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PHOTOSYNTHETIC PREPARATION AND CHARACTERIZATION OF ^{13}C-LABELED CARBOHYDRATES IN *Agmenellum quadruplicatum*

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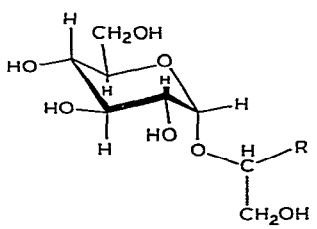
ABSTRACT

The blue-green alga *Agmenellum quadruplicatum* (strain PR6) has been used to prepare photobiosynthetically ^{13}C-labeled D-glucose, 2-*O*-(α-D-glucopyranosyl)-glyceric acid (glucosylglycerate), 2-hydroxy-1-(hydroxymethyl)ethyl α-D-glucopyranoside (glucosylglycerol), and α-D-glucopyranosyl β-D-fructofuranoside (sucrose). When grown to a cell density of 4.4 $g.L^{-1}$ (dry weight) under nitrate–nitrogen limiting growth conditions for 120 h, the algal cells contained 38% of the dry-cell weight as (1→4)-α-D-glucan (amylose). About 1% of the dry-cell weight was glucosylglycerol, glucosylglycerate, and sucrose. Glutamate was obtained, together with carbohydrates of low molecular weight, when the cells were extracted with chloroform–methanol; D-glucose was recovered from the extracted cells by acid hydrolysis of the starch. The algae were grown by using 20 mol% [^{13}C]carbon dioxide for preparation of labeled carbohydrates and for cellular component identification by whole-cell n.m.r. spectroscopy.

INTRODUCTION

We have described the biosynthesis of D-[U-^{13}C]glucose using the tobacco leaf[1]. This system gives a 50–60% recovery of ^{13}C as uniformly ^{13}C-labeled carbohydrates (starch, glucose, fructose, and sucrose); however, an 80- to 90-day plant growth-period is required, and the products contain some endogenous (unlabeled) material. The unlabeled material gives the labeled compounds a lower ^{13}C-enrichment



1 R = CO_2H
2 R = CH_2OH

level than the labeling precursor ([^{13}C]carbon dioxide). The blue-green alga *Agmenellum quadruplicatum* (strain PR6) has been shown to produce high levels of carbohydrate when grown on uric acid, although the composition and amounts of each type of carbohydrate present in the cell were not determined[2]. Here we report the biosynthesis of uniformly ^{13}C-labeled amylose from *A. quadruplicatum* (strain PR6) using nitrate-nitrogen, limiting-growth conditions. In addition, the minor carbohydrates [2-*O*-(α-D-glucopyranosyl)glyceric acid (glucosylglycerate, **1**), 2-hydroxy-1-(hydroxymethyl)ethyl α-D-glucopyranoside (glucosylglycerol, **2**), and α-D-glucopyranosyl β-D-fructofuranoside (sucrose)] have been identified by using ^{13}C n.m.r. spectroscopy.

The appearance and accumulation of carbohydrates of low molecular weight in *A. quadruplicatum* grown under nitrogen-limiting conditions are first described here; however, the presence of such other low-molecular-weight carbohydrates as galactosylglycerol[3], mannosylglycerate[4], sucrose[5], and polyhydroxy alcohols[6] has been reported for other algal types. The presence of these low-molecular-weight carbohydrates in algae may be a direct result of nitrogen depletion in the cell that is accompanied by a loss of cellular protein and an increase in intracellular salt content. These carbohydrates have been shown by Craigie and McLachlan[7] to play a role in osmoregulation; they have also shown that the intracellular level of carbohydrates of low molecular weight depends on the composition of the extracellular materials.

The preparation of uniformly ^{14}C-labeled glycogen (mg amounts) using *Anacystis nidulans* has been described by Lehman and Wöber[8]. Using nitrogen-limited incubation methods, they reported an accumulation of cell glycogen between 30 and 90 h; however, the glycogen content of the cells is related to the chlorophyll concentration which, as stated by the authors, cannot be measured accurately during the reported glycogen accumulation-period. They further reported a yield of 5% by dry weight for glycogen from cells after incubation. As discussed by Hough *et al.*[9], this level is within the normal concentration range when fresh-water blue-green algae are grown under the conditions described. In practice, we have not found the carbohydrate-to-chlorophyll ratio useful when algae are incubated and/or grown under nitrogen-limiting conditions.

EXPERIMENTAL

Apparatus. — The algae were grown in an apparatus consisting of three 40-L polycarbonate chambers joined together on a common oscillating mechanism[1,10]. The temperature of the medium (39°) in each chamber was controlled by a temperature controller that operated solenoid valves regulating the flow of coolant (at 0°) from external refrigerated baths. Air circulation around the growth chambers was provided by fans. The chambers were illuminated from above and below the plane of oscillation with very high-intensity natural–outdoor fluorescent lamps. The light intensity was 70,000 lux at the chamber surfaces. The chambers were oscillated horizontally (28 oscillations per min) to provide the necessary culture agitation. The pH of the

culture medium was maintained by the automatic addition of [^{13}C]carbon dioxide. The external pH meters and controllers were set to maintain the pH of the growth medium between 8.2 and 8.4. As carbon dioxide was fixed by the algae, the pH of the growth medium rose to 8.4, and additional carbon dioxide was added to decrease the pH of the medium to 8.2. A positive pressure (10 cm of water) was maintained inside the chambers by venting the contained gases through gas-washing bottles filled with carbon dioxide-free, 5M sodium hydroxide. Unused [^{13}C]carbon dioxide was recovered in this manner.

Cell culture. — The culture of blue-green algae was aseptically transferred from an agar slant to a 500-mL Bellco spinner flask and grown in liquid culture medium[11] for 3 days. When the culture density in the spinner flask reached 0.5 g.L^{-1} (dry weight), the culture was transferred to a 2.4-L flask and diluted with fresh growth medium. After 3 days of growth in the 2.4-L spinner flask, the algal suspension was transferred to 18 L of fresh growth medium. This solution was used to inoculate the growth chambers. Each of the three growth chambers was filled with 6 L of algal suspension and 14 L of fresh growth medium. Growth was monitored by measuring the culture density at 550 nm with a Bausch and Lomb Spectronic Model 20 spectrophotometer and by determining the dry weight of cells contained in 200 mL of growth medium. The change in culture density (dry weight of cells per liter of growth medium)

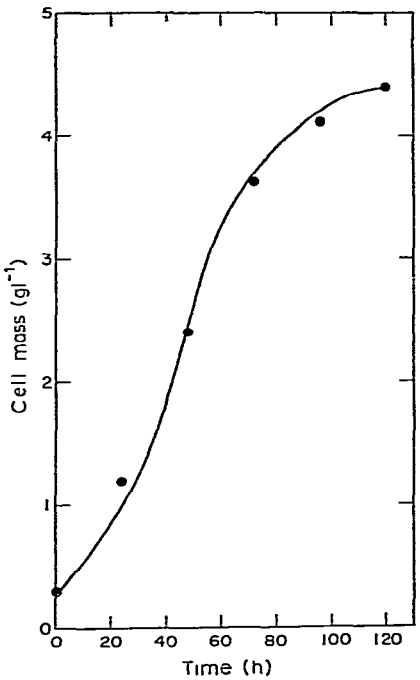


Fig. 1. *Agmenellum quadruplicatum* (strain PR6) cell mass per unit volume for a 120-h growth period. The dry-cell weight was determined by removing 200 mL of algal suspension from each growth chamber. The cells were removed from the growth medium by centrifugation (5000 × g for 10 min), resuspended in cold (4°) distilled water, washed twice, and lyophilized.

is shown as a function of time in Fig. 1. Growth was terminated after 120 h of incubation. The cell suspension was removed from the growth chambers and rapidly cooled to 4° in cold, physiological saline. The cells were collected by centrifugation with a Sharples Model 16 open-type, continuous-flow supercentrifuge and were lyophilized and stored at —20°.

Isolation of carbohydrates. — The labeled simple carbohydrates (glucosylglycerol, glucosylglycerate, and sucrose) were extracted from the dry algae with 2:1 (v/v) methanol–chloroform. The cells (25 g dry wt) were suspended in 1 L of the methanol–chloroform solution. The suspension was refluxed (65°) in a nitrogen atmosphere for 2.5 h, cooled to 25°, and filtered; the cell debris was washed with 500 mL of 2:1 (v/v) methanol–chloroform. The extract and wash solutions were cooled to 4° and set aside. The cell residue was lyophilized to give 21 g of cell material which was suspended in 900 mL of 0.25M trifluoroacetic acid (TFA). The solution was refluxed (95°) for about 24 h. When the maximum concentration of D-glucose was achieved, as measured by enzyme assay (Calbiochem glucose reagent), the mixture was cooled to 4° and the unhydrolyzed cell debris removed by centrifugation. The crude glucose solution was treated with 200 g of Dowex-1 × 2 (50–100 mesh, OH^-) to remove TFA, and the pH was adjusted to 6.5 with 10 g of Dowex-50W × 2 (50–100 mesh, H^+). The solution was concentrated to a thick syrup, and the glucose crystallized by adding ethanol. The crystalline glucose, m.p. 146–150°, weighed 13 g, and the specific rotation was $[\alpha]_D^{25}$ +51.2° (*c* 2.5, water).

The solvent was removed from the methanol–chloroform extract, which was dissolved in 25 mL of water, and chromatographed on a column of Sephadex G-15 (5 × 50 cm, water eluant, flow rate 60 $mL.h^{-1}$). The order of elution was salt, sugars, and chlorophyll–lipid. The eluant was monitored with a Waters Associates differential refractometer Model R404. The carbohydrate fractions were collected, concentrated to about 1 mL, and separated by chromatography on a 1 × 150-cm column of Dowex-50W × 8 (200–400 mesh, Ba^{2+}); the flow rate was[12] 20 $mL.h^{-1}$. The order of elution from the column of Dowex-50W with water was sucrose, glucosylglycerol, glucosylglycerate; the elution volumes were 7, 16, and 32 mL, respectively.

The carbohydrates were identified by their n.m.r. spectra and by enzymic assay. Sucrose was identified and the amount present determined enzymically by placing 1 mL of Calbiochem glucose reagent in a 1-mL quartz cuvette; a Gilford Model 240 spectrophotometer was nulled (340 nm) by using this solution. The sample to be assayed (5 μL) was added to the glucose reagent, and the absorbance (340 nm) was measured after 5 min. An increase in absorbance indicated the presence of glucose in the sample. Then 2 μL of phosphohexose isomerase (2000 units/mL) were added, and the absorbance (340 nm) was measured after 15 min. An increase in absorbance over that previously measured indicated that fructose was present in the solution. Additionally, 5 μL of invertase (23,600 units/mL in 0.1M acetate buffer at pH 4.6) was added to the cuvette, and a third absorbance (340 nm) measurement was made after 15 min. An increase in absorbance over the second measurement indicated the presence of sucrose in the sample. Comparison of the absorbance changes for the

addition of each enzyme, with standard absorbance curves for D-glucose, D-fructose, and sucrose, permitted quantification of each of the carbohydrates. This assay is specific for D-glucose, D-fructose, and sucrose, respectively, with the addition of each enzyme. The glucose and glycerol moieties of glucosylglycerol and glucosylglycerate were determined by Calbiochem glucose and glycerol reagents after 2 h of hydrolysis in 0.25M TFA. The glycerate moiety was determined by n.m.r. spectroscopy.

Nuclear magnetic resonance spectroscopy. — The ^{13}C-enriched cells of *A. quadruplicatum* that were to be used for n m.r. spectroscopy were taken aseptically from the growth chambers and rapidly cooled to 4°. They were removed from the

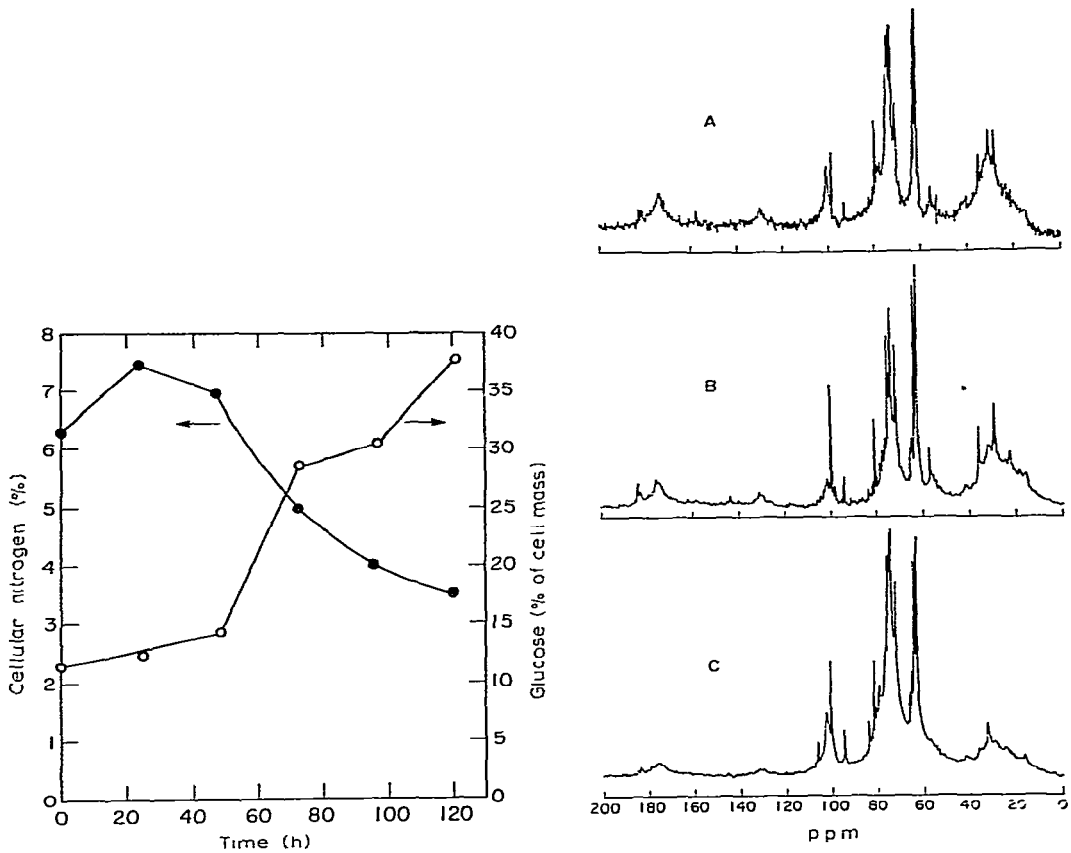


Fig 2. Cellular nitrogen (%) ●—● and cell D-glucose (% of dry cell weight) ○—○ plotted as a function of growth time. Cell nitrogen was determined by combustion and cell D-glucose concentration by enzymic assay after acid hydrolysis (0 25M trifluoroacetic acid) of the cellular starch.

Fig 3. Proton-decoupled n m r. spectra of washed whole cells of *A. quadruplicatum* (strain PR6) enriched to the 20 mol% level with ^{13}C The spectra correspond to (A) elapsed growth-time of 24 h, spectrum obtained at 50°, 20 000 pulses, (B) elapsed growth-time of 24 h, spectrum obtained at 27°, 69 000 pulses; and (C) elapsed growth-time of 116 h, spectrum obtained at 27°, 23,000 pulses Comparison of the intensity ratio of the aliphatic protein and lipid peaks ($\delta \sim 30$ p p.m.) to the carbohydrate peaks ($\delta \sim 70$ p p m.) between spectra (B) and (C) indicates a significant increase in the carbohydrate-to-protein ratio.

growth medium by centrifugation at 10,000 × g for 10 min. The cells were washed twice with 40-mL volumes of cold, normal, saline solution. After each wash, the cells were removed from the solution by centrifugation; they were resuspended by vortexing for 3 min. Following the second wash, the cells were suspended in 2.5 mL of cold, normal saline and placed in a 12-mm n.m.r. tube. A few drops of D_2O were added to the cell sample to provide a lock. The cell concentration in the n.m.r. sample was 0.25 g (dry weight) per mL of solution.

Pulsed ^{13}C n.m.r. spectra were obtained with a Varian Model XL-100-15 spectrometer (25 MHz) interfaced to a Nova Model 1210 computer. The spectra were recorded with a spectral width of 5000 Hz and 2048 spectral points. To better observe the broad resonances at high and low field, some spectra were recorded with a spectral width of 6666 Hz. The chemical shifts are reported relative to external tetramethylsilane.

RESULTS AND DISCUSSION

The growth rate of the algae is shown in Fig. 1. The cell mass produced during the 120-h growth period was 260 g (4.4 $g.L^{-1}$). The nitrate-nitrogen provided during growth was 0.2 mol for each of the three growth chambers; the nitrogen concentration was μM after 48 h of growth. Intracellular accumulation of nitrogen and glucose is shown as a function of elapsed growth time in Fig. 2. The intracellular nitrogen content decreased throughout the growth period after the first 24 h of culture (Fig. 2). Cell nitrogen changed from 7.5 to 3.5% during the nitrogen-limiting growth period. The cell glucose (amylose) concentration showed little change during the first 48 h of growth; however, when the *in vitro* nitrate-nitrogen was reduced to less than μM, the rate of cell glucose (amylose) accumulation increased. Glucose levels, as accumulated starch, increased from about 11% of the dry cell weight at 48 h to about 38% of the dry cell weight at 120 h; at this time, the growth period was terminated. The cell concentration and cellular carbohydrates did not change significantly with increased culture times up to 220 h.

In addition to monitoring the changes in carbohydrate content of the cells by enzyme assay, progress of the experiment was also monitored by n.m.r. spectroscopy. The experiments were carried out using 20 mol% ^{13}C cells to enhance sensitivity while minimizing complications due to ^{13}C–^{13}C coupling which is observed at higher enrichment levels. The general appearance of the proton-decoupled, ^{13}C whole-cell spectra of *A. quadruplicatum* (Fig. 3) resembles that of the ^{13}C spectra obtained for the blue-green alga *Anacystis nidulans*[13]. In Fig. 4, the region between 0 and 40 p.p.m. relative to external tetramethylsilane is dominated by resonances of aliphatic hydrocarbons corresponding to saturated portions of lipid molecules and saturated amino acid residues from cellular protein; the region of the spectrum between 60 and 110 p.p.m. is dominated by carbohydrate resonances arising from mono-, oligo-, and polysaccharides; the broad peak centered at 130 p.p.m. corresponds to unsaturated carbons of fatty acids, as well as aromatic carbon atoms of protein residues; and the broad

resonances in the region 170 to 180 p.p.m. correspond to carbonyl resonances of esterified fatty acids and peptide carbonyl groups. The general appearance of the spectrum (Fig. 3A) is similar to that for *A. nidulans* and is in striking contrast to the spectra obtained for cells having a high proportion of polyunsaturated fatty acids, which lead to sharp resonances in the aliphatic and unsaturated regions of the spectrum.

The carbohydrate regions of both the *A. quadruplicatum* and *A. nidulans* spectra are dominated by broad polysaccharide resonances which, on the basis of chemical shift[14], indicate the composition of an α-(1→4)-linked glucose. The primary small carbohydrates in the spectrum of *A. nidulans* are α- and β-D-glucose which appear to be present in the ratio expected at thermodynamic equilibrium. On the other hand, the primary low-molecular-weight carbohydrates in *A. quadruplicatum* are glucosylglycerol and sucrose. Although many of the carbohydrate resonances are poorly resolved in the whole-cell spectra (Fig. 3C), characteristic resonance shifts corresponding to the anomeric carbon atoms are readily observed. These include a resonance peak at 98.8 p.p.m., corresponding to the C-1 resonance of α-D-glucose in glucosylglycerol, and resonance peaks at 93.2 and 104.7 p.p.m., corresponding to the glucose and fructose anomeric carbons of sucrose, as well as the broad peak centered at 100.9 p.p.m., which corresponds to the anomeric carbon atoms of the polysaccharide.

Although some aspects of the progress of the nitrogen-depletion experiment can be monitored by n.m.r. spectroscopy, it is worth emphasizing that quantitating the various classes of cellular constituents from these spectra can lead to erroneous results. This reflects primarily underestimating the concentration of those constituents having very broad resonances, as well as lower NOE values due to slow molecular tumbling. A particularly good illustration of this effect is the disappearance of the ^{13}C resonances of dipalmitoyllecithin bilayers or vesicles below the phase-transition temperature of 41° as described by Levine *et al.*[15]. These effects are illustrated in the present case by a comparison of whole-cell spectra obtained prior to nitrogen depletion (24 h into the experiment) and those run at 27° (Fig. 3B) and 50° (Fig. 3A). At the higher sample-temperature, the anomeric-carbon resonance corresponding to the polysaccharide has increased dramatically relative to the corresponding resonances of the small metabolites. This is primarily due to the greater internal mobility and consequent sharpening of the oligosaccharide resonances. Nevertheless, by obtaining spectra at a constant temperature, it is possible to follow qualitatively the changes in protein and carbohydrate composition of cells and to obtain information about metabolic processes involving the small metabolites.

Following depletion of the available nitrogen, a significant decrease in intensity of the protein resonances and a corresponding increase in intensity of the polysaccharide resonances were observed (Fig. 3C). These changes appear to parallel the changes in cellular carbohydrate concentration as determined by acid hydrolysis and enzymic assay. The presence of an additional small carbohydrate metabolite having resonance shifts close to those of glucosylglycerol became apparent in the

TABLE I

^{13}C CHEMICAL SHIFTS OF LOW-MOLECULAR-WEIGHT CARBOHYDRATES

Compound	*Carbon position (p.p m.)*[a]								
	Glucose moiety						*Glycerol-derived moiety*		
	1	*2*	*3*	*4*	*5*	*6*	*1*	*2*	*3*
Glucosylglycerol[b,c]	98.9	(73.1)	(74 2)	70.8	(72.7)	(61.7)	(62.6)	79.9	(61.6)
Glucosylglycerate[c]	98.7	(73 3)	(74 5)	70.7	(72 8)	61.7	178.0	80.3	64 3
Glucosylglyceric acid[c]	98.7	(73.6)	(74.1)	70 7	(72 6)	61 7	174.8	76.9	63.7
Methyl α-D-glucopyranoside	100.1	72 2	74 2	70.7	72 5	61.7	—	—	—
Glycerol	—	—	—	—	—	—	63.9	73.4	63 9
Glycerate	—	—	—	—	—	—	179 8	74 5	65.1
Glyceric acid	—	—	—	—	—	—	176.9	—	64.5

[a]Spectra were obtained at 25°, and the chemical shifts are in p.p m. downfield from external Me_4Si. [b]Assignments in parentheses are uncertain. [c]Assignments of glucose C-2, C-3, and C-5 were made based on the expectation that only C-2 would be appreciably affected by substitution at C-1.

later stages of the experiment. Although a separate anomeric carbon resonance was resolvable at t = 120 h, it is probable that a significant quantity was present at earlier times but could not be resolved. This metabolite was subsequently isolated and purified and was identified as glucosylglycerate using n.m.r.

The n.m.r. spectrum of the isolated ^{13}C-enriched sucrose was the same as that described by Johnson and Jankowski[16]. The ^{13}C chemical shifts of glucosylglycerol (Table I) were similar to those of galactosylglycerol[3]; these shifts clearly indicate that an α-glucosidic bond is formed with C-2 of glycerol. The glucose moiety of glucosylglycerol has a chemical shift for C-1 of 98.9 p.p.m.; this is similar to methyl α-D-glucopyranoside. The glycerol moiety alternatively has a resonance at 79.9 p.p.m., which indicates substitution at C-2; C-1 and C-3 of the glycerol each produces a single resonance (62.6 and 61.6 p.p.m.) which suggests that they are inequivalent as expected and that neither C-1 nor C-3 is substituted. The chemical shifts for glucosylglycerol differ markedly from those reported by DeBoer *et al.*[17] for 2,3-dihydroxypropyl β-D-glucopyranoside in which the β-glucosidic bond is formed with the C-1 of glycerol. In that compound, the chemical shift for the C-1 of glucose was 103.1 p.p.m. and the substituted glycerol carbon (C-1) was at 71.4 p.p.m.

The chemical shifts obtained for glucosylglycerate (Table I) were similar to those obtained for glucosylglycerol except that C-1 of glycerol was shifted to 178.0 p.p.m., which is typical for a carboxylate ion; C-3 was shifted 2.7 p.p.m. downfield. Acid hydrolysis of the glycosylglycerate produced a mixture of α- and β-glucose and glyceric acid, which were identified by comparison of their chemical shifts with authentic compounds.

In addition to the small metabolites already discussed above, sharp resonances at 28.1, 34.6, 55.8, 176.4, and 182.8 p.p.m. were also observed and assigned to free glutamate (Fig. 3). The glutamate resonances were most intense during the period from 0–48 h, during which time the cells had sufficient nitrogen for exponential

growth. Subsequent to nitrogen depletion these resonances decreased in intensity and were difficult to discern at $t \geq 68$ h. The high concentration of glutamate presumably reflects its role as a substrate for glutamine synthetase, an important step in the incorporation of ammonia (derived from nitrate) into cellular components. After depletion of the nitrate-nitrogen in the medium, catabolic processes presumably became the primary means of obtaining nitrogen and the glutamate concentration decreased.

Growth of 260 g of cells enriched to the 20 ml% ^{13}C level resulted in the use of 9.6 mol of 20 mol% [^{13}C]carbon dioxide. The cell material, which was 38 2% carbon, contained 8.1 mol of 20 mol% ^{13}C carbon: this represents 84% of the carbon administered. The administered carbon not fixed by the cells was trapped in sodium hydroxide traps attached to the off-gas stream or was lost in the culture medium.

The D-[U-^{13}C]glucose isolated from the cells comprised 38% of the dry-cell weight. D-[U-^{13}C]Glucose (99 g) was isolated from 260 g of cells; the carbon isolated as D-[U^{13}C]glucose represents a 40% recovery of fixed carbon. About 1% of the dry cell mass consisted of sucrose, glucosylglycerol, and glucosylglycerate; the amounts isolated were 0.69, 1.8, and 0.09 g, respectively. ^{13}C-Labeled lipids, which consisted of 8% of the dry-cell weight, were extracted with and separated from the sugars. The salt, which was about 39% of the dry-cell weight, was obtained during the isolation procedures; it was quantified and discarded.

The investigation of carbohydrate synthesis in algae was undertaken to find a suitable method for producing large quantities of ^{13}C-labeled carbohydrates in shorter periods of time than with plant-leaf systems. Also, emphasis was specifically on obtaining compounds that have the same level of ^{13}C-enrichment as the carbon source administered. Using the blue-green alga *A. quadruplicatum* (strain PR6), we have obtained several carbohydrates containing the same level of ^{13}C-enrichment as the carbon source, and we have reduced the time for preparation from 100 days, using the plant leaf method, to 10 days.

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