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Chemical methods for the analysis of sulphated galactans from red algae

Thomas T. Stevenson and Richard H. Furneaux*
DSIR Chemistry, Private Bag, Petone (New Zealand)

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ABSTRACT

Methods are reported that facilitate the structural characterization of complex sulphated galactans of the red algae. Two procedures have been developed for the production of alditol acetates from carrageenans and agaroids. Both procedures generate 3,6-anhydrogalactitol acetate from the easily destroyed 3,6-anhydrogalactosyl residues in near quantitative yield. The "double hydrolysis–reduction" method involves preliminary hydrolysis under conditions sufficient to cleave all of the 3,6-anhydrogalactosidic bonds, but mild enough to avoid significant further degradation. The "reductive hydrolysis" method uses the acid-stable 4-methylmorpholine–borane to reduce the 3,6-anhydrogalactose end groups as they are released during acid hydrolysis. An alditol acetate sample can be prepared from a polysaccharide in a single tube, ready for g.l.c. analysis, in less than 2.5 h, *i.e.* more quickly than by any previous procedure. Problems associated with incomplete methylation of sulphated carrageenans and agaroids by the Hakomori procedure have been overcome by first converting the sulphated polysaccharide into its triethylammonium salt form. The reductive hydrolysis method is effective for the production of partially methylated alditol acetates from the methylated polysaccharides, enabling the rapid determination of the substitution pattern of these polysaccharides. These improved analytical methods have been applied successfully to κ-, ι-, and λ-carrageenans, as well as some agars.

INTRODUCTION

Constituent-monosaccharide analysis, involving the conversion of the constituent sugars of a polysaccharide into their alditol acetate derivatives, and methylation analysis, involving the methylation of a polysaccharide and subsequent production of partially methylated alditol acetates, are two fundamental chemical procedures used for the structural characterisation of polysaccharides. The volatile derivatives produced are analyzed by g.l.c. and g.l.c.–m.s. to provide information about the compositions and structures of complex polysaccharides.

Red-algal galactans contain up to 50% 3,6-anhydrogalactosyl residues (see Fig. 1 for structures) and are not amenable to analysis by the above procedures directly because the 3,6-anhydrogalactosyl residues are rapidly destroyed (both in the native and in the methylated form) under the harsh acidic conditions typically used to hydrolyse the polysaccharides into their constituent monosaccharides. Thus, no in-

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* To whom correspondence should be addressed.

formation regarding the quantity or substitution of the 3,6-anhydrogalactosyl residue can be obtained. Attempts have been made to stabilize the 3,6-anhydrogalactosyl residues during acid-catalysed cleavage by application of mercaptolysis[1], methanolysis[1], acetolysis[1], formolysis[3], or bromine oxidation during hydrolysis[4], but these techniques have met with only limited success and none has been used widely.

An additional problem arises during the methylation of carrageenans and agaroids. Some of these polysaccharides are highly charged due to the presence of sulphate groups and thus are insoluble in dimethyl sulphoxide (DMSO), and thereby difficult to methylate fully by the Hakomori procedure[5]. To overcome this problem, sulphated polysaccharides have been acetylated to improve solubility for the subsequent Hakomori methylation[6], or methylated using the Haworth procedure (aqueous sodium hydroxide–dimethyl sulphate)[4,7]. Both of these procedures are difficult to apply and time consuming.

In the study reported here, new solutions to these old problems are presented.

RESULTS AND DISCUSSION

Constituent-sugar analysis by the double hydrolysis–reduction procedure. — Studies reported some thirty years ago on the structures of seaweed polysaccharides demonstrated that mild acidic conditions could be found whereby the 3,6-anhydrogalactosidic bonds could be cleaved with only minimal concomitant degradation of the sensitive

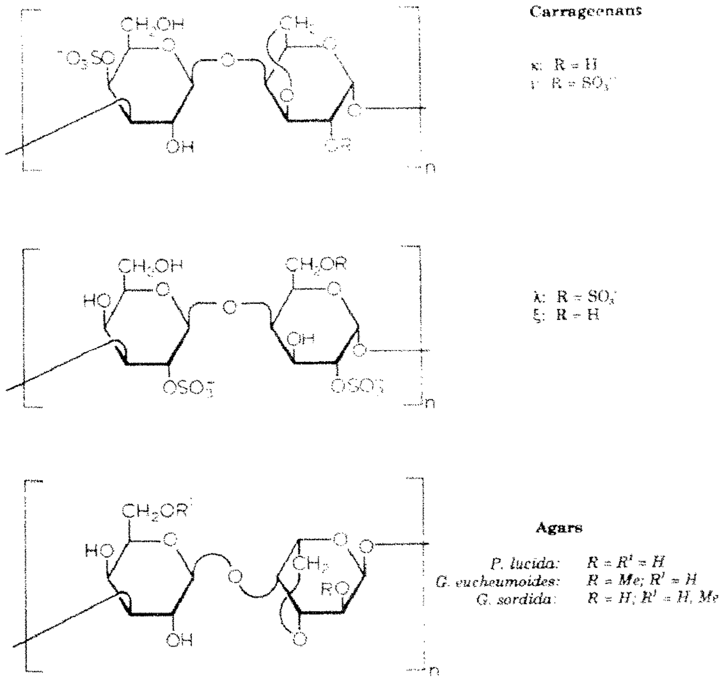

Fig. 1. Major structural features of various carrageenans and agars[1,2].

3,6-anhydrogalactose reducing end groups[8,9]. No further advantage appears to have been taken of these crucial observations. We have now incorporated such a preliminary, mild hydrolysis into a double hydrolysis–reduction procedure, and demonstrated that under standardised conditions this procedure affords alditol acetates from all of the neutral sugars, including the 3,6-anhydrogalactose, of carrageenans and agars.

(a) Optimisation of the procedure. The alkali-modified agar from *Pterocladia lucida* (Fig. 1) was an ideal substrate for the initial studies, since it is virtually an unsubstituted agarose[10]. Samples of this agar were hydrolysed in 0.1M trifluoroacetic acid (TFA) at 80° for varying lengths of time. The agar was not soluble under these conditions but it slowly dissolved as hydrolysis progressed. The resulting mixtures were reduced with aqueous sodium borohydride. The 3,6-anhydrogalactosyl residues whose glycosidic linkages were hydrolysed during the acid treatment were thereby converted into acid-stable 3,6-anhydrogalactitol derivatives*. The samples were then fully hydrolysed with 2M TFA for 1 h at 120°, reduced a second time, and acetylated. The resulting mixtures of alditol acetates were analysed by g.l.c. The molar ratio of 1,2,4,5-tetra-*O*-acetyl-3,6-anhydrogalactitol (AnGal) to hexa-*O*-acetylgalactitol (Gal) as a function of preliminary hydrolysis time is shown in Fig. 2. Since the galactosyl residues are not significantly degraded during the hydrolysis[11], the hexaacetylgalactitol produced served as an internal standard for determining the recovery of 3,6-anhydrogalactose. Without the preliminary hydrolysis and reduction, all 3,6-anhydrogalactose was destroyed during hydrolysis with 2M acid. However, with the preliminary hydrolysis and reduc-

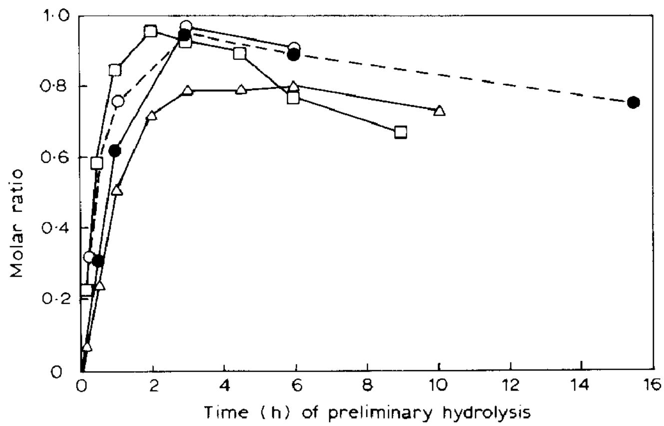

Fig. 2. Molar ratios of the acetylated derivatives of 3,6-anhydrogalactitol (solid lines) or 3,6-anhydro-2-*O*-methylgalactitol (dashed line) to galactitol as a function of time of preliminary hydrolysis (0.1M TFA, 80°) for *Pterocladia lucida* agar (○), *Gracilaria eucheumoides* agar (●), κ-carrageenan (□), and ι-carrageenan (△), all subjected to the double hydrolysis–reduction procedure.

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* The absolute configurations of the alditols described herein were not determined and are therefore not indicated. According to systematic nomenclature the alditol from 3,6-anhydro-L- (but not-D-)-galactose would be designated 1,4-anhydrogalactitol. However, to avoid confusion, the original galactose numbering has been used throughout the present paper.

tion, the stable 3,6-anhydrogalactitol acetate was obtained, the maximum yield resulting from a 3 h treatment. After 6 h some degradation of the 3,6-anhydrogalactose was evident. The AnGal:Gal molar ratio of 0.98 at 3 h is close to that required for a polysaccharide with equimolar amounts of 3,6-anhydrogalactosyl and galactosyl residues, such as agar.

It appeared likely that this method could be applied to other algal galactans. However, it first was necessary to determine whether 3,6-anhydrogalactitol was being formed under the double hydrolysis–reduction conditions by dehydration processes as well as from the constituent anhydrosugar, and also whether the presence of the commonly encountered methyl ether and sulphate half-ester substituents affected the procedure.

Anhydroalditols can be formed by intramolecular dehydration of alditols during acid treatment[12]. When galactitol and 6-*O*-methylgalactitol were separately subjected to the double hydrolysis–reduction procedure, only small amounts of the dehydration products 3,6-anhydrogalactitol (5.0 mol %) and 3,6-anhydro-1-*O*-methylgalactitol (1.6 mol %), respectively, were generated. Since only a small proportion of the galactosyl and 6-*O*-methylgalactosyl residues in an algal polysaccharide would be converted into the corresponding alditols during the preliminary hydrolysis–reduction sequence, it can be seen that the production of 3,6-anhydro-alditols due to acid catalysed dehydration is not going to be a concern with this methodology. To confirm this, methyl α-D-galactopyranoside was subjected to the double hydrolysis–reduction procedure using a 3 h preliminary hydrolysis, and the products were examined by g.l.c. for the presence of tetra-*O*-acetyl-3,6-anhydrogalactitol. Only the expected galactitol hexaacetate was found.

The alkali-modified agar isolated from *Gracilaria eucheumoides* has a minor proportion of its D-galactose residues methylated on O-6, but is almost entirely methylated on O-2 of its 3,6-anhydrogalactosyl residues[13] (Fig. 1). It seemed possible that methylation so close to the 3,6-anhydrogalactosidic bond could alter its rate of hydrolysis. However, when the double hydrolysis–reduction procedure was applied to the cold-water-insoluble *G. eucheumoides* agar, and the resulting alditol acetates were analyzed by g.l.c., the maximum yield of 3,6-anhydro-2-*O*-methylgalactitol again was obtained after 3 h (Fig. 2). The observed AnGal: 2-Me-AnGal: Gal : 6-Me-Gal ratio of 3:46:48:3 at 3 h (ref. 14) is consistent with the structure deduced from ^{13}C-n.m.r. spectroscopic studies[13].

Sulphate groups on algal galactans could adversely affect the analysis of these polysaccharides by the double hydrolysis–reduction procedure in three ways: 1) a high proportion of sulphate groups imparts water solubility to the polysaccharide and might alter its hydrolysis rate; 2) a sulphate group at O-2 of the 3,6-anhydrogalactosyl residues could alter the hydrolysis rate of the 3,6-anhydrogalactosidic bond through steric or electronic effects, or by intramolecular acid-catalysis; and 3) incomplete hydrolysis of the sulphate esters during the second hydrolysis step would result in sulphated alditols that could not be analyzed by g.l.c. To determine if problems arise from these considerations, the following experiments were conducted with κ-, ι-, and λ-carrageenan.

When the hydrolysis experiment was repeated with the sulphated, water-soluble polysaccharide κ-carrageenan (see Fig. 1), the maximum yield of 3,6-anhydrogalactitol resulted after 2 h (Fig. 2), but had not changed significantly by 3 h. The increased hydrolysis rate was probably due to the water solubility of the polysaccharide. However, because of the slow rate of degradation of 3,6-anhydrogalactose in 0.1M TFA at 80° a 3 h hydrolysis time could be used to analyse both cold-water-soluble κ-carrageenan and insoluble agar.

To ascertain the effect of a sulphate group attached to O-2 of the 3,6-anhydrogalactosyl residue, the hydrolysis experiment was repeated a fourth time on the water-soluble, sulphated polysaccharide ι-carrageenan (Fig. 1). The 3,6-anhydrogalactosidic bonds of polysaccharides bearing an O-2 sulphate substituent are hydrolysed more slowly than those of polysaccharides without such a substituent[15]. Our results agree with this observation (Fig. 2), however, a 3 h preliminary hydrolysis again gave the maximum yield of 3,6-anhydrogalactitol.

To produce volatile alditol acetates from sulphated sugar residues, the ionic sulphate groups must be removed during hydrolysis. To ascertain that sulphate groups were fully hydrolysed during the second (2M) acid hydrolysis, the amount of inorganic sulphate released from λ-carrageenan upon hydrolysis in 2M TFA at 120° was measured using a barium rhodizonate colourimetric assay[16] (Fig. 3). λ-Carrageenan was chosen because it contains primary sulphate esters (Fig. 1), which are relatively resistant to acid hydrolysis[17]. The colourimetric assay also detects sulphated polysaccharides, but with significantly lower sensitivity than for inorganic sulphate. An untreated sample of λ-carrageenan thus had an apparent inorganic sulphate content of 6.6% (left arrow in Fig. 3). To monitor sulphate release, aliquots of the hydrolysate were taken to dryness at room temperature prior to being assayed. Some hydrolysis of the half-ester sulphate groups must occur during this process, since an unheated sample (t = 0 in Fig. 3) had an apparent inorganic sulphate content of 14.2%. The maximum release of sulphate had occurred after hydrolysis for 15 min, at which time the 29.5% apparent sulphate content compared favourably to the 27.5% total sulphate determined after complete oxidation of the sample (right arrow in Fig. 3). Clearly, therefore, all sulphate groups would be removed during the double hydrolysis–reduction procedure, since it entails a treatment with 2M TFA for 1 h at 120°.

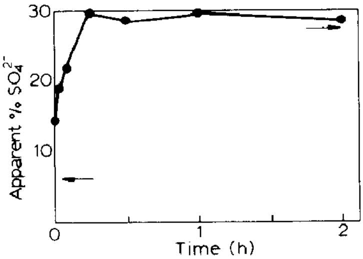

Fig. 3. Apparent content of inorganic sulfate during the hydrolysis (2M TFA, 120°) of λ-carrageenan in the Et_3NH^+ form. Left and right arrows mark the apparent SO_4^{2-} contents of untreated and fully oxidized λ-carrageenan, respectively.

We concluded that the double hydrolysis–reduction procedure, employing a preliminary treatment with 0.1M TFA for 3 h at 80°, is useful for analysing the glycosyl composition of any red-algal galactan, regardless of substitution. Although the rates of 3,6-anhydrogalactosidic-bond cleavage differ for polysaccharides with different substitutions, the rates of subsequent destruction of the 3,6-anhydrogalactosyl end groups and of the anhydrosugar itself are slow, being similar to those of pentoses in 2M TFA at 120° (ref. 11), the conditions typically used for "complete" hydrolysis of polysaccharides.

Application of the double hydrolysis–reduction procedure to samples containing significant levels of 3,6-anhydrogalactose generated two isomeric byproducts, each in a normalised yield of 1–1.5 mol % (Table I). These compounds, resulting from acid degradation of the 3,6-anhydrogalactosyl residues (Fig. 4), were deduced to be penta-*O*-acetyl-3-deoxy-*lyxo*- and -*xylo*-hexitol (**3**) from their e.i.-m.s. fragmentation patterns, which were similar to each other and to that of synthetic penta-*O*-acetyl-3-deoxy-D-*ribo*-hexitol. 5-Hydroxymethyl-2-furaldehyde (**2**) and its condensation products are the normal acid-degradation products of 3,6-anhydrogalactosyl residues[1]. The conditions of the preliminary hydrolysis are too mild to form appreciable amounts of **2**, but apparently a small amount of the intermediate 3-deoxy-2-keto compound (**1**) is formed (Fig. 4), and then reduced, hydrolysed, and acetylated during the subsequent reactions of the procedure. The diagnostic e.i.-m.s. fragment-ions for the hydrogen- and deuteri-

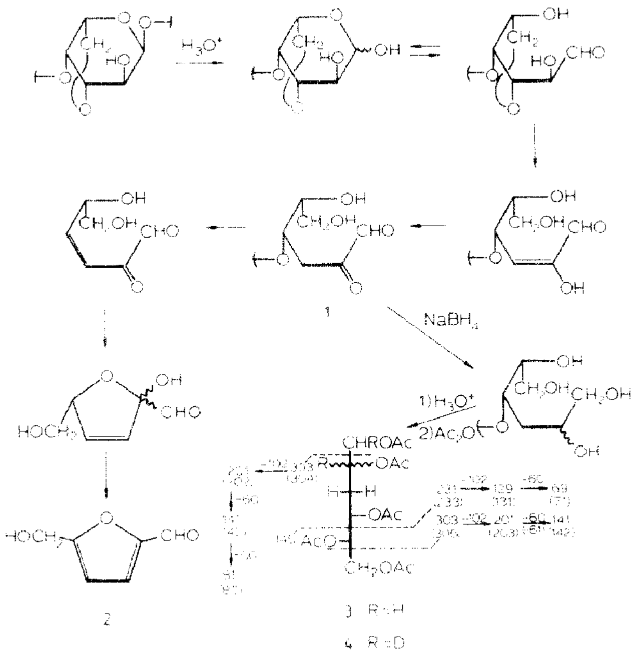

Fig. 4 Proposed mechanism for the production of 3-deoxyhexitol derivatives (**3**) by the degradation of 3,6-anhydro-L-galactosyl residues during the double hydrolysis–reduction procedure; 3,6-anhydro-D-galactopyranosyl residues would yield the enantiomeric 3-deoxyhexitols. The e.i.-m.s. fragmentation patterns of the hydride-reduced products (**3**) are shown, with those of the deuteride-reduced products (**4**) in parentheses.

TABLE I

Constituent-sugar analysis of various carrageenans, agars, and a seaweed

Sample	*Method*[a]	*Constituent sugars (mole %)*[b]									
		2-Me-AnGal	*AnGal*	*2-Me-Gal*	*3-DeoxyHex*[c]	*6-Me-Gal*	*Gal*	*4-Me-Gal*	*Xyl*	*Man*	*Glc*
κ-Carrageenan	A	–	44	–	3	2	48	–			3
	B	–	48	–	–	2	48	–	–		2
ι-Carrageenan	A	–	41	–	2		54	–	1	–	2
	B	–	39	–		–	57		2	–	2
λ-Carrageenan	A	–	5	–	–	–	94		–	–	1
	B	–	5	–	–	–	95	–	–	–	tr
P. lucida agar	A	1	47		2	1	48	–	–	–	1
	B	1	48	–	–	1	50		–	–	tr
Agarose Type VIII	A	1	33	–	2	8	35		–	21	tr
	B	1	36	–	–	8	34		–	21	tr
G. sordida agar	A	4	32	1	3	27	32	tr	tr		1
	B	4	36	1	–	26	30	tr	tr	–	1
G. sordida thaw water	A	3	15	4	3	26	36	2	5	1	5
	B	2	20	6	–	26	35	2	4	1	4
G. sordida dried seaweed	A	1	14	1	3	16	39	tr	1	2	23
	B	tr	21	2	–	18	37	tr	2	2	18

[a] Sugars determined by g.l.c. analysis of alditol acetates derived using A, the double hydrolysis–reduction, or B, reductive hydrolysis procedures. [b] 2-Me-AnGal, 3,6-anhydro-2-*O*-methylgalactitol; AnGal, 3,6-anhydrogalactitol; 2-, 4-, and 6-Me-Gal, 2-, 4-, and 6-*O*-methylgalactitol; Gal, galactitol; Xyl, xylitol; Man, mannitol; Glc, glucitol; –, not observed; tr, <0.8%. [c] 3-DeoxyHex, sum of penta-*O*-acetyl-3-deoxy-*lyxo*- and -*xylo*-hexitols, one coeluting with Man.

um-reduced analogues **3** and **4**, respectively, are shown in Fig. 4. Incorporation of deuterium at both C-1 and C-2 during borodeuteride reduction is consistent with the proposed structure and mechanism. 2-*O*-Methyl-3,6-anhydrogalactosyl residues, as found in *G. eucheumoides* and *Curdiea coriacea*, did not produce the 3-deoxyhexitol byproducts[14]. These residues are reported to be less susceptible to acid destruction than 3,6-anhydrogalactosyl residues[18], but the data in Fig. 2 indicate that the difference is not great.

(b) Application of the procedure. With the methodology for the double hydrolysis–reduction procedure now optimised, the constituent sugars of various samples whose compositions were known, or could be anticipated from previous work[1], were determined under standard conditions. The results are shown in Table I (Method A). As expected, κ- and ι-carrageenan were each shown to be composed of about equal amounts of anhydrogalactose and galactose, and 2–3% of 3-deoxyhexitols were produced during the procedure. λ-Carrageenan contained only a small proportion of 3,6-anhydrogalactosyl residues, and no 3-deoxyhexitols were evident. *P. lucida* agar was a very clean, base-treated sample, and as expected[10] it contained close to equimolar amounts of anhydrogalactose and galactose. Agarose Type VIII yielded derivatives characteristic of an agar with partial 6-*O*-methylation on D-galactose, along with a large amount of mannitol hexacetate. This material is offered commercially for use in isoelectric focusing and may contain substances (mannitol, mannose, mannosides, or mannan) added by the manufacturer for that purpose.

Samples of dried *G. sordida* and its agar (purified by freeze-thawing), and thaw-water polysaccharide were analysed. The thaw-water polysaccharide was treated with amyloglucosidase to digest floridean starch, dialysed, and freeze dried. These agar and thaw-water polysaccharides have previously been the subject of a semiquantitative ^{13}C-n.m.r. spectroscopic analysis, from which it was concluded that 47 and 46%, respectively, of the D-galactosyl residues were methylated on O-6 and that 10 and 46%, respectively, of the L-galactosyl residues existed as 6-sulphate "precursor" units, the remainder being in the 3,6-anhydride form[19]. The degree of methylation and proportion of precursor units can also be determined from the data in Table I if the following assumptions are made: 1) the polymer backbones are composed of equal numbers of D- and L-residues; 2) the first four components of Table I are derived from L-residues; 3) 6-Me-Gal is derived from a D-residue; 4) Gal is derived from a mixture of D- and L-galactosyl residues; 5) 2-Me-Gal and the L-portion of the Gal are derived from precursor units; and 6) the last four components in Table I are not derived from backbone residues. Thus it was calculated that 55 and 60% of the D residues were methylated on O-6, 10 and 16% of the L residues were methylated on O-2, and 20 and 52% of the L residues existed as precursor units in the agar and thaw-water polysaccharides, respectively. The occurrence of 2-*O*-methylation on the L-residues had not been recognised in the earlier study, but there is reasonable agreement between the two methods in respect of the other values.

The constituent-sugar composition of the dried seaweed was more complex and could not be interpreted in the same manner. The seaweed contained only 18%

3,6-anhydrogalactosyl residues, but also had significant proportions of glucosyl (from floridean starch[19] and possibly cellulose), 6-*O*-methylgalactosyl (from 3-linked residues of agar), and galactosyl residues (from floridoside[20], 3-linked residues of agar, and 4-linked precursor residues of agar).

Constituent-sugar analysis by the reductive hydrolysis procedure. — An alternative method for producing alditol acetates from carrageenans and agars was suggested by the work of Garegg *et al.*[12], who hydrolysed polysaccharides in acid containing 4-methylmorpholine–borane (MMB). MMB is somewhat acid stable[21], and can be used to reduce, and thus stabilise, newly released sugars *in situ*. Garegg *et al.* reported that on treatment of agarose with 0.5M TFA containing MMB for 15 h at either 65° or 100°, hydrolysis was complete and all the 3,6-anhydrogalactose was reduced to its alditol, but virtually no galactose was reduced[12]. We have since developed these initial observations into a useful method for analysing the glycosyl composition of carrageenans and agaroids.

(a) Development of the procedure. There are two limitations associated with the use of MMB as an *in situ* reductant. The degradation of MMB in aqueous acid is more rapid than the hydrolysis of "normal" glycosidic bonds, such as those of hexosyl residues. Furthermore, MMB is a relatively weak reductant under acidic conditions in comparison with sodium borohydride under alkaline conditions. Fortunately, those sugars that are both hydrolysed readily and destroyed easily also seem to be reduced readily. Because of these considerations, a three-step procedure, involving the addition of fresh MMB at each step, was required. The steps are (see Experimental for details): 1) a sample (1 mg) of carrageenan or agar is prehydrolysed in 2.4M TFA containing MMB at 80° for 5 min; 2) aqueous MMB is added, bringing the effective concentration of TFA down to ~2M, and the sample is fully hydrolysed for 1 h at 120°; 3) aqueous MMB is added and the sample is concentrated to dryness at 50°. Acetylation of the residue in acetic anhydride using TFA as a catalyst yields the alditol acetates, which are purified by extraction and analysed by g.l.c.

Pterocladia lucida agar was initially subjected to this procedure but the prehydrolysis step 1 was omitted, and the resulting sugars were converted to alditol acetates and analysed by g.l.c. The observed molar ratio of 1,2,4,5-tetra-*O*-acetyl-3,6-anhydrogalactitol to hexa-*O*-acetylgalactitol was close to that obtained using the double hydrolysis–reduction method. However, under the conditions of step 2 (2M TFA, 120°) MMB was hydrolysed within 2 min (data not shown), and thus the anhydrogalactosyl residues of the agar sample must also have been hydrolysed and reduced within 2 min. In such a rapid hydrolysis, the potential exists for the MMB to be depleted before the sample is fully dissolved, resulting in a low recovery of 3,6-anhydrogalactitol. To avoid this perceived problem a prehydrolysis step in the presence of MMB was included to ensure effective dissolution of the sample (step 1). Under the conditions used (2.4M TFA, 5 min, 80°) MMB was not fully depleted (data not shown).

The final MMB treatment (step 3) was necessary because reduction of hexoses by MMB is incomplete under the conditions required to completely hydrolyse polysaccharides (2M TFA, 120°, 1 h). In a control experiment, a sample of galactose was only

~20% reduced before the MMB was depleted. Thus, a protocol for the posthydrolysis reduction of sugars with MMB was required. The most effective method found was to add fresh aqueous MMB directly to the cooled hydrolysate, which was then concentrated to dryness at 50° in a stream of dry air. Significant reduction of galactose occurred only during the latter stages of the concentration, when both galactose and MMB were concentrated into a small volume. By the time the sample had been concentrated to a syrup, the reduction of galactose was complete. Rhamnose, fucose, arabinose, xylose, mannose, and glucose also were reduced satisfactorily under these conditions, as indicated by the absence of per-*O*-acetylated aldoses on g.l.c. analysis of the acetylated products.

Acetylation was carried out in TFA-Ac_2O (1:1, 50°, 10 min). Borate complexes do not form under acidic conditions, and thus the alditols were readily acetylated without removal of the byproducts from the reducing reagent. The conditions used by Garegg *et al.*[12] (100°, 10 min) were found to be too harsh, resulting in partial degradation of the alditols. The degradation was most evident with partially methylated alditols. 2,3,4-Tri-*O*-methylxylitol was completely acetylated at 50°, but at 100° was mostly degraded (data not shown). The alditol acetates were isolated by partitioning between CH_2Cl_2 and aqueous Na_2CO_3 followed by water. The entire procedure is carried out in one tube, without transferring the product at any stage, thus minimising material loss. A polysaccharide may be converted into alditol acetates in less than 2.5 h.

As was the case with the double hydrolysis-reduction method, dehydration of alditols to anhydroalditols was not a problem. No 3,6-anhydrogalactitol was found when methyl α-D-galactopyranoside was subjected to the reductive hydrolysis procedure.

(b) Application of the procedure. The constituent-sugar compositions of various carrageenans and agars determined using the reductive hydrolysis procedure are shown

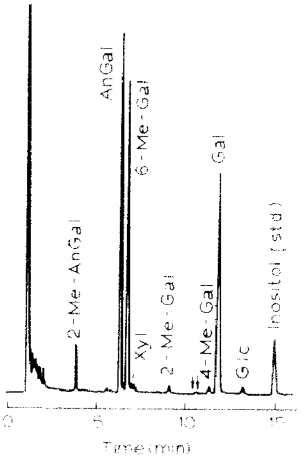

Fig. 5. Gas chromatogram of the alditol acetates derived from *Gracilaria sordida* agar using the reductive hydrolysis procedure. Arrows depict the retention times of penta-*O*-acetyl-3-deoxy-*lyxo*- and -*xylo*-hexitol, produced from 3,6-anhydrogalactosyl residues during the double hydrolysis-reduction procedure.

in Table I (Method B). A typical gas chromatogram is shown in Fig. 5. No 3-deoxyhexitols were produced. In most cases, the two methods for the conversion into alditol acetates gave comparable results. The yield of 3,6-anhydrogalactitol was higher using reductive hydrolysis, but when the yields of 3-deoxyhexitols and 3,6-anhydrogalactitol are summed, the two methods gave comparable results. The one case in which the two methods gave different results was the analysis of dried seaweed, in which the yield of 3,6-anhydrogalactitol was higher and the yield of glucitol was lower using the reductive hydrolysis procedure than with the double hydrolysis–reduction procedure.

It should be noted that while the reductive hydrolysis procedure is particularly useful for the analysis of red-algal polysaccharides, its use is not limited to these polysaccharides. For polysaccharides that do not contain easily degraded residues (such as those of 3,6-anhydrogalactose), the procedure can be abbreviated, in that both the prehydrolysis (step 1) and the addition of MMB in the second hydrolysis (step 2) can be omitted. This method is more rapid than other methods for the production of alditol acetates[11,22].

(c) Deuterium labeling. Alditols containing a deuterium label are produced when aldoses and ketoses are reduced with $NaBD_4$. Such labeling is useful in the identification of constituent sugars using mass spectrometry. Trideuterio-MMB is not commercially available, but borane amines are known to undergo rapid D–H exchange in D_2O under acidic conditions[23]. In an attempt to modify the reductive hydrolysis procedure to permit deuterium labeling, the alditol acetates from *P. lucida* agar were prepared as described, except that all reagents were prepared in D_2O rather than H_2O. The resulting alditol acetates were analyzed by g.l.c.–e.i.m.s. Approximately 85% deuterium label would be expected if the reductant had equilibrated with the D_2O and TFA. Disappointingly, while the 3,6-anhydrogalactitol tetraacetate had ~75% deuterium label on C-1, the galactitol hexaacetate had only ~50%. The disparity in the levels of deuterium incorporation arises because the 3,6-anhydrogalactose and galactose reductions occur in different steps of the procedure. Greater effective equilibration of MMB with D_2O evidently occurs at 80° in step 1. Thus, it is apparent that to get complete deuterium labeling, trideuterio-MMB must be prepared and used with D_2O and deuterio-TFA.

The two methods for the production of alditol acetates from red-algal polysaccharides gave comparable results (Table I). The method using MMB is preferred for routine analyses because it is much quicker than the double hydrolysis–reduction method, and does not produce 3-deoxyhexitols. However, the latter method is useful when deuterium labeling is required.

Methylation analysis. — As discussed in the Introduction, the major problems encountered with conventional methylation analysis applied to red-algal polysaccharides are the incomplete methylation of hydroxy groups of sulphated polymers and the degradation of methylated 3,6-anhydrogalactosyl residues during hydrolysis. We report here that complete methylation can be accomplished if the sulphate groups are first converted into the triethylammonium salts and demonstrate the near quantitative determination of 3,6-anhydrogalactosyl residues in the methylated polymer, using the methodology described in the first part of this paper.

The procedure we have used for the methylation analysis of sulphated agaroids and carrageenans is as follows (see Experimental for details). Polysaccharide (~ 1 mg) is dialysed against aqueous triethylamine hydrochloride, then against water, and finally freeze dried. The resulting polysaccharide is dissolved in DMSO and methylated using potassium methylsulphinylmethanide and methyl iodide in a manner similar to that described by Harris *et al.*[24]. The water-soluble, methylated polysaccharide is isolated by extensive dialysis against water followed by freeze drying. Nonsulphated agars are methylated directly using the procedure for DMSO-insoluble polysaccharides as described[24]. Partially methylated alditol acetates are prepared using the reductive hydrolysis procedure for semiquantitative analysis, and by a standard procedure that provided deuterium labeled products for qualitative analysis[25]. The products are analyzed by g.l.c. and g.l.c.-m.s. The experiments that led to the adoption of this procedure are described below.

(a) Methylation. First, an effective procedure for full methylation had to be developed. Samples of κ-, ι-, and λ- carrageenan in the cationic form provided by the supplier (mostly K^+, Ca^{2+}, and Na^+) were dissolved in water and freeze dried. The resulting κ-carrageenan sample was soluble in DMSO, while the ι- and λ-carrageenan samples appeared to be totally insoluble. All three samples were treated with methylsulphinyl carbanion followed by methyl iodide in the standard manner, in an attempt to methylate them. Partially methylated alditol acetates were prepared and analysed (Table II). Methylation of the DMSO-soluble κ-carrageenan sample was somewhat incomplete as demonstrated by the apparent presence of 5% of 2,3,4,6-tetrasubstituted galactosyl residues and of 9% of 2,4-disubstituted anhydrogalactosyl residues. The methylations of ι- and λ-carrageenans were very incomplete, as demonstrated by the apparent presence of 49 and 83%, respectively, of 2,3,4,6-tetrasubstituted galactosyl residues. Thus, the metal cationic forms of the carrageenans could not be completely methylated under these conditions.

The pyridinium salt of carboxyl-reduced heparin (a highly sulphated polysaccharide), was reported to be soluble in DMSO, and it was said that Hakomori methylation resulted in good *O*-methylation[26]. The pyridinium salts of κ- and ι-carrageenans were prepared, were soluble in DMSO, and were adequately methylated in a single procedure (data not shown). However, the pyridinium salts of some sulphated carrageenans and agaroids were not stable against degradation during storage (in weeks or months). The triethylammonium salts, however, proved to be considerably more stable, and were also soluble in DMSO. In the one direct comparison made, freeze-dried ι-carrageenan in the pyridinium form showed noticeable degradation after one week and had degraded to a dark liquid after three weeks when stored in a sealed container at room temperature. The corresponding triethylammonium salt did not display any sign of degradation after six months of storage. The difference in stability between the pyridinium and triethylammonium salt forms may be related to the acidity of pyridinium (pK_a 5.25) and triethylammonium ions (pK_a 11.01). The weakly basic pyridine may slowly dissociate from the pyridinium salt and evaporate, leaving acidic sulphate hemiesters which can catalyze degradation of the polysaccharide, whereas the strongly basic triethylamine forms a stable cation.

TABLE II

Glycosyl-linkage analysis of various carrageenans and *Pterocladia lucida* agar

Constituent sugar and deduced substitution[a]	*Proportion (mole %)*[b]										
	Carrageenans									P. lucida	
	κ-			ι-			λ-			*agar*	
	M^{+c}	*1*x[d]	*3*x[e]	M^+	*1*x	*3*x	M^+	*1*x	*3*x	*1*x	*3*x
T-AnGal	–	–	2	–	–	–				–	–
2-AnGal	–	–		–	–	26	–	–	–	–	–
4-AnGal	38	46	49		3	8	–	tr	tr	42	42
2,4-AnGal	9	4	2	37	37	18	5	5	5	1	–
T-Gal	tr	tr	tr	–	tr	tr	–	–	–	–	–
3-Gal	tr	1	1	–	1	4		1	1	54	56
4-Gal	tr	tr	tr	–	–	2		1	1		
2,3-Gal	–			–	–	–	7	36	36	–	
2,4-Gal	–		–				4	24	25	–	–
3,4-Gal	42	45	44	10	55	39				2	2
3,6-Gal		–	–							tr	tr
2,3,4-Gal	4	2	1	3	1	tr	1	2	1	–	
2,3,6-Gal[f] 2,4,6-Gal[f]	tr	tr	tr	–	1	1	–	7 14	22		
3,4,6-Gal	2	1	1	1	2	2	–	1	1		
2,3,4,6-Gal	5	1	tr	49	tr	tr	83	9	8	1	tr

[a] T-AnGal, nonreducing terminal 3,6-anhydrogalactopyranosyl residue, analysed as 1,5-di-*O*-acetyl-3,6-anhydro-2,4-di-*O*-methylgalactitol; 3,4-Gal, 3,4-disubstituted galactopyranosyl residue, analysed as 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methylgalactitol; etc. [b] Symbols: –, not observed; tr, <0.8%. [c] Freeze-dried samples in metal cationic form as supplied by Sigma, using single-methylation procedure. [d] Et_3NH^+ form, single-methylation procedure. [e] Et_3NH^+ form, triple-methylation procedure. [f] Enantiomeric alditols, origin in λ-carrageenan determined using deuterium labeling.

Triethylammonium salts are conveniently formed either by dialysis of the polysaccharide against aqueous triethylamine hydrochloride, then against water, or by passage of the polysaccharide through a cation-exchange column in the Et_3NH^+ form. Passage of sulphated carrageenans or agaroids through a cation-exchange column in the H^+ form followed by neutralization with triethylamine[26] is not recommended, because of the acid sensitivity of these polymers. The triethylammonium salts of κ-, ι- and λ-carrageenan were dissolved in DMSO and methylated satisfactorily. The results are described later.

Dialysis was chosen as the best method for isolation of the polysaccharide from the methylation mixture. κ-Carrageenan was methylated on a large scale, the excess of methyl iodide removed, and the remaining liquid dialysed against water. A fluid layer separated from the reaction mixture on dilution with water, but slowly dissolved over a period of several hours. We speculate that the half-ester sulphate groups in the polysaccharide were methylated during the methylation procedure, rendering the methylated polysaccharide initially insoluble in water, and that these sulphate methyl esters hydrolysed during the dialysis, to produce an ionic, water-soluble polysaccharide. Alternative procedures for isolating the methylated polysaccharide from the DMSO solution by either extraction or reverse-phase chromatography were judged inappropriate, in view of the ready hydrolysis of the initial hydrophobic product to a water-soluble material.

(b) Production of partially methylated alditol acetates. Next, the double hydrolysis–reduction and reductive hydrolysis procedures were tested to see if they were applicable to methylated polysaccharides. Methylated *P. lucida* agar is not water soluble, and application of the double hydrolysis–reduction procedure as used for nonmethylated polysaccharides resulted in very low yields of 1,4,5-tri-*O*-acetyl-3,6-anhydro-2-*O*-methylgalactitol (from 4-linked 3,6-anhydrogalactosyl residues, data not shown). However, preliminary hydrolysis in 0.5M rather than 0.1M TFA at 80° gave better results (Fig. 6). The yield of 1,4,5-tri-*O*-acetyl-3,6-anhydro-2-*O*-methylgalactitol relative to that of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol reached a maximum after 6 h and declined thereafter. Methylated κ-carrageenan is water soluble because of its polar sulphate groups. Hydrolysis of its 3,6-anhydrogalactosidic linkages is much faster than those of methylated agar (Fig. 6). The yield of 1,4,5-tri-*O*-acetyl-3,6-anhydro-2-*O*-methylgalactitol relative to that of 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methylgalactitol reached a maximum after preliminary hydrolysis for ~1 h, and slowly declined thereafter.

Because the rates of hydrolysis of methylated agar and methylated κ-carrageenan were significantly different, it was concluded that in order to use the double hydrolysis–reduction procedure to obtain quantitative data, the conditions of the preliminary hydrolysis would need to be optimised for each methylated polysaccharide. Fortunately, the reductive–hydrolysis procedure, with only a minor modification, yielded the expected proportion of 3,6-anhydrogalactitol derivatives from κ-carrageenan and close to the expected proportion from *P. lucida* agar (Table II).

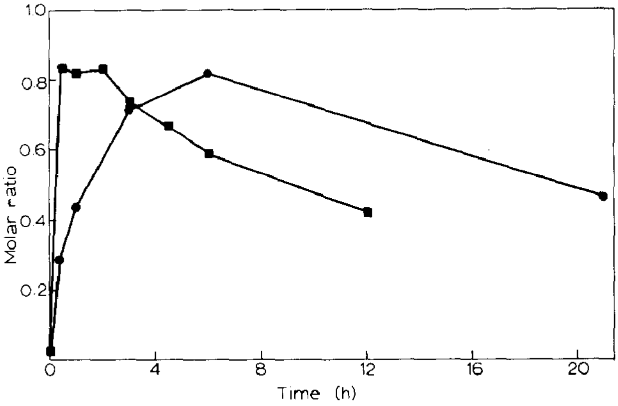

Fig. 6. Molar ratios of the acetylated derivatives of 3,6-anhydro-2-*O*-methylgalactitol to 2,4,6-tri-*O*-methylgalactitol for methylated *Pterocladia lucida* agar (●), and to 2,6-di-*O*-methylgalactitol for κ-carrageenan methylated in the Et_3NH^+ form (■), as a function of time of preliminary hydrolysis (0.5M TFA, 80°) in the double hydrolysis–reduction procedure.

(c) Deuterium labeling. Deuterium labeling is important for the mass spectral identification of partially methylated alditol acetates, even more so than for alditol acetates. However, as was the case with unmethylated *P. lucida* agar, the application to methylated *P. lucida* agar of the reductive hydrolysis procedure with all reagents dissolved in D_2O led to insufficient yields of deuterium-labeled products, giving ~80 and ~25% deuterium label on the anhydrogalactitol and galactitol derivatives, respectively. When deuterium labeling was required to identify partially methylated alditol acetates, a portion of the methylated polysaccharide was converted to such derivatives by a standard procedure using sodium borodeuteride reduction[25]. While this procedure yielded no useful 3,6-anhydrogalactitol derivatives, otherwise identical derivatives, such as those of 3-*O*-methyl- and 4-*O*-methyl-galactose, could be distinguished by m.s. analysis.

(d) Methylation analysis of κ-carrageenan. Methylation analyses were carried out on the freeze-dried triethylammonium salts of κ-, ι- and λ-carrageenans and on freeze-dried *P. lucida* agar (Table II). One portion (~1 mg) of each sample was methylated using single additions of base and methyl iodide ("1x" in Table II) as described in the Experimental section, while the other portion (~1 mg) was methylated using two preliminary methylations, followed by a final (standard) methylation[24] ("3x" in Table II). Partially methylated alditol acetates were prepared from each methylated polysaccharide by the reductive hydrolysis procedure. The triple-methylation procedure should result in complete methylation. Thus, the extent of substitution effected by the single-methylation procedure could be assessed by comparison.

The triethylammonium salt of κ-carrageenan from *Eucheuma cottonii* dissolved readily in DMSO, but formed a gel upon the addition of potassium methylsulphinylmethanide. Despite the gel formation, the single-methylation procedure gave the result expected from the structure of κ-carrageenan (see Table II and Fig. 1) and was in good agreement with the constituent-sugar analysis (Table I). The presence of a small proportion of 1,2,4,5-tetra-*O*-acetyl-3,6-anhydrogalactitol (2,4-AnGal in Table II) is in accord with the presence of a small proportion of ι-carrageenan units in this polysaccharide[27]. Yields of highly acetylated alditols (mostly derived from 3,4,6-tri-substituted galactosyl residues) were reduced only minimally by the triple-methylation procedure, indicating that incomplete methylation during the single methylation procedure was not a problem. The triple-methylation procedure resulted in some degradation of the carrageenan, as indicated by the presence of a small amount of 1,5-di-*O*-acetyl-3,6-anhydro-2,4-di-*O*-methylgalactitol (T-AnGal in Table II) among the products. The identity of this component was confirmed by comparison of its mass spectral and chromatographic properties with those of an authentic sample synthesised from methyl 3,6-anhydro-β-D-galactopyranoside. It derives from terminal anhydrogalactosyl residues, probably created by a small amount of base-catalyzed cleavage of the galactosyl residues during the methylation procedure, rather than from genuine end-groups.

(e) Methylation analysis of ι-carrageenan. ι-Carrageenan triethylammonium salt also gave a well methylated product by the single-methylation procedure (Table II), and the methylation analysis agreed well with the known structure (Fig. 1) and with the constituent-sugar analysis (Table I). There were only minor amounts of highly acetylated alditols, and these decreased only slightly in the triply methylated sample, indicating minimal undermethylation. Triple methylation must have severely degraded the ι-carrageenan, since a much lower total yield of alditol derivatives was obtained than was the case with the single-methylation procedure. Furthermore, a large amount of 1,2,5-tri-*O*-acetyl-3,6-anhydro-4-*O*-methylgalactitol (2-AnGal in Table II) was identified on the basis of its e.i.-m.s. fragmentation pattern and by analogy with the appearance of terminal 3,6-anhydrogalactosyl residues in κ-carrageenan (T-AnGal in Table II) and in the agaroid from *Chondria macrocarpa* during multiple-methylation procedures[28]. Presumably, base-catalysed cleavage at the glycosidic linkage of the 3-linked galactosyl residues during the methylation left terminal 2-*O*-sulphated 3,6-anhydrogalactosyl residues, and these were methylated at O-4 during the subsequent addition of methylating reagents. The sulphate groups were stable during the methylation procedure, but the glycosidic linkages were not.

(f) Methylation analysis of λ-carrageenan. The term "λ-carrageenan" is frequently used in a general sense and in commerce to describe non-gelling red-algal extracts, most correctly those from tetrasporophytes of the families *Gigartina*, *Chondrus*, and *Iridea*. It is also used in the context of polysaccharide structure to define a specific chemical entity, and for this purpose we have adopted the definition of λ-carrageenan and the related ξ-carrageenan (which lacks 6-sulphation) suggested by Bodeau-Bellion[2] (Fig. 1). Rees and co-workers concluded that "λ-carrageenan" from *Gigartina atropurpurea*, *G. canaliculata*, and *G. chamissoi* predominantly had the ξ-structure[29], while that from *Chondrus crispus* predominantly had the λ-structure but with ~30% of the 3-linked galactose residues lacking 2-sulphation[30]. Pyruvate ketals have also been observed as substituents of λ-carrageenans[31-34]. The structure of the commercially available λ-carrageenan from *Gigartina aciculaire* and *pistillata*, however, has not been reported.

Methylation analysis of the triethylammonium salt of this λ-carrageenan generated the major derivatives expected for a hybrid or mixture of λ- and ξ-structures (2,3-, 2,4- and 2,4,6-Gal in Table II; see Fig. 1) but revealed other residues to be present as well. The sample was methylated fully in the single-methylation procedure, since the proportions of the highly acetylated alditols did not decrease in the triply methylated sample and were thus not due to incomplete methylation. The presence of galactitol hexaacetate (2,3,4,6-Gal in Table II) is usually associated with incomplete methylation. However, by using an enzymatic analysis[35] this λ-carrageenan (Et_3NH^+ form) was shown to contain 1.8% pyruvate, corresponding to one pyruvate ketal for every 6 disaccharide repeat units. The presence of pyruvate ketals and the frequency of their occurrence was confirmed by solid-state ^{13}C-n.m.r. spectroscopy. Signals observed at 26.2 and 176.2 p.p.m. corresponded to the methyl and carboxylate carbons of the pyruvate group. The galactitol hexaacetate obtained from this methylated λ-carrageenan in an amount corresponding to $\sim$ 1 per 6 disaccharide repeat units was thus derived from 3-linked galactosyl residues bearing a 4,6-pyruvate ketal group, and a sulphate group on O-2.

λ-Carrageenan contained very few 3,6-anhydrogalactosyl residues (Table I), but a substantial proportion of "precursor" residues, *i.e.* 4-linked residues with a sulphate group attached at O-6, were identified. These precursor residues can form 3,6-anhydrogalactosyl residues under basic conditions[36], but they were stable during the methylation, since the amount of 3,6-anhydrogalactitol derivatives found in the methylation analysis was no higher than that found in the constituent-sugar analysis.

(g) Methylation analysis of agars. The nonsulphated, freeze dried agar from *P. lucida* was fully soluble in DMSO and methylated readily in the single-methylation procedure, showing only minimal undermethylation of O-2 of the 3,6-anhydrogalactosyl residues. Partial degradation of the 3,6-anhydro-2-*O*-methylgalactosyl residues during the preparation of partially methylated alditol acetates from this water-insoluble substrate was suspected, since the 4-linked anhydrogalactosyl residues constituted only 42% of the residues identified by the methylation analysis, whereas the glycosyl analysis confirmed that at least 49% were present. Thus, methylation analysis of water-insoluble nonsulphated agars using the methods described herein can only be regarded as semiquantitative.

In addition, quantitative differences were observed[28] in the partially methylated alditol acetates that were prepared from the methylated polysaccharide of *C. macrocarpa* by the reductive hydrolysis method and by the standard procedure[25]. The standard procedure gave lower yields of the alditol derived from terminal xylosyl residues, while the reductive hydrolysis procedure gave lower yields of the alditol derived from 4,6-disubstituted galactosyl residues (data not shown)[28].

These problems need to be further investigated. Considering the difficulties associated with methylation analyses and the lability of 3,6-anhydrogalactosyl residues, however, we regard these results as very promising.

CONCLUSIONS

The procedures described herein represent a significant advancement in the methodology for characterising carrageenans and agars. Reductive hydrolysis, using the acid-stable 4-methylmorpholine–borane to reduce 3,6-anhydrogalactosyl residues *in situ* is especially convenient for the quantitative determination of all of the neutral sugar components in the native polymers as their alditol acetate derivatives. The double hydrolysis–reduction method allows deuterated reduced derivatives to be prepared to assist in identification by g.l.c.–m.s.

Sulphated galactans in their stable, DMSO-soluble triethylammonium salt form are methylated effectively in a single Hakomori procedure, thereby avoiding the degradation associated with multiple methylation procedures. Water-soluble methylated galactan sulphates are recovered from the reaction mixture by dialysis. Application of the reductive hydrolysis procedure to the methylated polysaccharides provides semi-quantitative analyses of the glycosyl-linkage pattern of red-algal galactans. This protocol permits, for the first time, a ready identification of the linkage and substitution of the 3,6-anhydrogalactosyl residues.

EXPERIMENTAL

General methods. — All small-scale reactions were carried out in screw-cap tubes (13 × 100 mm) equipped with Teflon-lined caps. The sealed tubes were heated in aluminium-block heaters. Evaporations were carried out in a Pierce Reacti-Therm heating module, equipped with a Reacti-Vap evaporating unit, but a custom-built device will work just as well[25]. All evaporations were carried out at 50°, unless otherwise specified, with a stream of dry, filtered air. Extractions were also performed in the screw-cap tubes. The sealed tubes were shaken vigorously and the phases were separated by brief, low-speed centrifugation. Occasionally, the organic layer was cloudy, particularly in extractions with aqueous sodium carbonate. The cloudiness could usually be removed by more vigorous shaking, followed by centrifugation. The upper, aqueous layer was drawn off with a Pasteur pipette and discarded.

Procedures for constituent-sugar analysis. — *(a) Using double hydrolysis–reduction.* Polysaccharide (~1 mg) was placed in a tube, aqueous TFA (0.1M, 0.25 mL) was added and the tube was heated (3 h, 80°). The solvent was evaporated and two portions of toluene (~0.5 mL) were added and evaporated to remove residual traces of TFA. Aqueous $NaBH_4$ or $NaBD_4$ was added (0.25 mL, 10 mg/mL in aqueous 1M NH_4OH) and the tube left at room temperature for 1 h. Excess reductant was quenched with HOAc (~2 drops) and the solution evaporated to dryness. Boric acid was removed by evaporation with 3 portions of 10% HOAc in MeOH (~0.5 mL), followed by 3 portions of dry MeOH (~0.5 mL). Aqueous TFA (2M, 0.25 mL) was added and the tube heated (120°, 1 h). The procedure just detailed for the removal of TFA, reduction with $NaBH_4$ or $NaBD_4$, and removal of boric acid was then repeated, except that additional reductant was frequently added because residual amounts of TFA were difficult to remove. The alditols were acetylated in acetic anhydride (0.1 mL) and pyridine (0.1 mL) at 120° for 20 min. Toluene (~ 1 mL) was added and the solution evaporated to dryness. CH_2Cl_2 (~2.5 mL) was then added and the organic phase was extracted twice with H_2O (~ 2.5 mL), discarding the top layer each time. The CH_2Cl_2 layer was then evaporated to near dryness and excess water was removed azeotropically by adding CH_3CN (~0.5 mL) and evaporating to dryness. The residue was then dissolved in acetone (~ 0.1 mL), with the aid of mild heat if the residue was crystalline, and analysed by g.l.c. on a 15 m × 0.25 mm (i.d.) SP2330 fused silica column maintained at 230°.

(b) Using reductive hydrolysis. A fresh stock solution of aqueous MMB (80 mg/mL, 0.2 mL per sample) was made beforehand. MMB readily dissolves in water at ~ 50°, but will recrystallise if the solution is allowed to cool below ~ 20°. Polysaccharide (~ 1 mg) was placed in a tube. Aqueous MMB (0.05 mL) and aqueous TFA (3M, 0.2 mL) were added and the tube was heated (80°, 5 min), during which time all solid material dissolved. (WARNING: hydrolysis of MMB generates H_2. While no problem was encountered during the course of this study, due caution should be exercised). The tube was cooled, a second portion of aqueous MMB (0.05 mL) was added, and the tube was heated again (1 h, 120°). The tube was cooled, a third portion of aqueous MMB (0.1 mL) was added, and the solution was evaporated to dryness at 50°, taking about 15 min. Residual water was removed by adding CH_3CN (~0.5 mL) and concentrating to dryness again. Acetic anhydride (0.1 mL) and TFA (0.1 mL) were added and the tube heated (50°, 10 min). Toluene (~ 1 mL) was added and the solution evaporated to dryness. CH_2Cl_2 (~2.5 mL) was added and the organic phase extracted first with aqueous Na_2CO_3 (0.5M, ~ 2.5 mL) and then with water (~ 2.5 mL), discarding the upper, aqueous layer each time. The CH_2Cl_2 layer was then evaporated to near dryness and excess water was removed by adding CH_3CN (~ 0.5 mL) and evaporating to dryness. The residue was then dissolved in acetone and analysed as described above.

Experiments to monitor the reduction of galactose with MMB were performed as follows. A solution of aqueous TFA, MMB, and galactose was heated at the required temperature. Aliquots were periodically removed and the excess MMB quenched with acetone. The sample was acetylated and analysed by g.l.c. for galactitol hexaacetate and galactose pentaacetate. To monitor the reduction of galactose during concentration (step 3), small aliquots were removed periodically, quenched, and analysed as just described.

Procedures for methylation analysis. — (a) Methylation. Dry, sulphated polysaccharide (~ 1 mg per analysis) was placed in dialysis tubing with water. The sample was dialysed against aqueous $Et_3N \cdot HCl$ (~ 0.1M, pH 7) several times, then against distilled water, and finally freeze dried. The freeze-dried material (~ 1 mg) was transferred to a tube for further treatment similar to that described by Harris *et al.*[24]. First, the polysaccharide was dissolved in dry DMSO (0.2 mL) with stirring. Next, the tube was purged with argon and potassium methylsulphinylmethanide (2M in DMSO, 0.2 mL) was added. The solution was stirred at room temperature for 10 min, then cooled in an ice bath. After the addition of methyl iodide (0.15 mL), the mixture was allowed to warm to room temperature and stirred for an additional 20 min. Water (~ 2 mL) was added, and N_2 was bubbled through the liquid until the cloudiness (due to methyl iodide) had disappeared. The solution was transferred to a dialysis bag and dialysed sequentially against water, aqueous $Et_3N \cdot HCl$ (0.1M), and finally exhaustively against distilled water, until no noticeable odour remained. The aqueous solution of water-soluble polysaccharide was then freeze dried.

(b) Production of partially methylated alditol acetates. The reductive hydrolysis procedure described above was used, except that when acetylated products were concentrated the evaporations were performed at 30°, rather than 50°, to avoid the loss of volatile, highly methylated alditols. When deuterium labeling was necessary, partially methylated alditol acetates were produced from methylated polysaccharide by hydrolysis (2M TFA, 1 h, 120°), reduction ($NaBD_4$), and acetylation (Ac_2O, NaOAc) as described[25]. The partially methylated alditol acetates were dissolved in acetone (0.1 mL) and analysed on an SP2330 fused silica column (15 m × 0.25 mm) programmed to hold for 2 min at 170°, increase at 2°/min to 230°, and hold for 8 min.

Response factors. — Relative molar g.l.c. response factors were determined experimentally or were from theoretical calculations[illegible]. Those determined experimentally were for 1,4,5-tri-*O*-acetyl-3,6-anhydro-2-*O*-methylgalactitol, 0.64; and 1,2,4,5-tetra-*O*-acetyl-3,6-anhydrogalactitol, 0.72. Calculated response factors were for di-*O*-acetyltetra-*O*-methylhexitol, 0.70; tri-*O*-acetyltri-*O*-methylhexitol, 0.74; tetra-*O*-acetyl-di-*O*-methylhexitol, 0.80; penta-*O*-acetyl-*O*-methylhexitol, 0.84; hexa-*O*-acetylhexitol, 0.89; penta-*O*-acetylpentitol, 0.75; and penta-*O*-acetyldeoxyhexitol, 0.84. The relative molar response factors for 1,2,5-tri-*O*-acetyl-3,6-anhydro-4-*O*-methylgalactitol and 1,5-di-*O*-acetyl-3,6-anhydro-2,4-di-*O*-methylgalactitol were assumed to be 0.64 and 0.50, respectively.

Sulphate analysis. — Sulphate content was determined as described[16]. To obtain the data in Fig. 3, samples of λ-carrageenan (Et_3NH^+ form, ~75 μg/data point) were hydrolysed as indicated, then stored at 4°. The samples were concentrated *in vacuo* at room temperature and stored *in vacuo* over solid KOH overnight to remove residual acid. Each sample was then analysed for sulphate without oxidation.

Materials. — The source of κ- (no. C1263, from *E. cottonii*), ι- (no. C4014, from *Eucheuma "spinosa"*, presumably *E. spinosum*), and λ- (no. C3889, from *Gigartina aciculaire* and *G. pistillata*) carrageenans and agarose Type VIII (no. A4905) was the Sigma Chemical Co. Alkali-modified agars from *P. lucida*[10] and *Gracilaria eucheumoides*[13], and the seaweed, agar, and thaw-water polysaccharide from *Gracilaria sordida*[19] (then called *G. secundata* f. *pseudoflagellifera*, collected from the upper end of the Heathcote estuary) were samples remaining from previous studies.

G.l.c.–m.s. — Spectra were recorded on a Shimadzu QP-1000 quadrupole instrument, scanning from m/z 44 to 450.

*1,2,4,5-Tetra-*O*-acetyl-3,6-anhydro-*D,L*-galactitol.* — 3,6-Anhydro-D,L-galactitol was prepared from galactitol as previously described[28], and isolated by flash chromatography on silica gel, with 5:4:1 acetone–ethyl acetate–water as eluent. The product was acetylated (1:1 pyridine–Ac_2O, room temperature) and repurified by flash chromatography and vacuum distillation, giving a colourless, chromatographically pure oil having the expected e.i. mass spectrum[20].

Anal. Calc. for $C_{14}H_{20}O_9$: C, 50.6; H, 6.1. Found: C, 50.5; H, 6.0.

*1,2,4,5,6-Penta-*O*-acetyl-3-deoxy-*ribo-, -lyxo-, *and* -xylo-*hexitol.* — An authentic standard of the *ribo* isomer was prepared from 2-*O*-acetyl-1,6-anhydro-3-deoxy-β-D-*ribo*-hexopyranose[40], using the double hydrolysis–reduction procedure. Analysis by g.l.c.–e.i.m.s. gave a single peak having *m/z* (%) 303(4), 231(19), 201(22), 154(14), 145(27), 141(11), 129(57), 112(21), 103(36), 95(13), 94(23), 81(100), and 69(72). The two products from samples that contained significant levels of 3,6-anhydrogalactosyl residues and were processed using the double hydrolysis reduction procedure had e.i. mass spectra similar to that of the standard, differing only minimally in relative abundances. They were assumed to be the *lyxo* and *xylo* isomers of the title compound.

*1,4,5-Tri-*O*-acetyl-3,6-anhydro-2-*O*-methyl-*L*-galactitol.* — Agar was per-*O*-methylated, partially hydrolysed (0.5M TFA, 6 h, 80°), reduced ($NaBH_4$), fully hydrolysed (4M TFA, 4 h, reflux), reduced ($NaBH_4$), and acetylated in a manner similar to that described above. The partially methylated alditol acetates were separated by flash chromatography on silica gel, and the fractions containing the title compound were further purified by vacuum distillation to yield a chromatographically pure, colourless oil having the expected e.i. mass spectrum[3,39].

Anal. Calc. for $C_{13}H_{20}O_8$: C, 51.3; H, 6.6. Found: C, 51.0; H, 6.6.

*1,5-Di-*O*-acetyl-3,6-anhydro-1-deuterio-2,4,6-tri-*O*-methyl-*D*-galactitol.* — Methyl 3,6-anhydro-β-D-galactopyranoside was prepared from methyl β-D-galactopyranoside as described[41], except that CHI_3 was used[42], instead of tribromoimidazole. The product was acetylated and purified by column chromatography on silica gel. The deacetylated product (NaOMe, MeOH) was methylated (THF, NaH, CH_3I) and the resulting product purified by flash chromatography on silica gel. The methylated glycoside was hydrolyzed (0.1M TFA, 30 min, 80°), reduced ($NaBD_4$), and acetylated (NaOAc, Ac_2O). Analysis by g.l.c.–e.i.-m.s. gave a single peak having *m/z* (%) 217 (1), 213 (2), 189 (4), 185 (9), 175 (7), 171 (8), 159 (23), 143 (40), 118 (100), 99 (28), 87 (17), 71 (40), and 59 (49).

*1,2,5-Tri-*O*-acetyl-3,6-anhydro-4-*O*-methylgalactitol.* — In the analysis of triply methylated ι-carrageenan a major component was identified as the title compound on the basis of its e.i. mass spectrum: *m/z* (%) 212 (4), 170 (9), 159 (34), 153 (11), 130 (7), 129 (14), 125 (9), 124 (14), 115 (17), 111 (23), 110 (18), 99 (100), 87 (96), and 71 (90).

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