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Biosynthesis of agar polysaccharides in *Gracilaria chilensis* Bird, McLachlan *et* Oliveira

Jacqueline A. Hemmingson [a,*], Richard H. Furneaux [a], Virginia H. Murray-Brown [b]

[a] *Industrial Research Ltd., Gracefield Research Centre, P.O. Box 31-310, Lower Hutt, New Zealand*
[b] *Aquaculture Research Centre, National Institute of Water and Atmospheric Research, P.O. Box 14-901, Kilbirnie, Wellington, New Zealand*

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Abstract

The agar polysaccharides of the red seaweed *Gracilaria chilensis* Bird, McLachlan *et* Oliveira were labelled with the stable isotope ^{13}C by pulse feeding $NaH^{13}CO_3$ to samples of the seaweed acclimatised to indoor culture. An analytical method based on chemical derivatisation and GCMS was used to detect and monitor incorporation of the label into the dynamic carbon pool and individual units of the agar polysaccharides. Concurrent formation of three out of the four major units (D-galactopyranose, L-galactopyranose 6-sulfate, and 6-*O*-methyl-D-galactopyranose) was observed in frond tips. Formation of the ring-closed 3,6-anhydro-L-galactopyranosyl residues from 6-*O*-sulfo-L-galactopyranosyl residues was considerably slower. Similar behaviour was observed in the segment of the frond adjacent to the tips. Floridean starch may be utilised for agar polysaccharide biosynthesis in the absence of light and CO_2, when its level is high. © 1996 Elsevier Science Ltd.

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1. Introduction

The agar polysaccharides of the red seaweed *Gracilaria chilensis* Bird, McLachlan *et* Oliveira deviate from the basic agar structure, which has a (1 → 3)-β-D-galactopyranosyl-(1 → 4)-3,6-anhydro-α-L-galactopyranosyl repeating unit (I), by containing substantial amounts of methylated and sulfated units. The substitutions are

* Corresponding author.

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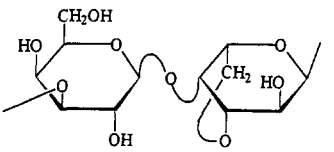
primarily at O-6 and are methylation giving 3-linked 6-*O*-methyl-β-D-galactopyranosyl residues and sulfation giving 4-linked 6-*O*-sulfo-α-L-galactopyranosyl residues.



I

The presence of these substituted units modifies the properties of the agar polysaccharides. For example, the native agar extracted from *Gracilaria chilensis* has poor gelling properties due primarily to the sulfated units present. To obtain a commercially valuable agar, the weed is treated with base prior to agar extraction in order to displace the sulfate group from the 6-*O*-sulfo-L-galactopyranosyl residues to give ring-closed 3,6-anhydro-L-galactopyranosyl residues. This removes the kinks from the polysaccharide chain which interfere with the gelation process [1]. The ring closure occurring during algal polysaccharide biosynthesis is an enzyme-mediated process [2,3].

Although the general biosynthetic pathway for the agar polysaccharides is fairly well established [4], knowledge is poor about the processes involved in transforming such precursor sugars as glucose and mannose, via D- and L-galactose, into the individual units of the agar polysaccharides, including the timing of formation and assembly of the different types of units [5,6]. It is generally believed that chains of alternating D- and L-galactopyranosyl residues are assembled on primer molecules in the Golgi apparatus [4]. Sulfation of L-galactopyranosyl residues is believed to occur in the Golgi at an early stage in the biosynthesis, while ring closure and methylation may occur somewhat later. At some stage in the biosynthesis, migration out of the Golgi into the cell-wall matrix takes place and further modification of the agar polysaccharides can occur as the new tissue ages [3,4,6,7]. Floridean starch is the major carbon storage polymer found in red algae [4]. It is a branched glucan similar to amylopectin, but it has some α-(1 → 3) branch points. Floridoside [α-D-galactopyranosyl-(1 → 2)-glycerol] is the principal low-molecular-weight photoassimilate [4], but its physiological roles in carbon storage and transport, and as a regulator of osmotic balance, are still to be fully established [8]. The formation of both of these compounds is reversible, so they act as a dynamic carbon pool for glucose and galactose, which can be used in the dark for cell processes, one of which may be agar biosynthesis [8–10]. A tentative pathway for this biosynthesis has been proposed by Yu and co-workers [9,10]. It involves degradation of floridean starch to its precursor glucose 1-phosphate, via a phosphorylase, with subsequent formation of UDP-D-galactose and GDP-L-galactose, the precursors of the agarobiose repeating unit. Glucose 1-phosphate is also a precursor of floridoside, which is formed from it via UDP-D-glucose and UDP-D-galactose, while floridean starch is formed from it via ADP-D-glucose [10].

In order to learn more about these processes, we have introduced $NaH^{13}CO_3$ into the biosynthetic pathway of *Gracilaria chilensis* Bird, McLachlan *et* Oliveira and used an analytical method based on chemical derivatisation and GCMS to follow the passage of

Fig. 1. *Gracilaria chilensis*.

the label into the component units of the agar polysaccharides. In this paper we report our findings on the formation and assembly of the individual units of these polysaccharides. The application of stable isotopic labelling with GCMS analysis to the study of carbohydrate metabolism in red algae is a fairly recent development [11,12].

We have examined both frond tips and the adjacent segment, as a prelude to studying biosynthetic activity in different regions of the thallus, such as along lateral filaments and in axial filaments (Fig. 1). This would give information on the type of growth undergone by this species, for example, diffuse, apical, or intercalary [4].

2. Experimental

Materials.—*Gracilaria chilensis* Bird, McLachlan *et* Oliveira was collected from Scorching Bay, Wellington, New Zealand and cultured indoors at NIWA Aquaculture Research Centre, Mahanga Bay, Wellington. A specimen of alga collected from this site in July 1971 is lodged in the herbarium at the Museum of New Zealand as sample WELT A 4437. The culture medium was filtered, natural seawater with or without added nutrients based on the F-medium of Guillard [13].

Labelling experiments.—Alga collected from the wild was acclimatised to low-growth-rate (LGR) indoor culture for 14 days prior to experiments, that is, weekly change of medium, compressed air aeration (500 mL min^{-1}), CO_2 provided by a gas bottle (20 mL min^{-1}), continuous irradiation with 30 μmol photons m^{-2} s^{-1} photosynthetically active radiation (PAR), and a temperature of 20 °C [13]. An algal sample was then suspended in an Erlenmeyer flask containing nutrient-enriched seawater (e.g., 9 g alga/3.6 L) and subjected to a short period (24 h) of irradiation with 50 μmol m^{-2} s^{-1} PAR. This was followed by 4 days in fresh, slowly aerated (compressed air) seawater with exclusion of light and without provision of CO_2 and nutrients. The alga was then fed $NaH^{13}CO_3$ (MSD Isotopes, Merck Frosst, Canada, 99 atom%, 3.7 mM) in a closed system comprised of TRIS buffered seawater (0.02 M, pH 7.5–8.0), continuously irradiated with 60 μmol m^{-2} s^{-1} PAR. The labelled $NaHCO_3$ was added in two

portions; 82% initially and the remainder 12 h into the pulse. The medium was agitated with a magnetic stirrer bar. The labelling pulse was followed by a chase period in the absence of light, nutrients, and a significant source of CO_2, by transferring the alga to fresh, slowly aerated seawater. The dark chase period was followed by an 8:16 h light:dark regime with 50 μmol m^{-2} s^{-1} irradiation, during which CO_2 and nutrients were either withheld or supplied. Samples of alga, typically 0.2–0.45 g, were removed periodically throughout the experiment, briefly rinsed, and lyophilised for analysis. The alga remained healthy throughout the experiment.

Polysaccharide composition.—Algal samples were subjected directly to a reductive hydrolysis procedure in order to convert their carbohydrate constituents into a mixture of alditol acetate derivatives [14]. Application of reductive hydrolysis procedures to galactans in situ was developed independently by the authors, and Usov and Klochkova [15]. Frond tip segments of 1–4 mm in length were removed from each dry sample and divided into two, ca. 1-mg portions, which were placed in screw cap tubes (100 × 12 mm o.d.). The adjacent segment was removed and treated likewise, except that it was washed in distilled water to remove floridoside before proceeding to the next step. One portion was then reductively hydrolysed and acetylated, while the second portion was alkali-modified before reductive hydrolysis, in order to convert 6-*O*-sulfo-L-galactopyranosyl residues (G6S units) into 3,6-anhydro-L-galactopyranosyl residues (AG units). The latter procedure was carried out by adding a solution (10 μL) containing NaOH (4%) and $NaBH_4$ (0.4%) to the algal sample and allowing it to steep for 30 min, inverting the reaction tube for a further 45 min to drain excess solution from the alga, removing the excess solution, and then heating the swollen algal sample to 80 °C for 3 h. The tube was then cooled and the product was neutralised with sodium dihydrogen phosphate (10 μL of a solution containing 179 g L^{-1}). After standing for 45 min, the algal sample was dried in a vacuum desiccator over silica gel.

GLC analysis for polysaccharide and floridoside composition was carried out on a Supelco SP™ 2330 capillary column (15 m × 0.25 mm i.d.) at 220 °C isothermal, or held at 185 °C for 2.5 min, then raised at 10 °C/min to 220 °C and held for 8 min. The carrier gas was H_2. A Hewlett–Packard 5890 series II instrument with FID detection was used. GLC response factors were determined using standard mixtures containing *myo*-inositol hexaacetate as the internal standard. Composition is expressed as normalised weight percent of hexose for the galactan components (Tables 1 and 2). The composition of non-galactan polysaccharide and other components is also normalised as a percentage of the sum of the galactan components. The content of G6S units in the algal polysaccharides is expressed in terms of AG units, that is, as being the difference between the AG content in alkali-modified and unmodified samples of alga. The content of D-galactopyranosyl residues (Gal units) in the algal polysaccharide of tip samples was determined from the galactopyranose content of alkali-modified samples (Table 1), by correcting for the small amount of galactopyranose derived from floridoside. To do this, the glycerol content was assumed to be a measure of the floridoside content. The adjacent segment samples were washed as described above, so this correction was not necessary.

Isotope enrichment.—GCMS analysis for isotope enrichment was carried out on a Hewlett–Packard MSD 5970 spectrometer fitted with a Hewlett–Packard Ultra-2 col-

Table 1
Constituent sugar composition of algal samples (tip samples, normalised weight percentage) [a]

Sample	G6S*	AG	6MG	Gal*	Glc	Gro
2	10.3	36.7	14.1	49.2	4.9	1.7
2B	–	47.0	15.1	37.9	5.1	
3	10.2	33.8	14.9	51.4	7.1	2.0
3B	–	44.0	15.2	40.8	7.7	
4	13.6	32.7	13.2	54.0	5.4	1.6
4B	–	46.3	13.9	39.8	5.8	
5	13.4	32.6	14.7	52.7	8.4	2.5
5B	–	46.0	16.9	37.2	7.0	
6	11.6	34.7	14.8	50.5	8.9	2.0
6B	–	46.3	13.8	39.9	11.1	
7	16.0	28.4	14.7	56.9	16.6	1.6
7B	–	44.4	16.0	39.6	17.6	
8	17.5	25.0	12.7	62.4	21.7	2.7
8B	–	42.5	15.4	42.1	30.7	
9	14.9	31.0	15.1	53.9	5.7	1.0
9B	–	45.9	16.4	37.8	6.8	
10	14.0	29.6	13.4	57.0	6.7	2.6
10B	–	43.6	13.6	42.8	9.2	
11	13.7	32.7	13.5	53.8	6.9	1.9
11B	–	46.4	14.6	39.1	7.9	
12	11.3	34.0	14.5	51.5	12.4	1.8
12B	–	45.3	14.5	40.2	10.1	
13	14.4	34.5	15.2	50.3	5.0	0
13B	–	49.0	15.0	36.0	5.1	
14	17.6	28.0	12.6	59.4	14.9	2.2
14B	–	45.6	13.3	41.1	12.3	
15	17.8	24.7	12.9	62.4	13.7	2.2
15B	–	42.5	12.1	45.5	12.3	
16	22.9	21.3	12.0	66.7	13.1	2.7
16B	–	44.2	11.7	44.1	19.5	

[a] G6S* = Galactopyranose 6-sulfate (determined as ΔAG between native and alkali-modified algal samples); AG = 3,6-anhydrogalactopyranose; 6MG = 6-*O*-methylgalactopyranose; Gal* = galactopyranose; Glc = glucopyranose; Gro = glycerol; B = alkali-modified algal sample.

umn (20 m × 0.2 mm i.d.), which was held at 50 °C (2 min), raised at 35 °C/min to 180 °C and held for 1 min, then raised at 50 °C/min to 220 °C and held for 15 min. The carrier gas was helium. Ionisation was by EI at 70 eV. Data were accumulated by scanning in a total ion abundance mode from m/z 44 to 350. The ion intensities of each component were integrated over the whole peak to allow for the small differences in elution times for species with differing isotopic compositions.

While 6-*O*-methyl-D-galactopyranosyl residues (6MG units) and AG units of the agar polysaccharides give alditol acetates that are well resolved by GLC, the reductive hydrolysis procedure converts both G6S units and Gal units into the same galactitol hexaacetate. In addition, floridoside is also reductively hydrolysed to give galactitol hexaacetate plus glycerol triacetate. The galactitol hexaacetate peak is therefore derived from two constituent units of the agar polysaccharides and one component of florido-

Table 2
Constituent sugar composition of algal samples (adjacent segment samples, normalised weight percentage) [a]

Sample	G6S*	AG	6MG	Gal*	Glc
2	11.9	37.8	13.1	49.1	5.0
2B	–	49.7	14.3	36.0	6.6
3	12.6	37.4	13.2	49.4	7.3
3B	–	50.0	13.4	36.6	7.7
4	12.4	37.6	13.4	49.0	5.7
4B	–	50.0	13.6	36.4	5.8
5	12.6	35.2	14.5	50.3	6.9
5B	–	47.8	15.0	37.3	9.9
6	14.1	35.4	13.8	50.9	9.6
6B	–	49.5	13.2	37.3	7.1
7	15.4	31.7	14.8	53.5	18.2
7B	–	47.1	15.6	37.3	16.0
8	14.9	33.3	13.7	53.1	32.7
8B	–	48.2	14.7	37.1	27.8
9	17.8	32.0	13.5	54.5	4.8
9B	–	49.8	14.2	36.0	6.0
10	14.4	35.9	13.5	50.5	6.0
10B	–	50.3	13.3	36.4	6.6
11	17.7	34.8	13.0	52.2	5.6
11B	–	52.5	12.4	35.2	6.7
12	15.4	37.1	13.6	49.4	10.7
12B	–	52.5	13.1	34.4	11.0
13	14.8	36.4	13.9	49.7	4.6
13B	–	51.2	13.7	35.2	5.8
14	18.9	31.2	13.4	55.4	13.9
14B	–	50.1	14.1	35.8	12.8
15	21.8	25.8	13.1	61.1	14.0
15B	–	47.6	14.6	37.8	16.8
16	23.5	24.7	14.5	60.8	16.1
16B	–	48.2	14.8	37.0	16.9

[a] G6S* = Galactopyranose 6-sulfate (determined as ΔAG between native and alkali-modified algal samples); AG = 3,6-anhydrogalactopyranose; 6MG = 6-*O*-methylgalactopyranose; Gal* = galactopyranose; Glc = glucopyranose; B = alkali-modified algal sample.

side. When the algal sample is alkali-modified prior to the reductive hydrolysis procedure (see above), the G6S units are converted into AG units and their contribution to the galactitol hexaacetate peak is removed. The floridoside contribution is either removed by washing the algal sample or corrected for as described below.

Under the EI conditions used, molecular ions are not detected for alditol acetate derivatives. Therefore, the following high intensity fragment ions were used to follow and determine [^{13}C] isotope enrichment of the various units of the agar polysaccharides, floridean starch, and floridoside: m/z 115 given by galactitol hexaacetate, 6-*O*-methyl-D-galactitol pentaacetate, and glucitol hexaacetate from floridean starch; m/z 129 given by 6-*O*-methyl-D-galactitol pentaacetate; m/z 85 given by 3,6-anhydro-L-galactitol tetraacetate; and m/z 103 given by glycerol triacetate from floridoside.

Primary ions are formed from alditol acetates by elimination of an acetoxyl group or

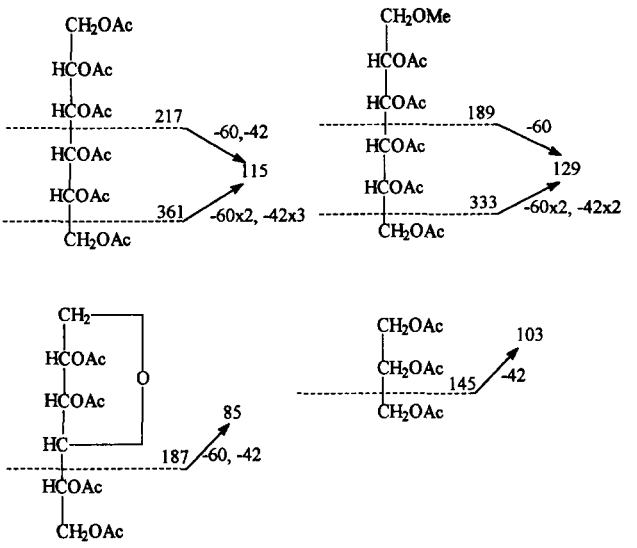


Fig. 2. EIMS fragmentation of alditol acetates.

by cleavage of the alditol chain [16]. The primary fragments are then degraded by elimination of acetic acid (60), ketene (42), or acetic anhydride (102).

Fragment ions m/z 115, 129, 85, and 103 can be formed as outlined in Fig. 2.

We define fractional isotope enrichment (FE) as the fractional increase in the ratio of [^{13}C]-labelled to unlabelled fragment ions relative to the natural abundance ratio. FE is a measure of the increase in the proportion of [^{13}C]-labelled molecules of a given type of polysaccharide unit, that is, of the biosynthesis of molecules of that type of unit. We calculate FE values and convert them into percentage isotope enrichment (%ER) values as shown below and then use these values to compare the timing of formation of the different types of polysaccharide units and to follow that biosynthesis as the culture conditions of the alga are changed. However, it should be noted that %ER values are not a quantitative measure of the amount of synthesis of a particular type of unit and they cannot be used to make quantitative comparisons between units. This is primarily because they are based on fragment ions and because the fragments given by the individual units have different structures.

FE values were calculated directly, or indirectly (see below), using the following formula, except in the case of 6MG units, where $x = 1$ instead of 1–4 (see below):

$$\mathrm{FE} = \left[\sum_{x=1}^{4} (I_{m+x}/I_m)_{\mathrm{L}} - \sum_{x=1}^{4} (I_{m+x}/I_m)_{\mathrm{N}} \right] \Bigg/ \sum_{x=1}^{4} (I_{m+x}/I_m)_{\mathrm{N}}$$

where L and N refer to [^{13}C]-enriched and natural abundance algal samples, respectively, I_m is the abundance of the ion containing only ^{12}C atoms, I_{m+x} is the abundance of the ion containing 'x' ^{13}C atoms, and m is the m/z of the ions containing only ^{12}C atoms. This method utilises the increased sensitivity gained by including, in the assessment of isotope enrichment, ions containing more than one ^{13}C atom. However, it

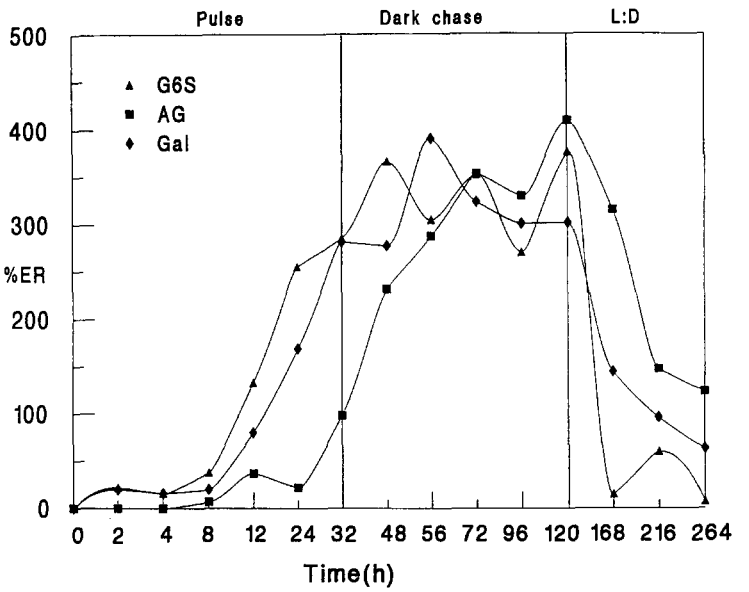


Fig. 3. Percentage ER of AG, G6S, and Gal in frond tip samples 2–16.

cannot be used to determine the FE of 6MG units, because the derivative prepared from this unit gives no fragment with ions of m/z greater than $m+1$ that are both sufficiently intense and free from overlap with ions given by another fragment. In this case, only $x=1$ was put into the above equation and the isotope enrichment of the fragment ion m/z 115 (see above), which is given by both 6-*O*-methylated and non-methylated Gal units was used to compare the enrichment of the 6MG units with that of the agar Gal units (see below). The enrichment of the 6-*O*-methylated units could then be related to that of the other major units (AG and G6S) via the $x=(1-4)$ calculations for the agar Gal units. Natural abundance ratios $(I_{m+x}/I_m)_N$ for all fragments were determined using samples taken prior to the labelling pulse. The enrichment values were finally 'normalised' with respect to the agar D-galactopyranose content of the sample and expressed as a percentage using the formula:

$$\%ER = 10^2\ FE \times (mol\%X/mol\%Gal)$$

where X = AG, G6S, 6MG. Although this operation does not, in general, give values that allow quantitative comparison of the amount of formation of one type of unit with that of a different type, it does allow the time courses for the formation of the different types of units to be displayed and compared on a single plot, as shown, for example, in Figs. 3 and 5. The enrichment of glucose and glycerol was not normalised and, therefore, $\%ER = 10^2$ FE for these compounds. The uncertainty in %ER is estimated to be $\pm 10\%$ for the units whose I_{m+x}/I_m ratios could be determined directly, that is, Gal (in washed, alkali-modified samples), AG, and 6MG units.

As noted above, the I_{m+x}/I_m ratio cannot be directly determined for G6S and agar Gal units. The following equation was used to determine the ratio for G6S units:

$$(I_{m+x}/I_m)_{G6S} = (A/Pr + 1)(I_{m+x}/I_m)_{ABT} - (A/Pr)(I_{m+x}/I_m)_{AUT}$$

where x is 1–4, A is the percentage of AG units in the polysaccharides, Pr is the percentage of G6S units in the polysaccharides (expressed in terms of AG units), and ABT and AUT refer to the ion abundance ratios for 3,6-anhydrogalactitol tetraacetate in alkali-modified and untreated algal samples, respectively.

Similarly, the following equation was used to correct the ratio measured for D-galactopyranosyl residues in alkali-modified tip samples for the galactopyranose component of floridoside.

$$(I_{m+x}/I_m)_{Gal} = (2GLY/G + 1)(I_{m+x}/I_m)_{GBT} - (2GLY/G)(I_{m+x}/I_m)_{GLYUT}$$

where G and GLY are the weight percentages of agar galactitol and glycerol, respectively, in the sample, as determined from the GLC analyses, and GBT and GLYUT refer to the ion abundance ratios for galactitol hexaacetate and glycerol triacetate in alkali-modified and untreated algal samples, respectively.

In this derivation, it was assumed that all the glycerol came from floridoside and that the labelling in the glycerol could be used as a measure of that in the D-galactopyranose component of this compound. Examination of the cold-water washings of algal samples showed that these assumptions were reasonable.

3. Results and discussion

Design and performance of algal labelling experiments.—Ekman et al. [8] found that transferring a growing sample of cultured *Gracilaria sordida* W. Nelson (now known as *G. chilensis* Bird, McLachlan *et* Oliveira) to aerated seawater and excluding light lowered the levels of floridean starch and floridoside. They also found that altering the salinity of the seawater during the dark treatment could affect agar yield and suggested that carbon from floridean starch and floridoside could be being used in agar biosynthesis.

We found that manipulating salinity killed our algal samples, but a dark treatment procedure (see Section 2) was used prior to labelling in order to lower the levels of floridean starch and floridoside, and reduce the potential for unlabelled carbon from these pools diluting the ^{13}C label going into polysaccharide biosynthesis. In the experiment described below a 32-h labelling pulse was followed by a dark chase of 88-h duration, during which CO_2 was withheld. This was designed to maximise isotope enrichment of newly synthesised agar polysaccharide material, as any carbon used for biosynthesis and other cell processes during this dark phase would have to come from storage compounds. (The slow aeration was calculated to be a negligible source of CO_2.) The dark chase was followed by an 8:16 h light:dark regime of 144 h, without reintroduction of CO_2, in order to observe the behaviour of the labelled products without diluting them with newly synthesised unlabelled material. This procedure was developed as it is easy to get label into storage compounds such as floridean starch and floridoside,

but it is more difficult to get detectable labelling of cell-wall galactan polysaccharide [11,12].

We examined two series of samples, frond tips and the segment adjacent to the tip, as a prelude to studying biosynthetic activity in different regions of the thallus.

Formation of agar D-*galactopyranosyl, 6-O-sulfo-*L-*galactopyranosyl, and 3,6-anhydro-*L-*galactopyranosyl residues (Gal, G6S, and AG units, respectively) in frond tips.*—A plot of percentage [^{13}C] isotope enrichment (%ER) of Gal, G6S, and AG units in frond tips (samples 2–16) versus pulse–chase time is shown in Fig. 3 for a labelling experiment carried out as described above and in Section 2. (Also see this section for our definition and determination of %ER and, in particular, the section describing the limitations on comparing %ER values.)

Isotope enrichment appears in both Gal and G6S units within 2 h of pulse-feeding $NaH^{13}CO_3$, but a significant increase was evident only after 12 h. Enrichment appears later in AG units, around 8 h into the 32-h pulse and is significant by 32 h. For both Gal and G6S units, enrichment peaks early in the dark chase period and then levels out for the remainder of the 88-h period. In contrast, enrichment of AG units increases throughout the pulse and dark chase periods, which, together with the later appearance of enrichment, is consistent with AG being formed from G6S units by intramolecular displacement of the sulfate group. During the 8:16 h L:D (light:dark) regime, enrichment falls sharply for all three types of unit when CO_2 is withheld. In contrast, if natural abundance CO_2 was not withheld, a much smaller fall was observed for Gal and G6S units and there was little change for AG units over a period of 96 h (data not shown). Formation of these units during the labelling pulse was accompanied by a rapid rise in both the weight percentage and isotopic enrichment of glucosyl residues in the algal sample (Fig. 4). These residues are assumed to be primarily in the storage polymer floridean starch.

The behaviour described above is consistent with relatively rapid, concurrent biosynthesis of Gal and G6S units during the labelling pulse and considerably slower formation of ring-closed AG units. Labelled AG units continue to be formed throughout the dark chase, which suggests that the cyclisation process does not require light, while formation of labelled Gal and G6S units appears to cease. Isotope enrichment in G6S and Gal units peaks early in the dark chase (Fig. 3), while at the same time the weight percentage of glucosyl residues and their isotopic enrichment fall rapidly (Fig. 4). This suggests that floridean starch formed during the pulse is being utilised for agar polysaccharide biosynthesis early in the dark chase, when light and CO_2 are being withheld. The rise in %ER of AG units is not accompanied by a fall in %ER of G6S units, an observation that is discussed in the next section.

During the 144-h L:D period the enrichment values fall sharply for all three types of agar constituent unit. This cannot be attributed to dilution with newly synthesised material made from natural abundance CO_2, as CO_2 was withheld, nor to utilisation of floridean starch as a carbon source, as glucose levels rise and enrichment falls. Destructive utilisation of the newly synthesised material in response to CO_2 deprivation is one possible explanation, but this is a surprising observation which merits further investigation. Although the plant cell wall is in general a dynamic structure, to our knowledge there is no evidence for metabolism of agar polysaccharides in the algae [7].

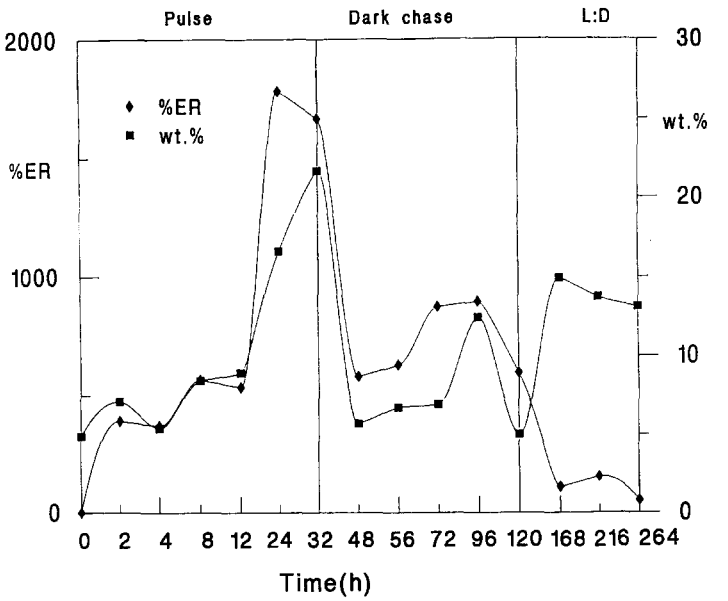


Fig. 4. Percentage ER and weight percentage glucose in frond tip samples 2–16.

In this respect, agar polysaccharides appear to resemble the fibrillar polysaccharides cellulose and xylans in being stable to endogenous enzymes, but degraded by enzymes from other sources such as bacteria [7]. In contrast, when natural abundance CO_2 is supplied during this L:D period, falls in the enrichment of Gal and G6S units are much smaller and the enrichment of AG units tends to reach a plateau, all of which can be attributed to dilution with newly synthesised material (data not shown). The carbon source for the glucose (floridean starch) biosynthesis observed during this period is unclear, but it is noteworthy that the synthesis occurs only during the first 48 h of the L:D chase.

Formation of Gal, G6S, and AG units in the frond segment adjacent to the tip.—The formation of all three types of unit in the segment adjacent to the frond tip was similar in general to that described above for the tips (Fig. 5), except that there appears to be a substantial fall in the enrichment of G6S units and a greater increase in the enrichment of AG units during the later part of the dark chase period. A considerably lower maximum %ER for Gal units relative to G6S and AG units is also observed.

When biosynthesis of the G6S units ceases, a fall in the enrichment of G6S units and concurrent rise in the enrichment of AG units might be expected to accompany formation of AG units from G6S units, if the intramolecular displacement of the sulfate group involves only newly formed, labelled units. However, if the total pool of G6S units is involved, no change would be expected. The reason for the observed fall in the %ER of G6S units with these samples and not the tip samples is not yet understood. In the case of the tip samples, it is possible that a fall was masked by experimental error, as the changes in %ER for AG units were considerably smaller over the same time interval

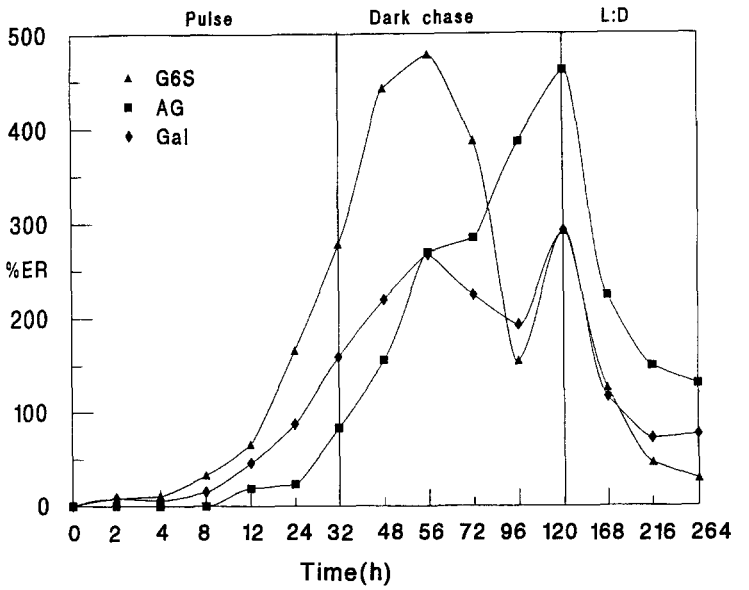


Fig. 5. %ER of AG, G6S, and Gal in adjacent segment samples 2–16.

and the uncertainty in the %ER values for G6S units is large ($> \pm 10\%$), due to the indirect method used to determine these values (see Section 2).

The trends in the formation of glucosyl residues (data not shown) were very similar to those found with the frond tips, so that the sharp fall in the %ER and weight percentage during the first 16 h of the dark chase again indicates biosynthesis of agar polysaccharide units from floridean starch.

The galactan compositions of the frond tips and the adjacent segment were much the same, but an apparently correlated rise in the level of G6S units and fall in the level of AG units during the 8:16 h L:D chase (samples 14–16 in Tables 1 and 2) is noteworthy. It is not clear what the latter signifies. This behaviour was not observed when CO_2 was reintroduced during the L:D chase (data not shown). These results, together with those described above, suggest that unusual processes may be initiated when CO_2 is withheld in the presence of light.

Formation of 6-O-methylated-D-galactopyranosyl residues (6MG units) in frond tips and the adjacent segment.—The plots shown in Fig. 6 are less sensitive than those for the other major units because they are based on I_{m+1}/I_m rather than $\Sigma_{x=1}^{4}(I_{m+x}/I_m)$ ratios (see Section 2). The enrichment measurements are based on the non-methylated fragment m/z 115, which is prominent in the mass spectrum of the alditol acetates of both 6MG and Gal units. Enrichment of the sugar carbons as opposed to the methyl carbon of 6MG units in frond tips follows that of Gal units quite closely throughout the experiment (Fig. 6). Formation of this unit is therefore concurrent with formation of Gal units and, therefore, also with formation of G6S units (see Section 2). Likewise, formation of 6MG units in the segment adjacent to the tips is concurrent with that of Gal units and, consequently, again concurrent with formation of G6S units (data not shown).

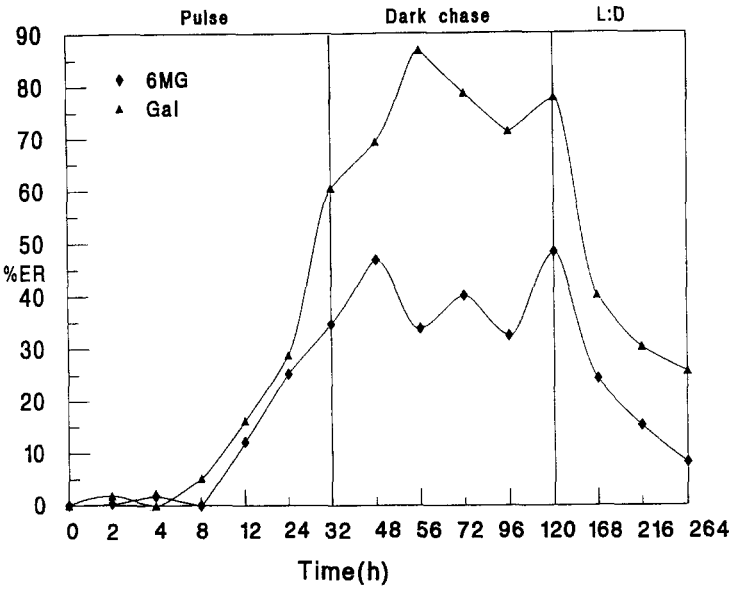


Fig. 6. %ER of 6MG and Gal in frond tip samples 2–16.

One pathway for formation of 6MG units in this algal species is therefore a process involving rapid partial methylation of newly forming Gal units. Variations in the timing of methylation between species have been reported by Lahaye et al. [17]. We attempted to probe for a pathway via methylation of older material, that is, methylation at a later stage in biosynthesis, by comparing enrichment of sugar carbon with that of methyl carbon. To do this, enrichment of the non-methylated fragment m/z 115 is compared with that of its methylated partner m/z 129, which can contain the label in either the methyl group or one of the sugar carbons of this fragment (see Section 2). Enrichment plots based on these two fragments are shown in Fig. 7 for frond tips. The plots for the adjacent segment samples are similar and therefore not shown. In both cases, enrichment based on either fragment follows a similar course, but that based on the methylated fragment attains a somewhat higher maximum level and tends to be consistently higher throughout the experiment. This suggests that a proportion of the 6-*O*-methylation activity could be methylation of older material, since with our method of calculation, if there was no methylation of older material, we would expect %ER values based on the methylated fragment to be less than those based on the non-methylated fragment, provided there is labelling of the methyl group of the methylating agent. Methylation activity ceases during the dark chase, so the process may require light, although it could also be due to depletion of the methylating agent, for example, lack of ATP, hence lack of *S*-adenosylmethionine, if this is the methylating agent [7]. Little is known about the mechanism or the *O*-methylating agent in red algae [3,5,7]. This strain of *Gracilaria chilensis* contains only trace amounts (ca. 1% and $< 1\%$, respectively) of 2- and 4-*O*-methylated units, so it is unsuitable for studying the biosynthesis of these units.

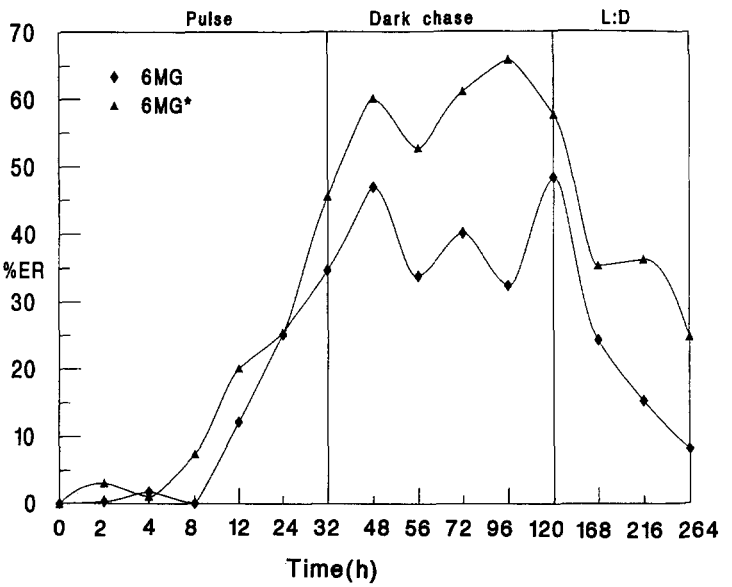


Fig. 7. %ER of sugar (6MG) [cf. sugar + methyl carbon (6MG *) of 6MG] in frond tip samples 2–16.

Early biosynthesis.—Only small amounts of the agar polysaccharide units appear to be formed during the first 8 h of the labelling pulse, after which the amounts tend to increase sharply. The formation of glucopyranosyl residues shows somewhat similar behaviour, although the amount produced in the first 8 h is estimated to be considerably greater than the amounts of the agar units. There seems to be no obvious explanation for this observation, other than that it represents a delay in the restart of their biosynthesis after a shutdown during the prepulse dark treatment. Throughout the labelling pulse, the amount of glucopyranosyl residues formed is also estimated to exceed that of galactopyranosyl residues by a considerable factor, which indicates a priority given to floridean starch synthesis, assuming that the majority of the glucopyranosyl residues are contained in this polymer.

4. Conclusions

We have developed a procedure for achieving and monitoring [^{13}C]-labelling of the individual units of the agar polysaccharides of *Gracilaria chilensis*. Using this procedure, sulfation to form 4-linked 6-*O*-sulfo-L-galactopyranosyl residues has been shown to be an early process in the biosynthesis of agar polysaccharide units and ring closure converting these residues into 4-linked 3,6-anhydro-L-galactopyranosyl residues has been shown to occur later. Methylation was also found to be an early process in this algal species, although some methylation of older material may also occur. There was little difference between these processes in frond tips and the adjacent segment. Utilisation of floridean starch for agar polysaccharide biosynthesis in the absence of

light and CO_2 may be significant when its level is high, while unusual processes may occur when the alga is CO_2-starved in the presence of light.

Acknowledgements

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