15.31.

Found C 47.69, H 5.11, S 8.27, N 3.61, OCH$_3$ 14.92.

N.m.r. data (conditions as above, in deuteriochloroform) were in agreement with the structure proposed: T 2.52-3.04 (3-proton multiplet corresponding to the phenylcarbamoyl protons), 6.43, 6.54 (two 3-proton singlets corresponding to two methoxy groups), 6.91 (3-proton singlet corresponding to the methyl of the sulphonyl ester).

Stage 11: Methyl 2-O-methanesulphonyl-3,6-di-O-methyl-4-O-N-methylphenylcarbamoyl-β-D-galactopyranoside.

The product from the previous stage (3.06 g.), in N,N-dimethylformamide (36 ml.), was methylated with methyl iodide (12 ml.) and silver oxide (12 g.), by shaking in ice for 2 hours then at room temperature for 4 days. The mixture was diluted with chloroform, filtered, and washed with sodium cyanide solution (1% in water). The cyanide solution was back-extracted with chloroform (five times), then the combined chloroform layers were washed with water and dried over sodium sulphate. After evaporation to dryness, the above procedure was repeated and the product isolated as a syrup which could not be crystallised.

Yield/...
Yield 2.90 g. (88.7%). $\left[\alpha\right]_D + 54.72$ (c 0.55 in acetone).

Calculated for $C_{18}H_{27}O_{3}NS$ C 49.99, H 6.24, S 7.37, N 3.23, OCH$_3$ 21.28.

Found C 50.14, H 6.33, S 6.93, N 3.21, OCH$_3$ 22.03.

N.m.r. data (conditions as above, in deuteriochloroform) were in agreement with the structure proposed: $\tau$ 2.58-2.62 (5-proton multiplet corresponding to the phenylcarbamoyl protons), 6.50, 6.63, 6.66 (three 3-proton singlets corresponding to three methoxy groups), 6.56 (3-proton singlet corresponding to the N-methyl group. This signal can be distinguished due to broadening by the quadruple coupling of the nitrogen atom), 6.92 (3-proton singlet corresponding to the methyl of the sulphonyl ester).

Stage 12: Methyl 3,6-di-0-methyl-$\alpha$-methyl-$\beta$-$D$-galactopyranoside.

The product from the previous stage (2.73 g.) in redistilled tetrahydrofuran (300 ml.) was refluxed with lithium aluminium hydride (5 g.) for 3 hours. Ethyl acetate (40 ml.) and water (10 ml.) were added to remove excess reagent and the tetrahydrofuran was removed by evaporation. Sulphuric acid (1.0 N, 150 ml.) was added and aniline was removed by extraction (thrice) with benzene. The solution was treated with Amberlite IR 120 (H$^+$ form) and IR 45 (OH$^-$ form) resins and evaporated to dryness. The product was isolated as a syrup which could not be crystallised.

Yield 1.27 g. (90.7%). $\left[\alpha\right]_D -11.48$ (c 1.86 in acetone).

Calculated for $C_{17}H_{18}O_{6}$ C 48.6, H 8.11, OCH$_3$ 41.8.

Found C 47.66, H 9.04, OCH$_3$ 40.71.

N.m.r. data (conditions as above, in deuteriochloroform) were in agreement with the structure proposed: $\tau$ 6.46, 6.52, 6.61 (three 3-proton singlets corresponding to three methoxy groups).

Characterisation/
Characterisation of methyl 3,6-di-O-methyl-β-D-galactopyranoside.

(a) Part of the product (10 mg.) in N,N-dimethylformamide (3 ml.) was shaken for 16 hours with methyl iodide (1 ml.) and silver oxide (0.7 g.). After dilution with chloroform and filtration, the solution was evaporated to dryness and the residue methanolysed and examined by g.l.c., column (b). This showed 2,3,4,6-tetra-O-methylgalactosides as the main product with traces of 2,3,6-tri-O-methylgalactosides.

(b) A mixture of 2,4,6-tri-O-methylgalactosides, 4,6-di-O-methylgalactosides and the synthetic β-glycoside was made up in chloroform. The solution was examined by g.l.c., column (b), and the ratio of the peak heights measured. The solution was evaporated to dryness and the residue dissolved in aqueous periodic acid (0.1M, 20 ml.), and left for 2 days. The solution was neutralised with saturated aqueous barium hydroxide, centrifuged, evaporated to dryness and the residue dissolved in chloroform and examined by g.l.c., column (b). This showed the complete disappearance of the 4,6-di-O-methylgalactoside peaks whereas the relative ratio of peaks due to 2,4,6-tri-O-methylgalactosides and the synthetic β-glycoside remained unchanged.

(c) The β-glycoside (50 mg.) in aqueous formic acid (45%, v/v, 4 ml.) was heated on a boiling water bath for 16 hours. After cooling the solution was evaporated repeatedly to remove formic acid. The residue was dissolved in water (5 ml.) and sodium metaperiodate (1 g.) added and the solution adjusted to pH 7.3 with saturated sodium bicarbonate solution. After leaving the solution to stand in the dark for 24 hours, it was acidified with hydrochloric acid (1.0 N) and hydrochloric acid (1.0 N, 10 ml.) and aqueous sodium arsenite (20%, 20 ml.) added. The solution/..
solution was distilled in vacuo and 2,4-dinitrophenylhydrazine (saturated solution in 2 N hydrochloric acid, 100 ml.) was added to the distillate. The solid which formed was filtered on a sintered glass disc and crystallised from ethanol; it had m.p. and mixed m.p. with the 2,4-dinitrophenylhydrazone of methoxyacetaldehyde 115-120° (lit. 124°). The latter was prepared by periodate oxidation of 6-0-methylgalactose.

\[
\begin{align*}
\text{CHO} & \quad \text{HCOOH} \\
\text{CH}_3\text{O} & \quad + \quad \text{CHO} \\
\text{HO} & \quad + \quad \text{CHO} \\
\text{CH}_2\text{OCH}_3 & \quad (1) \quad \text{distil} \\
\text{CH} & \quad \text{NNH} \\
\text{CH}_2\text{OCH}_3 & \quad \text{2,4 DNP} \\
\text{3H}_2\text{OCH} & \\
\end{align*}
\]

(Fig. 14.)

An n.m.r. spectrum (conditions as above in deuteriochloroform) was consistent with structure (2) (Fig.): T -0.6 (broad one proton singlet corresponding to N-H), 0.89 (one proton doublet, J 3c/s, corresponding to an aromatic proton), 1.65 (one proton quartet, J 3c/s, 10 c/s, corresponding to an aromatic proton), 2.05 (one proton doublet, J 10c/s, corresponding to an aromatic proton), 2.42 (one proton triplet, J 5c/s, corresponding to CH), 5.78 (two proton doublet, J 5c/s corresponding to CH\_2), 6.57 (three proton singlet corresponding to OCH\_3).

(d) A sample of the β-glycoside (5 mg.) was treated with methanolic hydrogen chloride (2.3% w/v, 100° for 16 hours) and the products examined by g.l.c. column (b). This showed peaks with retention times 4.38 (weak), 6.40 (strong), 8.81 (weak) and 10.90 (medium), identical to the pattern of g.l.c. peaks attributed to 3,6-di-0-methylgalactosides in the methanolsates of methylated G. canaliculata, G. chamisson and G. atropurpurea( λ fractions).
Stage 13: 3,6-di-O-methylgalactose.

Part of the product from the previous stage (0.20 g.) in sulphuric acid (2N, 10 ml.) was heated at 100° for 16 hours, neutralised with saturated barium hydroxide solution and treated with Amberlite IR 120 (H⁺) and IR 45 (OH⁻) resins. The product was examined by paper chromatography solvent (c), spray (1), which showed spots corresponding to a di-O-methyl and a mono-O-methylgalactose in the approximate ratio of 10:1 respectively. The di-O-methylgalactose was purified by chromatography on two sheets of Whatman 3MM filter paper, solvent (c). The sugar was eluted from the filter paper with water and the solution evaporated to a syrup (75 mg.) which partially crystallised (needles) on standing. Unfortunately, this material could not be characterised due to lack of time.
DISCUSSION

All possible partially methylated derivatives of D-galactopyranose, with the exception of 3,6-di-<sup>0</sup>-methylgalactose, were characterised by the early 1950's.<sup>50</sup> One of the reasons why 3,6-di-<sup>0</sup>-methylgalactose has never previously been characterised is that there is only one prior recorded instance of it being a constituent in the hydrolysate of a methylated polysaccharide,<sup>44</sup> and the evidence for this is very tentative. A second reason is that due to the configuration of the hydroxyl groups on the galactose ring, 3-<sup>0</sup>-substituted galactose derivatives are difficult to synthesise. This is in marked contrast to the glucose series where the orientation of the hydroxyl groups precludes the formation of cyclic acetal derivatives involving C-3. Thus by condensing 2 moles of acetone with one of glucose to give 1,2 : 5,6-diisopropylidene-D-glucofuranose, followed by methylation and hydrolysis, 3-<sup>0</sup>-methylglucose is easily obtained.<sup>51</sup> Galactose, on condensation with acetone, gives 1,2 : 3,4-diisopropylidene galactose and 3-<sup>0</sup>-methylgalactose is therefore much more difficult to synthesise than its glucose analogue. The first synthesis of 3-<sup>0</sup>-methylgalactose<sup>52</sup> was achieved by 2-tosylation of methyl 3-<sup>0</sup>-carbethoxy-4,6-benzylidene β<sub>IN</sub>-galactopyranoside followed by removal of the carbethoxy group, methylation and systematic removal of the blocking groups to give methyl 3-<sup>0</sup>-methyl-β<sub>IN</sub>-galactopyranoside, the free sugar was obtained on hydrolysis.

The synthetic route to 3,6-di-<sup>0</sup>-methylgalactose reported in the Experimental Section is a very long one and two alternative routes were unsuccessfully attempted in these laboratories before embarking upon it. The plan of the first route was to synthesise the ditosyl ester of methyl 3,6-anhydro-α<sub>IN</sub>-galactopyranoside, then to open the 3,6-anhydro ring/..
ring by acetylolation to give 2,4-di-O-tosyl galactose. Methylation, de-
tosylation and hydrolysis would then give 3,6-di-O-methylgalactose. This
method was unsuccessful as the acetylolation conditions necessary to open
the 3,6-anhydro ring caused extensive degradation of the sugar.

The plan of the second route was to synthesise methyl-4-O-mesyl
β-D-galactopyranoside,\(^{53}\) a known compound, which on hydrolysis and
condensation with acetone would give 1,2-isopropyldene-4-O-mesylgalactose.
Methylation, removal of the blocking groups and hydrolysis would give
3,6-di-O-methylgalactose. This was unsuccessful as the axial 4-O-mesyl
group could not be removed by reductive de-esterification. Another
attempt was made using a phenylurethane group in place of the mesyl group
but it was found to be too acid-labile, being removed simultaneously with
the glycosidic methyl group.

Although the synthetic route which was finally used is a long one,
each stage in it can be accomplished fairly easily and most of the yields
are over 60%. Due to the previous difficulty in removing axial 4-O-
mesyl groups a phenylcarbamoyl group was used to block the hydroxyl group
at C-4 in Stage 9. Erimacomb\(^{54}\) has reported an instance of phenyl-
carbamoyl migration under the methylation conditions used in Stage 11 but
methylation of carbohydrates partially esterified by phenylcarbamoyl groups
has been achieved under comparable conditions without significant migration
of the ester group\(^{55,56}\) and migration does not occur in the present
synthesis. Both the 2-O-mesyl and the 4-O-N-methyl-phenylcarbamoyl
groups were very easily removed on reduction with lithio aluminium
hydrida which is in marked contrast to the difficulty of removal of the
4-O-mesyl group.

The structure of methyl 3,6-di-O-methylgalactoside is proved by the
following evidence:

(a)/..
(a) chromatography, analysis and the n.m.r. spectrum are all consistent with a methyl di-α-methylglycoside.

(b) methylation followed by methanolysis to methyl 2,3,4,6-tetra-α-methylgalactosides shows that it is a galactose derivative.

(c) hydrolysis and periodate oxidation yielded methoxyacetaldehyde identified as its 2,4-dinitrophenylhydrazone. The only possible structures consistent with all the evidence are methyl 2,6- or 3,6-di-α-methylgalactosides. That the sugar is in fact methyl 3,6-di-α-methylgalactoside is proved by its resistance to periodate oxidation.
SECTION B

THE LOCATION OF SULPHATE ESTER GROUPS ON CARRAGEENANS

PART ONE

Oxidative Hydrolysis of κ-Carrageenan from Chondrus Crispus

INTRODUCTION

In the General Introduction, it was stated that the sulphate ester in κ-carrageenan from Chondrus crispus occurs mostly as 1,3 linked galactose 4-sulphate with smaller amounts of 1,4 linked 3,6-anhydrogalactose 2-sulphate, galactose 6-sulphate and 2,6-disulphate. Although there is a great deal of evidence to support this sulphation pattern, it is not absolutely unambiguous and is open to other possible interpretations which, although unlikely, cannot be rigorously excluded.

Evidence for 1,3 linked galactose 4-sulphate units comes from several sources. 2,6-di-α-methylgalactose is a major product of hydrolysis of the methylated polysaccharide and this indicates either a 1,3 linked 4-sulphate or a 1,4 linked 3-sulphate. These two alternatives, together with the possibility of branching through either the 3 or 4 position, cannot be distinguished by methylation and hydrolysis of the desulphated polysaccharide as the normal conditions for desulphation cause cleavage of the 3,6-anhydrogalactosyl linkages. The infrared spectrum of the polysaccharide shows a strong peak at 850 cm\(^{-1}\), very similar to the behaviour of monosaccharide derivatives of galactose 4-sulphate. The interpretation involving 1,3 linked galactose 4-sulphate residues is also preferred because degradation of κ-carrageenan, with a κ-carrageenase isolated from the culture medium of Pseudomonas carrageenovora, leads to a homologous series of sulphated oligosaccharides from...
from which a sulphated oligosaccharide, 2-3,6-anhydro-α-D-galacto-
pyranosyl-(1→3)-D-galactose 4-sulphate (neocarrabiose 4-sulphate) was isolated.\(^57\)

![Chemical structure of neocarrabiose 4-sulphate](image)

**Fig. 15.** neocarrabiose 4-sulphate.

Further evidence for the presence of galactose 4-sulphate units in the polymer is the alkaline stability of most of the sulphate ester in \(\kappa\)-carrageenan. This rules out galactose 3-sulphate as a major structural feature as sulphate groups on C-3 would undergo base catalysed elimination, via a 2,3 epoxide, probably to 3,6-anhydrogalactose.

Although all the above data are consistent with 1,3 linked galactose 4-sulphate being a major structural feature in \(\kappa\)-carrageenan, some of the 3-linked 4-sulphate could be replaced by 4-linked 3-sulphate or by branching through position 3 or 4, and not be detected by any of the present evidence.

The yield of carrabiose dimethyl acetal on methanolysis of alkali modified \(\kappa\)-carrageenan, which approaches 100\%, shows that all the 3,6-anhydride units in the molecule are linked through position 4. Thus the 3,6-anhydrogalactonic acid isolated after depolymerisation of the methylated polysaccharide could have arisen from the presence of 3,6-anhydride units sulphated on position 2 or branched through position 2 provided that the branches did not alter the 'carrabiose content' of the/..
the polymer. There is also a possibility that the 3,6-anhydrogalactonic acid could be due to undermethylation as it is a minor component in the hydrolysate and therefore at a level which could arise from incomplete methylation. Infrared studies indicate the presence of sulphated 3,6-anhydrogalactose units. (See Section B, Part Two for more details.)

κ-Carrageenan, on treatment with alkali, shows an increase in 3,6-anhydride which is diminished by prior periodate oxidation. This evidence is consistent with the presence of 1,4 linked galactose 6-sulphate units in the molecule but the possibility of some other leaving group on C-6, cannot be overlooked. The small increase in 3,6-anhydride on alkali treatment of the periodate oxidised polymer could be due to either 1,4 linked galactose 3-sulphate or 2,6-disulphate or even a natural epoxide which rearranges on alkali treatment to the anhydride.

If κ-carrageenan could be cleaved into sulphated oligosaccharides, the characterisation of these fragments would provide good evidence for the positions of the sulphate ester groups in the polysaccharide. Unfortunately, most methods for cleaving κ-carrageenan into smaller fragments, such as methanolation, mercaptolysis and acetolysis have the disadvantage that they remove sulphate ester groups at least as rapidly as they cleave 3,6-anhydride linkages. A method has been developed in these laboratories for hydrolysing 3,6-anhydride linkages using bromine-sulphuric acid at 60°; the bromine converts the 3,6-anhydride to 3,6-anhydrogalactonic acid which is stable to further attack by acid. These very mild hydrolysis conditions do not cause any cleavage of galactosyl linkages and seem unlikely to cause much sulphate ester hydrolysis. Thus, if the presently accepted sulphation pattern for κ-carrageenan is correct then hydrolysis of the polysaccharide by the bromine-sulphuric acid method should produce fragments such as (1), (2), (3), (4) and (5), Fig. 16.
(1) carrabionic acid 4-sulphate.

(2) carrabionic acid 2,4-disulphate.

(3) $R = R' = H$

(4) $R = H, R' = SO_3^-$

(5) $R = SO_3^- , R' = H$

Fig. 16.
Prior to the commencement of the research reported in this Thesis, attempts were made in these laboratories, by Dr. T.C.S. Dolan, to oxidatively hydrolyse \( \kappa \)-carrageenan and to isolate fragments such as those shown in Fig. 16. The method used was as follows: dried \( \kappa \)-carrageenan (20 g.) in sulphuric acid (0.5 N, 600 ml.) and bromine (2 ml.) was heated at 60° for 24 hours. After removal of bromine by aeration, the solution was neutralised with saturated aqueous barium hydroxide, filtered and treated with Amberlite IR 120 (H\(^+\)) resin then \( N,N \) dioctylmethylamine (5% v/v in chloroform). The solution was concentrated to a syrup (5.1 g.), dissolved in water (25 ml.) and applied to a DEAE Sephadex column in the formate form (prepared from 100 g. of 'Sephadex-A25'). The column was eluted with a linear gradient of aqueous formic acid (0-3% over 5 litres), then with 5 litres each of 5%, 10% and 15% formic acid solutions. Column fractions were combined into larger fractions on the basis of chromatography and each of these larger fractions was analysed for ester sulphate using the chloroaminodiphenyl method. The analyses showed that none of the fragments contained any ester sulphate.

Three explanations were put forward as the possible reason for the failure to isolate sulphated oligosaccharides after oxidative hydrolysis of \( \kappa \)-carrageenan.

1. The ester sulphate was more labile to hydrolysis than expected with the result that the sulphate was lost during the bromine-sulphuric acid hydrolysis.

2. The acid conditions of elution from the column caused hydrolysis of the ester.

3. The sulphated oligosaccharides were so strongly held to the column that they could not be eluted even with 15% formic acid. This could also account for the low recovery from the column.
The research reported in the Experimental Section describes the isolation and characterisation of sulphated oligosaccharides from κ-carrageenan and shows that none of these explanations is correct.
EXPERIMENTAL

All references to \( \kappa^- \) and \( \lambda^- \)-carrageenans in this Section refer to the \( \kappa^- \)-fraction from *Chondrus crispus* and the \( \lambda^- \)-fraction from *Eucheuma spinosum* unless otherwise stated.

**EXPERIMENT 1**

**Reaction Rates for Ester Sulphate Hydrolysis and 3,6-Anhydralactosyl Bond Cleavage on Oxidative Hydrolysis of \( \kappa^- \) and \( \lambda^- \)-carrageenans.**

Dried \( \kappa^- \)-carrageenan (0.35 g.) was dissolved in water (10 ml.) and hydrochloric acid (1.0N, 10 ml.) and bromine (sufficient to give a small separate layer) were added and the solution stirred on a water bath at 60°. Samples were withdrawn at intervals and freed from bromine by aeration. Residual (i.e. nonoxidised) 3,6-anhydralactose was determined by the resorcinol method and free sulphate was determined spectrophotometrically with chloroaminodiphenyl. After 48 hours the bulk solution was aerated to remove bromine and then heated at 100° for 16 hours to complete the hydrolysis of sulphate ester. Measurement of the free sulphate concentration in this solution gives the infinity value for sulphate hydrolysis.
RESULTS

Rate of disappearance of 3,6-anhydrogalactose

0.025 ml. aliquots were used for determinations.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Colorimeter readings (duplicate)</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>6.30, 6.30</td>
<td>6.30</td>
</tr>
<tr>
<td>4.0</td>
<td>3.02, 3.10</td>
<td>3.06</td>
</tr>
<tr>
<td>6.5</td>
<td>2.65, 2.79</td>
<td>2.72</td>
</tr>
<tr>
<td>11.5</td>
<td>1.97, 1.95</td>
<td>1.96</td>
</tr>
<tr>
<td>24.5</td>
<td>1.02, 1.01</td>
<td>1.02</td>
</tr>
<tr>
<td>48.5</td>
<td>0.58, 0.52</td>
<td>0.55</td>
</tr>
</tbody>
</table>

These results are shown on the graph in Fig. 17.

Sulphate Ester Release.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Aliquot (ml.)</th>
<th>Spectrophotometer reading (duplicate)</th>
<th>Average</th>
<th>Removed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>blank</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>0.25</td>
<td>0.726, 0.722</td>
<td>0.724</td>
<td>10.91</td>
</tr>
<tr>
<td>4.0</td>
<td>0.10</td>
<td>0.421, 0.408</td>
<td>0.415</td>
<td>16.34</td>
</tr>
<tr>
<td>6.5</td>
<td>0.10</td>
<td>0.530, 0.542</td>
<td>0.539</td>
<td>20.32</td>
</tr>
<tr>
<td>11.5</td>
<td>0.10</td>
<td>0.496, 0.491</td>
<td>0.494</td>
<td>19.43</td>
</tr>
<tr>
<td>24.5</td>
<td>0.050</td>
<td>0.512, 0.507</td>
<td>0.510</td>
<td>37.85</td>
</tr>
<tr>
<td>48.5</td>
<td>0.050</td>
<td>0.389, 0.389</td>
<td>0.389</td>
<td>59.20</td>
</tr>
<tr>
<td></td>
<td>0.025</td>
<td>0.446, 0.435</td>
<td>0.441</td>
<td>100.00</td>
</tr>
</tbody>
</table>

These results are shown on the graph in Fig. 18.

This/..
This experiment was repeated using \( \alpha \)-carrageenan.

RESULTS

Rate of disappearance of 3,6-anhydrogalactose

0.025 ml. aliquots were used for determinations.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Colorimeter readings (duplicate)</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>5.66, 5.66</td>
<td>5.66</td>
</tr>
<tr>
<td>2.0</td>
<td>4.66, 4.76</td>
<td>4.71</td>
</tr>
<tr>
<td>4.0</td>
<td>3.55, 3.31</td>
<td>3.43</td>
</tr>
<tr>
<td>6.75</td>
<td>2.36, 2.27</td>
<td>2.31</td>
</tr>
<tr>
<td>11.5</td>
<td>1.42, 1.47</td>
<td>1.45</td>
</tr>
<tr>
<td>24.75</td>
<td>0.86, 0.95</td>
<td>0.90</td>
</tr>
<tr>
<td>48</td>
<td>0.61, 0.68</td>
<td>0.65</td>
</tr>
</tbody>
</table>

These results are shown on the graph in Fig. 17.

Sulphate Ester Release.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Aliquot (ml.)</th>
<th>Spectrophotometer reading (duplicate)</th>
<th>Average</th>
<th>% SO₄₂⁻ Removed</th>
</tr>
</thead>
<tbody>
<tr>
<td>blank</td>
<td></td>
<td>0.744, 0.750</td>
<td>0.747</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>0.10</td>
<td>0.387, 0.397</td>
<td>0.392</td>
<td>24.23</td>
</tr>
<tr>
<td>2.0</td>
<td>0.10</td>
<td>0.410, 0.409</td>
<td>0.410</td>
<td>22.87</td>
</tr>
<tr>
<td>4.0</td>
<td>0.05</td>
<td>0.575, 0.602</td>
<td>0.589</td>
<td>21.50</td>
</tr>
<tr>
<td>6.75</td>
<td>0.05</td>
<td>0.551, 0.559</td>
<td>0.555</td>
<td>26.28</td>
</tr>
<tr>
<td>11.5</td>
<td>0.05</td>
<td>0.498, 0.490</td>
<td>0.494</td>
<td>34.46</td>
</tr>
<tr>
<td>24.75</td>
<td>0.025</td>
<td>0.548, 0.550</td>
<td>0.549</td>
<td>54.26</td>
</tr>
<tr>
<td>48.0</td>
<td>0.025</td>
<td>0.449, 0.447</td>
<td>0.448</td>
<td>81.56</td>
</tr>
<tr>
<td></td>
<td>0.020</td>
<td>0.458, 0.450</td>
<td>0.454</td>
<td>100.00</td>
</tr>
</tbody>
</table>

These results are shown in the graph in Fig. 18.

The/..
Fig. 17.

Decrease in 3,6-anhydrogalactose content of kappa (——) and iota (— —) carrageenans during bromine-hydrochloric acid hydrolysis at 60°.

Fig. 18.

Release of free sulphate from kappa (——) and iota (— —) carrageenans under bromine-hydrochloric acid hydrolysis at 60°.
The graphs show that 90% of the 3,6-anhydrogalactosyl linkages have been broken after hydrolysis of κ-carrageenan for 16 hours, whilst 70% of the ester sulphate remains. It should therefore be feasible to isolate oligosaccharide sulphates at this stage of the reaction.

EXPERIMENT 2

Preparation of Reference Compounds

(a) Hexa-α-acetyl carrabiose dimethyl acetal.

Carrabiose dimethyl acetal was isolated from commercial furcellaran (20 g; Litex) using the method of Painter except that the methanolysis products were separated on a cellulose column (60 x 7 cm) using as solvent butanol-ethanol-water (6 : 2 : 1). The fraction which contained carrabiose dimethyl acetal was evaporated to a syrup (3.1 g.) and dissolved at 0 in a mixture of pyridine (65 ml.) and acetic anhydride (50 ml.). The mixture was shaken at room temperature for 7 days and then poured into iced water. The solid which formed was crystallised from ethanol.

Yield 2.78 g. m.p. 148-149° (lit. 154°).

(b) Carrabionic acid.

A solution of methanol (25 ml.) and 2,2 dimethoxypropane (2.5 ml.) was shaken for 1 hour then sodium methoxide solution (2.5 ml., containing 5 mg./ml. sodium) was added followed by hexa-α-acetyl carrabiose dimethyl acetal (0.5 g.). The solution was left for 24 hours at room temperature when t.l.c. showed the deacetylation to be complete. After neutralisation with solid carbon dioxide the solution was evaporated to dryness and the residue dissolved in sulphuric acid (0.5 N, 10 ml.). Bromine (sufficient to give a small separate layer) was added and the mixture heated at 50° until oxidation was complete (resorcinol test on an aliquot after removal of/..
of the bromine by aeration). This required 8 hours. After cooling and removal of the bromine by aeration the solution was neutralised with barium carbonate and silver carbonate added to the suspension which was left in the dark, with occasional shaking, for 72 hours. After filtration and treatment with Amberlite IR 120 (H⁺ form) resin, the solution was concentrated to a syrup.

EXPERIMENT 3

Comparison of the Products of Bromine-Sulphuric Acid Cleavage of \( \kappa \) - and \( \iota \) -Carrageenans.

The experimental procedure was the same for both polysaccharides. Dried polysaccharide (0.5 g.) was dissolved in water (15 ml.) and sulphuric acid (1.0 N, 15 ml.) and bromine (sufficient to give a small separate layer) added. The mixture was heated at 60° for 16 hours. After this time the reaction was shown to be virtually complete by the resorcinol test. Excess bromine was blown off and the solution neutralised with saturated aqueous barium hydroxide and the resulting barium sulphate removed by centrifugation. Infrared spectra on the products, on silver chloride film, showed peaks at 1240 cm\(^{-1}\), 850 cm\(^{-1}\) and 618 cm\(^{-1}\). The absence of a peak at 805 cm\(^{-1}\) does not in itself show the absence of 2-sulphate as this ester, if it remained, would be attached to an acyclic side chain rather than a pyranoside ring.

Electrophoresis on the products, spray (2), showed only two spots from each product. These had \( \mu \)\( \text{GA} \) values 1.00 and 1.55 respectively. Carrabionic acid (prepared in Experiment 2) had \( \mu \)\( \text{GA} \) 1.00. The spots were thus taken to be carrabionic acid and its 4\( \text{X} \) sulphate respectively.

EXPERIMENT 4/
Early Unsuccessful Attempts to Separate Carrabionic Acid from its Sulphates.

Dried \( \kappa \)-carrageenan (10 g.) was dissolved in water (300 ml.) and sulphuric acid (1.0 \( N \), 300 ml.) and bromine (sufficient to give a small separate layer) added. The mixture was heated at 60° for 16 hours, cooled, aerated to remove bromine and neutralised with sodium hydroxide solution. The solution was evaporated to a small volume and applied to a charcoal-celite column (3 parts of May and Baker's charcoal to 2 of celite, 30 cm. x 3.9 cm. diameter) which was eluted with a linear gradient from 0-5% ethanol over 10 litres and fractions (50 ml.) collected automatically. The column was then eluted with 10% ethyl methyl ketone (5 litres) to remove any material which remained adsorbed. Fractions were analysed qualitatively for sulphate (with barium chloride) and quantitatively for carbohydrate (phenol-sulphuric acid). The following larger fractions were obtained on combining the contents of the tubes:

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Tube Numbers</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>28 - 37</td>
<td>Inorganic salts</td>
</tr>
<tr>
<td>B</td>
<td>38 - 123</td>
<td>Carbohydrate (plus traces of inorganic salts)</td>
</tr>
<tr>
<td>C</td>
<td>Eluted with aqueous ethyl methyl ketone</td>
<td>Carbohydrate</td>
</tr>
</tbody>
</table>

Electrophoresis, spray (2), showed that the contents of tubes 44, 53 and 62 were similar and gave two spots of equal intensity having \( \lambda_{\text{GA}} \) 1.00 and 1.55.

**Fraction B** - This seems to be a mixture of carrabionic acid and its 4 sulphate which does not seem to be separable by charcoal column chromatography.

**Fraction C/..**
**Fraction C** was evaporated to a syrup a small sample of which showed a significant increase in 3,6-anhydrogalactose content when treated with alkaline borohydride.

In a further attempt to separate carrabionic acid from its sulphate, Fraction B was evaporated to a syrup, redissolved in water (50 ml.) and applied to a DEAE Sephadex column in the phosphate form (prepared from 136 g. of Sephadex - A25). Elution was with a linear gradient of phosphate buffer (pH 7.5; 0-0.6M over 20 litres) with fractions (25 ml.) being collected automatically. Analysis of the fractions by the phenol-sulphuric acid method showed three fairly sharp peaks which corresponded to tube numbers (40-62) (77-95) and (118-180) respectively. The third peak, which was thought to contain carrabionic acid sulphate, was significantly larger than the other two.

This separation technique had to be abandoned however, as attempts to separate the products from phosphate buffer proved unsuccessful. Two methods were tried:

(a) Passage through a charcoal-celite column (3 parts of M & B charcoal to 2 of celite, 40 cm. x 5.5 cm. diameter). Only partial separation of carbohydrate and phosphate could be achieved by this technique.

(b) Gel filtration on a Sephadex column (prepared from 50 g. of Sephadex G-10). This proved unsuccessful as both the carbohydrate and phosphate were excluded from the column.

Due to the difficulty in separation of phosphate buffer from the oxidative hydrolysis products, it was decided to re-hydrolyse K-carrageenan and to separate the products on a DEAE Sephadex column using a volatile buffer, ammonium carbonate, as eluant.

Dried/..
Dried \( \kappa \)-carrageenan (10 g.) was hydrolysed with bromine-sulphuric acid in the usual manner. After removal of the bromine by aeration, the solution was neutralised with saturated aqueous barium hydroxide, centrifuged, treated with Amberlite IR 120 (H\(^+\)) resin and N,N diocetyl-methylamine (5% v/v in chloroform). The solution was concentrated to 50 ml. and examined by electrophoresis, spray (2), which showed only one spot with \( M_{GA} \) 1.00. This loss of carrabionic acid sulphate must have occurred in the work-up of the hydrolysate.

**Experiment 5**

**Demonstration that Carrabionic Acid Sulphates are Partitioned from Water to N,N-Dioctylmethylamine in Chloroform.**

Dried \( \kappa \)-carrageenan (50 mg.) was hydrolysed with bromine-sulphuric acid in the usual way and the hydrolysate was aerated and neutralised with barium hydroxide. After removal of precipitated barium sulphate on the centrifuge, the solution was de-cationised by passage through an Amberlite IR 120 (H\(^+\)) resin column and examined by electrophoresis, spray (2). Two spots were observed having \( M_{GA} \) 1.00 and 1.57. The solution was shaken with N,N-dioctylmethylamine in chloroform (5% v/v) then with chloroform to remove any amine from the aqueous phase. Electrophoresis showed only the spot with \( M_{GA} \) 1.00, thought to be carrabionic acid. The faster moving spot, thought to contain sulphate ester, was totally absent.

**Experiment 6**

**To Establish Optimum Work-up Conditions after Bromine-Sulphuric Acid Hydrolysis.**

Dried \( \kappa \)-carrageenan (50 mg.) was hydrolysed in the usual manner and the neutralised hydrolysate passed through a column of IR 120 (H\(^+\)) resin//.
resin then stirred in the dark with silver carbonate for several days. The solution was filtered, passed through an IR 120 (H⁺) resin column, and examined by electrophoresis, sprays (2) and (4), which showed spots with $M_{GA}$ 0.00, 0.53, 0.69, 1.00, 1.26, 1.59.

If the deionised solution was left standing for several days and then re-examined by electrophoresis a marked increase in the intensity of the neutral spot, mobility 0.00, occurred, which was attributed to lactone formation. Thus, the possibility of preventing lactone formation was examined as follows:

The aerated hydrolysate was neutralised with barium hydroxide, then excess barium hydroxide was added to approximately 0.1N concentration. The solution was left at room temperature for 2 hours before neutralisation with solid carbon dioxide, followed by treatment with silver carbonate and passage through an ion exchange column (IR 120 NH₄⁺ form). Examination by electrophoresis showed only very small amounts of neutral material and the ferric chloride-hydroxylamine spray for esters and lactones confirmed that no trace of lactone was present. Unfortunately, the relative intensity of the spot with $M_{GA}$ 1.59, thought to be carrabionic acid 2,4-disulphate, was also diminished. It thus appeared that the 2-sulphate was being eliminated on exposure to alkali. A further disadvantage of this procedure became apparent when the product was analysed for galactose 6-sulphate residues, with resorcinol reagent before and after treatment with hot alkali. This showed that most of the 6-sulphate residues present in the mixture of fragments appeared to have been lost by conversion to 3,6-anhydride. In view of these drawbacks, it was thought impracticable to use alkali to prevent or reverse lactone formation.

EXPERIMENT 7/..
EXPERIMENT 7

Oxidative Hydrolysis of $\kappa$-Carrageenan and Separation of the Products

Dried $\kappa$-carrageenan (10 g.) was hydrolysed with bromine-sulphuric acid for 16 hours at 60°. Excess bromine was removed by aeration and the pH was adjusted to 7.5 with saturated barium hydroxide. After removal of precipitated barium sulphate on the centrifuge, the pH was adjusted to 5.5 by addition of solid carbon dioxide. Silver carbonate (30 g.) was added and the mixture stirred in the dark for several days, then filtered and passed through a column of IR 120 ($\text{NH}_4^+$) resin. The solution was concentrated to 500 ml. and examined by electrophoresis, sprays (2) and (4). The following patterns of spots were observed.

<table>
<thead>
<tr>
<th>Silver Nitrate</th>
<th>Aniline-Xylose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobility</td>
<td>Intensity</td>
</tr>
<tr>
<td>0.00</td>
<td>weak</td>
</tr>
<tr>
<td>0.53</td>
<td>weak</td>
</tr>
<tr>
<td>1.00</td>
<td>++++</td>
</tr>
<tr>
<td>1.26</td>
<td>weak</td>
</tr>
<tr>
<td>1.59</td>
<td>++</td>
</tr>
</tbody>
</table>

* relative to carrabionic acid ($\text{M}_{\text{CA}}$).

An infrared spectrum (silver chloride film, no plates) showed a strong broad peak in the range 1240 cm$^{-1}$, a strong peak at 850 cm$^{-1}$ and a weak peak at 820 cm$^{-1}$. The hydrolysate (500 ml.) was applied to a DEAE Sephadex column in the carbonate form (prepared from 140 g. of Sephadex-A50). Elution was with a concentration gradient from 0.02M to 0.3M ammonium carbonate over 20 litres: the pH was kept as low as possible by/\ldots
by addition of solid carbon dioxide to the solutions (pH 6.4 to 7.2).

Fractions (50 ml.) were collected automatically and analysed by the
phenol-sulphuric acid method. Residual material was stripped from the
column with 3.0M ammonium carbonate (5 l.). The elution diagram is
shown in Fig. 19.

Solutions corresponding to the peaks were examined by electrophoresis and, on the basis of the results, combined into the following
fractions:

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Tube Numbers</th>
<th>Yield (g.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>23 - 55</td>
<td>0.05</td>
</tr>
<tr>
<td>B</td>
<td>56 - 98</td>
<td>1.80</td>
</tr>
<tr>
<td>C</td>
<td>108 - 134</td>
<td>3.10</td>
</tr>
<tr>
<td>CII</td>
<td>135 - 178</td>
<td>1.35</td>
</tr>
<tr>
<td>D</td>
<td>220 - 290</td>
<td>0.80</td>
</tr>
<tr>
<td>E</td>
<td>360 - 430</td>
<td>0.17</td>
</tr>
<tr>
<td>F</td>
<td>material removed with 3.0M ammonium carbonate</td>
<td>0.28</td>
</tr>
</tbody>
</table>

Total yield was 7.55 g. from 10 g. of K-carrageenan.

**EXPERIMENT 8**

**Preliminary Examination of the Fractions**

Each fraction was examined in three ways, (a) infrared spectroscopy
using a film prepared by evaporation on silver chloride, (b) electrophoresis in pyridine-acetate buffer, (c) measurement of the increase in
combined 3,6-anhydrogalactose content when heated with alkali.

Fraction A/
SEPARATION OF OXIDATIVE HYDROLYSIS PRODUCTS OF KAPPA.

TUBE NO. (50 ml.).
Fraction A  (a) No sulphate ester was indicated by the infrared spectrum, (b) no spots appeared on the electrophoretogram when sprayed with aniline-xylose; alkaline-silver nitrate showed a neutral spot only.

Fraction B  (a) No sulphate ester absorption was observed in the infrared spectrum, (b) aniline-xylose spray revealed components with $M_{GA} 0.58$ (+) and 1.00 (+++). This fraction was thought to be mainly carrabionic acid.

Fraction CII  (a) Strong sulphate absorption was observed at 1240 cm$^{-1}$ and 848 cm$^{-1}$, (b) aniline-xylose spray showed a single spot with $M_{GA} 1.26$, (c) a negligible increase in 3,6-anhydrogalactose content was observed after treatment with alkali. This fraction was therefore thought to be carrabionic acid 4-sulphate.

Fraction D  (a) Strong sulphate absorption was observed at 1240 cm$^{-1}$, 850 cm$^{-1}$ and 820 cm$^{-1}$, (b) aniline-xylose spray revealed two components in about equal concentrations, with $M_{GA} 1.20$ and 1.59, (c) a negligible increase in 3,6-anhydrogalactose content was observed after treatment with alkali. This fraction was thought to be a mixture of carrabionic acid 4-sulphate and 2',4-disulphate.

Fraction E  (a) Strong sulphate absorption was observed at 1240 cm$^{-1}$ and 850 cm$^{-1}$, (b) aniline-xylose spray showed spots with mobilities 1.34 (+) and 1.64 (+++), (c) an approximately 30% increase in 3,6-anhydrogalactose content was obtained on alkali treatment.

Fraction F  (a) Sulphate ester absorption was observed in the infrared spectrum at 1240 cm$^{-1}$ but the 800-850 cm$^{-1}$ region was too diffuse to allow any definite conclusions to be drawn from it, (b) no spots were observed/...
observed with aniline-xylose spray, (c) an approximately 420% increase in 3,6-anhydrogalactose content was obtained on alkali treatment. This fraction was thought to contain 6-sulphated material of rather high molecular weight.

EXPERIMENT 9

Characterisation of Carrabionic Acid 4-Sulphate.

On the basis of preliminary classification, Fraction CI was thought to contain carrabionic acid 4-sulphate. This conclusion is supported by the following evidence:

(a) **Electrophoresis** - Electrophoresis for more prolonged periods than before showed one spot only, spray (4), $M_{CA}$ 1.29.

(b) **Galactose Content** - A weighed amount of CI (0.2108 g.) was made up to 10 ml. with water. Phenol-sulphuric acid determination with reference to a calibration curve, showed that the galactose concentration was 78.6 micrograms in 0.01 ml. This would fit a carrabionic acid 4-sulphate sample of 93.9% purity.

(c) **Elementary Analysis**

Calculated for $C_{12}H_{26}O_{14}N_2S$  C 31.72, H 5.73, N 6.17, S 7.05.

Found  C 29.27, H 6.06, N 5.66, S 6.75.

The discrepancy between the calculated and found values would appear to be due to the moisture content of the hygroscopic, syrupy material. If the figures are expressed as ratios the agreement is much better

Calculated ratio (C : N : S) 5.13 : 1.00 : 1.14.

Found  5.17 : 1.00 : 1.19.

(d) **Optical Rotation** $[\alpha]_D + 15.3$ (c 1.98 in water). The concentration was determined by the phenol-sulphuric acid method to avoid errors from the moisture present.

(e)/
(e) **Action of Alkali** CI (10 mg.) in water (1 ml.) and saturated aqueous barium hydroxide (2 ml.) was heated on a water bath at 80° for 3 hours. The solution was cooled and neutralised with dilute sulphuric acid. After removal of the barium sulphate by centrifugation the solution was examined by electrophoresis, spray (4). The starting material was still present, but no other component. This result is only consistent with sulphate ester on position 4 of carrabionic acid.

(f) **Methylation** CI (100 mg.) in water (10 ml.) was stirred vigorously while dimethyl sulphate (6 ml.) and sodium hydroxide solution (30% v/v; 18 ml.) were added dropwise and simultaneously over 6 hours. This treatment was repeated twice, then the solution made alkaline and applied to a charcoal-celite column (24 x 2.2 cm.) which was eluted with water. Most of the inorganic ions were removed from the carbohydrate product in this way and the product was isolated by evaporation and freeze-drying. A small portion was hydrolysed by heating in 2N sulphuric acid on a boiling water bath for 16 hours. After neutralisation with barium hydroxide the filtrate was treated with Amberlite IR 120 (H⁺) resin and examined by paper chromatography in solvents (a), (b) and (i). Solvent (a) showed a tri-Ω-methylgalactose as the only spot detectable with spray (1); solvent (b) with the same spray, showed that this was 2,3,6-tri-Ω-methylgalactose. Solvent (i), with spray (4), showed 3,6-anhydro-2,5-di-Ω-methylgalactonic acid as the only detectable product, identical with authentic material which was kindly supplied by Professor Choji Araki, and clearly distinguishable from 3,6-anhydro-2,4-di-Ω-methylgalactonic acid.

A further sample of the methylated product was treated with methanolic hydrogen chloride (3% v/v; 100° for 16 hours). The products were examined by g.l.c., column (b). This showed 2,3,6-tri-Ω-methylgalactosides, (2.15 (strong), 2.64 (weak), 2.69 (medium)) and a derivative, presumably/...
presumably the methyl ester, of 3,6-anhydro-2,5-O-methylgalactonic acid (5.46).

(g) **Smith Degradation** - A Smith Degradation of carrabionic acid 4-sulphate should yield threitol 2-sulphate as the only charged product.

![Chemical Reaction](image)

Fig. 20.

Periodic acid (0.3 g.) was dissolved in water (5 ml.) and added to CI (100 mg.). After leaving the solution in the dark at room temperature for 24 hours, aqueous barium hydroxide was added to precipitate inorganic ions which were removed by centrifugation. Potassium borohydride (100 mg.) was added and the solution left at room temperature for 24 hours then acidified with dilute sulphuric acid, and excess acid added to a final overall concentration 1.0N. After 24 hours at room temperature, the solution was neutralised with barium hydroxide, centrifuged, and treated with Amberlite IR 120 (H⁺) resin. It was applied to a column of DEAE Sephadex in the carbonate form (prepared from 10 g. of A-25). The column was eluted with water (500 ml.) and then 0.5M ammonium carbonate, pH 8.2 (300 ml.). The fractions were examined by electrophoresis, spray (2). The fraction eluted with water showed a strong immobile spot with traces of a spot having \( R_{GA}^{\text{m}} 1.05 \). The fraction eluted with ammonium carbonate showed an immobile spot (+), and a spot having \( R_{GA}^{\text{m}} 1.05 (+) \).

The/..
The fraction eluted with ammonium carbonate was examined by paper chromatography, solvent (e), spray (2), which showed a spot with the same mobility as threitol (+) and a component with R\textsubscript{threitol} 0.43 (++)

The latter component was purified by electrophoresis on thick paper and re-examined by paper chromatography in the same solvent. No threitol was observed but spots having R\textsubscript{threitol} 0.29, 0.62 and 1.24 were present. Hydrolysis with 45\% formic acid at 100\(^\circ\) for 16 hours followed by re-chromatography gave spots having R\textsubscript{threitol} 1.00 and 1.38. The latter is probably formyl ester as authentic threitol gave the same two spots when similarly treated with formic acid. The properties of the material eluted with ammonium carbonate are therefore consistent with threitol 2-sulphate: its contamination with threitol in the column fractions is explained by alkaline elimination at the pH of the ammonium carbonate, and the several spots after electrophoresis probably correspond to different salt forms and/or anhydrides.

All the evidence is therefore consistent with CI being carrabionic acid 4-sulphate.

EXPERIMENT 10

Characterisation of Carrabionic Acid 2',4-Di-sulphate.

On the basis of the infrared spectrum and electrophoretic mobility Fraction CII was thought to be a mixture of carrabionic acid 4-sulphate and 2',4-di-sulphate. In an attempt to obtain pure carrabionic acid 2,4-disulphate, Fraction CII (1.20 g.) was dissolved in ammonium carbonate (0.02M, 50 ml.) and applied to a column of DEAE Sephadex in the carbonate form (prepared from 140 g. of Sephadex-A50). Elution was with a concentration gradient from 0.02M to 0.15M over 20 litres. Fractions (50 ml.) were collected automatically and analysed by the phenol-sulphuric acid method/...
method which showed that tubes 180-250 contained carbohydrate. Selected
tubes were examined by electrophoresis and on the basis of the results
combined into the following fractions:

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Tube Numbers</th>
<th>Yield (g)</th>
<th>Suspected Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>180 - 208</td>
<td>0.62</td>
<td>Carrabionic acid 4-sulphate</td>
</tr>
<tr>
<td>II</td>
<td>209 - 217</td>
<td>0.15</td>
<td>Mixture of carrabionic acid 4-sulphate and 2,4-disulphate.</td>
</tr>
<tr>
<td>III</td>
<td>218 - 250</td>
<td>0.19</td>
<td>Carrabionic acid 2,4-disulphate.</td>
</tr>
</tbody>
</table>

Fraction III was characterised by the following experiments:

(a) **Electrophoresis** - Electrophoresis, spray (4), showed one spot only with \( M_{CA} 1.55 \).

(b) **Infrared Spectrum** - An infrared spectrum, on silver chloride film, showed a broad strong peak at 1240 cm\(^{-1}\) with peaks of approximately equal intensity at 850 cm\(^{-1}\) and 820 cm\(^{-1}\).

(c) **Elementary Analysis**

Calculated for \( C_{12}H_{29}O_{17}N_{3}S_{2} \) C 26.10, H 5.26, N 7.61, S 11.60.


(d) **Hydrolysis** - Fraction III (20mg.) was hydrolysed in 2\( \text{N} \) sulphuric acid on a boiling water bath for 16 hours. After neutralisation with barium hydroxide, the filtrate was treated with Amberlite IR 120 (H\(^+\)) resin and examined by paper chromatography. Solvent (f), spray (1), showed galactose as the only spot. Solvent (i), spray (4), showed 3,6-anhydrogalactonic acid as the only spot.

(e) **Periodate Oxidation followed by Hydrolysis** - Periodic acid (0.2 g.) was dissolved in water (5 ml.) and Fraction III (30 mg.) added. The mixture was left in the dark for 24 hours then neutralised with aqueous barium hydroxide, centrifuged and sulphuric acid (4\( \text{N} \)) added to
a final concentration of 2N. The solution was hydrolysed and deionised as above and examined by paper chromatography. Solvent (f), spray (1), showed no galactose. Solvent (i), spray (4), showed 3,6-anhydrogalactonic acid as the only spot.

(i) Action of Alkali - Fraction III (10 mg.) in water (1 ml.) and saturated aqueous barium hydroxide (2 ml.) was heated on a water bath at 80° for 3 hours. The solution was cooled, neutralised with dilute sulphuric acid and examined by electrophoresis, spray (4). This showed spots with $M_{GA} 0.00 (++)$, 0.75 (+) and 1.00 (+++).

All the evidence is thus consistent with Fraction III being carrabionic acid 2,4-disulphate.

EXPERIMENT 11

Characterisation of Galactose 6-Sulphate Containing Tetrasaccharide.

On the basis of preliminary classification (Experiment 8), fractions D, E and F were thought to contain galactose 6-sulphate residues. The galactose to 3,6-anhydride ratios for these fractions were obtained using the following procedure:

Part of each fraction (10 mg.), in water (5 ml.), was mixed with sodium hydroxide (2N, 5 ml.) and heated at 80° for 3 hours. The relative proportions of galactose and 3,6-anhydrogalactose residues were then determined by reference to calibration graphs which were prepared for the phenol-sulphuric acid determinations using standard solutions of galactose and methyl 3,6-anhydro-$\alpha$-D-galactoside, and for the resorcinol determinations using the latter compound only. The concentration of combined 3,6-anhydrogalactose in each solution was then determined directly (resorcinol method), and the galactose content was determined by/...
by difference (by subtraction of the calculated contribution due to the known amount of 3,6-anhydrogalactose in each solution from the total optical density in the phenol-sulphuric acid determination, and reading this difference off the calibration curve).

This procedure was first checked by analysis of carrabiose dimethyl acetal when replicate determinations gave 1.02 and 1.05 for the galactose : anhydride ratio.

The results obtained for the fractions were:

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Galactose : 3,6-Anhydride Ratio (Duplicate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>3.10, 3.15</td>
</tr>
<tr>
<td>E</td>
<td>1.93, 2.04</td>
</tr>
<tr>
<td>F</td>
<td>2.28, 2.31</td>
</tr>
</tbody>
</table>

The results, combined with previous evidence, seemed to indicate that Fraction D contained a tetrasaccharide of the type

Gal-4-S—Gal-6-S—Gal-4-S—3,6-anhydrogalactonic acid,

contaminated with some carrabionic acid derivative.

Fraction E seemed to contain tetrasaccharide only. Fraction F probably contained tetrasaccharide with higher oligosaccharides and sephadex contaminants. From previous evidence, all fractions contained some 3,6-anhydrogalactose residues before heating with alkali. It was therefore decided to combine the three fractions and attempt re-separation to give an oligosaccharide in pure form containing a galactose 6-sulphate residue.

The combined fractions, in 0.1M ammonium carbonate (50 ml.), were applied to a DEAE sephadex column in the carbonate form (prepared from 140 g. of Sephadex-A50) and eluted with a linear gradient from 0.1M to 0.3M ammonium carbonate over 10 litres (pH 7.0 - 8.1). Fractions (50 ml.) were/...
were collected automatically and analysed by the phenol-sulphuric acid method which showed two main peaks. The tubes were also analysed for 3,6-anhydrogalactose residues before and after treatment with alkali and on this basis, they were combined to give two larger fractions.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Tube Numbers</th>
<th>Yield (g)</th>
<th>% Increase in 3,6-anhydride on Alkali Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>80-120</td>
<td>0.69</td>
<td>35</td>
</tr>
<tr>
<td>II</td>
<td>145-215</td>
<td>0.16</td>
<td>450</td>
</tr>
</tbody>
</table>

These results suggested that Fraction II contained the higher proportion of 6-sulphate residues. It was therefore examined as follows:

(a) **Infrared Spectrum** - An infrared spectrum, on silver chloride film, showed peaks at 1240 cm\(^{-1}\), 850 cm\(^{-1}\) and 620 cm\(^{-1}\).

(b) **Elementary Analysis**

Calculated for (i) \(\text{C}_{24}\text{H}_{52}\text{O}_{30}\text{N}_{3}\text{S}_{3}\)  
\(N\) 5.75,  S 9.88

(ii) \(\text{C}_{24}\text{H}_{47}\text{O}_{26}\text{N}_{3}\text{S}_{2}\)  
\(N\) 4.90,  S 7.45

Found  
\(N\) 5.35,  S 8.06.

The calculated values correspond to:

(i) \(\text{Gal-4-S—Gal-6-S—Gal-4-S—AGA}\)

(ii) \(\text{Gal-4-AG—Gal-4-S—AGA}\)

in their ammonium salt forms, where AG = 3,6-anhydrogalactose and AGA = 3,6-anhydrogalactonic acid.

The figures found lie between these two possibilities and are therefore consistent with the possibility that the two tetrasaccharide sulphates are separating on the column but that elimination of 6-sulphate occurs during subsequent isolation.

(c)/..
(c) Sulphate Release and 3,6-Anhydride Formation During Treatment with Alkali. Fraction II (40 mg.) was dissolved in sodium hydroxide (0.1N, 7 ml.) and heated at 80° on a water bath. Samples were withdrawn at intervals and kept at 0°. Aliquots were taken from these samples for 3,6-anhydrogalactose analysis by the resorcinol method, and, after exact neutralisation with the calculated amount of 0.1N hydrochloric acid, for sulphate analysis with chloroaminodiphenyl. (Preliminary experiments showed that there was no interference with sulphate determination by NH₄⁺ or Cl⁻ at these concentrations).

The results were as follows:

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Sulphate Release (micrograms/ml. reaction mixture)</th>
<th>Sulphate Release Expected from the observed increase in 3,6-anhydride (micrograms/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>65</td>
<td>68</td>
</tr>
<tr>
<td>4</td>
<td>127</td>
<td>122</td>
</tr>
<tr>
<td>6</td>
<td>318</td>
<td>230</td>
</tr>
<tr>
<td>24</td>
<td>908</td>
<td>586</td>
</tr>
</tbody>
</table>

(d) Alkali Treatment Before and After Periodate Oxidation - A sample of II (5 mg.) was dissolved in water (5 ml.) and aliquots (1.00 ml.) were withdrawn for the following determinations:

(A) To the above solution (1.00 ml.) was added periodic acid (0.02M, 1.00 ml.) and the solution left to stand in the dark at room temperature for 48 hours. The excess of periodate was precipitated by adding saturated aqueous barium hydroxide (3.00 ml.). After centrifugation, a portion (3.00 ml.) was withdrawn, sodium hydroxide (4N, 1.00 ml.) added and the solution heated at 80° for 3 hours. Samples (1.00 ml.) were removed for analysis of 3,6-anhydrogalactose content.

(B)/..
(B) The above solution (1.00 ml.) was added to a mixture of periodic acid (0.2N, 1.00 ml.) and saturated aqueous barium hydroxide (3.00 ml.). The solution was centrifuged and a portion (3.00 ml.) withdrawn, sodium hydroxide (4N, 1.00 ml.) added and the solution heated at 80° as above. Samples (1.00 ml.) were removed for analysis.

(C) As for (B) except that water (1.00 ml.) was used in place of 4N sodium hydroxide.

Results:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Colorimeter Reading (triplicate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.20, 0.21, 0.20</td>
</tr>
<tr>
<td>B</td>
<td>1.10, 1.18, 1.20</td>
</tr>
<tr>
<td>C</td>
<td>0.20, 0.17, 0.19</td>
</tr>
</tbody>
</table>

The reading for C gives the amount of 3,6-anhydride present originally (A-C) gives the amount of galactose 2,6-disulphate present and (B-A) the amount of galactose 6-sulphate. These results show that in the tetrasaccharide there are no 2,6-disulphate residues and very little 3,6-anhydride. The 1,4 linked component is almost entirely galactose 6-sulphate.

(e) Methylation - Fraction II (40 mg.) in water (10 ml.) was stirred vigorously while dimethyl sulphate (6 ml.) and sodium hydroxide solution (30% w/v, 18 ml.) were added dropwise and simultaneously over 6 hours. The treatment was repeated three times then the solution was made alkaline and applied to a charcoal-celite column (14 x 4 cm.) which was eluted with water. Most of the inorganic ions were removed from the carbohydrate product in this way and the product was isolated by evaporation and freeze-drying. Hydrolysis of a small portion showed that the product was undermethylated. Thus the above methylolation and separation procedure was repeated. A small portion was hydrolysed by/...
by heating in 2N-sulphuric acid on a boiling water bath for 16 hours. After neutralisation with barium hydroxide, the filtrate was treated with Amberlite IR 120 (H\(^+\)) resin and examined by paper chromatography in solvents (b), (c) and (i). Solvent (b), spray (1), showed 2,3,6-tri-\(\beta\)-methylgalactose, solvent (c), same spray, showed 2,6- and 2,3-di-\(\beta\)-methylgalactoses in approximately equal intensity. Solvent (i), spray (4), showed 3,6-anhydro-2,5-di-\(\beta\)-methylgalactonic acid as the only detectable spot.

A further sample of the methylated material was treated with methanolic hydrogen chloride (3% w/v, 100°C for 16 hours). The products were examined by g.l.c., column (b). This showed:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,6-tri-(\beta)-methylgalactosides</td>
<td>2.09, 2.62, 2.88</td>
</tr>
<tr>
<td>2,6-di-(\beta)-methylgalactosides</td>
<td>5.48, 6.44, 7.15, 8.51</td>
</tr>
<tr>
<td>2,3-di-(\beta)-methylgalactosides</td>
<td>5.48, 6.44, 7.45</td>
</tr>
<tr>
<td>methyl ester of 3,6-anhydro-2,5-di-(\beta)-methylgalactonic acid</td>
<td>5.48</td>
</tr>
</tbody>
</table>

(f) Desulphation followed by Methylation - Fraction II (15 mg.)

was dissolved in methanolic hydrogen chloride (0.8%, 10 ml.) and the solution stirred magnetically for 16 hours. After neutralisation with diazomethane and evaporation to dryness, the residue was dissolved in water and examined by electrophoresis, spray (3). This showed a neutral spot and no electrophoretically mobile material indicating that desulphation was complete. After evaporation to dryness, the residue was methylated by shaking with dimethylformamide (5 ml.), silver oxide (1.5 g.) and methyl iodide (10 ml.) for 5 days. The mixture was filtered and washed with a large volume of methanol, the silver salts so formed were isolated on the centrifuge.

A/..
A small portion of the product was hydrolysed with 2N sulphuric acid and examined by paper chromatography in solvents (b) and (i). Solvent (b), spray (1), showed 2,3,4,6-tetra-O-methyl, 2,3,6-tri-O-methyl and 2,4,6-tri-O-methylgalactoses in approximately equal concentration. Solvent (i) showed 2,5-di-O-methyl 3,6-anhydrogalactonic acid as the only detectable spot with spray (4).

A further sample of the methylated material was methanolysed and the products examined by g.l.c., column (b).

Results:

<table>
<thead>
<tr>
<th>Component</th>
<th>Retention Times</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>tetra-O-methylgalactosides</td>
<td>1.48</td>
<td>strong</td>
</tr>
<tr>
<td>2,3,6-tri-O-methylgalactosides</td>
<td>2.20, 2.74, 2.94</td>
<td>strong</td>
</tr>
<tr>
<td>2,4,6-tri-O-methylgalactosides</td>
<td>2.74, 2.94</td>
<td>strong</td>
</tr>
<tr>
<td>2-O-methyl 3,6-anhydrogalactose derivatives</td>
<td>4.19, 4.64</td>
<td>weak</td>
</tr>
<tr>
<td>methyl ester of 2,5-di-O-methyl 3,6-anhydrogalactonic acid</td>
<td>5.46</td>
<td>strong</td>
</tr>
</tbody>
</table>

All the evidence is consistent with Fraction II being

Gal-4-S——Gal-6-S——Gal-4-S——AGA

contaminated with a small amount of

Gal-4-S——AG——Gal-4-S——AGA.
DISCUSSION

Measurement of the rates of hydrolysis of 3,6-anhydrogalactosidic bonds and of sulphate ester in $\kappa$-carrageenan, with bromine-sulphuric acid, shows that after a reaction time of 16 hours 90\% of the 3,6-anhydrogalactosyl linkages are broken whilst 70\% of the ester sulphate remains. The galactosyl linkages are unaffected by these very mild hydrolysis conditions and the 3,6-anhydrogalactose end units are protected from further attack by acid, by conversion to acid stable aldonic acids. Thus, using this technique $\kappa$-carrageenan has been hydrolysed to sulphated disaccharide, tetrasaccharide, and oligosaccharide units.

Comparison of the sulphate ester release curves for $\kappa$- and $\lambda$-carrageenans (Fig. 18) shows that for both polysaccharides there is initial rapid removal, followed by more gradual removal, of sulphate. Hydrolysis of sulphate is more rapid from $\lambda$- than from $\kappa$-carrageenan indicating that the 2-sulphate is being hydrolysed more rapidly than the 4-sulphate. In general, it is thought that the sulphate esters derived from axial secondary hydroxyl groups are relatively acid stable and both the 2- and 4-sulphates are axial. However, the 3,6-anhydrogalactosidic linkages in the polysaccharide are very acid labile and, as shown in Fig. 17, are cleaved very quickly in the reaction. Once the 3,6-anhydrogalactosyl linkage is broken, the 2-sulphate is attached to an open chain sugar and therefore it is not inconsistent with the generalisation to attribute the rapid removal of sulphate to the 2-ester. Indeed, rapid removal of sulphate, on position 2 of the 3,6-anhydrogalactose units, on acid hydrolysis ($0.25\text{M}$ hydrochloric acid at 100°C) of alkali-modified $\lambda$-carrageenan has been reported. The irregularity in the curve for sulphate release from $\lambda$-carrageenan is due to interference from oligosaccharide sulphates of high molecular weight.

Dr./
Dr. T.C.S. Dolan put forward three possible reasons for his failure to isolate sulphated oligosaccharides after oxidative hydrolysis of \( \kappa \)-carrageenan.

1. The sulphate was lost during the hydrolysis.
2. The acid conditions of elution from the column caused hydrolysis of the ester.
3. The sulphated oligosaccharides were so strongly held to the column that they could not be eluted.

The reaction rate curve for ester sulphate hydrolysis shows that explanation (1) is incorrect. To avoid the conditions outlined in (2) and (3), an attempt was made to separate the hydrolysis products on a charcoal-celite column using aqueous ethanol as eluant but no separation was achieved. However, it was found, using neutral buffers, that the hydrolysis products could be eluted from DEAE Sephadex and that the actual reason for loss of the oligosaccharide sulphates is that these substances are partitioned into \( N,N \)-dioctylmethyamine in chloroform during deionisation of the hydrolysate. This accounts for the low recovery which Dr. Dolan obtained from his column separation. This partitioning is very surprising; no other carbohydrate is known to behave in this way, and must be due to the high 'charge to hydroxyl' ratio for the sulphated oligosaccharides.

In the Introduction to this Section, it was stated that although all prior evidence is consistent with 1,3-linked galactose 4-sulphate as a major structural feature in \( \kappa \)-carrageenan, minor structural features such as 4-linked 3-sulphate and/or branching through position 3 or 4 could not be dismissed. To be consistent with the methylation evidence, the terminal units of these branches would have to be sulphated. The isolation of carrabionic acid 4-sulphate, in high yield, on oxidative hydrolysis...
Hydrolysis provides the first definitive chemical proof for the presence of 4-sulphate units in \( \kappa \)-carrageenan. No carrabionic acid 3-sulphate or any sulphated fragments consistent with terminal units from branch points were found.

Carrabionic acid 4-sulphate was characterised by methylation analysis, using methanolation followed by g.l.c., and also hydrolysis and paper chromatography; 2,3,6-tri-O-methylgalactose and 3,6-anhydro-2,5-di-O-methylgalactonic acid were the products obtained. That the sulphate occurred on position 4 of the carrabionic acid residue was confirmed by its alkaline stability because sulphate ester on any other position of carrabionic acid would be eliminated by alkali. This is further confirmed by the infrared spectrum, which showed a strong peak at 850 cm\(^{-1}\), indicative of axial 4-sulphate, and by Smith Degradation to threitol 2-sulphate.

Pure carrabionic acid 2',4-di-sulphate was isolated in very small yield, perhaps due to the preferential removal of sulphate on position 2 of the 3,6-anhydrogalactonic acid residue during acid hydrolysis. It had a faster electrophoretic mobility than carrabionic acid 4-sulphate and its infrared spectrum showed peaks at 850 cm\(^{-1}\), axial sulphate, and 820 cm\(^{-1}\) which is presumably due to the sulphate on position 2 of the open chain 3,6-anhydrogalactonic acid unit, but no reference compounds were available for comparison.

Hydrolysis and paper chromatography showed galactose and 3,6-anhydrogalactonic acid whereas periodate oxidation followed by hydrolysis showed only 3,6-anhydrogalactonic acid, indicating that on the original molecule the 3,6-anhydrogalactonic acid was substituted on position 2 as well as 4.

Alkali/..
Alkali treatment caused a reduction in electrophoretic mobility indicating sulphate elimination and as there was no increase in 3,6-anhydrogalactose content, the eliminated sulphate must have been on position 2 of the 3,6-anhydrogalactonic unit. The elimination could have taken place by one of the mechanisms shown in Fig. 21. The electrophoretogram of the alkali-treated material showed spots with $M_C/A$ values 0.00 (++), 0.75 (+) and 1.00 (+++) indicating that more extensive breakdown, than is indicated by the mechanisms shown in Fig. 21 has occurred with the loss of at least two of the three anionic groups. The alkaline solution was neutralised with sulphuric acid before being examined electrophoretically, and this would cause further rearrangement of the product from mechanism 3, which is an enol ether and thus acid labile, to give the ketose shown in Fig. 22.

As the molecule was unstable to alkali, methylation could not be used in the structure proof because this requires alkaline conditions.

The characterisation of the galactose 6-sulphate-containing tetrasaccharide was made more difficult by contamination with oligosaccharides containing 3,6-anhydrogalactose residues. The latter units are present because hydrolysis cleaves only 90% of the 3,6-anhydrogalactosidic bonds. The two components appeared to be separated on the column but there must have been some elimination of 6-sulphate during subsequent isolation.

The galactose : 3,6-anhydride ratios, after alkali-modification of the Fractions (D, E and F) from the first DEAE Sephadex column indicated that they contained tetrasaccharides. After further purification on a second DEAE Sephadex column, one fraction gave an increase in 3,6-anhydrogalactose on alkali treatment, and analysis figures, which were consistent/..
Mechanism 1.

Mechanism 2.

Mechanism 3.

Fig. 22.
consistent with a galactose 6-sulphate-containing tetrasaccharide contaminated with small amounts of a 3,6-anhydrogalactose-containing tetrasaccharide. The fraction was shown to be mainly the tetrasaccharide shown in Fig. 23 by the following experiments.

![Diagram of tetrasaccharide structure]

**FIG. 23.**

The infrared spectrum showed peaks at 850 cm⁻¹ (axial sulphate) and 820 cm⁻¹ (primary sulphate or sulphate on position 2 of 3,6-anhydrogalactonic acid). Comparison of the amounts of free sulphate liberated and 3,6-anhydrogalactose synthesised on treatment with alkali showed good correspondence after short reaction times, indicating the presence of galactose 6-sulphate residues. After longer reaction times, the sulphate released was in excess of the 3,6-anhydride synthesised indicating that some other reaction was taking place. If there was sulphate on position 2 of the 3,6-anhydrogalactonic acid unit then this would be removed with alkali; there is some evidence from the carrabionic 2',4'-disulphate characterisation that removal of this 2-sulphate with alkali leads to general breakdown of the molecule. This would cause cleavage of the linkage to 3,6-anhydrogalactonic acid and subsequent 'peeling' in alkali. This reaction sequence could therefore account for the high sulphate ester release on alkali treatment.

The/..
The negligible increase in 3,6-anhydrogalactose content on alkali treatment after periodate oxidation shows that the 1,4-linked component is galactose 6-sulphate and not 2,6-disulphate. Desulphation followed by methylation and analysis by g.l.c. after methanolysis, and by paper chromatography after hydrolysis, showed approximately equal proportions of 2,3,4,6-tetra-O-methylgalactose, 2,3,6-tri-O-methylgalactose, 2,4,6-tri-O-methylgalactose and 3,6-anhydro-2,5-di-O-methylgalactonic acid. Assuming that the linkage configurations are the same as in the original polysaccharide this indicates that the desulphated tetrasaccharide has structure 0-β-D-galactopyranosyl (1—> 4)-0-α-D-galactopyranosyl-
(1—> 3)-0-β-D-galactopyranosyl-(1—> 4)-3,6-anhydro-D-galactonic acid.

Methylation of the tetrasaccharide, with the sulphate groups intact, followed by analysis by g.l.c. after methanolysis, and by paper chromatography after hydrolysis, showed 2,3,6-tri-O-methylgalactose, 2,3-di-O-methylgalactose, 2,6-di-O-methylgalactose and 3,6-anhydro-2,5-di-O-methylgalactonic acid in approximately equal proportions. This pattern of methylated sugars combined with that obtained by methylation after desulphation shows that the tetrasaccharide has the structure shown in Fig.

Analysis of the desulphated tetrasaccharide by g.l.c. after methylation and methanolysis showed small amounts of 3,6-anhydro-2-O-methylgalactonic acid derivatives which were not observed on methanolysis of the methylated tetrasaccharide. This could be due to removal of 6-sulphate from a 1,4 linked galactose unit, under the acid conditions, leading to the formation of 3,6-anhydrogalactose. This reaction takes place very easily under alkaline conditions but has not previously been observed in acid.
SECTION B

PART TWO

Characterisation of Sulphate Esters by Infrared Spectroscopy

INTRODUCTION

The infrared spectra of carbohydrate sulphates have been classified according to whether the ester is attached to a secondary axial, a secondary equatorial, or a primary hydroxy group: characteristic bands are supposed to appear at 850, 830 and 820 cm\(^{-1}\) respectively.\(^4\) The main esters in \(\kappa\) - and \(\lambda\) -carrageenans from *Chondrus crispus* and *Eucheuma spinosum* are galactose 4-sulphate and 3,6-anhydrogalactose 2-sulphate, both of which would be expected, from this classification, to give bands at 850 cm\(^{-1}\). Such a band is indeed observed for each polysaccharide but there is also an absorption at 805 cm\(^{-1}\) which is much stronger for \(\lambda\) - than \(\kappa\) -carrageenan. This band at 805 cm\(^{-1}\) is observed in many other carrageenans and its intensity seems to parallel the proportion of 3,6-anhydrogalactose 2-sulphate which is shown to be present by methylation analysis and which can be inferred from the galactose : sulphate ratio of the polysaccharide. It therefore seems likely that the band at 805 cm\(^{-1}\) is due to a sulphate ester on position 2 of a 3,6-anhydrogalactose ring even though this ester is axial. The research reported in the Experimental Section describes the synthesis of some sulphated derivatives of methyl 3,6-anhydro-\(\alpha\)-galactopyranoside to check this assignment.

In the past, infrared spectroscopy has proved very useful in providing a quick means of preliminary classification of carrageenan structure. It would be very useful if p.m.r. spectroscopy could provide
a specific means for analysis of each type of sulphate ester, thus providing a quick method for characterising carrageenans which would be complementary to infrared spectroscopy. In this Section, experiments, to ascertain the effect the sulphate group has on the chemical shift of the proton attached to the same carbon atom, are described for model monosaccharide compounds. Preliminary experiments have shown that a number of difficulties have to be overcome before interpretable spectra can be obtained at the polysaccharide level.
SYNTHESIS OF 3,6-ANHYDROGALACTOPYRANOSE SULPHATES

The 2-O-methyl and 4-O-methyl ethers of methyl 3,6-anhydro-α-D-galactopyranoside used in this experiment were prepared by Mrs. D.B. Davy by partial methylation of methyl 3,6-anhydro-α-D-galactopyranoside followed by separation of the products on a Dowex (OH⁻) resin column. The methods were based on those developed by Turvey and co-workers.

Methyl 3,6-anhydro-α-D-galactopyranoside (200 mg.) was dissolved in dry pyridine (4 ml.; dried over type 4Å molecular sieve and shown by ¹H n.m.r. to contain less than 0.1% water) and treated with pyridine-sulphur trioxide reagent (freshly prepared; 4 moles). The mixture was stirred on a water bath (65-70°C) under anhydrous conditions for 24 hours, and water (4 ml.) was added. Stirring was continued for 1 hour and aqueous barium hydroxide was added to give a pH of 9. Barium sulphate was removed by centrifugation and the solution was carefully (bath temperature 35°C) evaporated with continuous addition of water to maintain the volume until all pyridine had been removed. After removal of cations with Amberlite IR 120 (H⁺) resin and neutralisation to pH 8 with ammonia, the solution was evaporated and freeze-dried. Electrophoresis, spray (1), showed a major product, with a mobility similar to that of glucose disulphate, which was therefore methyl 3,6-anhydro-α-D-galactopyranoside 2,4-disulphate. Its identity as a sulphate ester was confirmed by its infrared spectrum, on silver chloride film, which showed a peak at 1200-1250 cm⁻¹ which was not present before treatment with pyridine-sulphur trioxide.
trioxide. There was no trace of the peak at 850 cm\(^{-1}\) which would be expected on the basis of existing assignments\(^4\) for a compound containing axial sulphate ester, but there was instead a broad band at 600-630 cm\(^{-1}\). Similarly, sulphation of each of the mono-\(\beta\)-methyl ethers of methyl 3,6-anhydro-\(\alpha\)-L-galactopyranoside (80 mg.) gave a product with the electrophoretic mobility of a sugar monosulphate which showed an infrared band at 1240 cm\(^{-1}\). These products were, therefore, methyl 3,6-anhydro-2-\(\beta\)-methyl-\(\alpha\)-L-galactopyranoside 4-sulphate and methyl 3,6-anhydro-4-\(\beta\)-methyl-\(\alpha\)-L-galactopyranoside 2-sulphate. Each showed an infrared absorption band in the region 790-820 cm\(^{-1}\) which was not present in the spectrum of the corresponding parent glycoside before sulphation; for the 2-sulphate this was at 795 cm\(^{-1}\) and for the 4-sulphate it was 815 cm\(^{-1}\). Fig. 24.

**EXPERIMENT 13**

Identification of the \(H(2)\) and \(H(4)\) Signals in the Proton Magnetic Resonance Spectrum of the 3,6-Anhydrogalactopyranoside Ring.

By comparison of the spectra of several methyl 3,6-anhydrogalactopyranosides, it has been possible to identify the \(H(2)\) and \(H(4)\) signals. All \(\tau\) values are listed after correction using tert-butanol (8.77 \(\tau\)) as internal standard, unless otherwise stated.

(a) Methyl 3,6-anhydro-\(\alpha\)-L-galactopyranoside in \(D_2O\) (all labile protons exchanged for deuterons). This spectrum showed the following features:

\[\tau/\ldots\]
Fig. 24. The Infra-red Spectra of 3,6-anhydrogalactosyl sulphates.

A vertical line is drawn at 805 cm\(^{-1}\) for comparison.
\[ \tau 5.21 \] one proton doublet, $J \ 3c/s$, corresponding to the anomeric proton.

\[ \tau 5.54 - 5.74 \] 3 proton multiplet.

\[ \tau 5.84 - 6.09 \] 3 proton multiplet.

\[ \tau 6.52 \] 3 proton singlet corresponding to the glycosidic methyl group.

(b) Methyl 2,4-di-$\beta$-acetyl-3,6-anhydro-$\alpha$-$\beta$-galactopyranoside in CDCl$_3$, tetramethylsilane (10.0 $\tau$) as internal standard. This spectrum showed the following features:

\[ \tau 4.61 \] 1 proton doublet, $J \ 1.5c/s$.

\[ \tau 4.85 \] 1 proton quartet, $J \ 6c/s, 3c/s$.

\[ \tau 5.15 \] 1 proton doublet, $J \ 3c/s$.

\[ \tau 5.50 - 5.70 \] 2 proton multiplet.

\[ \tau 5.78 - 5.98 \] 2 proton multiplet.

The doublet at \[ \tau 5.15 \] is assigned to the anomeric proton, by analogy with spectrum (a). The effect of acetylation has been to shift two bands, each corresponding to 1 proton, downfield below the anomeric proton signal. Thus the doublet at \[ \tau 4.61 \] and the quartet at \[ \tau 4.85 \] must be due to $H(2)$ and $H(4)$ as these would be the protons most affected by the introduction of the deshielding acetyl groups. No distinction can be made between them on the basis of this spectrum alone.

(c) Methyl 3,6-anhydro-2-$\beta$-methyl-$\alpha$-$\beta$-galactopyranoside in D$_2$O (all labile protons exchanged for deuterons). This spectrum showed the following features:
1 proton doublet, J 3c/s, corresponding to the anomeric proton.

$\tau$ 5.45 - 5.65 3 proton multiplet.

$\tau$ 5.85 - 6.15 2 proton multiplet.

$\tau$ 6.32 1 proton quartet, J 6c/s, 3c/s.

The quartet at 6.32 $\tau$ can be assigned to $H(2)$ which is shifted upfield relative to spectrum (a) by the electron releasing effect of the methoxyl group.

(d) Methyl 3,6-anhydro-4-$\eta$-methyl-$\alpha$-$D$-galactopyranoside in $D_2O$ (all labile protons exchanged for deuterons). This spectrum showed the following features:

$\tau$ 5.17 1 proton doublet, J 3c/s, corresponding to the anomeric proton.

$\tau$ 5.44 - 6.09 Very complex multiplet (approximately 5 protons).

$\tau$ 6.44 - 6.64 Two large singlets with a smaller doublet superimposed (totalling 7 protons).

The two large singlets at 6.50 $\tau$ and 6.59 $\tau$ are assigned to the two methyl groups. Superimposed upon them at 6.51 $\tau$ it is possible to distinguish a doublet, assigned to $H(4)$, which is shifted upfield by introduction of the methyl group at $C(4)$.

**Experiment 14**

The Proton Magnetic Resonance Spectra of 3,6-Anhydrogalacto-
pyranoside Sulphates.

The 3,6-anhydrogalactoside sulphates prepared in Experiment 13 were /..
were examined in D$_2$O solution, after exchanging the labile protons for deuterons by repeated freeze-drying of a D$_2$O solution. It was found that some important parts of the spectrum were obscured by the signal of residual HOD which normally appears at about 5.3 T. This was therefore shifted downfield to about 4.6 T by addition of trifluoroacetic acid.

(e) Methyl 3,6-anhydro-α-D-galactopyranoside-2,4-disulphate in D$_2$O-trifluoroacetic acid. This spectrum showed the following features:

\[ \tau \ 5.17 \quad 2 \text{ proton irregular singlet.} \]
\[ \tau \ 5.29 \quad 1 \text{ proton multiplet, possibly a quartet.} \]

The irregular singlet at 5.17 appears to be a superimposition of two doublets and thus probably corresponds to $H_1^{(1)}$ plus $H_4^{(4)}$. The multiplet at 5.29 is probably $H_2^{(2)}$.

(f) Methyl 3,6-anhydro-4-O-methyl-α-D-galactopyranoside-2-sulphate in D$_2$O-trifluoroacetic acid. This spectrum showed the following features:

\[ \tau \ 5.07 \quad 1 \text{ proton doublet corresponding to the anomeric proton.} \]
\[ \tau \ 5.29 - 5.51 \quad 1 \text{ proton multiplet - presumably } H_2^{(2)} \text{, occurring at slightly higher field than } H_1^{(1)} \text{ as in the disulphate.} \]

(g) Methyl 3,6-anhydro-2-O-methyl-α-D-galactopyranoside-4-sulphate in D$_2$O-trifluoroacetic acid.

\[ \tau \ 4.74 - 5.04 \quad 1 \text{ proton multiplet which is probably a superimposition of the } H_1^{(1)} \text{ and } H_4^{(4)} \text{ signals as in the disulphate.} \]
EXPERIMENT 15

Proton Magnetic Resonance Spectra of Carrageenan Type Polysaccharides

Carrageenans do not give solutions which are sufficiently concentrated to give good spectra by direct measurement at 60 MHz. Dilute solutions were therefore examined using the CAT (time averaging computer) to accumulate the spectrum over a large number of scans (approximately 250) and to average out the noise.

Polysaccharide (20 mg.) and sodium acetate (25 mg.) were mixed and dissolved in deuterium oxide (99.7%), and exchangeable protons were removed by repeated freeze-drying. A solution of the residue in deuterium oxide (0.5 ml.) was used to record the spectrum. Unfortunately, the spectra were quite uninterpretable owing to the residual HOD signal which obscured the region 4.5-6.0 τ owing to its increased size by the large number of scans. This peak was shifted downfield to below 5 τ by addition of trifluoroacetic acid, and it was then possible to obtain better spectra for alkali modified λ- and κ-carrageenans. For both polysaccharides, the number of protons in the 5.2-5.6 τ region corresponded roughly with the total proportion of the types listed in Table 3, page 117.
When methyl 3,6-anhydro-2-0-methyl-\(\alpha-D\)-galactopyranoside and methyl 3,6-anhydro-4-0-methyl-\(\alpha-D\)-galactopyranoside were each treated with sulphur trioxide in pyridine, the products were the 4-sulphate and 2-sulphate, respectively, which showed new infrared bands at 815 and 798 cm\(^{-1}\). It is therefore concluded that 3,6-anhydrogalactopyranoside sulphates, both as monosaccharides and as polymer building units, show absorptions at about 795-820 cm\(^{-1}\). If the anhydride is assumed to be glycosidically linked through position 4, this band may be used to estimate the proportion of 3,6-anhydrogalactose 2-sulphate in a carrageenan.

Bowker and Turvey\(^6\) have prepared a number of other new sugar sulphates, and some of these also show infrared bands which deviate from the positions expected on the basis of whether the ester is axial, equatorial or primary. It seems that anomalies might be particularly common in bicyclic sugar systems, such as 3,6-anhydro and 4,6-ethyldene compounds, in which the sugar ring is held in a relatively rigid conformation.

It now seems doubtful whether the precise positions of the infrared bands of carbohydrate sulphates can in general be safely predicted on such simple grounds as whether the ester is axial, equatorial or primary. Until a new and more reliable generalisation emerges, it will be necessary to examine the spectra of model compounds for each sulphate ester of interest.

By comparison of the p.m.r. spectra of several methyl 3,6-anhydrogalactopyranosides carrying electron donating and withdrawing substituents the signals due to \(H(2)\) and \(H(4)\) have been identified. The \(H(4)\) signal is/...
is a quartet, the $H(2)$ signal is a doublet and the $H(4)$ signal is shifted further downfield by the substitution of an electron withdrawing group on the same carbon and further upfield by an electron donating group, than is the $H(2)$ signal.

The spectra of the 3,6-anhydrogalactopyranoside sulphates were not very satisfactory, but did indicate that, with improved technique, it might be possible to use p.m.r. spectroscopy for the analysis of mixtures of 2-sulphated and 4-sulphated derivatives. Sulphation of the 3,6-anhydrogalactose ring at $C(2)$ or $C(4)$ causes a downfield shift of the corresponding proton signal so that it may be distinguished from other protons of the sugar ring, although not necessarily from the anomeric proton. $H(4)$ is shifted further downfield by sulphation at the same carbon than is $H(2)$.

In the p.m.r. spectra of carrageenans two types of protons will occur at lower field than the normal ring protons; these are anomeric protons and protons attached to the same carbon atoms as sulphate esters. The chemical shifts and spacings to be expected for such protons in carrageenans are shown in Table 3.

The method of recording the polysaccharide spectra, using trifluoroacetic acid to shift the HOD peak downfield, was not satisfactory as the presence of the acid could cause hydrolysis. Several other solutes were tried in an attempt to shift the HOD peak out of the range 5-7 T without causing polysaccharide degradation. These included paramagnetic and diamagnetic ions and also compounds which affect the structure of water, such as guanidine hydrochloride. Only paramagnetic ions, such as Co$^{2+}$, shifted the HOD signal to any appreciable extent but they also caused broadening of the polysaccharide signals to such a degree that the spectra were uninterpretable.

Thus, although it seems feasible to use p.m.r. spectroscopy for carrageenan characterisation a much more satisfactory method for recording the spectra will have to be found.
<table>
<thead>
<tr>
<th>Proton</th>
<th>Chemical Shift, in $\text{D}_2\text{O}$</th>
<th>Spacings c/s</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anomeric proton, axial</td>
<td>5.56 $\pm$ 0.06</td>
<td>7</td>
<td>64</td>
</tr>
<tr>
<td>Anomeric proton, equatorial</td>
<td>4.9 $\pm$ 0.3</td>
<td>3-4</td>
<td>64</td>
</tr>
<tr>
<td>Anomeric proton, 3,6-anhydro-galactosyl*</td>
<td>5.15 $\pm$ 0.05</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>$\text{H}(2)$ on 3,6-anhydrogalactosyl-2-</td>
<td>5.4 $\pm$ 0.1</td>
<td>6,3</td>
<td></td>
</tr>
<tr>
<td>sulphate unit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\text{H}(4)$ on galactosyl 4-sulphate unit</td>
<td>5.2</td>
<td>2</td>
<td>65</td>
</tr>
</tbody>
</table>

*Note: 3,6-Anhydrogalactose units are often assumed to exist in the Reeves 1C conformation, and on this basis the anomeric signal would be expected at about 5.56 as for other axial anomeric protons. However, the strain in this bicyclic residue causes substantial flattening of the ring and movement of the proton (and its signal) away from the idealised axial position.
GENERAL METHODS OF INVESTIGATION

Isolation of Polysaccharides. Polysaccharides were isolated by freeze-drying of aqueous solutions.

Evaporations were effected on a rotary evaporator at reduced pressure at about 40°.

Dialyses of solutions were carried out in sections of dialysis tubing suspended in running water for four days.

Drying. Samples were dried by leaving overnight in a vacuum oven, at 50°, in the presence of phosphorous pentoxide.

Complete Hydrolysis of Polysaccharides. Unless otherwise stated, the material (10-20 mg.) was heated, in a quickfit test tube, with aqueous formic acid (45%, 2 ml.) at 100° for 16 hours. The formic acid was removed by repeated evaporation.

Methanolysis of Samples for Gas-Liquid Chromatography. Samples (10-20 mg.) were heated in sealed tubes at 100° with methanolic hydrogen chloride (2.3%; prepared by adding 4.5 ml. acetyl chloride to 100 ml. dry methanol) for 16 hours. The solutions were neutralised with silver carbonate, filtered, and concentrated to a syrup.

Gas-Liquid Chromatography was carried out on a Pye Argon Chromatograph with a $^{90}$Sr detector and 4 ft. columns. The liquid phases were

(a) Polyethylene glycol adipate (10% on Gas Chrom F) Run at 175° with a gas flow of 80 ml./min.

(b)/..
(b) Neo-pentyl glycol adipate (3% on Gas Chrom P)

Run at 175° with a gas flow of 80 ml./min.

Retention times ($R_T$) of the methyl glycosides of the monosaccharide methyl ethers were expressed relative to the faster of the two methyl 2,3,4,6-tetra-$O$-methylglucoside peaks.

**Melting Points.** These were measured on the Kofler hot stage and are uncorrected.

**Optical Rotation Measurements.** These were made with the Perkin-Elmer 141 Automatic Polarimeter with 1 dm. cells.

**Proton Magnetic Resonance Spectra.** These were recorded with the Perkin-Elmer R10 (60 MHz) and Varian HA100 (100 MHz) spectrometers. The internal standards were tetramethylsilane, for organic solvents, and tertiary butanol, for spectra in deuterium oxide.

**Infrared Spectra.** These were recorded with the Perkin-Elmer model 237 spectrometer (sample as film). For polysaccharides, films were prepared by evaporation of an aqueous solution (0.5%) on mercury in a vacuum desiccator at room temperature; for sugar sulphates, they were prepared similarly but on a piece of silver chloride which was then placed, carrying the sample, in the beam.

**Analysis.** The galactose : 3,6-anhydride : sulphate ratios for the unmethylated polysaccharides were supplied by Marine Colloids Incorporated. All other analyses were carried out by Alfred Bernhardt, Elbach uber Engelakerchen, West Germany.
Carbohydrate Contents. These were determined by the phenol-sulphuric acid method, using the procedure outlined in reference 66 page 388.

3,6-Anhydrogalactose Contents. These were determined using Rees's modification of Yaphe's resorcinol method.  

Sulphate Contents. These were determined as described by Rees, based on the spectrophotometric method of Jones and Letham.

Electrophoresis. For electrophoresis, the paper was stretched between two buffer compartments in a closed chamber with no special cooling arrangement; the electrolyte was 0.1M acetic acid adjusted with pyridine to pH 6.5, and the potential gradient was about 9 volts/cm. for periods of up to 2 hours. Migration values were expressed relative to D-glucuronic acid, after allowance for migration due to endosmosis.

Thin Layer Chromatography was carried out using micro-plates coated with silica gel (Kieselgel G, nach Stahl, Merck). The spots were developed using p-anisaldehyde-sulphuric acid spray. The dried plate was sprayed lightly with a solution which was prepared by dissolving p-anisaldehyde (1 ml.) and Analar concentrated sulphuric acid (1 ml.) in ethanol (20 ml.). The plate was then heated at 150°C for about 5 minutes.

Paper Chromatography. Qualitative separations were carried out on Whatman No. 1 paper, and preparative separation on Whatman 3 MM filter sheet. The following solvent systems were used:

(a) n-Butanol-ethanol-water (upper phase) 4 : 1 : 5
(b) Ethyl methyl ketone-water-ammonia (S.G. 0.86) 200 : 17 : 1
   Double development 2 x 4 hours.
(c) The same conditions as in (b) but with double development 2 x 8 hours 169 : 47 : 15

(d) Benzene-ethanol-water (upper phase) triple development 2 x 16 hours, 1 x 8 hours 169 : 47 : 15

(e) n-Butanol-ethanol-water 3 : 1 : 1

(f) Ethyl acetate-pyridine-water 10 : 4 : 3

(g) Ethyl acetate-acetic acid-formic acid-water 18 : 3 : 1 : 4

(h) Ethyl methyl ketone-water-formic acid 20 : 17 : 1

(i) A mixture of solvents (g) and (h) in the ratio 3 : 2 respectively.

The following detection sprays were used:

(1) **p-Anisidine hydrochloride**⁷⁰ (for reducing sugars and dimethyl acetalts).

The dried chromatogram was sprayed with a 3% solution of p-anisidine hydrochloride in water saturated n-butanol, and heated at 120° for 5 minutes.

(2) **Ammoniacal Silver Nitrate**⁷¹ (for reducing sugars and glycol groups).

A saturated aqueous solution of silver nitrate (0.1 ml.) was diluted to 20 ml. with acetone, and by the dropwise addition of water, the precipitated silver nitrate was redissolved. The dried chromatogram was quickly dipped in this reagent and dried. The chromatogram was then sprayed with 0.5N sodium hydroxide in aqueous ethanol, and the spots developed by holding the paper over a boiling water bath. Excess background colour was removed with thiosulphate.

(3) **Periodate-benzidine**⁷² (for compounds containing glycol groups).

The dried chromatograms were sprayed with an aqueous solution of sodium/..
sodium periodate (0.3%, w/v) and left for 10 minutes to dry. The chromatogram was then dipped in a solution made by dissolving benzidine (184 mg) in a mixture of glacial acetic acid (0.6 ml.), water (4.4 ml.) and acetone (95 ml.).

(4) **Aniline-xylose** (for acids and sulphates). The dried chromatograms were sprayed with the reagent and heated at 110°C for 5 minutes. The reagent was prepared as follows: xylose (1 g.) was dissolved in the minimum amount of water, then aniline (1 ml.) was added and the solution dissolved in ethanol (100 ml.).

(5) **Potassium iodide-methyl red** (for vicinal epoxides). The dried chromatograms were sprayed with a solution of sodium iodide (5 g.) and Methyl Red (0.01 g.) in n-butanol (100 ml.) and heated at 140°C for a few minutes.

(6) **Hydroxylamine-ferric chloride** (for lactones and esters). The dried chromatogram was sprayed with a freshly prepared solution of alkaline hydroxylamine (made by mixing equal volumes of 1.0N methanolic hydroxylamine hydrochloride and 1.1N methanolic potassium hydroxide). After drying in air for about 10 minutes the paper was sprayed with an aqueous solution containing 1-2% ferric chloride and 1% hydrochloric acid.
BIBLIOGRAPHY

   b) E. Percival and J.K. Wold, ibid., 1963, 5459.
   b) A.G. Lloyd and K.S. Dodgson, ibid., p. 116.
6. J.R. Turvey in 'Advances in Carbohydrate Chemistry', 1965, 29,
   1968, 596.
11. N.S. Anderson and D.A. Rees, in 'Proceedings of the V International
    Seaweed Symposium', E.G. Young and J.L. Mclachlan, ed. Pergamon,
13. N.S. Anderson, T.C.S. Dolan, C.J. Lawson, A. Penman and D.A. Rees,
14. //
   and earlier papers in the same series.
19. C. Araki in *Proceedings of the V International Seaweed Symposium*,
   Samuel, *J. Mol. Biol.*, 00,000.
   Part C, 00, 000.
   2958.
33. J.S. Brimacombe and J.M. Webber, *'Nucopolysaccharides', Elsevier 
34./**


50. D.J. Bell in 'Advances in Carbohydrate Chemistry', 1951, 6, 11.


55./*


68. A.S. Jones and D.S. Letham, Chem. and Ind., 1954, 662.


74./*
Carrageenans. Part IV. Variations in the Structure and Gel Properties of $\kappa$-Carrageenan, and the Characterisation of Sulphate Esters by Infrared Spectroscopy

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Reprinted from
JOURNAL
OF
THE CHEMICAL SOCIETY
SECTION C
Organic Chemistry
1968
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Structures of the "masked repeating" type seem to occur generally in $\kappa$-carrageenans from Chondrus crispus and Gigartina species and will account for the analytical variation between one sample and another. Most—possibly all—of the variation is in the 4-linked units which occur as 3,6-anhydrogalactose, 3,6-anhydrogalactose 2-sulphate, galactose 6-sulphate, and galactose 2,6-disulphate. In $\kappa$-carrageenan from G. stellata, as from C. crispus, all galactose units are believed to have the D-configuration. Samples from Chondrus were similar to each other and had a lower content of both types of 2-sulphate than those from Gigartina. The separation of a Chondrus sample into a series of subfractions which differed in the relative proportions of the various 4-linked units, suggests that $\kappa$-carrageenan might normally contain a mixture of related molecules. Evidence is given that absorption bands shown by sulphated 3,6-anhydrogalactose units in the 800—850 cm.$^{-1}$ region of the infrared spectrum cannot be interpreted in the simple stereochemical terms used previously. However, many carrageenans show a band at about 805 cm.$^{-1}$ which is characteristic of 3,6-anhydrogalactose sulphate and potentially useful because it is easily recognised, even in the presence of other sulphate esters. Gel formation by $\kappa$-carrageenan is enhanced by removal of 6-sulphate with conversion into 3,6-anhydrogalactose, but is much less sensitive to an excess of 2-sulphate. The variation of gel strength with polysaccharide structure provides some evidence on the molecular basis of gel formation.

The masked repeating structure of $\kappa$-carrageenan, for which evidence is given in the preceding paper, suggests in outline a path for biosynthesis. $\beta$-Galactose units could be assembled into a polymer with alternately $\alpha$-1,3- and $\beta$-1,4-linkages, and subsequently sulphated and a proportion of them converted into 3,6-anhydro. Biosynthesis of 3,6-anhydro-rings is known to occur after polymerisation in the L-galactose series. Variation in composition of $\kappa$-carrageenans need not then reflect any deviation from the alternating structure which has been established for one specimen, but merely a quantitative change in the pattern of sulphation and anhydro formation as occurs in other polysaccharides and for which biological reasons have been suggested. We now describe the structural variations in detail and show that infrared spectroscopy is a particularly convenient method for characterising individual samples once the pattern of variation is established.

$\kappa$-Carrageenan samples were given by Dr. E. T. Dewar. They were compared by methylation analysis by use of the methods previously devised, and also by measurement of the 3,6-anhydrogalactose formed by alkaline borohydride before and after periodate oxidation. This was assumed to reflect respectively the sum of the 4-linked galactose 6-sulphate and galactose 2,6-disulphate units, and the 4-linked galactose 2,6-disulphate only. 2,6-Di-O-methylgalactose was the only important acid-stable hydrolysis product from each methylated polysaccharide, except for sample 5 which gave some 4,6-di-O-methylgalactose. This preparation, from Gigartina pistillata, had been difficult to fractionate, and contamination by $\lambda$-carrageenan was suspected. The 4,6-di-O-methylgalactose was therefore attributed to this contaminant. The results then show that the $\kappa$-carrageenans did not vary in their 3-linked units, virtually all of which were present as galactose 4-sulphate. This might be generally true for samples from Chondrus and Gigartina species, although $\kappa$-fractions from some other sources, at least from Fucus, might be incompletely 4-sulphated.

Variation in the 4-linked units was more noticeable, as shown in Table I. The three Chondrus samples were similar to each other and to the sample characterised earlier, but there were significant differences between $\kappa$-carrageenans from different species. Gigartina species, particularly G. pistillata and G. radula, seem to yield $\kappa$-carrageenans in which a higher proportion of 3,6-anhydrogalactose units are sulphated. A sample of G. acicularis was found to contain sulphated and non-sulphated 3,6-anhydrogalactose in similar proportions to one from G. stellata; however, there was not sufficient of this material for analysis for galactose 6-sulphate and 2,6-disulphate, and the results are therefore omitted from the Table. Since this work was completed, the $\kappa$-carrageenan from G. skottsbergi has been reported to contain a high proportion of 3,6-anhydrogalactose 2-sulphate, and it is therefore similar in at least this respect to the Gigartina polysaccharides described here. It is also reported to contain 1-galactose units but, as in the case of $\kappa$-carrageenan from Chondrus crispus, we have been unable to detect this sugar in our G. stellata polysaccharide. We have not examined any other Gigartina polysaccharides for the possible presence of L-galactose.

To investigate structural variation within Chondrus

In more detail, another sample was prepared (sample 7, Table 2) and separated into subfractions by graded precipitation from aqueous solution with propan-2-ol. Table 2 shows a comparison of the two extreme subfractions and the original polysaccharide. The whole K-carrageenan (sample 7) was very similar to the other Chondrus samples. However, the subfractions contained, respectively, a much smaller and a rather larger proportion of 3,6-anhydrogalactose 2-sulphate chain of galactose 4-sulphate and 3,6-anhydrogalactose units is not only an idealised representation of κ-carrageenan but probably also a literal one for a small proportion of molecules in any one Chondrus preparation. This structure may be compared with that of chondroitin 4-sulphate, another alternately 1,3- and 1,4-linked polysaccharide sulphate. This differs in origin (animal connective tissue instead of seaweed), in both sugar units (N-acetylgalactosamine 4-sulphate and glucuronic acid) and in the configuration of one linkage. Nevertheless, the chains have very similar skeletons [compare (I) and (II)] and it is tempting to suggest that similar chain conformations are produced in each despite the use of different building units. This possibility is being investigated by physical and theoretical methods.

**Table 1**

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Source</th>
<th>Analysis (molar ratio, galactose : 3,6-anhydrogalactose : sulphate)</th>
<th>(i) 3,6-anhydrogalactose</th>
<th>(ii) 3,6-anhydrogalactose</th>
<th>(iii) galactose</th>
<th>(iv) galactose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Chondrus crispus (Northumberland Strait, Nova Scotia, May)</td>
<td>1 : 0.76 : 1.26</td>
<td>80—85</td>
<td>10—15</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Chondrus crispus (Northumberland Strait, Nova Scotia, November)</td>
<td>1 : 0.84 : 1.40</td>
<td>85—90</td>
<td>10</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>Chondrus crispus (Ketch Harbour, Nova Scotia, August)</td>
<td>1 : 0.72 : 1.44</td>
<td>65—70</td>
<td>25</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>Gigartina stellata (Millport, August)</td>
<td>1 : 0.73 : 1.36</td>
<td>33</td>
<td>40</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>5</td>
<td>Gigartina pistillata * (Povoa do Varzim, Portugal, April/July)</td>
<td>1 : 0.41 : 1.67</td>
<td>35—45</td>
<td>40—50</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>6</td>
<td>Gigartina radula (South Africa)</td>
<td>1 : 0.90 : 1.68</td>
<td>70—80</td>
<td>10—20</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

* Contaminated with small amounts of λ-carrageenan.

**Table 2**

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Source</th>
<th>Analysis (molar ratio, galactose : 3,6-anhydrogalactose : sulphate)</th>
<th>(i) 3,6-anhydrogalactose</th>
<th>(ii) 3,6-anhydrogalactose</th>
<th>(iii) galactose</th>
<th>(iv) galactose</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>Chondrus crispus (Yarmouth, Nova Scotia); before subfractionation</td>
<td>1 : 0.82 : 1.25</td>
<td>70—80</td>
<td>10—20</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>First subfraction of sample 7</td>
<td>1 : 0.89 : 1.07</td>
<td>94</td>
<td>5</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>Final subfraction of sample 7</td>
<td>1 : 0.67 : 1.20</td>
<td>60</td>
<td>25</td>
<td>13</td>
<td>2</td>
</tr>
</tbody>
</table>
Infrared Spectra of Sulphate Esters.—The infrared spectra of carbohydrate sulphates have been classified according to whether the ester is attached to a secondary axial, a secondary equatorial, or a primary hydroxy-group: characteristic bands are supposed to appear at 850, 830, and 820 cm$^{-1}$, respectively. The main esters present in samples 1—9 are galactose 4-sulphate and 3,6-anhydrogalactose 2-sulphate, both of which would be expected from this classification to give a band at 850 cm$^{-1}$ [see (I)]. Such a band was indeed observed for each preparation, but there was also an absorption at about 805 cm$^{-1}$, the intensity of which paralleled the proportion of 3,6-anhydrogalactose 2-sulphate shown by methylation analysis (Tables 1 and 2). We have observed this band in many other carrageenans, always with an intensity which seems to be related to the proportion of 3,6-anhydrogalactose 2-sulphate. When methyl 3,6-anhydro-2-O-methyl-$\alpha$-D-galactopyranoside and methyl 3,6-anhydro-4-O-methyl-$\alpha$-D-galactopyranoside were each treated with sulphur trioxide in pyridine, the products were the 4-sulphate and 2-sulphate, respectively, which showed new infrared bands at 815 and 798 cm$^{-1}$. We therefore conclude that 3,6-anhydrogalactopyranoside sulphates, both as monosaccharides and as polymer building units, show absorptions at about 795—820 cm$^{-1}$. If the anhydride is assumed to be linked glycosidically through position 4, this band may be used to estimate the proportion of 3,6-anhydrogalactose 2-sulphate in a carrageenan. Galactose 4-sulphate units show a band at 850 cm$^{-1}$, and the presence of this feature in the spectra of samples 1—9 confirms our interpretation of the presence of 2,6-di-O-methylgalactose in the hydrolysis products after methylation (cf. ref. 1). Simple derivatives of galactose 3-sulphate show an absorption at 830 cm$^{-1}$. Although the samples showed, in varying amounts, some broadening between the 850 and 805 cm$^{-1}$ bands, this is attributed to galactose 6-sulphate and 2,6-disulphate units and there is therefore no clear indication of 3-sulphate in $\kappa$-carrageenan.

It now seems doubtful whether the precise positions of the infrared bands of carbohydrate sulphates can in general be safely predicted on such simple grounds as whether the ester is axial, equatorial, or primary. Until a new and more reliable generalisation emerges, it will be necessary to examine the spectra of model compounds for each sulphate ester of interest.

Gel Properties and Molecular Structure.—When $\kappa$-carrageenans are in the ammonium, potassium, rubidium, or caesium salt forms, their aqueous solutions may form thermally reversible gels which have widespread industrial uses. The sodium and lithium salts do not behave in this way. Although reversible gel formation by polymers is not very well understood as a general phenomenon, two main types of mechanism are recognised, at least in outline. In the first, perhaps typified by glycosaminoglycans from animal tissues, the chain conformation of the polymer is so expanded through position 4, this band may be used to estimate the proportion of 3,6-anhydrogalactose 2-sulphate in a carrageenan. Galactose 4-sulphate units show a band at 850 cm$^{-1}$, and the presence of this feature in the spectra of samples 1—9 confirms our interpretation of the presence of 2,6-di-O-methylgalactose in the hydrolysis products after methylation (cf. ref. 1). Simple derivatives of galactose 3-sulphate show an absorption at 830 cm$^{-1}$. Although the samples showed, in varying amounts, some broadening between the 850 and 805 cm$^{-1}$ bands, this is attributed to galactose 6-sulphate and 2,6-disulphate units and there is therefore no clear indication of 3-sulphate in $\kappa$-carrageenan.

* Note added in proof: Similar conclusions have been reached independently by J. R. Turvey, D. M. Bowker, and M. J. Harris, Chem. and Ind., 1967, 2081.


** Table 3 **

Table 3: Physical properties of $\kappa$-carrageenan subfractions and derivatives

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Source</th>
<th>Proportion of 6-sulphated units (%)</th>
<th>Proportion of 2-sulphated units (%)</th>
<th>Viscosity (cp)</th>
<th>Gel strength (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>See Table 2</td>
<td>4</td>
<td>5—10</td>
<td>217</td>
<td>355</td>
</tr>
<tr>
<td>8</td>
<td>See Table 2</td>
<td>0.6</td>
<td>2—3</td>
<td>69</td>
<td>420</td>
</tr>
<tr>
<td>9</td>
<td>See Table 2</td>
<td>7</td>
<td>10—15</td>
<td>59</td>
<td>113</td>
</tr>
<tr>
<td>10</td>
<td>Lime modification of sample 7</td>
<td>0</td>
<td>5—10</td>
<td>77</td>
<td>328</td>
</tr>
<tr>
<td>11</td>
<td>Lime modification of sample 8</td>
<td>0</td>
<td>2—3</td>
<td>16</td>
<td>500</td>
</tr>
<tr>
<td>12</td>
<td>Lime modification of sample 9</td>
<td>0</td>
<td>10—15</td>
<td>11</td>
<td>223</td>
</tr>
<tr>
<td>13</td>
<td>Alkaline borohydride modification of sample 7</td>
<td>0</td>
<td>5—10</td>
<td>15</td>
<td>405</td>
</tr>
<tr>
<td>14</td>
<td>Alkaline borohydride modification of sample 8</td>
<td>0</td>
<td>2—3</td>
<td>28</td>
<td>550</td>
</tr>
<tr>
<td>15</td>
<td>Alkaline borohydride modification of sample 9</td>
<td>0</td>
<td>10—15</td>
<td>14</td>
<td>305</td>
</tr>
</tbody>
</table>


in microcrystallites, because the most elementary requirement for polymer crystallisation is regularity in the covalently-bonded structure and, as we have shown, removal of 6-sulphate converts the 'masked regularity' of native \(\kappa\)-carrageenan into more genuine regularity. Because gelation is so noticeably inhibited at low levels of structural irregularity, we conclude that stable microcrystallites contain a number of monomer units in the chain direction. There is probably a similar condition for formation of pectin gels.\(^{17,20}\)

Gel formation by \(\kappa\)-carrageenan is less sensitive to 2-sulphation of the 3,6-anhydride units (cf., for example, the properties of samples 10, 11, 13, and 14) than to 6-sulphation of the 4-linked units. The dramatic effect of the latter change can no doubt be traced to the conformational difference between galactose 6-sulphate (C1) and 3,6-anhydrogalactose (1C), i.e., an isolated 6-sulphate unit represents a severe 'kink' in the polymer chain. The commercially important process\(^{21}\) in which the gel properties of \(\kappa\)-carrageenan are improved by treatment with lime, is therefore likely to act by 'de-kinking' polymer chains. On the other hand, the general contour of the chain is probably unaffected by 2-sulphation, so that crystallisation is not sterically obstructed if the chains are arranged with 2-sulphate on the outside of the microcrystallite. This would only be possible if microcrystallites did not contain very many chains. In a later paper (with M. M. Harding and J. W. B. Samuel), we will comment on these conclusions with evidence from X-ray diffraction.

**Experimental**

Most of the general and analytical (including chromatographic) methods have been described before.\(^{1,5,7,8,11}\) Infra-red spectra were recorded with the Perkin-Elmer model 237 spectrometer (sample as film). For polysaccharides, films were prepared by evaporation of an aqueous solution (0.5%) on mercury in a vacuum desiccator at room temperature; for sugar sulphates, they were prepared similarly but on a piece of silver chloride which was then placed (carrying the sample) in the beam.

**Methylation of \(\kappa\)-Carrageenan Samples and Examination of the Products.**—Samples (1 g.) were methylated as before.\(^1\) Yields were 0.8—1.1 g. and the following analytical figures were obtained:

<table>
<thead>
<tr>
<th>Sample no. (see Tables 1 and 2)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>OMe (%)</td>
<td>14.7</td>
<td>13.3</td>
<td>13.2</td>
<td>13.1</td>
<td>11.6</td>
<td>12.3</td>
<td>14.2</td>
<td>14.8</td>
<td>14.9</td>
</tr>
<tr>
<td>SO(_4) (%)</td>
<td>18.3</td>
<td>16.2</td>
<td>17.5</td>
<td>18.6</td>
<td>17.7</td>
<td>16.7</td>
<td>13.4</td>
<td>16.7</td>
<td></td>
</tr>
</tbody>
</table>

Hydrolysis and paper chromatography of each showed 2,6-di-O-methylgalactose in large amounts together with degradation products of 3,6-anhydrogalactose derivatives and traces of tri-O-methylgalactose and mono-O-methylgalactose. Sample 5 showed some 4,6-di-O-methylgalactose (see Discussion section). For identification of the 3,6-anhydro-units, methylated polysaccharide (60 mg.) was dissolved in 0.5 N-sulphuric acid (6 ml.), bromine was added\(^1\) and the solution was kept at 60°. When hydrolysis of 3,6-anhydrogalactosyl linkages was complete (23 hr.), the solution was aerated, the product was hydrolysed, and the mixture of methyl ethers was examined as before.\(^{1}\) A quantitative estimate of the proportions of 3,6-anhydrogalactonic acid and 3,6-anhydro-2-O-methylgalactonic acid was made by visual matching of spot intensities with those obtained after oxidative hydrolysis of known weights of the crystalline glycosides,\(^7\) when measured volumes of solutions were applied to the paper chromatograms with micropipettes (accuracy ca. \(\pm 10\%)\). The results are shown in Tables 1 and 2. In addition to these two major products, traces of an acid which might have been 3,6-anhydro-4-O-methylgalactonic acid were detected in samples 3—5, 7, and 8; and traces of an acid which might have been 3,6-anhydro-2,4-di-O-methylgalactonic acid were detected in these samples and in samples 2 and 6.

**Alkaline Borohydride Modification before and after Periodate Oxidation.**—The more direct of the two methods described earlier\(^1\) (i.e., without prior hydrolysis and reduction) was used. The results are given in Tables 1 and 2.

**Fractionation of \(\kappa\)-Carrageenan.**—\(\kappa\)-Carrageenan, isolated from hand sorted *Chondrus crispus* and purified by repeated precipitation (four times) with potassium chloride, was separated into four fractions of approximately equal weight by gradual addition of propan-2-ol to a dilute aqueous solution (0.2%) of the potassium salt in 3% w/w aqueous sodium chloride. The fractions were collected at 20, 32.5, 33.5, and 50% w/w propan-2-ol, respectively, washed with propan-2-ol, and dried. The absence of \(\lambda\)-carrageenan from the original polysaccharide and from the two extreme subfractions, was confirmed by methylation analysis (no 4,6-di-O-methylgalactose detected) and by the low content of galactose 2,6-disulphate (see Table 2). The first and last of the subfractions had \([\alpha]_D\) +60 and +65°, respectively, compared with +62° for the unfraccionated polysaccharide (all c. 0.15 in water). The other properties are listed in Tables 2 and 3.

**Gel Strength and Related Measurements.**—Gel strengths were measured with a plunger (11 mm. diam.) acting on an aqueous gel containing 1% of polysaccharide (potassium salt form) and 1% of potassium chloride, at 25°. The gel strength is here defined as the load that is required to break the gel. Viscosities were measured at 60° for a 1% solution of the polysaccharide (potassium salt form) with a Brookfield LVF Viscometer with a UL adaptor. The modification of carrageenans with alkaline borohydride\(^4\) and with lime\(^{11}\) has been described before.

**The Possible Presence of \(\beta\)-Galactose in \(\kappa\)-Carrageenan from *G. stella*.*—No \(\beta\)-galactose was detected in the hydrolysate when it was prepared and treated with galactose—adapted yeast as before.\(^1\)

3,6-Anhydrogalactopyranoside Sulphates.—The methods were based on those developed by Turvey and co-workers.\(^{14}\) Methyl 3,6-anhydro-\(\alpha\)-D-galactopyranoside (200 mg.) was dissolved in dry pyridine (4 ml.); dried over type 4A molecular sieve and shown by \(^1\)HN.m.r. to contain less than 0.1% water, and treated with pyridine—sulphur trioxide reagent (freshly prepared; 4 moles). The mixture was

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stirred on a water-bath (65—70°) under anhydrous conditions for 24 hr., and water (4 ml.) was added. Stirring was continued for 1 hr. and aqueous barium hydroxide was added to give a pH of 9. Barium sulphate was removed by centrifugation and the solution was carefully (bath temperature 35°) evaporated with continuous addition of water to maintain the volume, until all pyridine had been removed. After removal of cations (IR-120 resin, H+ form) and neutralisation to pH 8 with ammonia, the solution was evaporated and freeze-dried. Electrophoresis (pyridine-acetic acid buffer, p-anisidine hydrochloride spray) showed a major product, with mobility similar to that of glucose disulphate, which was therefore methyl 3,6-anhydro-α-D-galactopyranoside 2,4-disulphate. Its identity as a sulphate ester was confirmed by the infrared peak at 1200—1250 cm.⁻¹ which was not present before treatment with pyridine-sulphur trioxide. There was no trace of the peak at 850 cm.⁻¹ which would be expected on the basis of existing assignments for a compound containing axial sulphate ester, but there was instead a broad band at 800—830 cm.⁻¹. Similarly, sulphation of each of the monomethyl ethers of methyl 3,6-anhydro-α-D-galactopyranoside (80 mg.) gave a product with the electrophoretic mobility of a sugar monosulphate which showed an infrared band at 1240 cm.⁻¹. These products were, therefore, methyl 3,6-anhydro-2-O-methyl-α-D-galactopyranoside 4-sulphate and methyl 3,6-anhydro-4-O-methyl-α-D-galactopyranoside 2-sulphate. Each showed an infrared absorption band in the region 790—820 cm.⁻¹ which was not present in the spectrum of the corresponding parent glycoside before sulphation; for the 2-sulphate this was at 795 cm.⁻¹ and for the 4-sulphate it was at 815 cm.⁻¹.

We thank Professor Sir Edmund Hirst, C.B.E., F.R.S., and S. D. Upham, for their interest and encouragement, and E. T. Dewar and J. R. Turvey for samples.
CARRAGEENANS
PART V. THE MASKED REPEATING STRUCTURES OF λ- AND μ-CARRAGEENANS

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(Received January 1st, 1968)

ABSTRACT

For the potassium chloride-soluble fractions of five carrageenans, correlations have been established between 3,6-anhydrogalactose content and the products of methylation and fragmentation. After modification with alkaline borohydride, two subfractions were obtained, and shown to be distinct by methylation analysis and other methods.

Carrageenan is now believed to contain three polysaccharide components: κ-carrageenan, λ-carrageenan, and μ-carrageenan. All have masked repeating structures based on alternately (1→3)- and (1→4)-linked galactose residues, but there are important differences in sulphation pattern and 3,6-anhydride content.

INTRODUCTION

Carrageenan has long been known to contain at least two polysaccharides, of which one is selectively precipitated with potassium chloride and named κ-carrageenan. Many features of structure have been characterized for this component, but there has been less agreement on the nature of the potassium chloride-soluble fraction. In this paper, we show that there are two structurally distinct polysaccharides in the soluble fraction. Both are polymers of alternately β-(1→4)- and α-(1→3)-linked galactose derivatives, but they differ in sulphate ester distribution and certain other respects. On the principle that nomenclature should be related to structure rather than solubility properties, they are named λ-carrageenan and μ-carrageenan.

RESULTS AND DISCUSSION

Methylation and other methods have shown that the potassium chloride-soluble fraction of carrageenan (Sample I, Table I) contains both (1→3)- and (1→4)-linked galactose residues. Most of the former type are 2-sulphated, although some are 4-sulphated and others are nonsulphated; the (1→4)-type occurs chiefly as galactose 2,6-disulphate, but also as 3,6-anhydrogalactose (and/or its 2-sulphate) and galactose 6-sulphate. The variation of gross composition from one sample to another might indicate polymer heterogeneity. Several further samples (Table I), kindly supplied...
by Dr. E. T. Dewar\textsuperscript{7}, were therefore examined by methylation, followed by hydrolysis and paper chromatography. The products from each were 3-O-methylgalactose, 4,6-di-O-methylgalactose, 2,6-di-O-methylgalactose, 2,4,6-tri-O-methylgalactose, and traces of 2,3,4,6-tetra-O-methylgalactose. In addition, 3,6-anhydro-2-O-methylgalactose derivatives were detected by gas–liquid chromatography after methanolation. The relative proportions of 3,6-anhydro-2-O-methylgalactose and 2,6-di-O-methylgalactose increased from Sample 1 to Sample 5, whereas 3-O-methylgalactose, 4,6-di-O-methylgalactose, and 2,4,6-tri-O-methylgalactose decreased. With \(\lambda\)-carrageenan defined as a copolymer of 3-linked galactose 2-sulphate, 3-linked galactose, and 4-linked galactose 2,6-disulphate\textsuperscript{4}, and \(\kappa\)-carrageenan as a copolymer of 3-linked galactose 4-sulphate and 4-linked 3,6-anhydrogalactose with some 4-linked 3,6-anhydrogalactose 2-sulphate\textsuperscript{3}, the methylation results show that the samples are progressively less “\(\lambda\)-like” from Sample 1 to Sample 5 and progressively more “\(\kappa\)-like”. The variations in 3,6-anhydrogalactose content (Table I) point to a similar conclusion. The proportion of 2,3,4,6-tetra-O-methylgalactose increased progressively from Sample 1 to Sample 5, but the amounts were very small indeed and it is not possible to be certain of their significance at present.

### Table I

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Source</th>
<th>Time and place of collection</th>
<th>Molar ratio, galactose: 3,6-anhydrogalactose: sulphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Chondrus crispus</td>
<td>Prince Edward Island (Nova Scotia), August</td>
<td>1.00:0.01:1.25</td>
</tr>
<tr>
<td>2</td>
<td>Chondrus crispus</td>
<td>Northumberland Strait (Nova Scotia), May</td>
<td>1.00:0.11:1.22</td>
</tr>
<tr>
<td>3</td>
<td>Chondrus crispus</td>
<td>Northumberland Strait (Nova Scotia), November</td>
<td>1.00:0.10:1.22</td>
</tr>
<tr>
<td>4</td>
<td>Chondrus crispus</td>
<td>Ketch Harbor (Nova Scotia), August</td>
<td>1.00:0.29:1.36</td>
</tr>
<tr>
<td>5</td>
<td>Gigartina stellata</td>
<td>Millport (Scotland), August</td>
<td>1.00:0.43:1.07</td>
</tr>
</tbody>
</table>

The methylation data might therefore suggest that all samples are mixtures of \(\lambda\)-carrageenan and a component which, although not precipitable by potassium chloride, has some structural similarity to \(\kappa\)-carrageenan. This interpretation was confirmed by treatment of Sample 1 with alkaline borohydride to convert any 4-linked galactose 6-sulphate into 3,6-anhydrogalactose residues, followed by addition of potassium chloride. This gave a precipitate \(P\) (10\%) and a soluble polysaccharide \(Q\) (87\%), which were easily distinguished by methylation analysis: \(P\) gave 2,6-di-O-methylgalactose and 3,6-anhydro-2-O-methylgalactose as cleavage products with traces of 4,6-di-O-methylgalactose and 2,4,6-tri-O-methylgalactose, whereas \(Q\) gave 4,6-di-O methylgalactose and 2,4,6-tri-O-methylgalactose but no 2,6-di-O-methylgalactose.

or 3,6-anhydro-2-\(O\)-methylgalactose. After methanolysis of both \(P\) and \(Q\), carrabiose dimethyl acetal (3,6-anhydro-4-\(O\)-\(\beta\)-D-galactopyranosyl-\(D\)-galactose dimethyl acetal) was isolated as the crystalline hexa-acetate. Quantitative paper chromatography, in experiments published already\(^a\), has shown that the yields of carrabiose dimethyl acetal formed by methanolysis are close to those expected for a polymer built entirely of carrabiose segments. With the evidence from methylation, this shows that the structures of \(P\) and \(Q\) are based on alternately arranged 3-linked \(\beta\)-D-galactopyranosyl residues and 4-linked 3,6-anhydrogalactosyl residues, and are therefore of the "masked repeating"\(^2,3,9\) type. The 3,6-anhydrogalactosyl linkages probably have the \(\alpha\)-D configuration, by analogy with \(\kappa\)-carrageenan\(^2,10\), and because the \(\alpha\)-(1\(\rightarrow\)3)-linked disaccharide has been isolated from the acetolysis products of the potassium chloride-soluble fraction of carrageenan\(^11\). In \(Q\), the galactose residues are mostly 2-sulphated; the quantitative data\(^4\) suggest that 70\% are 2-sulphated and most of the remainder nonsulphated. Virtually all of the 3,6-anhydrogalactose residues in this subfraction were shown to be sulphated, because oxidative hydrolysis of the methylated polysaccharide gave preponderantly 3,6-anhydrogalactonic acid, with little of its 2-methyl ether. The peak at 808 cm\(^{-1}\) in the infrared spectrum also indicates\(^3\) a high proportion of 3,6-anhydrogalactose 2-sulphate residues. Before modification with alkaline borohydride, the structure therefore corresponds to 1, which, by definition\(^4\), is \(\lambda\)-carrageenan.

1. \(R^1 = H; R^2 = SO_3^-\) (70\%) and H (30\%); \(R^3 = SO_3^-\)
2. \(R^1 = SO_3^-; R^2 = R^3 = H\)

The galactose residues in \(P\) are largely 4-sulphated, since 2,6-di-\(O\)-methylgalactose was detected as the main, acid-stable hydrolysis product after methylation. The 3,6-anhydride is largely nonsulphated, because oxidative hydrolysis, after methylation, gave 3,6-anhydro-2-\(O\)-methylgalactonic acid with little 3,6-anhydrogalactonic acid, and because the anhydride 2-sulphate peak\(^3\) in the region of 805 cm\(^{-1}\) was virtually absent from the infrared spectrum. Those samples which gave a high yield of 2,6-di-\(O\)-methylgalactose after methylation and hydrolysis, and which were therefore rich in the precursor of \(P\), also had high contents of native 3,6-anhydrogalactose (Table I). This suggests that the 3,6-anhydride, present before modification of the potassium chloride-soluble fraction, occurs in the precursor of \(P\). The quantitative data\(^4\) for Sample 1 shows that there is insufficient 3,6-anhydrogalactose to account for more than about 20\% of the native 4-linked residues, but the proportion would be higher for Sample 5, even if \(\lambda\)-carrageenan were totally absent (Table I).
The structure of the precursor of \( P \) is therefore idealised as 2, with replacement of galactose 6-sulphate residues by 3,6-anhydrogalactose to an extent which is variable and dependent on source. The further deviations, \( R^1 = R^2 = H; R^1 = H, R^2 = SO_3^- \); and \( R^3 = SO_3 \), seem to occur to very minor extents and might be artefacts owing to incomplete separation. This polysaccharide represents a new type of carrageenan structure, and we therefore propose the name \( \mu \)-carrageenan. Its biological relationship to \( \kappa \)-carrageenan has been discussed elsewhere.

EXPERIMENTAL

General. — Poly(ethylene glycol adipate) (15% on Gas Chrom P) was the liquid phase for g.l.c. in 4-ft. columns at 175\(^\circ\), with the Pye-Argon Chromatograph fitted with a \( ^{90}\)Sr detector. For paper chromatography, the solvents were (A) butyl alcohol-ethanol-water (4:1:5, upper phase), (B) ammonia (sp. gr. 0.880)—water—ethyl methyl ketone (1:17:200), (C) butyl alcohol-ethanol-water (3:1:1), and (D) ethyl acetate-acetic acid-formic acid—ethyl methyl ketone—water \( ^2 \) (17:3:1:15:5). Sprays were \( \beta \)-anisidine hydrochloride \(^1\) for reducing sugars and their methyl ethers, and aniline-xylose \(^1\) for aldonic acids and their methyl ethers. Infrared spectra were recorded for polysaccharide films, which were prepared by evaporation of solutions (0.5% w/v) on silver chloride. The analytical methods for sulphate ester and 3,6-anhydrogalactose have been described earlier.

Methylation analysis of potassium chloride-soluble fractions. — The methylation analysis of Sample 1 (Table I) has been described before. Samples 2-5 (1 g of each) were separately dissolved in water (100 ml), and each solution was cooled in ice. Methyl sulphate (10 ml) and sodium hydroxide (30% w/v, 30 ml) were added over 6 h with vigorous stirring under nitrogen. After stirring for 16 h, the procedure was repeated five times at room temperature. The solution was dialysed, and concentrated under diminished pressure to 100 ml, and the entire cycle was repeated. The methylated polysaccharides were isolated in yields of 0.8–1.0 g by dialysis and freeze-drying (Found: SO\(_4\), 18.0; OCH\(_3\), 13.6 for Sample 2; SO\(_4\), 21.5; OCH\(_3\), 13.0 for Sample 3; SO\(_4\), 20.9; OCH\(_3\), 16.3 for Sample 4; SO\(_4\), 15.1; OCH\(_3\), 15.3 for Sample 5). Repeated methylation did not change the pattern of methyl ethers observed after subsequent hydrolysis and paper chromatography.

Each methyl ether, including that of Sample 1, was heated with aqueous formic acid (45% w/v) for 6 h at 100\(^\circ\). Paper chromatography (solvents A and B with \( \beta \)-anisidine hydrochloride spray) gave the results reported in the Discussion. Samples 3, 4, and 5 contained only traces of 2,4,6-tri-O-methylgalactose. The relative proportions of the two di-O-methylgalactoses were similar to each other in Samples 2 and 3; in Sample 5, there was an approximately ten-fold excess of the 2,6 isomer (contrast Sample 1, for which the 4,6 isomer is present in much the greater amount). Methanolysis (2.3% methanolic hydrogen chloride in a sealed tube for 15 h at 100\(^\circ\), followed by neutralisation with silver carbonate) and g.l.c. confirmed these trends and showed the presence of 3,6-anhydro-2-O-methylgalactose derivatives which,
for each sample with our apparatus and conditions, gave a total peak area quite
close to the corresponding total area for 2,6-di-O-methylgalactose derivatives.

Modification with alkaline borohydride, fractionation, and characterization of
the products. — Sample 1 (40 g) was dissolved in water (2 l), and potassium borohydride (2 g) was added. After 20 h at room temperature, 3N sodium hydroxide (1 litre) was added with a further quantity of borohydride (1 g), and the mixture was kept for 24 h at 80°, dialysed against running tap-water, concentrated, and freeze-dried (30.5 g). Part of the product (5 g) was dissolved in water (2 l), and potassium chloride (50 g) was added with stirring. After storage of the mixture overnight, a gelatinous precipitate had separated. This was collected by centrifugation, dissolved in 5% sodium chloride for dialysis against running tap-water, and isolated by freeze-drying (P, 0.5 g). The supernatant solution was dialysed against running tap-water and freeze-dried (Q, 4.4 g). In the infrared spectrum, Q showed strong absorption
for ester sulphate at 1220–1260 cm⁻¹, and peaks at 808 and 835 cm⁻¹; there was no peak at 850 cm⁻¹. After purification by reprecipitation with potassium chloride, washing thrice with 5% KCl at the centrifuge, dissolution in sodium chloride, and isolation as before, P showed absorption bands at 1240 and 850 cm⁻¹, but little, if any, at 805 and 830 cm⁻¹. Other details of these fractions have been published.

Fraction P (0.70 g) was refluxed with methanolic hydrogen chloride (0.2% w/v, 500 ml) for 40 min. After being kept over excess of silver carbonate for 20 h, the solution was filtered and concentrated to small volume. Separation on sheets of Whatman 3MM paper with solvent C gave syrupy carrabiose dimethyl acetal (0.40 g), which was acetylated by treatment with pyridine (5 ml) and acetic anhydride (3.3 ml) for 1 h at 80°. Evaporation of the reagents, and washing with water by decantation, left hexa-0-acetylcarrabiose dimethyl acetal which was crystallised from ethanol as needles (0.22 g, 33% overall), m.p. and mixed m.p. 152–154°, [α]D –14.0° (c 1.1, benzene). Similar treatment of Q (0.71 g) also gave syrupy carrabiose dimethyl acetal (0.47 g) and hence the same crystalline hexa-acetate (0.25 g, 34% overall), m.p. and mixed m.p. 153–154.5°, [α]D –15.5° (c 0.9, benzene).

Treatment with methyl sulphate and sodium hydroxide, as already described, converted Q purified and P (0.25 g of each) into their methyl ethers (0.2 g). Hydrolysis and paper chromatography, and methanalysis and g.l.c., were carried out by methods described above; the results are given in the Discussion. For oxidative hydrolysis, a sample of each (20 mg) was dissolved in water (2 ml), and 8N sulphuric acid (2 ml) was added with shaking followed by bromine (1–3 drops). The solution was kept at 50° with addition of more bromine as necessary to maintain the concentration. After 24 h, the bromine was removed by aeration, and 6N sulphuric acid (1 volume) was added, followed by heating for 15 h at 100° to hydrolyse the galactosyl bonds and sulphate groups. After neutralisation with barium carbonate, silver carbonate was added to the suspension which was left in the dark with occasional shaking for 72 h, and then filtered and treated with Amberlite resin IR-120 (H⁺ form). The solution was concentrated under diminished pressure and compared with 3,6-anhydrogalactonic acid and its 2-methyl, 4-methyl, and 2,4-dimethyl ethers, by paper chromatography.
in solvent $D$ with aniline-xylose spray. The product from $Q$ contained 3,6-anhydrogalactonic acid and its 2-methyl ether, in the approximate ratio 20:1. For $P$, the ratio was approximately 1:5.

ACKNOWLEDGMENTS

We thank Marine Colloids, Inc., for generous financial support, Dr. E. T. Dewar for samples, and Sir Edmund Hirst for encouragement and advice.

REFERENCES