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THE FINE STRUCTURE OF THE BRANCHED α-D-GLUCAN FROM THE BLUE-GREEN ALGA *Anacystis nidulans*: COMPARISON WITH OTHER BACTERIAL GLYCOGENS AND PHYTOGLYCOGEN

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ABSTRACT

The fine structure of the glycogen from the blue-green alga *Anacystis nidulans* has been examined. After selective hydrolysis of all (1→6)-α-D linkages by a bacterial isoamylase, the resulting mixture of linear chains was subjected to gel-permeation chromatography. For purposes of comparison, the glycogens from *Escherichia coli* and *Arthrobacter* sp., amylopectin, phytoglycogen from sweet corn, and shell-fish glycogen were treated similarly. The profiles of the unit chains of *A. nidulans* glycogen and phytoglycogen were closely similar. There was no close resemblance in the size distribution of unit chains for *A. nidulans* glycogen, other bacterial glycogens, and amylopectin.

INTRODUCTION

Blue-green algae and bacteria have many properties of cellular organization and biochemical apparatus in common, but blue-green algae are distinguished from other photosynthetic bacteria in having two systems for the photolysis of water. In this respect, they resemble eucaryotic plants.

Under appropriate culture conditions, many bacteria accumulate glycogen as a storage material. If a utilizable carbon source is present in excess, glycogen is built up mainly in the stationary phase of growth when cell multiplication has ceased due to exhaustion of the nitrogen, sulphate, or phosphate source in the medium[1]. Although, based on electron-microscopic observation, blue-green algae are generally thought to be capable of glycogen formation, unequivocal identification of the isolated material has been established only occasionally. α-Granules, as apparent in electron micrographs of *Nostoc muscorum*, were correlated[2] with a glycogen having an average chain-length ($\overline{CL}$) of 11. On the other hand, an amylopectin-like fraction from *Oscillatoria* sp. appeared[3] to be a branched α-D-glucan with a $\overline{}$ of 23–26. A physiological function, in blue-green algae, of glycogen as a reserve material was

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demonstrated in one case by electron microscopy[4]. Appropriate enzyme studies are lacking.

A study of the physiology and biochemistry of reserve polysaccharide metabolism in *Anacystis nidulans* first requires identification of the polymer type. Various parameters are frequently determined for the structural characterization of amylaceous polysaccharides, including λ_{max} of the iodine–glucan complex, extent of hydrolysis by β-amylase, $\overline{CL}$, or yield of material resistant to the attack by α-amylase. With the advent of purified, debranching enzymes, a new and powerful tool is now available. The branched polymer is treated with isoamylase or pullulanase for the selective cleavage of all (1→6)-α-D linkages, thereby liberating a mixture of the component linear-chains containing (1→4)-α-D linkages which may be fractionated by gel-permeation chromatography[5]. A characteristic profile of unit chains may thus be obtained.

We now report on the nature of the α-D-glucan from *A. nidulans*.

RESULTS AND DISCUSSION

A. nidulans was selected for experiments on the accumulation of reserve material under controlled environmental conditions, since it may be obtained from Culture Collections as an axenic strain and hence interference by other bacteria that might also store glycogen can be avoided. Also, as with most other members of the order *Chroococcales*, *A. nidulans* is incapable of reducing gaseous nitrogen. Therefore, by exploiting the need for nitrate or ammonium salts as a nitrogen source in the culture medium, experimental conditions can be devised to arrest cell multiplication at any desired population density. The stationary phase of growth thus induced leads to glycogen accumulation, the rate and extent of which are reproducible. This approach was preferred to simple ageing of a culture in the presence of light and CO_2-enriched air, because, working with *N. muscorum*, Chao and Bowen[2] observed a change in the $\overline{CL}$ of the glycogen depending on the age of the culture. An additional advantage of *A. nidulans* is that it is by far the fastest growing alga known and can be cultivated in a chemically defined medium.

The technique of cultivation under nitrogen-limiting conditions has been employed previously in an investigation of the enzymic machinery of glycogen mobilization in *E. coli*[6]. The growth and kinetics of glycogen formation in *E. coli* under nitrogen-limiting culture conditions are depicted in Fig. 1. The arrow 1 indicates exhaustion of ammonium chloride, the sole nitrogen source in the culture medium. The apparent increase in cell mass beyond arrow 1 is not due to further cell division but is caused by augmentation of viable cells as shown by the simultaneous increase in glycogen content. After ~10 hours, the culture had reached its maximal amount of glycogen content (arrow 2). Care was taken to ensure an excess of the carbon source in all phases of growth; the data for the continuous monitoring of D-glucose concentration in the culture medium are not shown. An analogous pattern of growth and glycogen accumulation was obtained with *Arthrobacter* sp. and *A. nidulans*, the

appropriate modifications with respect to the nitrogen source being indicated in the Experimental. For each organism, arrow 2 in Fig. 1 indicates the time of harvest of a culture. Intracellular glycogen was then isolated and purified; Table I lists some relevant analytical data.

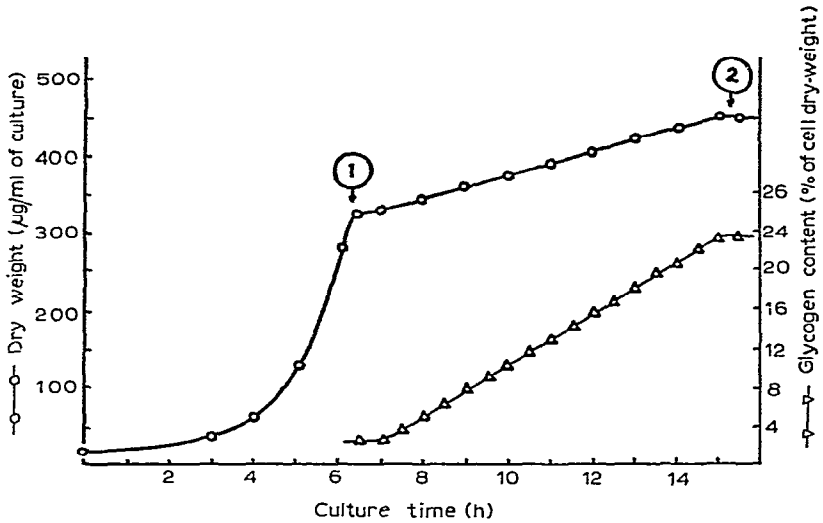

Fig. 1. Growth and rate of glycogen accumulation of *E. coli* under nitrogen-limiting culture conditions. (1) Cells entering the stationary phase of growth due to exhaustion of the nitrogen source in the medium; (2) plateau value of glycogen content.

TABLE I

DATA ON GLYCOGENS EXTRACTED FROM *E. coli*, *Arthrobacter* sp., AND *A. nidulans*

Organism	*Cell mass (mg, dry weight)*	*Glycogen purified (mg)*	*Glycogen content of cells (% of dry weight)*	*Purity of glycogen (as polyglucose) (%)*
E. coli	2,700	460	17	99
Arthrobacter sp.	7,200	1,100	15.2	97
A. nidulans	3,100	430	13.8	98

Isoamylase causes complete debranching of glycogen and amylopectin[7,8]. A partially purified isoamylase preparation from *Pseudomonas amyloderamosa* devoid of α-amylase and other interfering activities was used throughout this study. The resulting mixtures of linear maltose oligosaccharides were separated by gel-permeation chromatography. The elution profiles of debranched, shell-fish glycogen and debranched amylopectin are shown in Fig. 2; Acrylex P-10 is a locally available polyacrylamide molecular sieve, but similar results were obtained[9] with Biogel P-10. These two debranched polysaccharides also served to calibrate the column. For each fraction,

polyglucose content and reducing power were determined, thus correlating elution volume with the molecular weight of the oligosaccharides expressed as $\overline{CL}$ (reproducible to within 0.5 unit).

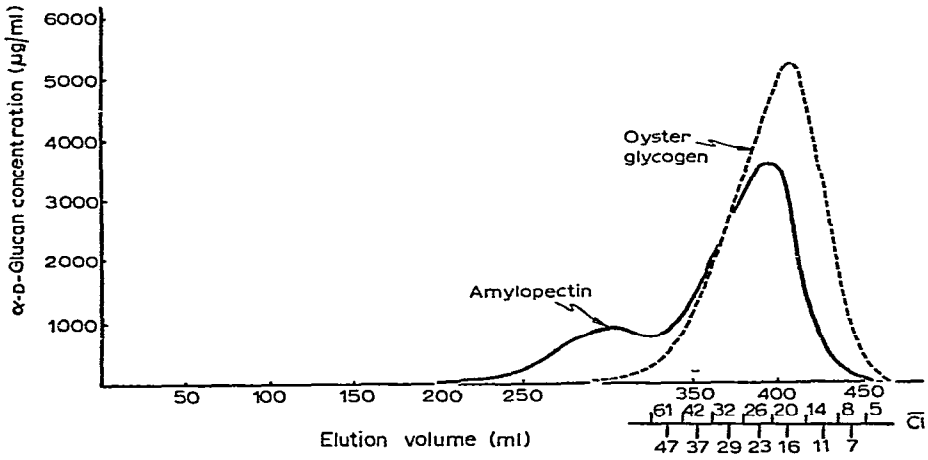

Fig. 2. Elution profile on Acrylex P-10 of debranched, oyster glycogen and amylopectin.

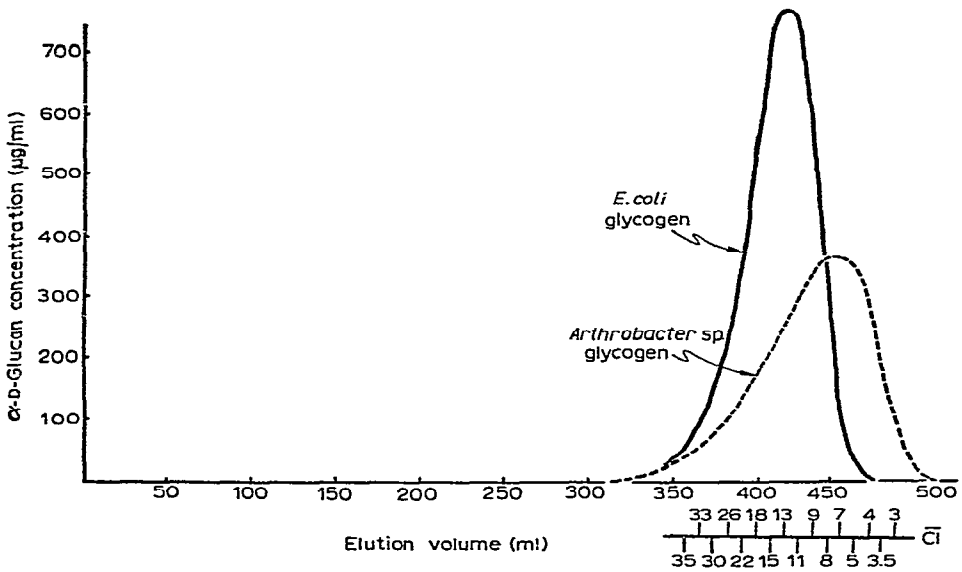

Fig. 3. Elution profile on Acrylex P-10 of debranched *E. coli* glycogen (———) and *Arthrobacter* sp. glycogen (— — —).

Fig. 3 represents the size distribution of the unit chains of the glycogens from *E. coli* and *Arthrobacter* sp. The profiles show a near-symmetrical distribution, but

with differences in peak width and elution position ($\overline{CL}$ of 14 and 8 for *E. coli* and *Arthrobacter* sp., respectively). To demonstrate that an elution profile was an intrinsic property of a branched α-D-glucan and not an artefact produced by the particular debranching enzyme used, a sample of *Arthrobacter* sp. glycogen was incubated with *Cytophaga* isoamylase[8] and processed on a column of different size calibrated separately; the elution patterns coincided.

Complete debranching is a prerequisite for a reasonable interpretation of the elution profile of unit chains. In all incubations with isoamylase, including *A. nidulans* glycogen and phytoglycogen, complete debranching was checked by consecutive

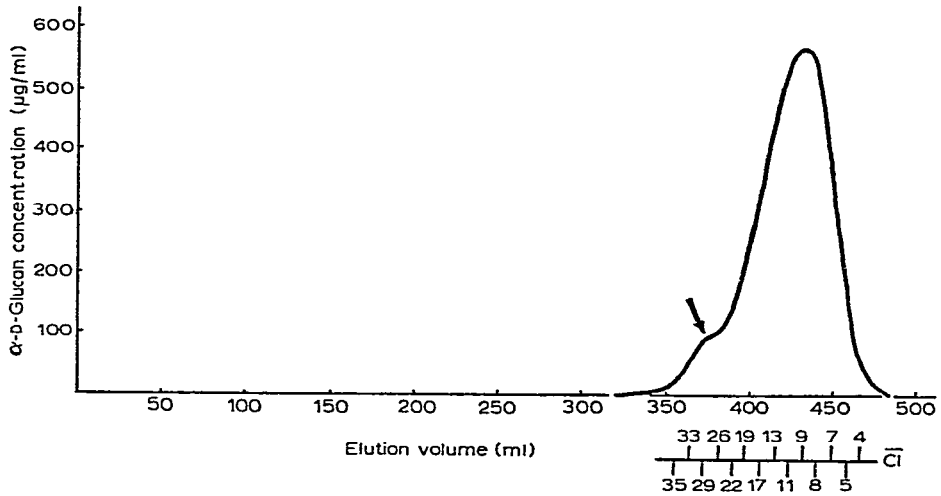

Fig. 4. Elution profile on Acrylex P-10 of debranched phytoglycogen from sweet corn.

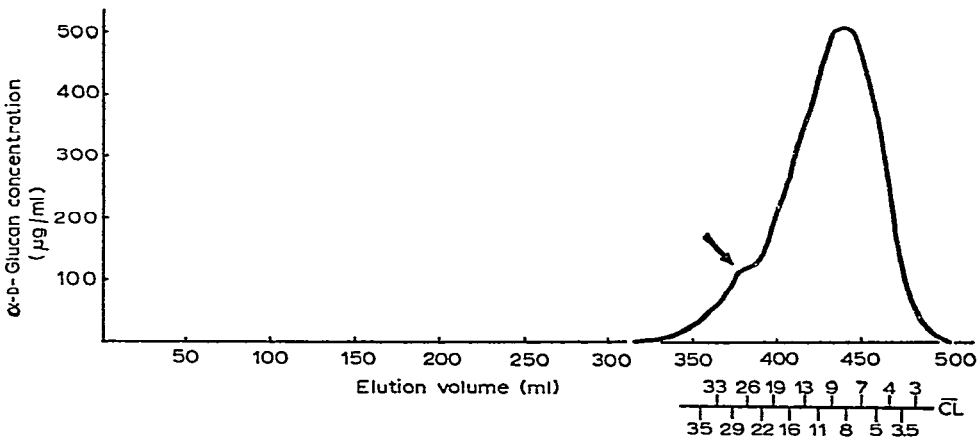

Fig. 5. Elution profile on Acrylex P-10 of debranched *A. nidulans* glycogen.

addition of β-amylase. When an enzymic digest had turned from an opalescent to a clear solution, an aliquot was removed and incubated with an excess of β-amylase. Quantitative conversion into maltose, as measured by the increase in reducing power, was indicative of complete debranching of the digest, since β-amylase cannot by-pass a branching point.

Figs. 4 and 5 show the elution patterns of the unit chains of phytoglycogen and *A. nidulans* glycogen, respectively. The CL of *A. nidulans* glycogen was found to be 9, and the value of 10 for phytoglycogen is lower than that published earlier[10]. On the basis of the size distribution of chains, the reserve polysaccharide of *A. nidulans* is identified as a glycogen. The shoulder in the elution profile (marked with an arrow) is also found in phytoglycogen, but is absent from the other glycogen samples. Thus, *A. nidulans* glycogen is similar to phytoglycogen and only superficially similar to other bacterial glycogens. The reason for the apparent lack of a close resemblance of this blue-green "bacterium" to other bacteria, with respect to the fine structure of the glycogen, is not known.

EXPERIMENTAL

Analytical methods. — D-Glucose concentration in the culture fluids of micro-organisms (see below) was monitored with the specific D-glucose oxidase reagent[11], comprising D-glucose oxidase (grade II) and peroxidase (grade II) (both products of Boehringer Mannheim), and *o*-dianisidine dihydrochloride. α-D-Glucan was measured with a combined glucoamylase/D-glucose oxidase reagent[9]. Reducing power was determined with the alkaline copper reagent[12]. Glucoamylase was a partly purified[13], commercial preparation ("Diazyme", Miles Labs., Elkhart, Ind.). In the standard purification of β-amylase from sweet potatoes[14], a step for the removal of traces of α-D-glucosidase was included[15]. Isoamylase from *Ps. amyloderamosa*[7] ATCC 21262 was partially purified by somewhat altering the original procedure[16]. Instead of isolating an extracellular isoamylase from the culture fluid of *Ps. amyloderamosa*, the cell-bound enzyme was solubilized by sonication in buffer of a washed-cell suspension. A step for the removal of nucleic acids by precipitation with streptomycin sulphate was included in an otherwise analogous, enzyme-purification procedure. A cell-free preparation from *Cytophaga* sp. containing isoamylase is commercially available from British Drug Houses (Poole, Dorset, England) and was used without further purification[8]. Both preparations of isoamylase, when tested with oxidized amylose[17], were shown to be free from endo-(1→4)-α-D-glucanase activity.

Growth of micro-organisms. — A stock culture of *E. coli* NCTC 5928 was maintained on a synthetic salts medium[18] solidified with 1% (w/v) of agar. For the isolation of glycogen from this organism, cells were grown aerobically at 37° in 10 litres of a nitrogen-limiting medium (180 mg of ammonium chloride/litre) in a fermenter (Jungkeit, Göttingen, Germany). Growth was followed by measuring turbidity at 540 nm (EEL-Colorimeter 222, Evans Electroselenium Ltd., Halstead, Essex, England) and related to the dry weight by means of a calibration curve. At

regular intervals, samples of the culture fluid were removed aseptically for the determination of dry weight and glycogen content.

Arthrobacter sp. NRRL B-1973 (U.S. Department of Agriculture, Peoria, Ill.) was maintained and cultivated as previously described[19]. Under nitrogen-limiting conditions, the liquid medium contained 2.2 g of peptone/litre. Otherwise, growth of the organism and monitoring of glycogen accumulation were performed as described above.

A. nidulans L 1420-1 (Pflanzenphysiologisches Institut der Universität Göttingen, Germany) was grown on a synthetic salts medium[20] with CO_2-enriched air (0.5%) under continuous illumination at 41°. The nitrogen-limiting medium contained 100 mg of KNO_3/litre. For a mass culture of cells, the fermenter was illuminated with a bank of daylight and warm white fluorescent tubes (Osram 40 Watt 25-1 and 40 Watt 30-1, respectively) delivering approximately 10,000 lux at the surface of the culture vessel. Growth was followed by measuring turbidity at 710 nm.

Isolation and/or purification of branched polysaccharides. — The procedure was based on that described by Strange *et al.*[21]. Cells high in glycogen content (*cf.* Fig. 1) were harvested with the SZ-14 GK continuous-flow zonal rotor in a refrigerated centrifuge (RC 2-B, I. Sorvall Inc., Newtown, Conn.) at 10,000 *g*. The extracellular, slimy polysaccharide of *Arthrobacter* sp. could be removed by repetitive washing of the cells with cold physiological saline. To prevent endogenous degradation of the glycogen, all further steps were performed at 0°. The cells were suspended in cold distilled water and sonically ruptured (Model J-22, Branson Sonic Power Co., Danburg, Conn.). Proteins in the resulting suspension were precipitated with cold, aqueous trichloroacetic acid (final concentration, 5%) and centrifuged (30 min, 30,000 *g*). The opalescent supernatant was neutralized with solid $NaHCO_3$, dialyzed, and concentrated (Amicon ultrafiltration cell, PM 10 Diaflo membrane, Amicon Corp., Lexington, Mass.). Glycogen could be precipitated by adding 3 vol. of ethanol and a few drops of saturated, aqueous potassium acetate. After storage overnight, the precipitate was collected and dried *in vacuo* over anhydrous $CaCl_2$.

A commercially available amylopectin from waxy maize (Koch–Light, Colnbrook, Bucks, England) was freed[22] from small amounts of amylose. Phytoglycogen was isolated from sweet corn (Golden Bantam) and purified as described in the literature[23]. Oyster glycogen, type II, was purchased from Sigma Chemical Co., St. Louis, Mo.

All branched α-D-glucan samples, irrespective of the source, were subjected to further purification by ion-exchange chromatography before use. A solution of the polysaccharide in 10mM phosphate buffer (pH 7) was passed through a column of DEAE-Sephadex (Pharmacia Fine Chemicals, Uppsala, Sweden), equilibrated with the same buffer. Material emerging in the void-volume range was collected, dialyzed, and free-dried.

Enzymic digestion with isoamylase. — Debranching of polysaccharides was performed with 30-mg samples, each dissolved in 100mM acetate buffer (pH 3.5), and an excess of isoamylase at 37°. The reaction appeared to be complete after a few hours

as judged by the disappearance of the opalescence, but was allowed to proceed for 15 h (for criteria of completion, see Results and Discussion). The incubation was protected from microbial growth by adding a few drops of toluene. Samples (~500 mg) of shell-fish glycogen and amylopectin were incubated with appropriate amounts of isoamylase to bring about complete debranching, when a calibration of the molecular-sieve column was intended (see below). After inactivation of the enzyme by boiling for 15 min, the maltose oligosaccharides were separated on a column (2.5 × 80 cm) of Acrylex P-10 (0.05–0.1 mm, Macherey, Nagel & Co., Düren, Germany). The column was eluted with distilled water at room temperature, fractions of 8 ml being collected. The elution volume could be correlated with the molecular weight of the oligosaccharides by measuring the glucan content and reducing power of each fraction.

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