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Location of the Sulfate Groups in κ-Carrageenan from *Gigartina Tenella*

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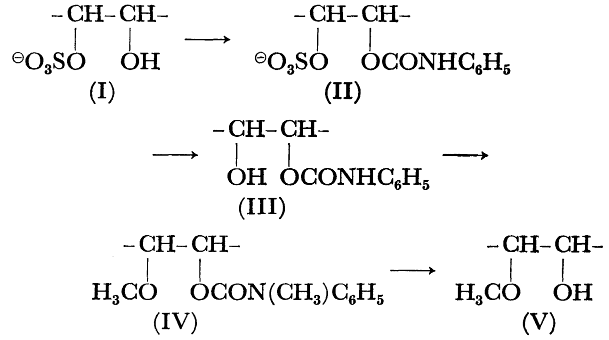
Location of the sulfate groups in κ-carrageenan from *G. tenella* has been re-investigated by adapting the method, which was devised by Bouveng for locating the acetyl groups in hemicellulose. In this method, the sulfate groups were replaced by methyl groups through a series of reactions consisting of blocking the free hydroxyl groups with phenylcarbamoyl groups, removing the sulfate groups and methylating the newly formed hydroxyl groups. The blocking substituents were then removed, and the positions of the methoxyl groups were investigated by the standard method. The results have shown, in accordance with the conclusion based on the methylation data, that one forth of the 1,4-linked 3,6-anhydro-D-galactose residues are 2-sulfated and that the 1,3-linked D-galactose residues are 4-sulfated. No evidence was obtained to indicate the presence of D-galactose 2,6-disulfate, which had been presumed by other workers. The problem concerning the acyl migration of the blocking groups during the process has also been discussed.

In our paper,[1] the structure of κ-carrageenan from *Gigartina tenella* has been investigated mainly by the methylation method. It has been concluded that the κ-carrageenan is composed of the residues of 1,3-linked β-D-galactose 4-sulfate (95 mol), 1,4-linked α-D-galactose 6-sulfate (5 mol), and 1,4-linked 3,6-anhydro-α-D-galactose (67 mol) and its 2-sulfate (24 mol), the 1,3- and 1,4-linked residues being repeated alternatingly to form a chain of the macro-molecule. This structure has been in approximate accordance with those reported by Anderson and his co-workers[2,3] for κ-carrageenans obtained from other species of *Gigartina* as well as from *Chondrus crisps*. The only difference is that they suggested the presence of a small amount of 1,4-linked D-galactose 2,6-disulfate residues in their polysaccharide samples. With this in mind, the location of the sulfate groups has been re-examined by the different method of investigation.

In the present work, the method devised by Bouveng for locating *O*-acetyl groups in hemicellulose[4,5] has been adapted for the purpose of locating sulfate groups. The method involves a series of reactions consisting of blocking the free hydroxyl groups with phenylcarbamoyl groups (I→II), removing the sulfate groups (II→III), methylating the newly formed hydroxyl groups (III→IV), removing the blocking groups (IV→V), breaking down the macro-molecule, and then identifying the fragments. The positions of methoxyl groups in the final products have been expected to indicate the positions of sulfate groups in the native polysaccharide.



$$\underset{\text{(I)}}{\begin{array}{cc}\multicolumn{2}{c}{\text{-CH-CH-}}\\ | & | \\ {}^{\ominus}O_3SO & OH\end{array}} \longrightarrow \underset{\text{(II)}}{\begin{array}{cc}\multicolumn{2}{c}{\text{-CH-CH-}}\\ | & | \\ {}^{\ominus}O_3SO & OCONHC_6H_5\end{array}} \longrightarrow \underset{\text{(III)}}{\begin{array}{cc}\multicolumn{2}{c}{\text{-CH-CH-}}\\ | & | \\ OH & OCONHC_6H_5\end{array}} \longrightarrow \underset{\text{(IV)}}{\begin{array}{cc}\multicolumn{2}{c}{\text{-CH-CH-}}\\ | & | \\ H_3CO & OCON(CH_3)C_6H_5\end{array}} \longrightarrow \underset{\text{(V)}}{\begin{array}{cc}\multicolumn{2}{c}{\text{-CH-CH-}}\\ | & | \\ H_3CO & OH\end{array}}$$

In applying the Bouveng's procedure to our own purpose, modification was needed in several respects. In the step I→II, κ-carrageenan in sodium or potassium salt was difficult to react with phenylisocyanate because of its insolubility in the reaction medium. This difficulty was overcome by converting the metal salt into cetyl pyridinium salt, which was found soluble in pyridine so that the complete phenylcarbamoylation was effected. For removing sulfate groups (the step II→III), on the basis of some preliminary experiments, the phenylcarbamoate(II) was heated in a mixture of dioxane and methanol containing 0.5% hydrogen chloride at 80°C for 3 hr. By this treatment, the sulfate groups were mostly released. But the macro-molecule was degraded by the concurrent methanolysis of the

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[1] S. Hirase and K. Watanabe, This Bulletin, **45**, No. 6 (1972) (in the press).

[2] N. S. Anderson, T. C. S. Dolan, and D. A. Rees, *J. Chem. Soc., C*, **1968**, 596.

[3] N. S. Anderson, T. C. S. Dolan, A. Penman, D. A. Rees, G. P. Mueller, D. J. Stancioff, and N. F. Stanley, *ibid.*, **1968**, 602.

[4] H. O. Bouveng, *Acta Chem. Scand.*, **15**, 87 (1961).

[5] H. O. Bouveng, *ibid.*, **15**, 96 (1961).

anhydro-galactoside linkages to form a mixture of oligosaccharide derivatives, whose average degree of polymerization was about six as determined by the methoxyl content. Examination of a part of the product by paper chromatography, after removal of the blocking substituents, showed the presence of carrabiose dimethyl acetal as well as higher oligosaccharide derivatives. The desulfated, partially degraded phenylcarbamoate(III) was next treated with trityl chloride to protect the primary hydroxyl groups, which might have been formed from galactose 6-sulfate residues, otherwise the phenylcarbamoyl groups would be migrated⁴⁾ from C_4 to C_6 during the succeeding step (III→IV) of methylation. The methylated product was then subjected to reductive removal of the blocking groups followed by detritylation and subsequent complete methanolysis.

TABLE 1. THE SUGARS IDENTIFIED IN THE FINAL PRODUCTS OF METHANOLYSIS

D-Galactose	Mol ratio^a)	3,6-Anhydro-D-galactose	Mol ratio^a)
non-methylated	1.0	non-methylated	1.0
3-*O*-methylated	0.66	2-*O*-methylated	0.24
4-*O*-methylated	1.64	5-*O*-methylated	0.84
3,4-di-*O*-methylated	2.44	2,5-di-*O*-methylated	0.38

a) The molar ratios are separately based on the respective non-methylated sugars.

The final product of methanolysis was a mixture of methyl glycosides or dimethyl acetals of the sugars listed in Table 1. These compounds were identified by isolation after separation on columns as well as by paper chromatography, thin layer chromatography, gas liquid chromatography and paper electrophoresis.

From these results, location of the sulfate groups in the polysaccharide is to be discussed. In the first place, the discussion is focussed to the distribution of the sulfate groups on anhydro-galactose residues. The methoxyl groups of C_5 in the final products are ascribed to the opening of pyranose rings during the process. When, as has already been described, anhydro-galactoside linkages were cleaved during the step II→III, the anhydro-sugar residues concerned would be undoubtedly changed from the pyranose structure to dimethyl acetal with the open chain structure,⁶⁾ forming thereby the free C_5-hydroxyl groups available for methylation in the succeeding step. While, the methoxyl groups on C_2 in the final products are ascribed to 2-sulfate groups. Thus, the relative yields of the anhydro-sugars (Table 1) show that non-sulfated and 2-sulfated anhydro-galactose residues exist in the proportion of 3: 1 in the native polysaccharide in exact agreement with the conclusion reported in our paper.¹⁾ This agreement proves that the present carbamoylation-methylation analysis is effective in locating the sulfate groups on anhydro-galactose residues.

The discussion is now turned to the distribution of the sulfate groups on D-galactose residues. The presence of 3,4-di-*O*-methyl-D-galactose in the final products provides evidence that 1,3-linked D-galactose 4-sulfate residues exist in the polysaccharide in accordance again with the result reported in our paper.¹⁾ In similar consideration, the positions of methoxyl groups in 3-*O*- and 4-*O*-methyl-D-galactoses may appear to indicate the positions of glycoside linkages and sulfate linkages, respectively, of D-galactose residues. However, no definite conclusion is obtainable from these mono-*O*-methyl-galactoses, because a part of the *O*-phenylcarbamoyl groups has been migrated between *cis* hydroxyl groups at C_3 and C_4 by the action of silver oxide during the methylation (III→IV). The occurrence of this migration has been proved by treating agarose from *Gelidium amansii* in exactly the same manner as in the case of κ-carrageenan. Since the agarose is a practically sulfate-free polysaccharide consisting of alternatingly repeated residues of 1,3-linked β-D-galactose and 1,4-linked 3,6-anhydro-α-L-galactose,⁷⁾ cleavage of the anhydro-galactoside linkages during the step II→III should produce free hydroxyl groups only at C_3 of D-galactose residues. Nevertheless, in the final products, a considerable amount of 4-*O*-methylgalactose was detected in addition to 3-*O*-methylgalactose. This result can be accounted for by the occurrence of migration of a part of the carbamoyl groups from C_4 to C_3. As for the non-methylated D-galactose in the final products from κ-carrageenan, the interpretation thereof is again ambiguous, because the sugar may be derived from any of the D-galactose non-sulfate, 4-sulfate and 6-sulfate residues, if these residues have survived the step II→III.

Thus the analysis by the present carbamoylation-methylation method seems to be of limited value as to the distribution of the sulfate groups on D-galactose residues. However, an important information would be obtainable as to the 2-sulfation of D-galactose residues. Anderson and his co-workers pointed out the presence of a small amount of the sugar sulfate residue, which formed 3,6-anhydro-galactose by alkaline treatment after periodate oxidation of their samples of κ-carrageenan.²,³⁾ They presumed this residue to be 1,4-linked D-galactose 2,6-disulfate. Analysis of our polysaccharide sample by their method showed that there existed a very small amount of a similar residue. If this residue is presumed to be 1,4-linked D-galactose 2,6-disulfate as Anderson and his coworkers did, 2-*O*-methylgalactose is then expected to be present in the final products of the carbamoylation-methylation method. In spite of careful examination, no trace of this sugar was detected. It follows from this result that neither galactose 2-sulfate nor 2,6-disulfate exist in our polysaccharide sample. The contradiction in the result between the two methods of analysis might be ascribed to an unavoidable experimental error of either method, because the amount of the residue under question is very small, if present. Another possible explanation is to presume the residue under question to be 1,4-linked D-galactose 3-sulfate, which would behave in the same manner as the 2,6-disulfate in periodate

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6) C. Araki and S. Hirase, This Bulletin, **27**, 109 (1954).

7) C. Araki, Proceedings of the Fifth International Seaweed Symposium, Halifax, August 25—28, 1965, Pergamon Press-Oxford & New York, 1966.

oxidation followed by alkaline treatment, and which would not give 2-*O*-methylgalactose in the carbamoylation-methylation experiment. Further work will be needed to solve this problem.

Experimental

Abbreviation. For simplifying the description, the names of the compounds are abbreviated as shown in Table 2.

TABLE 2. ABBREVIATIONS OF THE NAMES OF COMPOUNDS

Compounds	Abbreviations
3-*O*-methyl-D-galactose	3MG
4-*O*-methyl-D-galactose	4MG
3,4-di-*O*-methyl-D-galactose	34MG
3,6-anhydro-D-galactose	AG
2-*O*-methyl-3,6-anhydro-D-galactose	2MAG
5-*O*-methyl-3,6-anhydro-D-galactose	5MAG
2,5-di-*O*-methyl-3,6-anhydro-D-galactose	25MAG

General and Analytical Methods. General and analytical methods have been described in our paper[1] except those described below. The solvent I) benzene-ethyl acetate (3: 2) was used for paper chromatography (pc), and L) benzene-ethyl acetate-water (40: 15: trace) for thin layer chromatography (tlc), in addition to those (A—H and J—K, respectively) descrived before.

Gas Liquid Chromatography (glc). The columns used for glc were (a) 5% neopentylglycol succinate-chromosorb W, (b) 2.5% SE-30-chromosorb W and (c) 5% polyethylene glycol-chromosorb W, the length being 1 m unless otherwise stated. The final methanolysis product to be analysed was a complicated mixture consisting of methyl glycosides and dimethyl acetals of partially methylated galactoses and 3,6-anhydro-galactoses so that it was needed to analyse anhydro-galactose derivatives separately from galactose derivatives. For identifying the former derivatives was used the method B described in our paper,[1] in which anhydro-sugar derivatives were selectively converted into diethyl dithioacetal trimethylsilyl ethers. Analyses were carried out on the columns b at 160°C and c at 180°C as well as on the column a described before. For identifying galactose derivatives, the method D and E described below were devised. Furthermore, the method F described below was used for the sample of the relatively simple composition as in the case of a fraction obtained from column chromatography.

Method D: The sample to be analysed was hydrolysed with 1N-sulfuric acid at 100°C for 16 hr. During this treatment, anhydro-galactose derivatives were completely degraded. The remaining partially methylated galactoses were converted into diethyl dithioacetal trimethylsilyl ethers,[8] and analysed on the columns a at 160°C, b at 190°C and c at 180°C.

Method E: The partially methylated galactoses obtained in the method D were converted into diethyl dithioacetal acetates,[8] and analysed on the columns a at 215°C and b at 200°C.

Method F: Methyl glycosides or dimethyl acetals of partially methylated sugars were trimethylsilylated in the usual manner, and analysed on the columns a at 135°C, and b (2 m) at 160°C or 190°C.

Cetyl Pyridinium Salt of κ-Carrageenan. To a solution of κ-carrageenan (8 g) in water (240 m*l*), cetyl pyridinium chloride (8 g) in water was added with stirring. The precipitates, which formed, were collected by centrifugation, washed successively with water, methanol and again water, and dried *in vacuo.* The cetyl pyridinium salt was obtained as colorless powder (11.3 g). Found: N, 2.1%. Calcd for the polymer composed of galactose (1.0), 3,6-anhydro-galactose (0.91) and sulfate (1.24):[1] N, 2.26%.

Phenylcarbamoylation (I→II). To the cetyl pyridinium salt (11.3 g), which had been dried over phosphorus pentoxide at 40°C in vacuum overnight, dry pyridine (300 m*l*) and phenylisocyanate (25 g) were added. The mixture was heated at 100°C, when the salt was gradually dissolved within 1 hr. After being heated for 6 more hr, the reaction solution was left for 24 hr at room temperature. Ethanol (50 m*l*) was then added to destroy the excess of the reagent. The solution was centrifuged to remove small amounts of insoluble matter, and evaporated. The product was precipitated with 99% ethanol, the phenylcarbamoate (II) being obtained as slightly colored powder (14 g); $[\alpha]^{28}_{D}+75°$ (*c* 1.0 in pyridine). Found: SO_3, 8.9; N, 5.5%. Calcd for the phenylcarbamoate of the polymer with the composition described above: SO_3, 9.14; N, 5.04%.

Desulfation (II→III). A solution of the above-obtained phenylcarbamoate (14 g) in a mixture of dry dioxane (280 m*l*) and 1% methanolic hydrogen chloride (280 m*l*) was refluxed for 3 hr. The reaction mixture was neutralized with barium carbonate, filtered, evaporated to a small volume, and re-dissolved in chloroform. The solution was washed 3 times with water, dried with magnesium sulfate, and evaporated to a small volume, to which was added dry hexane. The product (III) was precipitated as powder (12.5 g); $[\alpha]^{24}_{D}+28.0°$ (*c* 0.78 in pyridine). Found: CH_3O, 3.3; SO_3, 0.5%. The methoxyl content indicated that the polymer was degraded to *O*-phenylcarbamoylated hexasaccharide dimethyl acetal in average. A portion of the product in dry dioxane was treated with lithium aluminum hydride to remove the phenyl-carbamoyl groups. Examination of the product by pc (solvent A, spray c) showed the presence of carrabiose dimethyl acetal as well as higher oligosaccharide derivatives.

Tritylation of III. The above-obtained product (12.5 g), which had been completely dried over phosphorus pentoxide, was dissolved in dry pyridine (75 m*l*). Trityl chloride (5.7 g) was added, and the mixture was left at 40°C for 2 days. After destroying the excess of the reagent by addition of water (10 m*l*), the mixture was disolved in chloroform, and the solution was washed successively with potassium hydrogen sulfate solution, sodium bicarbonate solution and water, dried with magnesium sulfate, and evaporated to a syrup, which was then dissolved in methanol and left aside in a refrigerator. Trityl carbinol, which crystallized, was filtered off, and the filtrate was evaporated to dryness (11.0 g); $[\alpha]^{24}_{D}+26.7°$ (*c* 0.72 in pyridine).

Methylation (III→IV). The tritylated product (11.0 g) obtained above in *N,N*-dimethylformamide[9] (130 m*l*) was shaken with iodomethane (50 g) and silver oxide (20 g) for 0.5—1.0 hr. Additional iodomethane (50 g) and silver oxide (20 g) were added, and the mixture was shaken for 24 hr. This methylation was repeated two more times. The reaction mixture was filtered, and evaporated at 90°C to a syrup, which was then dissolved in chloroform. The solution was washed successively with 10% potassium cyanide solution and water, dried with magnesium sulfate, and evaporated to a

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[8] Retention times of reference compounds and other experimental details will be published later.

[9] R. Kuhn, H. Trischmann, and I. Löw, *Angew. Chem.*, **67**, 32 (1955).

thin syrup. Addition of hexane precipitated the product (IV) (8.4 g), which was filtered off, and dried *in vacuo* over phosphorus pentoxide at 40°C; $[\alpha]_{\mathrm{D}}^{26}+30.8°$ (*c* 0.64 in pyridine).

Decarbamoylation (IV→V). The methylated product (8.4 g) obtained above was dissolved in dry tetrahydrofuran (200 m*l*), lithium aluminum hydride (3 g) added slowly, and the mixture was refluxed for 4 hr. Methanol (10 m*l*) and water (10 m*l*) were added slowly, and aluminum hydroxide was filtered off, and washed with methanol-water (1:1). The combined filtrate and washings were neutralized with dilute phosphoric acid and filtered again. The filtrate was extracted with hexane to remove an oily reduction product, and the residual solution was evaporated to dryness (7.0 g).

Detritylation and Methanolysis. The product (7.0 g) obtained above was refluxed with 1% methanolic hydrogen chloride (50 m*l*) for 2 hr, neutralized with silver carbonate and filtered. The filtrate was deionized with Amberlite IR-120 (70 m*l*) and IR-45 (150 m*l*), and evaporated to a syrup (3.6 g), $[\alpha]_{\mathrm{D}}^{24}+9.3°$ (*c* 0.4 in methanol), which was again treated with 2% methanolic hydrogen chloride (50 m*l*) at 80°C for 22 hr. The reaction solution was treated in the usual manner, and the product was obtained as a syrup (3.4 g); $[\alpha]_{\mathrm{D}}^{24}+60.1°$ (*c* 0.7 in water).

Analysis of the Methanolysis Products. Examination of the syrup obtained above by pc (solvent D, I, spray c) and tlc (solvent K) indicated the presence of dimethyl acetals of AG, 2MAG, 5MAG, and 25MAG and methyl glycoside of 2MAG. Quantitative determination was carried out by glc (method B, column a and b), and the result was expressed in the molar ratio (Table 1). On the other hand, a portion of the syrup was hydrolysed with 1N-hydrochloric acid at 100°C for 16 hr. The hydrolysate contained galactose, 3MG, 4MG, and 34MG as shown by pc (solvent A, B, C, E, G, and H, spray a and b), paper electrophoresis, and glc (method D and E). The results of quantitative determination by glc using the columns a, b and c were in good mutual agreement, and the mean values expressed in the molar ratio were given in Table 1.

Separation of the Methanolysis Products. A part of the methanolysis products (2.0 g), obtained above, was applied to a starch-Celite 545 column (5.5×46 cm), which was then eluted with butanol-water (6:1). The effuluents were checked by a resorcinol reagent, an anthrone reagent, pc (solvent D and I) and/or tlc (solvent J and K). Fractions showing similar contents were combined, and evaporated to give Fractions I, II, and III. These fractions were combined with similar fractions obtained from the remaining portion of the methanolysis products (1.4 g).

Fraction I. Yield 0.99 g. This fraction was shown to be a mixture of dimethyl acetals of 25MAG, 2MAG, and 5MAG and methyl glycoside of 2MAG by pc (solvent D and I, spray c) and tlc (solvent K and L). Separation and isolation of these components was achieved only when chromatography was repeated several times using different columns and different solvent mixtures. The yields were low mainly because of mechanical loss during the repeated treatment. Fraction I (0.99 g) was applied to a Dowex 1×4 column (OH form, 5×80 cm) and eluted by distilled water to give three fractions, I-1, I-2, and I-3 which were checked by pc (solvent D and I), tlc (solvent K and L) and glc (method F, column a, b and c).

Fraction I-1 (80 mg) was a mixture of dimethyl acetals of 25MAG and 5MAG. The mixture was separated on an alumina column (2×26 cm). 25MAG dimethyl acetal was eluted with benzene-ethanol (15:1), and obtained as a syrup (57 mg); $[\alpha]_{\mathrm{D}}^{26}+22.9°$ (*c* 4.21 in water). It was converted into crystalline 3,6-anhydro-2,5-di-*O*-methyl-D-galactonic acid in the usual way;[10] mp and mmp 165—166°C, $[\alpha]_{\mathrm{D}}^{25}+59.0°$ (*c* 0.64 in water). (Found: C, 46.42; H, 6.33; OCH_3, 30.18%). The alumina column was then eluted with benzene-ethanol (2:1) to give 5MAG dimethyl acetal (5 mg).

Fraction I-2 (0.44 g) was shown by pc to be 5MAG dimethyl acetal contaminated with a small amount of 2MAG dimethyl acetal. Seeding with a synthetic sample[11] afforded crystalline 5MAG dimethyl acetal, which was then recrystallized twice from ethyl acetate-benzene-hexane (1:1:2), yield 0.33 g; mp and mmp 53.5—54.5°C, $[\alpha]_{\mathrm{D}}^{20}+42.6°$ (*c* 0.59 in water), (Found: C, 48.79; H, 8.23; OCH_3, 40.99%). This compound was further identified by conversion into its *p*-nitrobenzoate;[11] mp and mmp 143—145°C, $[\alpha]_{\mathrm{D}}^{20}+66.1°$ (*c* 1.15 in chloroform), (Found: C, 52.92; H, 4.66; N, 5.26; OCH_3, 16.52%). The mother liquor of the crystallization showed the presence of 2MAG dimethyl acetal in addition to 5MAG dimethyl acetal by pc, tlc, and glc. Then the mixture was separated on a Sephadex LH-20 column (2×24 cm) with chloroform as a solvent. 5MAG (4 mg) was the faster-moving component, which was followed by 2MAG (15 mg); $[\alpha]_{\mathrm{D}}^{26}+27.4°$ (*c* 1.48 in water). This compound was identified by conversion into crystalline 3,6-anhydro-2-*O*-methyl-D-galactonic acid in the usual manner; mp 142°C, $[\alpha]_{\mathrm{D}}^{25}+65.2°$ (*c* 0.31 in water),[2] (Found: C, 42.98; H, 7.15%).

Fraction I-3 (53 mg) was a mixture of dimethyl acetal of 5MAG and methyl glycoside of 2MAG. The mixture was separated on an alumina column (2×26 cm) with benzene-ethanol (15:1) as solvent. The 2MAG derivative was the faster moving component, and was obtained as a syrup (13 mg), $[\alpha]_{\mathrm{D}}^{26}-65.1°$ (*c* 1.06 in water), which was identified again by conversion into crystalline anhydro-2-*O*-methyl-3,6-D-galactonic acid;[2] mp and mmp 141.5°C, $[\alpha]_{\mathrm{D}}^{25}+63.9°$ (*c* 0.15 in water).

Fraction II. Yield 1.32 g. This fraction was shown to be a mixture of dimethyl acetal of AG and methyl glycoside of 34MG by pc (solvent A, D and I) and tlc (solvent J and K). The mixture (1.32 g) was placed on a Dowex 1×4 (OH form, 3×30 cm) column. The methyl glycosides of 34MG were readily eluted with water, and obtained as a syrup (0.85 g); $[\alpha]_{\mathrm{D}}^{29}+146°$ (*c* 1.2 in water). The syrup was hydrolysed with 1N-sulfuric acid at 100°C for 4 hr, and the product was twice recrystallized from methanol-ethyl acetate, 34MG being obtained as prisms;[12,13] mp and mmp 169—171°C, $[\alpha]_{\mathrm{D}}^{21}+105.8° \rightarrow +126.3°$ (*c* 0.43 in water), (Found: C, 46.02; H, 7.65; OCH_3, 30.1%).

The Dowex column was then eluted with 0.2M-ammonium carbonate solution, and the effluent was deionized by Amberlite IR-120 and IR-45, and evaporated to dryness. AG dimethyl acetal was obtained as a syrup (0.45 g); $[\alpha]_{\mathrm{D}}^{29}+38.9°$ (*c* 0.90 in water). It was converted into AG diphenyl hydrazone in the usual manner;[10] mp and mmp 154—156°C, $[\alpha]_{\mathrm{D}}^{19}+24.5°$ (*c* 0.66 in methanol), (Found: C, 65.61; H, 6.24; N, 8.37%).

Fraction III. Yield 0.96 g. This fraction was shown to be a mixture of methyl galactosides and their mono-*O*-methyl derivatives and a small amount of oligosaccharide derivatives by pc (solvent A) and tlc (solvent J and K). The mixture (0.96 g) was hydrolysed with 1N-sulfuric acid for 4 hr in the usual manner to give a mixture of reducing sugars (0.92 g). Crystallization from methanol afforded D-galactose (0.36 g); mp and mmp 164—165°C, $[\alpha]_{\mathrm{D}}^{20}+118.1°$—

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[10] C. Araki and S. Hirase, This Bulletin, **29**, 770 (1956).

[11] S. Hirase and K. Watanabe, *ibid.*, **45**, No.6(1972) (in the press).

[12] J. S. D. Bacon and D. J. Bell, *J. Chem. Soc.*, **1939**, 1869.

[13] S. Hirase and K. Watanabe, This Bulletin, **45**, 1569 (1972).

+80.4° (c 0.91 in water), (Found: C, 40.14; H, 6.63%). The mother liquor was evaporated to a syrup (0.45 g), which was then separated on a starch-Celite 545 column (3×30 cm) with butanol-water (6:1). Three fractions III-1, III-2, and III-3 were obtained as checked by pc (solvent A, B, C, and E).

Fraction III-1 (42 mg) showed a single spot of 34MG by pc and crystallized on standing.

Fraction III-2 (0.19 g) was shown to be a mixture of 3MG and 4MG by pc (solvent A, E, G, and H), glc (method D, column a and b; method E, column a, and b), and paper electrophoresis. On standing, 4MG crystallized. It was recrystallized from water-methanol (36 mg);[14] mp and mmp 209—211°C, $[\alpha]_D^{20}+64.76\rightarrow+89.46°$ (c 0.43 in water). (Found: C, 43.16; H, 7.20; OCH_3, 15.8%).

The mother liquor was evaporated to a syrup (0.14 g), which was then placed on a Dowex 1×4 (borate form, 2×16 cm) column. Elution with water afforded 4MG (13 mg). The eluant was then replaced by 0.03M-potassium borate solution. The effluent was treated with Amberlite IR-120 to remove potassium ion, and the resulting acidic solution was evaporated to dryness. Boric acid was removed by repeated addition of methanol to the residue followed by evaporation. The final product was obtained as a syrup (0.1 g); $[\alpha]_D^{31}+80.7°$ (c 0.82 in water). This syrup was indistinguishable in pc (solvent G, spray a and b), glc (method D, column a and b), and paper electrophoresis[15] from the sample of 3MG, which was synthesized by the method of Ball and Jones.[16] Crystallization of either of the syrup obtained above and the synthesized one was unsuccessful.

Fraction III-3 (0.14 g) was shown to be a mixture of galactose and a small amount of oligosaccharide by pc (solvent A, B and C). On standing, D-galactose crystallized (70 mg); mp and mmp 164—166.5°C.

Alkaline Treatment of κ-Carrageenan after Periodate Oxidation. According to the method of Anderson and his co-workers,[2] κ-carrageenan (0.5 g) was treated with 0.05M-sodium periodate solution (100 m*l*) at room temperature for 75 hr in the dark. After destroying the excess of the oxidant with ethylene glycol, potassium borohydride (1.5 g) was added. The solution was diluted to 500 m*l*, left for 2 days, and dialysed against running water. The dialysate was divided into two halves, one of which was used to analyse the sugars, and the other one was subjected to the treatment with potassium hydroxide at 80°C for 4 hr in the usual manner and subsequent analysis of the sugars. Comparison of the analytical results showed that 3% of the total galactose residues existed as the periodate-resistant sulfate residues, which were convertible into 3,6-anhydro-galactose by the alkaline treatment.

Experiments on Agarose from G. amansii. A series of reactions was performed in a similar manner to the case of κ-carrageenan. Dry agarose (2.0 g) suspended in dry *N*,*N*-dimethylformamide (70 m*l*) was heated with phenylisocyanate (10 m*l*) at 100°C for 10 hr. The phenylcarbamoate (4.6 g), obtained as powders, was treated with a boiling mixture of dioxane (40 m*l*) and 2% methanolic hydrogen chloride (40 m*l*) for 2 hr. The product (4.2 g), a part of which on removal of the carbamoyl group showed the presence of agarobiose dimethyl acetal as well as higher oligosaccharide derivatives by pc (solvent D, spray c), was methylated repeatedly by the Kuhn's method.[9] The methylated product (4.6 g) in dry tetrahydrofuran (50 m*l*) was refluxed with lithium aluminum hydride (1.0 g) for 4 hr, and the resulting decarbamoylated product (1.1 g) was subjected to complete methanolysis. The final product was obtained as a syrup (0.9 g).

Examination by pc (solvent D and I, spray c) and tlc (solvent K) showed the presence of dimethyl acetals of 3,6-anhydro-galactose and its 5-methyl ether. While, hydrolysis of the above final product followed by examination by pc (solvent C, E, G and H, spray a and b) showed the presence of galactose and its 3- and 4-methyl ether. Quantitative glc (method D, columns a, b and c) showed that galactose and its 3- and 4-methyl ethers existed in the molar proportions of 100: 43: 13.

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