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PREPARATION OF [U-^{14}C]-LABELLED GLYCOGEN, MALTOSACCHARIDES, MALTOSE, AND D-GLUCOSE BY PHOTOASSIMILATION OF $^{14}CO_2$ IN *Anacystis nidulans* AND SELECTIVE ENZYMIC DEGRADATION

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ABSTRACT

A procedure is described for the isolation from the phototrophic procaryote *Anacystis nidulans* of [U-^{14}C]-labelled glycogen, with high specific radioactivity, formed when $NaH^{14}CO_3$ was added to non-dividing cells that continued to photoassimilate CO_2. [U-^{14}C]-Labelled glycogen was then treated with isoamylase (EC 3.2.1.68), isoamylase plus beta-amylase (EC 3.2.1.2), or glucoamylase (EC 3.2.1.3) to give [U-^{14}C]-labelled maltosaccharides, maltose-*U*-^{14}C, or D-glucose-*U*-^{14}C, respectively.

INTRODUCTION

[U-^{14}C]-Labelled starch has been prepared from bean[1], tobacco[2], sweet potatoes[3], green algae, and red algae[4]. The plant material was exposed to an atmosphere of $^{14}CO_2$ in the presence of light. Glucose, fructose, sucrose[5], and galactose[6] were similarly obtained in radioactive form. The isolation of labelled carbohydrates was achieved by extraction of the plant with boiling ethanol, and the residue was often used for a preparation of [U-^{14}C]-labelled amino acids and nucleotides[7]. The polysaccharides were usually decomposed by acid hydrolysis, and the sugars purified by ion-exchange and paper chromatography. Nonspecific isolation of the carbohydrate fraction and the laborious purification of the degradation products are disadvantages of these procedures.

We now describe the isolation of soluble, [U-^{14}C]-labelled glycogen (as opposed to starch granules) and its selective enzymic conversion into [U-^{14}C]-labelled maltosaccharides, maltose, or D-glucose. The photosynthetic organism used was the blue-green bacterium *Anacystis nidulans*, which accumulates glycogen[8] under certain environmental conditions[9] up to a constant amount per cell.

The purpose of our investigation was (*a*) to find conditions of growth for *A. nidulans* which allow a biosynthesis of glycogen of high specific radioactivity, (*b*) to develop an easy and rapid method for the isolation of pure [U-^{14}C]-labelled

glycogen in reasonable yield, suitable for large and small quantities, and (*c*) to devise a convenient procedure for purification of the enzymic degradation products.

RESULTS AND DISCUSSION

Growth and glycogen accumulation. — The growth pattern of *Anacystis nidulans* cultivated in a nitrogen-limiting medium in the presence of light and air plus CO_2 is shown in Fig. 1. Cell division stopped after exhaustion of the nitrogen source in the medium, and the colour of the culture began to change from blue-green to yellow. During this time (dotted line), determination of chlorophyll was not possible, because the blue chromophore of the phycobilins was also solubilized in addition to chlorophyll. Glycogen accumulated (*cf.* Fig. 1) if cell division was prevented and if an excess of a utilizable carbon source was present. The accumulation phase began after the end of logarithmic growth (30 h), and the amount of glycogen became maximal

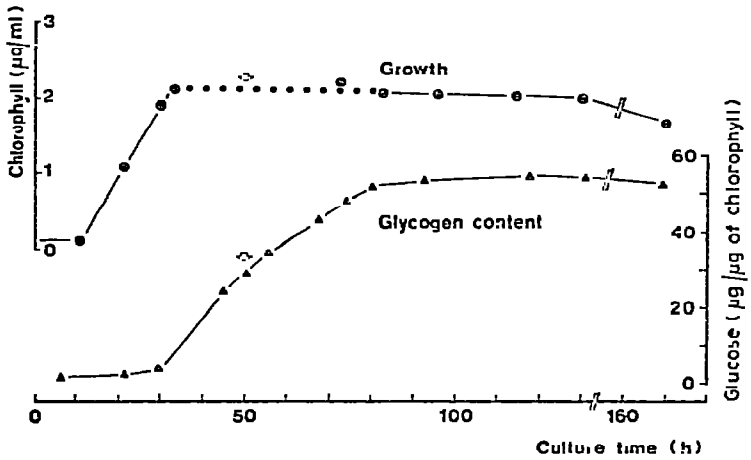

Fig. 1. Growth and glycogen accumulation of *Anacystis nidulans* cultivated in a nitrogen-limiting medium in the presence of light, and air plus CO_2; ↓ indicates initiation of contact with $^{14}CO_2$.

and remained constant after ~90 h. Therefore, cells were transferred to an atmosphere of radioactive CO_2 in the middle of the glycogen-accumulation phase (50 h, marked with an arrow in Fig. 1), because the organism may be expected to direct a considerable portion of its metabolic activities towards a synthesis of α-D-glucan. After an assimilation period of 24 h, >90% of the radioactivity had been taken up by the cells. Provided that facilities for mass cultivation of *A. nidulans* are available, large amounts of [U-^{14}C]-labelled glycogen may easily be isolated by the method described.

Purification of [U-^{14}C]-labelled glycogen. — The method for isolation and purification of [U-^{14}C]-labelled glycogen from *A. nidulans* is basically different from those described elsewhere[1–6]. It involves lysozyme digestion of the cells, followed by

absorption of coloured substances and proteins on DEAE-cellulose, and isolation of [U-^{14}C]-labelled glycogen by using concanavalin A covalently bound to Sepharose 2B. The well-known ability of concanavalin A to bind glycogen[10] can be exploited for the purification of small amounts of glycogen. The concanavalin A–glycogen complex is stable to washing with aqueous sodium chloride, whereas the addition of 0.1M sucrose causes elution of [U-^{14}C]-labelled glycogen. The elution profile consisted of a single peak of [U-^{14}C]-labelled glycogen which contained >90% of the total radioactivity loaded onto the column (*cf.* Table I). Dialysis of the eluate to remove salt and sucrose, followed by lyophilization, yielded ~2 mg of [U-^{14}C]-labelled glycogen (from 40 mg dry-cell weight) with a specific radioactivity of ~5 mCi/mmol of D-glucose residue.

A higher specific radioactivity of glycogen may be expected by the use of more sophisticated photosynthetic apparatus[11–13], where $^{14}CO_2$ is assimilated under steady-state conditions. Alternatively, if photoassimilation of $^{14}CO_2$ was allowed to continue for more than 24 h, the [U-^{14}C]-labelled glycogen content per cell increased further, but the mechanical stability of the cells decreased and, hence, the radiochemical yield of step 2 was smaller than that noted in Table I.

TABLE I

PURIFICATION OF [U-^{14}C]-LABELLED GLYCOGEN FROM *Anacystis nidulans*

Procedure	*Vol.* (*ml*)	*Total protein* (*μg*)	[*U*-^{14}*C*]-*Labelled glycogen* (*μg*)	*Total radioactivity* × 10^8 (*d.p.m.*)	*Radiochem. yield* (%)
1. Photoassimilation of $NaH^{14}CO_3$	—	—	—	3.2	—
Medium (discarded)	100	n.d.[a]	n.d.	0.3	—
2. ^{14}C-Labelled cells	2	n.d.	n.d.	2.9	100
3. Spheroplasts	2	n.d.	n.d.	2.6	89.7
Extrusion medium (discarded)	50	n.d.	n.d.	0.3	10.3
4. Crude extract	5	1,000	2,500	1.9	65.6
5. Void volume of DEAE-cellulose effluent	6	<10	2,400	1.7	58.6
6. Concanavalin A–Sepharose					
Wash (discarded)	5	<10	200	0.1	3.5
Sucrose elution	10	<10	2,200	1.55	53.5
Freeze-dried [U-^{14}C]-labelled glycogen	0	—	2,000	1.5	51.7

[a]n.d. = not determined.

Enzymic degradation of [*U*-^{14}*C*]-*labelled glycogen.* — Treatment of [U-^{14}C]-labelled glycogen with isoamylase cleaves all (1→6)-α-D linkages, yielding a mixture of [U-^{14}C]-labelled maltosaccharides, which may be fractionated by paper or gel-permeation chromatography. However, beta-amylase gives a limit dextrin and

[U-^{14}C]-labelled maltose. The concomitant action of isoamylase and beta-amylase causes complete conversion into [U-^{14}C]-labelled maltose; furthermore, degradation with glucoamylase affords [U-^{14}C]-labelled D-glucose.

Although ^{14}C-labelled D-glucose and maltose are commercially available, if large amounts are needed, the method of preparation described here would be useful.

Fractionation of the maltosaccharide mixture up to [U-^{14}C]-labelled maltoheptaose was achieved by paper chromatography. Saccharides of higher molecular weight contained ~50% of the total radioactivity of the maltosaccharide mixture. The yields of separated saccharides were maltotriose, 1.9%; maltotetraose, 5.1%; maltopentaose, 9.1%; maltohexaose, 13.1%; and maltoheptaose, 18.1%. These saccharides should be useful for investigating the maltosaccharide transport system in certain bacteria[14].

EXPERIMENTAL

Materials. — Concanavalin A was extracted from jack-bean meal and purified as described by Agrawal and Goldstein[10]. Glucoamylase (specific activity, 100 IU/mg of protein) was purified[15] to apparent homogeneity by repeated chromatography (DEAE-cellulose) of a commercial preparation (Diazyme, Miles Labs., Elkhart, Ind.). beta-Amylase was isolated from sweet potatoes[16]. A step for the removal of traces of α-D-glucosidase was added[17]. Isoamylase (specific activity, 50 IU/mg of protein, British Drug Houses, Poole, Dorset, U.K.) was a cell-free preparation from *Cytophaga* sp., and was used without further purification. The enzyme was free from endo-(1→4)-α-D-glucanase activity when tested with oxidized amylose[18]. Crystalline lysozyme (EC 3.2.1.17) was obtained from Serva, Heidelberg, Germany.

Attachment of concanavalin A to Sepharose. — Sepharose 2B (100 ml, Pharmacia, Frankfurt, Germany) activated[19] with CNBr (15 g) was incubated for 24 h at 4° with a solution of concanavalin A[20] (1.25 g) in 0.1M sodium phosphate buffer (pH 7.4) containing 0.1M sucrose. The resin was washed with distilled water (20 ml/h, 4°) for 3 days.

The capacity of the conjugate was determined as follows. An aliquot of concanavalin A–Sepharose was incubated (gentle shaking) with an excess of glycogen for 5 h at room temperature in the presence of mM calcium chloride and mM manganese chloride. The material was freed from excess of glycogen by washing with 0.5M sodium chloride. 0.1M Sucrose was used to elute the adsorbed glycogen. A capacity of 30 mg of glycogen/ml (bed volume of concanavalin A–Sepharose 2B) was found.

Analytical methods. — The growth of *Anacystis nidulans* was followed by measuring the chlorophyll content of cells[21]. Chlorophyll was extracted from wet cells with acetone/0.05M NH_3 (8:2), and the absorbances at 650 and 665 nm were determined.

The glycogen content of cells was determined as follows. Cells of 10-ml culture were harvested by centrifugation (5 min, 5,000 *g*), suspended in 10 ml of 10mM

sodium acetate buffer (pH 5.0), sonicated for 1 min (Branson Sonifier, 30% relative output) at 4°, and centrifuged (10 min, 10,000 *g*). The clear supernatant (0.5 ml) was incubated for 1.5 h with a glucoamylase–D-glucose oxidase–peroxidase reagent[23], followed by determination of the absorbance at 540 nm.

Radioactivity of aqueous samples was measured by liquid scintillation counting with a Packard TRI-CARB liquid scintillation spectrometer (counting efficiency for ^{14}C, ~90%). An aqueous sample (0.1 ml) was added to 5 ml of a scintillation mixture [500 ml of toluene, 2.5 g of 2-(4-*tert*-butylphenyl)-5-(4-biphenylyl)-1,3,4-oxadiazole, and 200 ml of Scintisol solubilizer from Isolab, Akron, Ohio]. Paper chromatograms were scanned for radioactivity with a Packard Radiochromatogram Scanner (counting efficiency for ^{14}C, ~15%).

Cultivation of A. nidulans. — *A. nidulans*, strain 1402-1 (Algensammlung der Universität Göttingen, Germany), was grown at 41° in a synthetic salt medium[23] (where the nitrogen source was diminished to 0.1 g of potassium nitrate/l) contained in thermostated glass vessels (~300 ml, with a small lateral tube for the introduction of gas) and illuminated with several day-light fluorescent tubes. Each vessel was continuously illuminated with 2,000 lux at the surface, and gassed with ~4 litres of air plus CO_2 (99:1)/h.

After 50 h of growth, 100 ml of the culture was transferred into a sterile, 500-ml Erlenmeyer flask. The pH of the medium was 7.5–8.0. After the addition of 0.2 ml of aqueous $NaH^{14}CO_3$ (specific radioactivity, 58.5 mCi/mmol), the flask was sealed, and the cells were continuously illuminated and gently agitated at room temperature for 24–36 h.

Preparation of spheroplasts. — Radioactive cells from 100 ml of culture (40 mg, dry-cell weight) were harvested by centrifugation (1 min, 1,000 *g*), and a suspension in 50 ml of 50mM sodium phosphate buffer (pH 6.8; containing 20 mg of EDTA, 100 mg of lysozyme, and 0.4M D-glucitol) was gently agitated for 3 h at 37°. To prevent glycogen degradation in the dark, the sample was illuminated during lysozyme digestion. The spheroplasts were then harvested by centrifugation (1 min, 1,000 *g*), and lysed by the addition of water (3 ml) and 0.1M sodium acetate buffer (pH 5.0; 2 ml) with vigorous stirring for several min. Cell debris was removed by centrifugation (10 min, 10,000 *g*) to give the clear supernatant as the crude [U-^{14}C]-labelled-glycogen-containing extract.

Purification of [U-^{14}C]-labelled glycogen. — A Pasteur pipette was filled with DEAE-cellulose, equilibrated in 50mM sodium acetate buffer (pH 5.0). Nonspecific adsorption of glycogen was reduced by pre-incubation of the DEAE-cellulose with a solution of 2 mg of phytoglycogen in equilibration buffer, followed by washing of the column with 5 ml of buffer. The crude [U-^{14}C]-labelled-glycogen-containing extract was added, and glycogen was eluted with 6 ml of buffer. The eluate was free of coloured substances and showed the typical opalescence of a glycogen solution.

The foregoing eluate was shaken with ~0.5 g (wet weight) of concanavalin A–Sepharose in the presence of mM calcium chloride and mM manganese chloride. Mechanical stirring was avoided. Incubation was continued until >90% of the radio-

activity had been adsorbed (5 h). Then, after transfer of the conjugate to a Pasteur pipette and washing with 5 ml of 0.5M sodium chloride, the glycogen was eluted with 0.1M sucrose (10 ml). The eluate was dialysed exhaustively against distilled water and then lyophilised. The purity of the glycogen so obtained was determined enzymically by using an excess of glucoamylase and D-glucose oxidase to effect quantitative degradation to ^{14}C-labelled D-gluconic acid, which was then adsorbed onto an anion-exchange resin. By this method, >95% of the purified glycogen was shown to consist of ^{14}C-labelled polyglucose.

[U-^{14}C]-Labelled maltosaccharides, maltose, and D-glucose. — Solutions of [U-^{14}C]-labelled glycogen and 2 mg of phytoglycogen, as a carrier, in 10mM sodium acetate buffer (pH 5.0) were severally incubated under aseptic conditions with an excess of isoamylase (to give a mixture of [U-^{14}C]-labelled maltosaccharides), isoamylase plus beta-amylase (to give maltose-*U*-^{14}*C*), beta-amylase (to give [U-^{14}C]-labelled limit-dextrin), and glucoamylase (to give D-glucose-*U*-^{14}*C*). The digests with beta-amylase also contained 5mM 2-mercaptoethanol and human serum albumin (1 mg/ml). The reaction products of each incubation were separated by p.c. (1-butanol–pyridine–water, 6:4:3; multiple ascent technique[24]) and scanned for radioactivity. A standard mixture of maltosaccharides was cochromatographed, and revealed with the $AgNO_3/NaOH/Na_2S_2O_3$ reagent[25] after spraying with glucoamylase solution.

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