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# Structure of Agars from *Gracilaria tikvahiae* Rhodophyta: Location of 4-*O*-Methyl-L-Galactose and Sulphate*

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*ABSTRACT*

*Agars from young and old tissues of* Gracilaria tikvahiae *were compared by $^{13}C$ NMR spectroscopy. An alkali-modified agar containing 4-O-methyl-L-galactose was degraded by β-agarase. Methylation analysis combined with $^{13}C$ NMR studies established the presence of single 4-O-methyl-α-L-galactopyranosyl-(1→6) residues attached as side branches to the 3-linked galactose of the main chain. Repeating units of 6-O-methyl-agarobiose-4-O-sulphate were detected in agars from old tissues.*

*Enzymolysis with β-agarase released 26·8% of the agar from mature algae, mostly as neoagarobiose, neoagarotetraose and their $6^3$-mono-O-methyl and $6^1$, $6^3$-di-O-methyl derivatives. No 4-O-methyl-L-galactose-containing fragments were detected among the low-molecular-weight products of enzymolysis.*

*The agar from young tissues grown at a suboptimal temperature differs from agars of mature tissues in that it contains low levels of 4-O-sulphate and only traces of 4-O-methyl-L-galactose. It is concluded that physiological condition and growth regimen can induce wide variations in the structure of agar within the same species.*

## INTRODUCTION

Red algae of the genus *Gracilaria* are important commercial sources of agar (Lewis *et al.*, 1988). Evidence is accumulating that considerable interspecific variation exists in the chemical structures of their agars. It is generally agreed that the underlying repeating structure is agarobiose (Fig. 1); however, this pattern may be masked by the replacement of 3,6-

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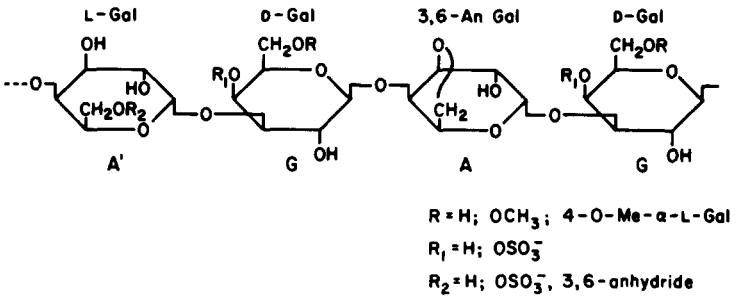


**Fig. 1.** Structures of agars from *G. tikvahiae*. The G-A repeating unit is agarobiose when $R = R_1 = H$. Neoagarobiose is the A-G repeating unit when $R = R_1 = H$.

anhydro-L-galactose by L-galactose and by the presence of sulphate, pyruvate, and methoxyl substitutents (Painter, 1983; Craigie, in press). When a sulphate hemi-ester occurs at C-6 of the 4-linked unit, it is alkali-labile and is the precursor of the 3,6-anhydride both *in vitro* and *in vivo* (Rees, 1961). Ester sulphate at C-3 of the same residue is similarly alkali-labile (Brasch *et al.*, 1983). Alkali-stable sulphate esters may occur at C-2 of the 4-linked residue and at C-2, C-4 and C-6 of the D-galactose residues. Pyruvate, when present, exists as 4,6-*O*-(1-carboxyethylidine)-D-galactose (Painter, 1983).

The presence of 6-*O*-methyl-D-galactose is widespread in *Gracilaria* spp., but methoxyl may occur elsewhere in their agars. The anhydrogalactose residues may be partially replaced by their 2-*O*-methyl ethers (Brasch *et al.*, 1983; Lahaye *et al.*, 1986) or virtually completely replaced as in *Gracilaria eucheumoides* agar (Ji *et al.*, 1985). In addition, 4-*O*-methyl-L-galactose occurs in significant quantities in agars from *Gracilaria tikvahiae* (Craigie *et al.*, 1984). This unusual sugar was detected earlier in three other *Gracilaria* spp. (Duckworth *et al.*, 1971) and in several red algal genera (Araki *et al.*, 1967; Nunn & Parolis, 1969).

Due to the low quantities of 4-*O*-methylgalactose usually encountered in agars, it was not deemed to be of structural significance (Araki, 1966). Chemical studies of the carrageenan-related polysaccharide, aeodan, revealed that 4-*O*-methyl-L-galactose was attached as a single branch residue through an $\alpha$-glycosidic bond to C-6 of the 4-linked D-galactose in the main chain (Allsobrook *et al.*, 1974). However, analytical data for agars from *G. tikvahiae* indicated that 4-*O*-methyl-L-galactose was attached at C-6 of the 3-linked residue (Craigie & Wen, 1984). Our present communication confirms this structure and, further, provides

evidence that 6-*O*-methylagarose-4-sulphate is a constituent of agar from mature tissues of *G. tikvahiae*.

## EXPERIMENTAL

### Agar sources

The agars investigated were from *G. tikvahiae* McLachlan as described in Craigie and Wen (1984). The wild type (clone MP-2) cultivated at 17°C and 27°C was the source of both the young tissue (stipe branches, SB-17) and old tissue (old stipe, OS-27) used for agar extraction. A moderately branched mutant (clone MP-44) grown at 28°C was the source of the agar used for enzymic degradation. Agaroses with 6-*O*-methyl-D-galactose (Calbiochem) and without (Baker) were used directly as references for $^{13}C$ NMR and to prepare authentic oligosaccharide fractions following digestion with $\beta$-agarase.

### Analytical procedures

Alkali modification of the agars and 3,6-anhydrogalactose analysis by resorcinol followed standard procedures (Craigie & Leigh, 1978). Sulphate was measured tubidimetrically as $BaSO_4$ in hydrolysates (Craigie *et al.*, 1984).

### $^{13}C$ Nuclear magnetic resonance

Sugars and oligosaccharides were dissolved in $D_2O$ and examined at ambient temperature with a Varian XL-100 spectrometer at 25·2 MHz. The chemical shifts of the $^{13}C$ $^{1}H$-broadband-decoupled spectra were referenced to external tetramethylsilane (TMS) using DMSO-$H_6$ ($\delta$ 39·5) as an internal standard.

Polysaccharide fractions were dissolved (1–5%) in 3 ml distilled water in 10-mm NMR tubes. Either DMSO-$d_6$ or sodium-3-trimethylsilyl-propionate-2,2,3,3-$d_6$ (TSP) was added as an internal standard and spectra (90·8 MHz) were recorded at 90°C on the Nicolet 360 NB spectrometer of the Atlantic Region Magnetic Resonance Centre, Halifax, N.S. Chemical shifts are reported relative to external TMS. Neat TMS in a concentric tube had a chemical shift of 2·6 ppm relative to aqueous TSP at 90°C. Confirmation of the chemical shifts for the methylene carbons was obtained from an attached proton test (APT)

experiment. Single-echo ($\tau$ 8 ms) spectra were recorded at 90°C for alkali-modified agar (5% in $H_2O$) from clone MP-44 as described in Patt and Schoolery (1982).

### Anion-exchange chromatography

Alkali-modified MP-44 agar (24·8 mg) was fractionated at 60°C on a column (34 × 1·0 cm) of Sephadex DEAE-A50 using stepwise elution with distilled water, 0·5 M and 2·5 M NaCl, and 6 M urea (Ji *et al.*, 1985).

### Methylation and GC–MS analysis

Single Hakomori methylations were performed (Jansson *et al.*, 1976) on alkali-modified MP-44 agar and on the $\beta$-agarase-resistant fractions, gels I and II. Infrared scans (3600 to 3400 $cm^{-1}$) confirmed that methylation was complete. Following sequential hydrolysis with 90% HCOOH and 0·13 M $H_2SO_4$ (Jansson *et al.*, 1976), samples were reduced with $NaBH_4$ and acetylated ($Ac_2O$ in pyridine) for analysis by GC–MS. The sugar derivatives were separated using a DB-5 column coupled to a low-resolution commercial mass spectrometer (New Brunswick Research and Productivity Council, Fredericton, N.B.). Spectra were recorded according to manufacturer's specifications at an ionization potential of 70 eV. Identification of the individual substituted hexitols followed Jansson *et al.* (1976).

### Enzymolysis with $\beta$-agarase

The extracellular endo-$\beta$-agarases (EC 3.2.1.81) were isolated as described in Duckworth and Yaphe (1970). The crude enzyme preparation was stored as a lyophilized powder and used without further purification. The principal enzyme in our preparation was $\beta$-agarase I with only slight $\beta$-agarase II activity detected (Morrice *et al.*, 1983*b*).

Alkali-modified MP-44 agar (1·92 g) was dissolved in boiling 0·1 M phosphate buffer, pH 7·5 (400 ml). Crude $\beta$-agarase (50 mg) was added initially when the agar had cooled to 60°C and again after 30 min when the temperature reached 50°C. After 1 h the mixture was cooled to 40°C and further additions (30–50 mg) of $\beta$-agarase were made periodically over the next 20 h. When no further increase in reducing power (Somogyi, 1952) could be detected on adding fresh enzyme, the still gelatinous reaction mixture was centrifuged (14 000 × ***g***) to obtain the fraction gel I. Additional fractions were recovered as outlined in Fig. 2.

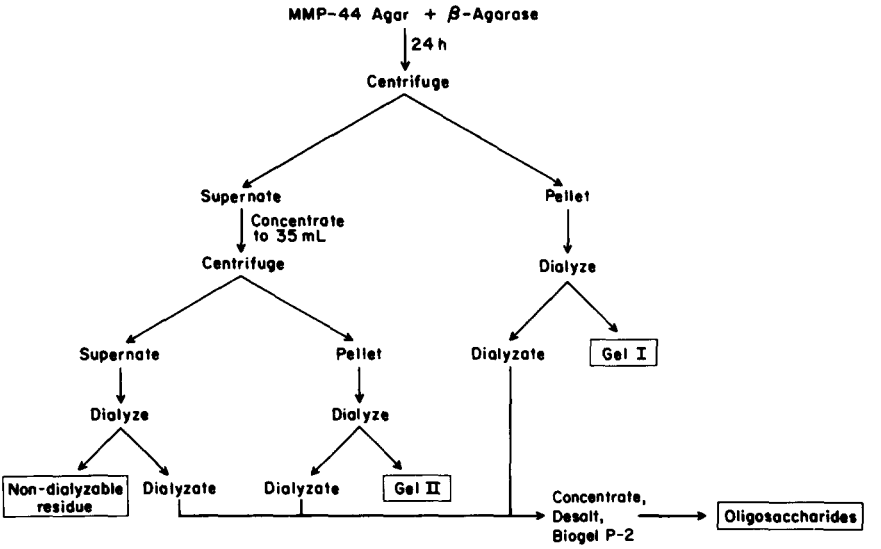


**Fig. 2.** Flow diagram for β-agarase enzymolysis of alkali-modified agar (MMP-44) from *G. tikvahiae* clone MP-44.

The combined oligosaccharides (Fig. 2) were placed on a Biogel P-2 column (1·5 m × 2·5 cm) and eluted with distilled water. The fractions were examined by $^{13}C$ NMR. Conclusive identification of the constituents was achieved using published data (Brasch *et al.*, 1981; Morrice *et al.*, 1983*a*; Rochas *et al.*, 1986) and by direct comparison with $^{13}C$ spectra of neoagaro-biose, -tetraose, and -hexaose isolated from β-agarase digests of commercial agaroses.

## RESULTS

Chromatography of alkali-modified MP-44 agar on DEAE Sephadex established that the preparation was heterogeneous. The overall recovery from the column was 84·2% with the following elution pattern: distilled water, 10·4%; 0·5 M NaCl, 41·9%; 2·5 M NaCl, 14·5%; and 6 M urea, 17·3%.

### Enzymolysis

β-Agarase released 26·8% of the alkali-modified agar as oligosaccharides (Table 1) which were shown by $^{13}C$ NMR to consist principally of neoagarotetraose and its $6^3$-*O*-methyl- and $6^1$, $6^3$-di-*O*-

**TABLE 1**
Recoveries and Analytical Data for Products Resulting from β-Agarase Enzymolysis of Alkali-modified MP-44 Agar[a]

| *Sample* | *Weight (g)* | *3,6-Anhydrogalactose* | | | *$OSO_3Na$* | |
|---|---|---|---|---|---|---|
| | | *(%)* | *(mmol.g$^{-1}$)* | *(%)* | *(mmol.g$^{-1}$)* | *(%)* |
| MMP-44 Agar | 1·920 | 100·0 | 2·49 | 100·0 | 0·37 | 100·0 |
| Oligosaccharides[a] | 0·513 | 26·8 | — | 34·3[b] | 0 | 0 |
| Gel I | 0·600 | 31·2 | 2·34 | 29·4 | 0·36 | 30·3 |
| Gel II | 0·433 | 22·6 | 2·08 | 18·8 | 0·51 | 31·2 |
| Nondialysable residue | 0·427 | | 1·66 | 14·8 | 0·59 | 35·8 |
| Total | | ≥ 80·6[c] | | 97·3 | | 97·3 |

[a]No anions were detected by either ion-exchange chromatography or paper electrophoresis.
[b]Calculated from theoretical values for mono-*O*-methyl-neoagarotetraose.
[c]A value for the nondialysable residue was not included. The 3,6-anhydrogalactose analysis suggests that only about 10% of the original agar was present in this residue.

methyl derivatives. Neoagarobiose and neoagarohexaose were only minor components; no ionic or branched chain oligosaccharides were detected. The enzyme-resistant gel I and gel II fractions together constituted 53·8% of the original agar with a further amount of undigested polymer (approx. 10%) associated with the enzyme debris in the nondialysable fraction (Fig. 2) (Table 1). The gel II fraction and the nondialysable residue were relatively enriched in sulphate and depleted in 3,6-anhydrogalactose.

## Methylation analysis

Six significant components were obtained after hydrolysis of the permethylated, alkali-modified MP-44 agar, and the β-agarase-resistant fractions, gel I and gel II. Their identities as established by GC–MS are given in Table 2 where it may be seen that only four (compounds I, II, III and V) were major constituents.

Compound I, the second most abundant, provides evidence for the presence of galactose end-groups or single-unit side chains with or without methoxyl substituents in the original agar. Compound II was the main constituent. Its presence establishes that much of the galactose or 6-*O*-methylgalactose was 3-linked and unsubstituted at C-2 and C-4. The third most abundant derivative, compound III, must have arisen from a 3-linked galactose (or its 6-*O*-methyl ether) residue which carried

**TABLE 2**
Galactitol Derivatives and Their Mole Ratios[a] in Hydrolysates of Permethylated, Alkali-modified MP-44 Agar and β-Agarase-resistant Fractions

| *Compound*[b] | *MMP-44* | *Gel I* | *Gel II* |
|---|---|---|---|
| (I) 1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-MeGal | 1·00 | 1·00 | 1·00 |
| (II) 1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-MeGal | 5·04 | 2·83 | 2·75 |
| (III) 1,3,4,5-Tetra-*O*-acetyl-2,6-di-*O*-MeGal | 0·79 | 0·81 | 0·79 |
| (IV) 1,2,3,5-Tetra-*O*-acetyl-4,6-di-*O*-MeGal | —[c] | —[c] | —[c] |
| (V) 1,3,5,6-Tetra-*O*-acetyl-2,4-di-*O*-MeGal | 0·37 | 0·34 | 0·37 |
| (VI) 1,3,4,5,6-Penta-*O*-acetyl-2-*O*-MeGal | —[c] | —[c] | —[c] |

[a]As determined by GC on an SE-30 packed column.
[b]In order of elution from a GC capillary column DB-5.
[c]Present at less than 5% of the total mass.

a substituent other than methoxyl at C-4. Compound IV was a minor constituent presumably arising from a 3-linked residue with the C-2 position substituted by other than methoxyl. The C-4 and C-6 positions were either methoxylated or unsubstituted. Compound V was the fourth most abundant constituent in the hydrolysates. It provides evidence for a glycosidic linkage at C-6 of a 3-linked galactose residue. Hydroxyls at C-2 and C-4 were either unsubstituted or methylated in the original agar. Compound VI occurred as a minor component and appears to have arisen from the 4-linked 3,6-anhydrogalactose in the polymer. Most of the anhydrogalactose would be destroyed during the hydrolysis (Brasch *et al.*, 1983).

## NMR of native agars

High-field $^{13}C$ spectra of *G. tikvahiae* agars from young and old tissues are shown in Fig. 3(a), (b). All major signals in agar from young tissue (Fig. 3(a)) were readily identified (Table 3) as originating from the repeating units of agarobiose and 6-*O*-methylagarobiose according to published assignments (Hamer *et al.*, 1977; Usov *et al.*, 1980, 1983; Brasch *et al.*, 1981; Morrice *et al.*, 1983a; Lahaye *et al.*, 1985; Lahaye, 1986). Resonances appearing near 82 and 70 ppm were partially overlapping (Fig. 3(a)); those at 82·0 and 82·1 were assigned to C-3 of agarose and 6-*O*-methylagarose, respectively, in accordance with the small downfield shift observed at this position caused by 6-*O*-methylation of the β-D-galactopyranose (Brasch *et al.*, 1981). Confirmation of this assignment was obtained by a direct comparison under our conditions of spectra of commercial agarose containing the 6-*O*-methyl-

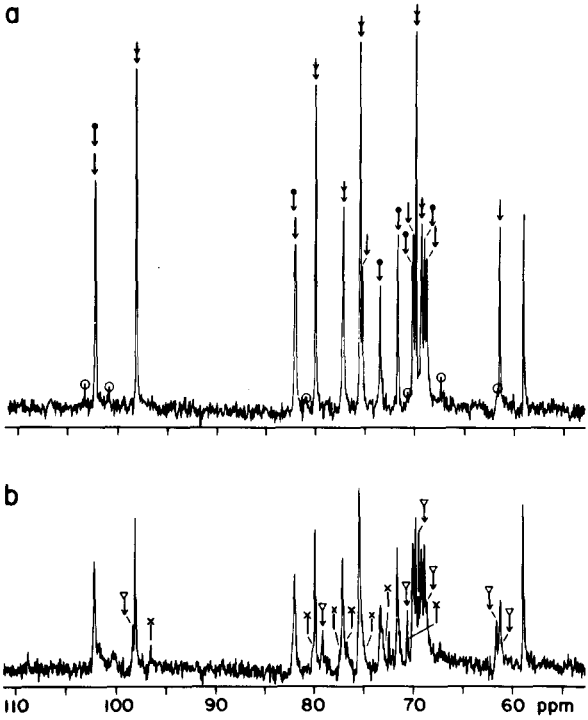


**Fig. 3.** $^{13}C$ NMR spectra of unmodified (native) *G. tikvahiae* agars extracted from (a) young tissue grown at 17°C (stipe branches, SB-17) and (b) old tissue grown at 27°C (old stipe, OS-27). D-galactose (↓), 6-*O*-methyl-D-galactose (↕), 3,6-anhydro-L-galactose (↕), agarose precursor (○), 6-*O*-methyl carbon (unmarked), 4-*O*-methyl-L-galactose (⊽), and galactose-4-sulphate (⊼).

**TABLE 3**

$^{13}C$ Chemical Shift Assignments for Repeating Structures in Agars from *Gracilaria tikvahiae*

| | | *C-1* | *C-2* | *C-3* | *C-4* | *C-5* | *C-6* | $OCH_3$ |
|---|---|---|---|---|---|---|---|---|
| Agarobiose | G | 102·2 | 70·1 | 82·0 | 68·6 | 75·2 | 61·3 | |
| | A | 98·1 | 69·8 | 80·0 | 77·2 | 75·5 | 69·2 | |
| 6-*O*-Methylagarobiose | G | 102·2 | 70·2 | 82·1 | 68·9 | 73·4 | 71·6 | 58·9 |
| | A | 98·1 | 69·8 | 80·0 | 77·2 | 75·5 | 69·2 | |
| 4-*O*-Methyl-α-L-galactose[a] | | 98·4 | 68·6 | 69·5 | 79·2 | 70·7 | 61·2 | 61·6 |

[a]A methylene carbon resonance at 71·4 ppm originates with C-6 of 3-linked galactose forming the glycosidic bond with 4-*O*-methyl-L-galactose.

galactose residue with those of agarose devoid of methoxyl. Similarly, the two resonances for C-2 at 70·1 and 70·2 were assigned to agarose and 6-*O*-methylagarose, respectively, when it was observed that the C-2 resonance in reference 6-*O*-methylagarose consistently appeared ≈ 0·06 ppm downfield relative to that in agarose, whether examined in $D_2O$ or $H_2O$ under our conditions. The minor resonances appearing at 103·5, 101·1, 81·0, 70·0, 67·4, and 61·5 ppm (Fig. 3(a)) are assignable to G-1, A*-1, G-3, A*-5, A*-6 and G-6 of the precursor to agarobiose (Lahaye, 1986; Lahaye *et al.*, 1986) as would be expected from the known presence in this sample of the alkali-labile, 4-linked L-galactose 6-sulphate (Craigie *et al.*, 1984).

The $^{13}C$ spectrum of agars from old tissues (Fig. 3(b)) was considerably more complex in that 14 new resonances of moderate to low intensities were recorded. While these resonances did not appear in agar from young tissues, most of them could be recognized in spectra from alkali-modified MP-44 agar and its gel I and gel II fractions (Fig. 4(a)–(c)). Among the new resonances of moderate intensity, those at 98·4, 79·2 and 61·6 ppm could be assigned immediately to the α-anomeric, C-4 and methoxyl carbons of 4-*O*-methyl-L-galactose known to be present in this agar (Craigie *et al.*, 1984). The correct assignments for C-6 and the methoxyl carbons were established in an APT experiment (Fig. 5). Of the five positive resonances due to methylene carbons (Fig. 5), four were assigned as shown in Table 3. The remaining methylene resonance (71·4 ppm) was therefore assignable to C-6 of a 3-linked galactose involved in the glycosidic linkage with 4-*O*-methyl-α-L-galactopyranose. As part of our study of reference carbohydrates, the $^{13}C$ spectrum of crystalline 4-*O*-methyl-L-galactose was recorded and the assignments are presented in Table 4.

The most obvious of the remaining resonances, all of minor intensity, was the anomeric signal at 96·5 ppm (Fig. 3(b)). It has been pointed out that sulphation at C-4 of agarose results in an upfield shift of 2·0 ppm for the C-1 resonance of the adjacent 3,6-anhydro-L-galactose residue (Usov *et al.*, 1980). Additional minor resonances (ppm) consistent with the presence of 4-*O*-sulphate are A-3, 80·1; A-4, 77·5; G-4, 76·8; A-5, 75·4; and G-2, 70·6. The resonance near 75·0 ppm for G-5 of agarose 4-sulphate (Lahaye, 1986) appears upfield near 73·0 ppm as would be expected for a 6-*O*-methyl-galactose 4-sulphate residue. The presence of such a residue would be consistent with the evidence obtained from methylation analysis.

The main structural features, agarobiose and 6-*O*-methylagarobiose (Fig. 3(a)), are obvious in the $^{13}C$ spectrum of alkali-modified agar from

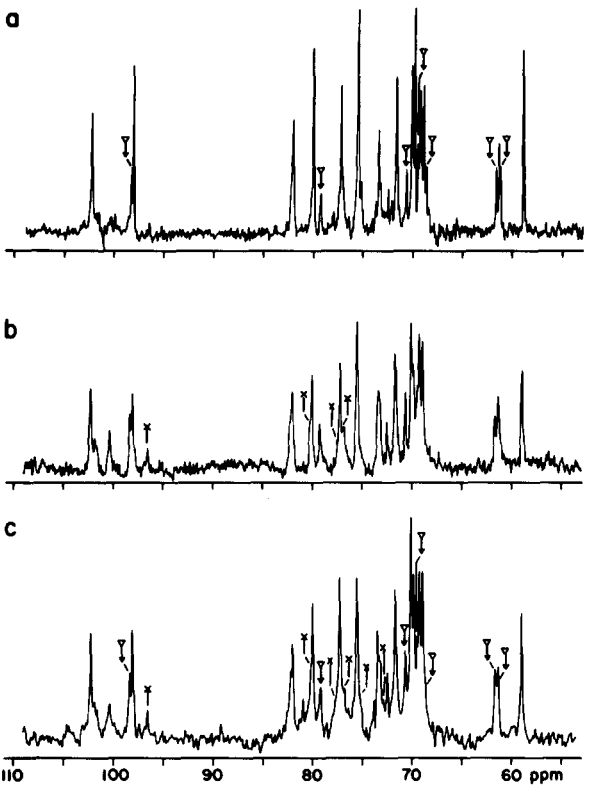


**Fig. 4.** $^{13}C$ NMR spectra of (a) alkali-modified agar from *G. tikvahiae* clone MP-44, (b) gel I and (c) gel II fractions resistant to $\beta$-agarase. Symbols are as in Fig. 3.

clone MP-44 (Fig. 4(a)). Conspicuous also in the latter are the seven resonances for 4-*O*-methyl-L-galactose (Table 3). Enzymolysis with $\beta$-agarase resulted in fractions gel I and gel II, both of which were enriched in 4-*O*-methyl-L-galactose and 4-*O*-sulphated galactose residues (Fig. 4(b), (c)) relative to the original agar. The chemical shift assignments are as reported for the unmodified agars. While the gel I and gel II fractions are similar to each other, they are not identical (Fig. 4(b), (c)). The methylene resonance arising from the $\alpha$-(1 → 6) linkage with 4-*O*-methyl-L-galactose is visible at 71·4 ppm in the spectra of alkali-modified MP-44 agar and gel I, but is obscured by excessive line broadening in the spectrum of gel II (Fig. 4(c)).

The $^{13}C$ resonances appearing at 100·3 and 72·4 ppm (Fig. 4(b), (c)) remain unassigned. The former is unlikely to be C-1 of floridean starch because the other expected starch resonances are absent. They may be

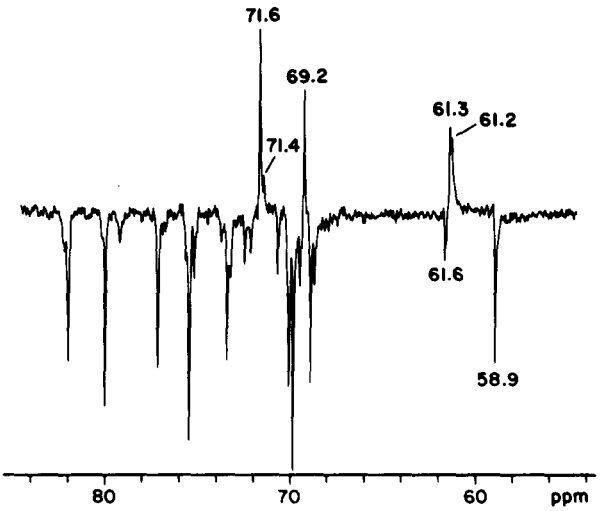


**Fig. 5.** $^{13}C$ APT spectrum of alkali-modified *G. tikvahiae* MP-44 agar.

**TABLE 4**
$^{13}$NMR Chemical Shifts (ppm) for 4-*O*-Methyl-L-galactose[a]

| *Anomer* | *C-1* | *C-2* | *C-3* | *C-4* | *C-5* | *C-6* | $OCH_3$ |
|---|---|---|---|---|---|---|---|
| $\alpha$ | 93·0 | 69·4 | 70·4 | 80·6 | 71·4 | 61·4 | 62·1 |
| $\beta$ | 97·1 | 72·4 | 73·9 | 79·9 | 76·0 | 61·2 | 62·1 |

[a]Spectra were recorded in $D_2O$ at ambient temperature using DMSO-$H_6$ as internal standard with a chemical shift of 39·5 ppm relative to tetramethylsilane.

due to undetermined substituent effects (Usov *et al.*, 1980) in these complex fractions.

## DISCUSSION

Knowledge of the structure of the agarocolloids has improved immensely since the pioneering studies using $^{13}C$ NMR (Hamer *et al.*, 1977; Usov *et al.*, 1980). Although the main repeating unit of *Gracilaria* agars is agarobiose, many variations exist in the patterns of the methoxyl, sulphate and pyruvate substituents commonly encountered. The presence of small quantities of other sugars such as glucose and xylose is well known, but only recently have they been reported as side branches

attached to C-6 of the 3-linked galactose in agars (Hirase *et al.*, 1983). Chemical investigation of aeodan, an unusual red algal polysaccharide related to the carrageenans, led to the conclusion that 4-*O*-methyl-L-galactose was glycosidically linked to the polysaccharide backbone through C-6 of the 4-linked galactose residue (Allsobrook *et al.*, 1974). The agar from some strains of *G. tikvahiae* contains more than 8% of 4-*O*-methyl-L-galactose and, because the agars showed the normal L:D galactose stoichiometry (near unity) expected for agarose, it was suggested (Craigie & Wen, 1984) that this methylated sugar was attached at C-6 of the 3-linked residue rather than as in aeodan.

In the case of *G. tikvahiae*, the same clone can produce agars that vary by an order of magnitude in their content of 4-*O*-methyl-L-galactose (Craigie & Wen, 1984). $^{13}C$ spectra of these preparations (Fig. 3(a), (b)) clearly illustrate that the same alga can produce agars of widely differing structure depending on its physiological state and the environmental conditions used for growth. Data from methylation studies (Table 2) confirm that the galactose end groups are either unsubstituted or methoxylated and therefore are consistent with the presence of 4-*O*-methylgalactose as a single unit side chain. The presence of 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl galactitol (Table 2) establishes that the 3-linked galactose bears a substituent other than methoxyl at C-6. The NMR spectrum (Fig. 3(b)) demonstrates the $\alpha$-glycosidic linkage for 4-*O*-methylgalactose and further confirms that C-6 of 3-linked galactose is the methylene carbon giving the resonance at 71·4 ppm. It must therefore be concluded that the structural linkage for 4-*O*-methyl-L-galactose is as shown in Fig. 6 and not as is reported for aeodan (Allsobrook *et al.*, 1974).

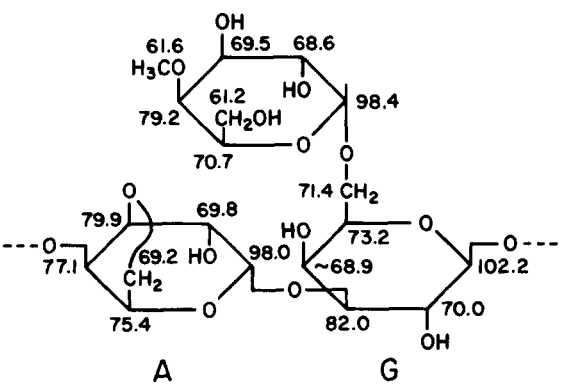


**Fig. 6.** Structure of neoagarobiose substituted with 4-*O*-methyl-$\alpha$-L-galactose and the $^{13}C$ chemical shift assignments relative to tetramethylsilane.

Three types of evidence lead to the conclusion that *G. tikvahiae* agar carries ester sulphate at C-4. Firstly, the proportion of alkali-stable sulphate increases from approximately one-third of the total sulphate in agar from young tissue to four-fifths in that from old tissue (Craigie & Wen, 1984). Secondly, the methylation analysis (Table 2) establishes that some 3-linked galactose residues are substituted at C-4 with a substituent other than methoxyl. Thirdly, $^{13}C$ NMR spectra revealed several resonances (Figs 3(b) and 4(b), (c)) that have been assigned to agarose 4-sulphate (Usov *et al.*, 1980; Lahaye, 1986). The lack of a C-5 resonance near 75·0 ppm and its appearance at 73·0 ppm indicates the presence of a substituent on C-6 of the 3-linked residue. The demonstration of 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methyl galactitol among the products of hydrolysis of permethylated agars (Table 2) is consistent with the occurrence of 6-*O*-methylgalactose-4-sulphate residues. It is concluded that these repeat units are present in *G. tikvahiae* agars.

The NMR evidence for the presence of *O*-β-D-galactopyranosyl-(1→4)-6-*O*-sulphate-α-L-galactopyranosyl-(1→3), the precursor of agarobiose, in agar from young tissues (Fig. 3(a)) is consistent with the observations on alkali-labile sulphate in this agar (Craigie & Wen, 1984). The amount of the agarobiose precursor may be underestimated because of possible losses of non-gelling agaroids during the preparation of our samples (Wen & Craigie, 1984; Lahaye *et al.*, 1986).

The 4-*O*-methyl-L-galactose and sulphate residues appear to be grouped in long chains to give the β-agarase-resistant fractions, gel I and gel II. Whether the failure to release low-molecular-weight oligosaccharides containing these groups is due to the C-6 substitution or to sulphation is unclear. It is known that β-agarase activity is reduced if neighbouring agarobiose residues are methylated or sulphated (Morrice *et al.*, 1983).

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