PHOTOSYNTHETIC PREPARATION OF GALACTOSE-$^{13}C_6$ AND GLYCEROL-$^{13}C_3$ USING A MARINE RED ALGA

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SUMMARY

The photosynthetic preparation of D-galactose-$^{13}C_6$ and glycerol-$^{13}C_3$ from *Gigartina corymbifera* is described. The red marine algae biosynthesize the galactoside 2-hydroxy-1-(hydroxymethyl)ethyl α-D-galactopyranoside (galactosylglycerol) during photosynthesis. The galactoside was isolated from an alcohol extract by an acetylation procedure and hydrolyzed to D-galactose and glycerol. The average enrichment of the isolated products was 55 mol % ^{13}C while the actual enrichment of newly synthesized material, determined by ^{13}C nmr, was 80 mol % ^{13}C after a photosynthetic period of 48 hr during which 90 mol % $^{13}CO_2$ was administered. Thirty percent of the administered carbon was recovered in the products. The distribution of ^{13}C in the galactose and glycerol was shown to be uniform by ^{13}C nmr.

Key Words: *Gigartina*, Algae, Carbon-13, ^{13}C-nmr, Carbohydrates.

INTRODUCTION

Methods for the preparation of small quantities of ^{14}C labeled compounds using photosynthetic organisms have been described by Putman *et al.* (1,2) and Bean and Hassid (3). More recently, these techniques have been modified and applied to the photosynthetic

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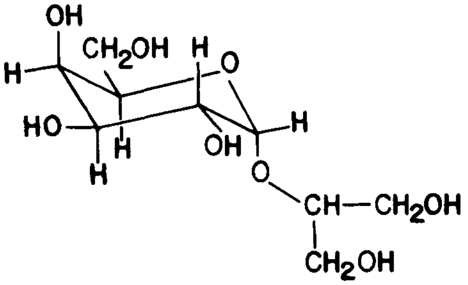
preparation of small amounts of labeled material using the short-lived isotope ^{11}C (4). Bidwell (5) and Bean and Hassid (6) have described the products of photosynthesis using ^{14}C labeling for several types of brown, red, and green algae. The primary carbohy-hydrates obtained from the red algae were galactosylglycerol and/or a glycoside of mannose. Here we describe the biosynthesis of ^{13}C labeled 2-hydroxyl-1-(hydroxymethyl)ethyl α-D-galactopyranoside (galactosylglycerol) from *Gigartina corymbifera*.



This organism has been used to obtain multigram quantities of D-galactose-$^{13}C_6$ for use in clinical studies in the evaluation of carbohydrate metabolism (7).

RESULTS AND DISCUSSION

Several parameters were investigated to maximize conversion of $^{13}CO_2$ to D-galactose-$^{13}C_6$ and glycerol-$^{13}C_3$. Among these were: use of various light-dark cycles during photosynthetic incubation; different photosynthetic periods, dark incubation periods, and incubation temperatures. Only the incubation temperature and length of the photosynthetic period affected the conversion of CO_2 to galactosylglycerol. Pretreatment of the thalli (broad leaf-like portions of the organism which consists of undifferentiated cells) prior to photosynthetic incubation was not necessary. Experiments were done using continuous illumination for 48 hr while the thalli were kept at 15°C.

The rate of $^{13}CO_2$ uptake was 0.5 mg per hr per g of thalli during the first 20 hr of photosynthesis, 0.3 mg per hr per g of thalli from 24 to 26 hr, and 0.2 mg per hr per g of thalli for the next 23 hr. The CO_2 uptake measurements, which were taken at 4 hr intervals, reflect a decrease in the photosynthetic rate of the algae as the experiment progressed. A total of 60 g of $^{13}CO_2$ (90 mol % ^{13}C) was supplied to 4.6 kg of *G. corymbifera*. The galactosylglycerol-$^{13}C_9$ isolated (19.5 g, 55 mol % ^{13}C) represents a 30% recovery of ^{13}C. While ^{13}C recovery was improved (ca. 43%) by shorter incubation periods, the product was not uniformly labeled. The lower average enrichment level of the isolated galactosylglycerol, as compared with the $^{13}CO_2$ administered, reflected either the presence of unlabeled endogenous material, or dilution of the $^{13}CO_2$ by natural abundance CO_2 produced by oxidative metabolism of the thalli. The ^{13}C nmr analysis of product samples taken at various times during the photosynthetic period showed that the first process was dominant.

In biosynthetic labeling, where the enrichment of the precursor differs significantly from that of the product, the distribution of labeled atoms within the product is important. Tracer studies with ^{14}C (6,8) indicated that galactosylglycerol-$^{14}C_9$ was rapidly labeled; after 4 min, the galactose-$^{14}C_6$/glycerol-$^{14}C_3$ activity ratio was approximately 2:1. As was pointed out by Bean and Hassid (6), however, a number of nonequivalent labeling schemes can lead to the same ratio.

^{13}C nmr spectra of galactosylglycerol-$^{13}C_9$ showed that the intensities of the ^{13}C resonances corresponding to the galactose and glycerol carbons for highly enriched material (i.e. samples incubated with 90 mol % $^{13}CO_2$ for $\geq$ 12 hr) are consistent with equivalent labeling of all carbons in the galactosylglycerol molecule. A more detailed analysis of the labeling pattern was done using the C-2 resonances of glycerol since these resonances are always well resolved from other ^{13}C resonance peaks. The analyses can be performed on either glycerol, galactosylglycerol, or the hexa-O-acetylgalactosylglycerol and, as described below, analysis of this peak allows a separate determination of the labeling pattern only in the newly synthesized galactoside. The ^{13}C spectrum of the glycerol

C-2 carbon is a sum of the multiplets each of which corresponds to a specific isotope isomer (Fig. 1). Although the two central lines of the quartet, which correspond to the isomer having all three glycerol carbons labeled, overlap the singlet resonance corresponding to labeling of glycerol C-2 only, the intensity of the former can be calculated from the intensity of the two outer quartet lines (Fig. 1). Subtraction from the observed intensity of the central resonances then gives the singlet intensity.

Since endogenous unlabeled galactosylglycerol makes a negligible contribution to the intensities of the doublet and quartet peaks, the intensities of these multiplets can be used to estimate the enrichment of the newly synthesized galactosylglycerol. The analysis is based on the assumption of uniform random labeling of all carbon atoms. Several other nmr parameters, notably the average intensities of the galactose and glycerol carbon resonances are consistent with this assumption. With the $^{13}C/^{12}C$ ratio (55 mol %) established by combustion for the galactoside, it was then possible, by analysis of the central resonance of the C-2 multiplet, to obtain a ratio for the amounts of newly synthesized and endogenous galactosylglycerol. At 48 hr this ratio was found to be 0.51.

The kinetics of ^{13}C enrichment can be followed by obtaining nmr spectra of the enriched galactoside from algae samples taken at various incubation times. Such spectra confirm the presence of significant levels of endogenous galactosylglycerol. Thus, the early labeling of galactosylglycerol is significantly below the level of the administered $^{13}CO_2$ but gradually increases toward isotopic equilibrium. Average enrichment of the newly synthesized material after 40 hr of incubation is 80 mol % ^{13}C (9). Multiplet analysis is also useful for obtaining information on the distribution of the ^{13}C label. In a series of samples incubated under different lighting conditions, significant alterations in the labeling patterns were apparent although the average ^{13}C enrichment of the galactoside, based on the $^{13}C/^{12}C$ ratio, indicated equivalent average labeling (10). Comparison of the doublet and quartet intensities for the glycerol C-2 carbon from samples taken after 48 hr of continuous illumination, alternating 12 hr light/12 hr dark cycles, and alternating 18 hr light/6 hr dark cycles, indicates that continuous illumination results in the highest quartet/doublet ratio, and consequently the highest ^{13}C enrichment of the newly synthesized galactoside (10). This is consistent with the expected increase in catabolic processes that produce unlabeled CO_2 during the dark periods.

Previous work by Bean *et al.* (2) using $^{14}CO_2$ and by Palmer and Goulding (4) using $^{11}CO_2$ involved the incubation of 1 g to 3 g of thalli. Due to the greater sensitivity of detection for ^{14}C and ^{11}C only small amounts of labeled material were necessary to meet their requirements. Since ^{13}C is usually detected by mass spectrometry or nmr larger amounts are required for analysis. To obtain the necessary quantities of D-galactose-$^{13}C_6$ for use in our clinical applications, it was necessary to use kg amounts of thalli per incubation and also to increase the yield of labeled galactoside per gram of thalli over that reported (17%) (8). To increase the average isotope enrichment of the product, we extended the continuous photosynthetic period from 11 hr (Bean *et al.*) (8) to 48 hr. The amount of galactoside obtained was about 0.5% of the fresh weight of the thalli.

EXPERIMENTAL

Materials and Methods

The algae were obtained fresh in 6-kg lots (Pacific Biomarine Supply Company, Venice, CA) within 24 hr of harvest from the ocean. The temperatures of the Pacific ocean along the southern California coast are suitable for vigorous growth of the algae from March through June of each year. Dormant algae give low yields of labeled product; therefore, experiments were done during periods when the algae were growing rapidly. Upon arrival in our laboratory, the thalli were cut into strips about 8-cm wide, and most of the reproductive nodules were removed. The algae were placed in polycarbonate incubation chambers. Illumination (70,000 lux) from above and below the chambers was by Sylvania VHO fluorescent lamps. The chambers were oscillated horizontally (28 cpm) to keep the nutrient medium mixed. The temperature within each chamber was maintained by an external cooling bath at 15°C. Construction details of the apparatus have been described (11).

Glycerol concentrations were determined by enzyme assay (Calbiochem Glycerol Stat Pack). Galactose concentrations were determined as follows: Tris-acetate buffer (0.9 ml, pH 8.6) was placed in a 1-ml quartz cuvette followed by addition of NAD solution (0.1 ml, 14 mM) and galactose dehydrogenase (5 μl, U/ml). The galactose solution (containing about 10 μg galactose) was added to the cuvette and the well mixed solution was allowed to stand for 30 min at room temperature. The absorbance at 340 nm was recorded and compared with standard solutions.

Pulse ^{13}C nmr spectra were obtained with a Varian XL-100-15 spectrometer (25 MHz) which was interfaced to a Nova 1210 computer.

Preparation of D-Galactose-$^{13}C_6$ and Glycerol-$^{13}C_3$

Fresh thalli strips (20-cm long x 8-cm wide) were placed in each incubation chamber (ca. 1.5 kg/chamber); the chambers were closed and each was partially filled with 12 liters of CO_2-free enriched seawater medium (12) that was precooled to 15°C. To start the automatic addition of $^{13}CO_2$ (90 mol %), 10 ml of aqueous potassium hydroxide (5 N) was injected into each chamber causing the pH to rise from 8.3 to 9.0. As $^{13}CO_2$ was added, the pH decreased to 8.2 (the operational pH). When each chamber contained $^{13}CO_2$, the incubation chambers were illuminated. As the algae fixed $^{13}CO_2$, the pH rose, and additional amounts of $^{13}CO_2$ were added automatically. A positive pressure was maintained inside the chamber by venting the gases through gas-washing bottles filled with CO_2-free 5N sodium hydroxide. The 4.6 kg of thalli fixed 60 g of $^{13}CO_2$ during the incubation period.

After 48-hr incubation, the thalli were removed from the chambers, washed with cold water, frozen in liquid nitrogen, ground into small pieces, and stored at -20°C. Wet-frozen thalli (4.6 kg) were placed in 16 ℓ of 95% ethanol and refluxed for 30 min. The extract was filtered and the extraction process repeated once using 80% ethanol. The cell debris was washed with 80% ethanol (2 x 1.5 ℓ) and discarded. The combined extracts and washings were concentrated to about 200 ml and filtered through Celite. Pyridine (200 ml) was added and the pyridine-water solution was evaporated under reduced pressure to give a thick brown syrup. The syrup was taken up in pyridine (300 ml); the precipitated salts were removed by filtration; and the solution evaporated to a syrup under reduced pressure. This procedure was repeated once. The syrup was dissolved in pyridine (400 ml) and cooled in an ice bath. Acetic anhydride (100 ml) was slowly added while keeping the temperature below 25°C. The resulting solution was placed in a refrigerator overnight and then kept in the dark at 25°C for 3 days. The solution which contained the acetylated galactoside, was poured into ice water (1.5 ℓ) producing an insoluble oil that solidified after stirring 2-3 hr. The tan solid was filtered, washed with cold water, and dried to give 47.4 g of tan needles, mp 99-100°C. Recrystallization from ethanol (Norit) gave, in three crops, 42.2 g of hexa-O-acetylgalactosylglycerol-$^{13}C_9$ as white needles, mp 102-103°C [reported (13) 100°C].

A solution of sodium methoxide [prepared from 12.6 g (0.55 mol) of sodium and 250 ml of methanol (14)] was added to a slurry of 42.2 g (82.6 mmol) of the acetylated galactoside suspended in methanol, and the mixture was stirred for 3 hr. Water (50 ml) was added and the solution was treated with 700 ml of Dowex 50 (H^+). The mixture was filtered and the resin was washed with water (4 x 250 ml). The filtrate was treated with Norit, filtered, and evaporated to a syrup.

The syrup was taken up in ethanol, concentrated and allowed to crystallize to give 19.5 g (91%) of galactosylglycerol-$^{13}C_9$ as white needles, mp 131-132°C [reported (13) 129°C]. The ^{13}C content of the galactosylglycerol, determined by combustion to CO_2 with sodium dichromate-sulfuric acid and mass spectrometric measurement of the $^{13}C/^{12}C$ ratio, was 55 mol %.

Galactosylglycerol-$^{13}C_9$ (19.5 g) was dissolved in trifluoracetic acid (780 ml, 0.1 N) and refluxed under nitrogen for 12 hr. Progress of the hydrolysis was monitored by measuring optical rotation at 589 nm and by enzymatic assays for galactose and glycerol. When the hydrolysis was completed, the solution was cooled, 500 ml of Dowex 1 (OH^-) was added and the solution was stirred for 15 min. The solution was filtered, and the resin was washed with water (5 x 500 ml). The pH of the filtrate was adjusted to 6.5 with 25 ml of Dowex 50 (H^+). The solution was filtered and the resin was washed with water (4 x 100 ml). The filtrate, which contained galactose and glycerol, was concentrated to a thick syrup. Galactose was crystallized from the mixture by the addition of ethanol to yield 12.7 g of colorless crystals, mp 167°C [reported (15) 167°C]. The filtrate from the crystallization was concentrated to a thick syrup and dissolved in water (15 ml). The solution, which contained mostly glycerol with small amounts of galactose, was placed on a column (5 x 100 cm) of Dowex 50 (X8, 200-400 mesh, Ba^{++}) resin (16) and eluted with water (flow rate 40 ml/hr, 6.7 ml/fraction). Galactose was eluted in a 50-ml volume after 870 ml of eluant had passed through the column and glycerol was eluted in 50 ml after 1 ℓ of eluant had been collected. The amount of glycerol and galactose obtained was 6.5 g and 0.7 g, respectively. The average $^{13}C/^{12}C$ ratio for each compound was 54 mol %.

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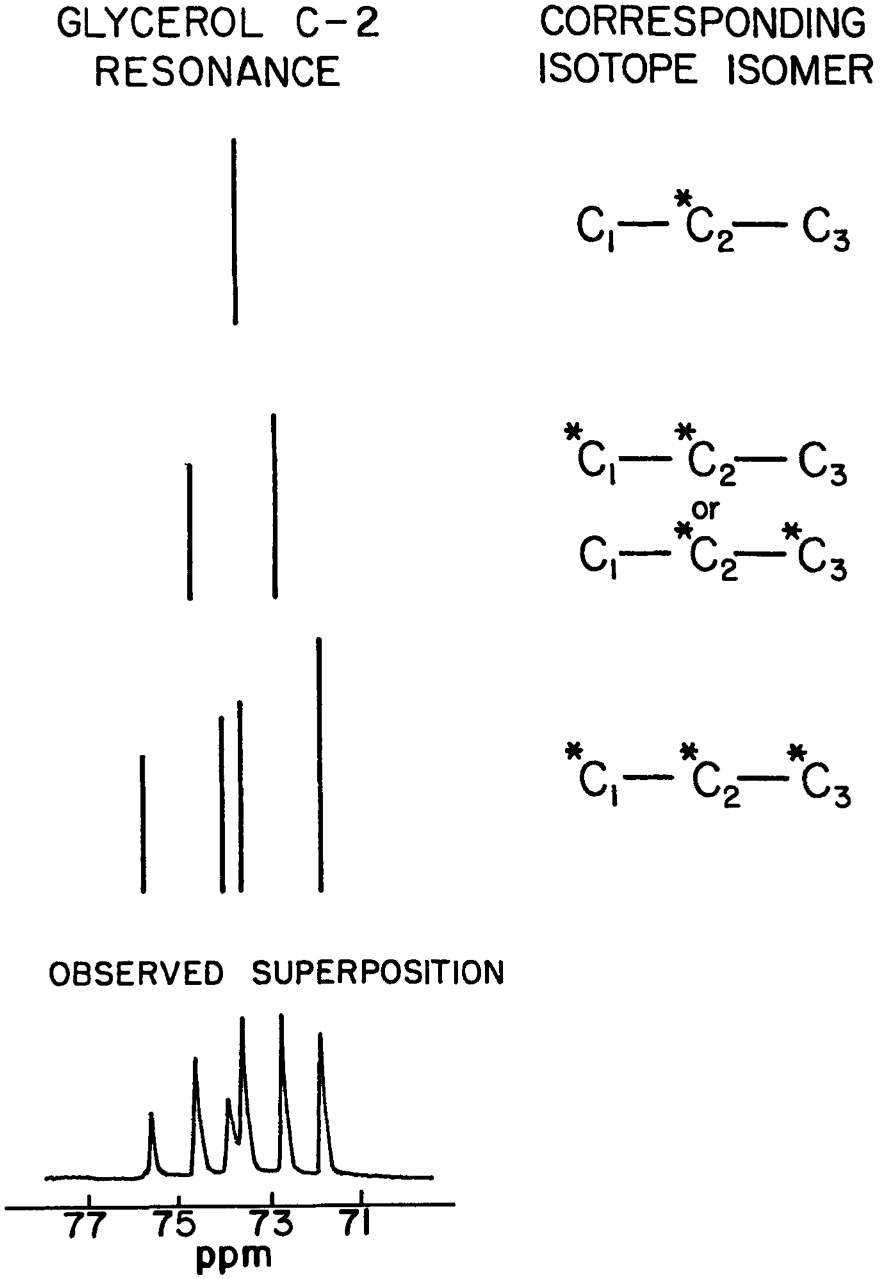

Fig. 1. The observed glycerol C-2 resonance is a superposition of the singlet, doublet, and quartet corresponding to the isotope isomers illustrated in the figure. The relative intensities of the lines in each multiplet were calculated using the J and Δν values obtained from the spectrum.