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The Biosynthesis of Granulose by *Clostridium pasteurianum*

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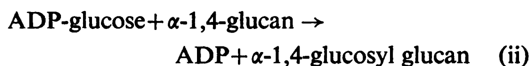
1. Mutant strains of *Clostridium pasteurianum* were obtained, which are unable to synthesize granulose (an intracellularly accumulated amylopectin-like α-polyglucan). 2. These mutants lacked either (*a*) ADP-glucose pyrophosphorylase (EC 2.7.7.27), or (*b*) granulose synthase (i.e. ADP-glucose–α-1,4-glucan glucosyltransferase, EC 2.4.1.21). 3. Although both of these enzymes were constitutively synthesized by the wild-type organism, massive deposition of granulose in a sporulating culture coincided with a threefold increase in the specific activity of ADP-glucose pyrophosphorylase. 4. The soluble ADP-glucose pyrophosphorylase was partially purified (33-fold). Its ATP-saturation curve was not sigmoidal and its activity was not enhanced by phosphorylated intermediates of glycolysis, pyruvate, NAD(P)H or pyridoxal 5′-phosphate. ADP at relatively high concentrations acted as a competitive inhibitor (K_i = 19mM). 5. The dependence of granulose synthase on a suitable polyglucan primer was demonstrated by using enzyme obtained from a granulose-free mutant strain (lacking ADP-glucose pyrophosphorylase). 6. Partial purification of granulose synthase from wild-type strains was facilitated by its being bound to the native particles of granulose. No activator was discovered, but ADP, AMP and pyridoxal 5′-phosphate were competitive inhibitors, ADP being most effective (K_i about 0.2mM). 7. It would appear that the synthesis of granulose in *Cl. pasteurianum* is not subject to the positive, fine control that is a feature of glycogen biosynthesis in most bacteria.

Several species of *Clostridium* synthesize granulose, an α-polyglucosan whose structure resembles that of amylopectin (Gavard & Milhaud, 1952; Whyte & Strasdine, 1972), or may be less branched (Laishley *et al.*, 1974). Electron-microscopic studies have shown this material to be accumulated intracellularly in the form of large granules (Mackey & Morris, 1971; Rousseau *et al.*, 1971; Krasilnikov *et al.*, 1972; Laishley *et al.*, 1973), whose presence in these obligately anaerobic bacteria is readily evidenced by their intense blue-black staining with I_2.

In batch culture in a defined, minimal medium containing only glucose, NH_4^+ ions, salts and vitamins, sporulation of a wild-type strain of *Clostridium pasteurianum* ATCC 6013 was accompanied by massive deposition of granulose (Mackey & Morris, 1971). When it was further found that (*a*) *Clostridium pasteurianum* NCIB 9486, which sporulated poorly under the same batch culture conditions, did not accumulate granulose in such large quantity, and (*b*) granulose-negative mutant strains were virtually unable to sporulate in the usual glucose-rich minimal medium (Morris & Robson, 1973), it seemed likely that knowledge of the route and means of regulation of granulose biosynthesis could contribute to our understanding of the metabolic state of the organism at that time when sporulation is initiated.

In prokaryotes, biosynthesis of the related polyglucosan, glycogen, proceeds via synthesis and utilization of ADP-glucose, i.e.:

$$\text{Glucose 1-phosphate} + \text{ATP} \rightleftharpoons \text{ADP-glucose} + \text{PP}_i \quad \text{(i)}$$



$$\text{ADP-glucose} + \alpha\text{-1,4-glucan} \rightarrow \text{ADP} + \alpha\text{-1,4-glucosyl glucan} \quad \text{(ii)}$$

Reaction (i) is catalysed by ADP-glucose pyrophosphorylase (i.e. glucose 1-phosphate adenyltransferase, EC 2.7.7.27) and reaction (ii) by glycogen synthase (i.e. ADP-glucose–α-1,4-glucan 4-glucosyl transferase EC 2.4.1.21). The elegant studies of Preiss and co-workers (Preiss, 1969; Preiss *et al.*, 1973) have shown bacterial synthesis of glycogen to be regulated by fine control of ADP-glucose pyrophosphorylase activity. This contrasts with the situation in eukaryotes where glycogen is synthesized from UDP-glucose and glycogen synthetase is the locus of regulation.

It is the chief purpose of the present paper to report that although granulose synthesis in *Cl. pasteurianum* proceeds via ADP-glucose, we have been unable to detect any allosteric fine control of the ADP-glucose pyrophosphorylase of this organism. Nor is the granulose synthase subject to sophisticated regulation.

Part of this work has been reported in a preliminary form (Robson *et al.*, 1972).

Materials

Organisms

The sporulating strain of *Cl. pasteurianum* (W-5) A.T.C.C. 6013 was kindly supplied by Mrs. Winifred Ego, University of Hawaii, Honolulu. *Cl. pasteurianum* N.C.I.B. 9486 was obtained from the National Collection of Industrial Bacteria, Aberdeen, U.K.

Enzymes

Alkaline phosphatase (EC 3.1.3.1, type IIIs from *Escherichia coli*), α-amylase (EC 3.2.1.1), deoxyribonuclease (EC 3.1.4.5) inorganic pyrophosphatase (EC 3.6.1.1), muramidase (EC 3.2.1.17, grade 1) and ribonuclease (EC 3.1.4.22, type X1-A) were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.

Chemicals

Crystalline bovine serum albumin, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, nucleotide sugars and Trizma base were obtained from Sigma (London) Chemical Co. Adenine nucleotides, coenzymes and sugar phosphates were purchased from Boehringer Corp. (London) Ltd., Ealing, London, U.K. DEAE-celluloses were obtained from Whatman Biochemicals Ltd., Maidstone, Kent, U.K., and Sephadex G-25 and G-200 were from Pharmacia Fine Chemicals, Uppsala, Sweden. All radioactive chemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Amylopectin, dithiothreitol, glycogen and all AnalaR grade reagents were purchased from BDH Chemicals Ltd., Poole, Dorset, U.K. Reinforced Clostridial Medium and agar were bought from Oxoid Ltd., London E.C.4, U.K.

Methods

Maintenance and growth of organisms

Washed spore suspensions of *Cl. pasteurianum* (strain W-5), sub-strains ATCC 6013 and NCIB 9486 were prepared as described by Mackey & Morris (1971) and were stored at 2°C. Samples of these spore suspensions were 'heat-activated' by incubation at 80°C for 10min in sterile, capped test-tubes, before being employed as inocula of suitable volumes of growth medium. The minimal medium contained (per litre), 40g of glucose, 3g of NH_4Cl, 0.1g of $MgSO_4,7H_2O$, 0.1g of NaCl, 0.01g of $NaMoO_4,2H_2O$, 0.01g of $CaCl_2$, 0.015g of $MnSO_4,4H_2O$, 0.275g of ferric sodium sequestrate, 0.121g of Trizma base, 2mg of *p*-aminobenzoic acid and 0.12mg of biotin. Some 2 drops of aq. 0.05% Methylene Blue were added immediately before sterilizing by autoclaving, and after cooling, 1M-potassium phosphate buffer, pH7, was aseptically added to a final concentration of 0.05M. Freshly prepared medium in an almost completely filled 500ml of Ehrlenmeyer flask was inoculated with heat-activated spores (to give 10^6 spores/ml). The flask was then sealed with a sterile 'Suba-Seal' stopper, and with a hypodermic needle attached to a sterile gas filter the atmosphere above the culture was aseptically replaced by O_2-free N_2+CO_2 (95:5). After overnight incubation at 37°C, this culture served as an inoculum for a similar 8litre anaerobic culture. For experiments in which the course of growth and sporulation were to be measured, a 350ml culture was incubated anaerobically in the fermenter vessel described by O'Brien & Morris (1971).

Mutagenesis

(i) *With N-methyl-N'-nitro-N-nitrosoguanidine.* Organisms harvested by aseptic centrifugation from anaerobic cultures in the mid-exponential phase of growth, were resuspended in 25mM-sodium citrate buffer, pH5.5, to a density of 10^7 cells/ml. The culture supernatant was transferred to a sterile McCartney bottle and was maintained anaerobically at 37°C. Then 1ml of a freshly prepared solution of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (1mg/ml) in citrate buffer was added to 10ml of the cell suspension, and the mixture was then incubated aerobically for 15min at 37°C in a 50ml centrifuge tube. The organisms recovered by centrifugation at 20000*g* for 10min at 5°C were resuspended in 40ml of the original culture supernatant and allowed to 'grow out' anaerobically at 37°C.

(ii) *With u.v. irradiation.* A 10ml sample of an exponentially growing, anaerobic culture (containing about 10^7 organisms/ml) was exposed to u.v. irradiation from a germicidal lamp (30W placed 10cm above the culture) until 99.9% of the population was killed. The survivors were allowed to resume anaerobic growth in the dark at 37°C.

Isolation of granulose-negative mutants

Cultures 'grown out' for at least two generations after mutagenesis, were plated on glucose minimal medium solidified with 2% (w/v) agar (Oxoid no. 1). The petri dishes were incubated anaerobically for 3 days at 37°C [in anaerobic jars containing an O_2-free atmosphere of $H_2+N_2+CO_2$ (approx. 5:43:2)]. When the opened dishes were then inverted over I_2 crystals, the colonies of granulose-negative mutants were identifiable by their being unstained by

the sublimed I_2 vapour. Provided that exposure to I_2 was not over-prolonged (generally less than 1 min), these mutant strains could be directly subcultured on to anaerobic slopes of agar-solidified minimal medium.

Preparation of cell extracts

Organisms harvested by centrifugation at 3°C (approx. 10000*g* for 10 min) were washed and resuspended in 0.05 M-Tris–HCl buffer, pH 8, containing 0.5 mM-dithiothreitol. The resultant suspension (about 0.5 g wet wt. of organisms/ml) was disrupted by anaerobic passage at 1.4×10^8 Pa through a chilled French pressure cell (Aminco, Silver Spring, Md., U.S.A.). The process was repeated until microscopic examination revealed that adequate disruption had been achieved.

Assay methods

Glucose. Glucose was assayed colorimetrically by the standard 'Glucostat' procedure (Teller, 1956).

Granulose. Granulose was determined as glucose liberated by acid hydrolysis of well-washed organisms. The suspension of organisms in 1 M-H_2SO_4 was heated at 100°C for 3 h and neutralized with KOH.

Growth measurements. (1) Cell counts were done microscopically by using an 'improved Neubauer' counting chamber. (2) Culture density was measured spectrophotometrically at 680 nm with a Unicam SP. 600 spectrophotometer. (3) Dry weight determinations were done after centrifuging the culture sample at 15000*g* for 5 min and twice washing the cell pellet in water. The washed suspension was then transferred to a pre-weighed foil container and dried at 105°C to constant weight. (4) Viable counts were done by the spread-plate technique by using plates of Reinforced Clostridial Agar (Oxoid Ltd.), suitable dilutions being prepared in sterile 'bacterial Ringer' solution (Wilson, 1922). The plates were incubated anaerobically at 37°C for 3 days before the colonies were counted.

Protein. Soluble protein was generally measured by the method of Lowry *et al.* (1951); a standard curve was prepared with crystalline bovine serum albumin (fraction V). Protein in fractions obtained by column chromatography was assayed by the method of Warburg & Christian (1941).

Radioactivity. Radioactivity was measured by scintillation counting with a Beckman LS-200B spectrometer. Aqueous samples (less than 0.5 ml) were counted for radioactivity with negligible quenching in 10 ml amounts of a toluene–Triton X-100–2,5-diphenyloxazole mixture consisting of 4 g of 2,5-diphenyloxazole (PPO) and 500 ml of Triton X-100 in toluene (to a final volume of 1 litre). Dried filter-paper or anion-exchange-paper discs were submerged in 10 ml amounts of a toluene–PPO mixture containing 4.8 g of PPO/litre of toluene.

Enzyme assays

ADP-glucose pyrophosphorylase. The rate of synthesis of ADP-[^{14}C]glucose from [^{14}C]glucose 1-phosphate and ATP was measured by the method described by Shen & Preiss (1964). The reaction mixture, which was incubated for 10 min at 37°C, contained (in 0.2 ml): 2 μmol of ATP, 4 μmol of $MgCl_2$, 0.172 μmol of D-[^{14}C]glucose 1-phosphate (sp. radioactivity 3 μCi/μmol), 20 μmol of Tris–HCl buffer, pH 8.0, 1 unit of inorganic pyrophosphatase and enzyme. The reaction was terminated by heating the mixture in a boiling water bath for 30 s. Alkaline phosphatase (10 units) was then added and the mixture was incubated for 1 h at 37°C. Duplicate 10 μl samples of the phosphatase-treated preparation were applied to discs (25 mm diam.) of DEAE-cellulose (Whatman DE-81). One disc was thoroughly washed with water by using a Millipore filtration system (Millipore Corp., Bedford, Mass., U.S.A.) and then both discs were dried under an i.r. lamp and counted for radioactivity.

Granulose synthase. The rate of incorporation of radioactivity from ADP-[^{14}C]glucose into α-1,4-glucan was measured by the method of Greenberg & Preiss (1965). The reaction mixture, incubated at 25°C for 10 min, contained (in 0.2 ml): 0.05 μmol of ADP-[^{14}C]glucose (0.1 μCi/μmol), 0.05 μg of amylopectin, 5 μmol of Tris–HCl, pH 8, 1 μmol of $MgCl_2$ and enzyme. The reaction was stopped by the addition of 2 ml of cold 75% (v/v) methanol containing 1% (w/v) KCl. The mixture was left for 10 min at 0°C and then centrifuged at 3000*g* for 10 min. The pellet was washed three times by suspension in, and re-centrifugation from, 5 ml volumes of methanol with KCl. It was finally resuspended in 50 μl of 0.1 M-NaOH for assay of its radioactivity.

Enzyme purification

ADP-glucose pyrophosphorylase from wild-type strains of Cl. pasteurianum. Cultures (8 l) were harvested during late exponential growth, by centrifugation at 10000*g* ($r_{av.}$ 10 cm) for 10 min at 3°C. The organisms were twice washed by suspension in, and re-centrifugation from, 25 mM-Tris–HCl buffer, pH 8. The washed cell pellet (approx. 30 g wet wt.) was resuspended under an atmosphere of H_2, in 50 mM-$MgCl_2$ containing 1 mM-dithiothreitol, to give a suspension containing 0.5 g wet wt. of organisms/ml. To this were added 20 mg of muramidase (approx. 4×10^5 units; 1 unit of muramidase will produce a ΔE_{450} of 0.001/min at pH 6.24 at 25°C in a 2.6 ml suspension of *Micrococcus lysodeikticus*), 5 mg of deoxyribonuclease (approx. 7×10^3 Kunitz units;

1 Kunitz unit will produce a ΔE_{260} of 0.001/min per ml at pH5.0 at 25°C) and 5mg of ribonuclease (approx. 500 Kunitz units). The suspension was incubated at 25°C for 30min and then centrifuged anaerobically at 20000g ($r_{av.}$ 7.6cm) for 15min at 3°C. The clear, brown supernatant was used as the source of the 'crude' ADP-glucose pyrophosphorylase and could be stored frozen at −30°C under H_2 without loss of activity. All subsequent operations were carried out at 0–4°C.

Step 1. To 40ml of the cell extract was slowly added 10.03g of finely ground solid $(NH_4)_2SO_4$ (low in heavy metals), the mixture being stirred throughout at 0°C. The final suspension [45% $(NH_4)_2SO_4$ satn.] was left at 0°C for 30min, and was then centrifuged at 20000g for 15min at 3°C. To the supernatant was slowly added a further 3.2g of $(NH_4)_2SO_4$ (to achieve 55% satn.). A precipitate was obtained which, after being left at 0°C for 30min, was recovered by centrifugation, dissolved in 10ml of 25mM-Tris–HCl buffer, pH8, containing 1mM-dithiothreitol (i.e. Tris–dithiothreitol buffer), and desalted by passage through a column (250mm × 15mm) of Sephadex G-25 previously equilibrated with Tris–dithiothreitol buffer.

Step 2. A 15ml sample of the Sephadex G-25 eluent was applied to a column (185mm × 20mm) of DEAE-cellulose (Whatman DE 23), which had previously been equilibrated with Tris–dithiothreitol buffer, pH8. Fresh buffer (60ml) was run in, and a linear gradient of increasing concentration of Mg^{2+} ions in Tris–dithiothreitol buffer, pH8 (0–0.4M-$MgCl_2$ in 500ml) was then applied. Fractions (5ml) of the eluent were serially collected, and those which contained ADP-glucose pyrophosphorylase activity (usually nos. 33–41, inclusive) were pooled and brought to 80% satn. with solid $(NH_4)_2SO_4$. The pellet obtained by centrifugation at 20000g for 15min was redissolved in 4ml of Tris–dithiothreitol buffer.

Step 3. The 4ml sample of partially purified enzyme from Step 2 was applied to a column (350mm × 25mm) of Sephadex G-200 previously equilibrated with Tris–dithiothreitol buffer. This buffer was also used as the eluent, which was applied at a rate of 30ml/h. Fractions (5ml) were serially collected, and those containing ADP-glucose pyrophosphorylase activity (usually nos. 16–21, inclusive) were pooled, brought to 80% satn. with solid $(NH_4)_2SO_4$ and centrifuged at 20000g for 15min. The pellet was dissolved in 5ml of Tris–dithiothreitol buffer and could be stored anaerobically at −30°C. The complete procedure, which effected a 33-fold purification of the enzyme, is summarized in Table 1.

Granulose synthase from wild-type strains of Cl. pasteurianum. Cultures (8l) were harvested during early stationary phase and the organisms were twice washed with Tris–dithiothreitol buffer, pH8. The resultant 50ml of suspension (containing about 35g wet wt. of cells) was disrupted by passage at 1.4×10^8 Pa through a chilled French pressure cell. The resultant crude extract could be stored in liquid N_2.

Approx. 95% of the granulose synthase activity was associated with the pellet obtained by centrifugation of this crude cell extract at 10000g for 15min at 3°C. This suggested that partial purification could be accomplished by differential centrifugation.

Step 1. Crude cell extract (50ml) equally distributed between 2 × 30ml 'Corex' centrifuge tubes was centrifuged for 30min at 3°C at 26000g ($r_{av.}$ 7.5cm). The resulting pellet was horizontally stratified in four distinct bands. The uppermost layer was removed by careful agitation and withdrawn with the supernatant fraction. The following (middle) two layers were carefully resuspended in Tris–dithiothreitol buffer and the residual (basal) layer containing unbroken organisms was discarded.

Step 2. Further centrifugation (as in Step 1) of the suspension derived from the 'middle layers', produced a stratified pellet consisting of three layers. Removal of the uppermost layer (by careful resuspension in Tris–dithiothreitol buffer) revealed the medial thin white layer which contained the bulk of the granulose. This was removed by suspension in Tris–dithiothreitol buffer and the remaining basal layer was discarded. The suspensions prepared from the two conserved layers were separately re-centrifuged (as above) and the granulose-rich, white layers in the resulting pellets were suspended in Tris–dithiothreitol buffer, pooled, and stored in liquid N_2.

Attempts were made to solubilize the enzyme by (*a*) hydrolysis of the 'carrier' polyglucan by using α-amylase at 10°C, (*b*) incubation with various solutions

Table 1. *Partial purification of ADP-glucose pyrophosphorylase from Cl. pasteurianum*

Full details are given in the text. One unit of enzyme activity equals 1nmol of ADP-glucose produced/min at 37°C.

Fraction	Volume (ml)	Activity (units/ml)	Protein (mg/ml)	Specific activity (units/mg of protein)	Yield (%)
Crude extract	40	290	31.0	9.36	100
$(NH_4)_2SO_4$ precipitate	15	545	24.2	22.50	70.5
DEAE-cellulose eluate	4	1635	16.9	96.80	56.4
Sephadex G-200 effluent	5	781	2.54	310.00	33.7

Table 2. *Partial purification of granulose synthase from Cl. pasteurianum*

Full details of the procedures used are given in the text. One unit of enzyme activity represents 1 nmol of glucose incorporated/min at 25°C.

Fraction	Volume (ml)	Activity (units/ml)	Protein (mg/ml)	Specific activity (units/mg of protein)	Yield (%)
Crude extract	50	266.4	23.8	11.2	100.0
First centrifugation					
Supernatant plus top layer	40	—	22.9	—	—
2nd layer	11	108.4	14.4	7.51	9.0
3rd layer	16	600.0	8.3	72.3	72.1
Bottom layer	13	65.5	1.3	50.4	6.4
Subsequent centrifugations					
Pooled granulose fractions	5	1137.0	4.9	232.0	42.7

of salts at high ionic strength (e.g. 2M-LiCl) and (*c*) brief exposure to mildly acid or alkaline conditions. None of these treatments yielded a soluble, granulose-free, enzymically active product.

The partial purification procedure used to obtain the granulose-bound preparation is summarized in Table 2. This material lost its enzymic activity quite rapidly at 37°C, and although addition of bovine serum albumin (5mg/ml) decreased the rate of deterioration, kinetic studies were performed at 25°C, at which temperature the enzyme proved to be adequately stable.

Results

Deposition of granulose

Cl. pasteurianum 6013, which undergoes near-synchronous sporulation in glucose minimal medium (Mackey & Morris, 1971), synthesized little granulose during its vegetative growth, but produced massive quantities of granulose at the end of growth and onset of sporulation (Fig. 1*a*). In contrast, *Cl. pasteurianum* 9486 in the same medium failed to complete the process of sporulation despite apparently successful forespore formation, and accumulated only half of the quantity of polyglucan laid down by strain 6013 (Fig. 1*b*). Both strains ceased to grow for reasons other than deprivation of exogenous sources of carbon and nitrogen since both glucose and NH_4^+ ions were present in excess in the final culture supernatants.

ADP-glucose is the precursor of granulose

Granulose-negative mutants derived from both wild-type strains of *Cl. pasteurianum* (see the Methods section) were found to lack either ADP-glucose pyrophosphorylase or granulose synthase. Revertants able to synthesize granulose had reacquired the previously missing enzyme (Table 3). It was therefore concluded that ADP-glucose is the sole precursor of granulose in *Cl. pasteurianum.*

Synthesis of the key enzymes of granulose biosynthesis

Both ADP-glucose pyrophosphorylase and granulose synthase were present in vegetatively growing cultures of *Cl. pasteurianum* even during the exponential phase of batch culture when little granulose deposition was observed. In strain 6013 both enzymes were present at high specific activity at the onset of the stationary phase of the culture, i.e. coincident with the period of most dramatic granulose accumulation. This was most marked in the case of ADP-glucose pyrophosphorylase whose specific activity increased threefold at this time (Fig. 2*a*). The specific activities of both enzymes were significantly lower in strain 9486 and underwent little change as growth ceased (Fig. 2*b*).

Properties of the ADP-glucose pyrophosphorylase

Nucleotide triphosphate specificity. Crude extracts of wild-type *Cl. pasteurianum* contained UDP-glucose pyrophosphorylase at approx. 50% of the specific activity of ADP-glucose pyrophosphorylase. The UTP-utilizing enzyme was significantly more heat-stable than the ATP-dependent enzyme, and the partially purified preparation of ADP-glucose pyrophosphorylase (see the Methods section) displayed little activity with UTP, GTP and CTP (Table 4).

pH optimum. The partially purified enzyme demonstrated activity over a broad range of pH values (pH 7–10); its optimum pH was about pH 8.0.

Effects of changes in substrate concentration. Primary plots of enzyme activity against substrate concentration were hyperbolic in the case of both ATP and glucose 1-phosphate; there was no evidence of sigmoidicity in these curves. Double-reciprocal plots (of $1/v$ against $1/[S]$) were linear, and the apparent K_m values for ATP and glucose 1-phosphate were 2.5mM and 70μM respectively.

Effects of various metabolites. In all, over 20 compounds were tested for their effect on the ADP-glucose pyrophosphorylase activity in (i) crude cell

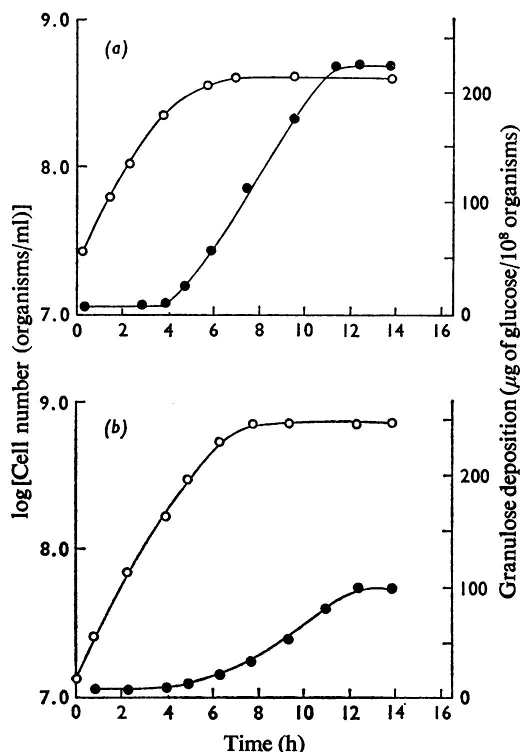


Fig. 1. *Granulose accumulation by wild-type strains of Clostridium pasteurianum*

(*a*) *Cl. pasteurianum* 6013. This sporulated well; forespores were produced by over 80% of the organisms (at 8h) and these gave rise to mature refractile spores (at 14h). (*b*) *Cl. pasteurianum* 9486, which sporulated very poorly. The organisms were grown anaerobically at 37°C in batch culture in minimal medium containing 4% (w/v) glucose. Towards the end of the exponential phase of growth, cell number (○) and the granulose content of the organisms (●) were determined at intervals by the procedures described in the Methods section.

extracts and (ii) partially purified preparations. These compounds included those metabolites reported to act as allosteric effectors of the ADP-glucose pyrophosphorylases of other glycogen-synthesizing bacteria (Preiss, 1969). None of the following substances enhanced the activity of the enzyme when supplied at 3mM final concentration: acetyl CoA, acetyl phosphate, ADP, AMP, butyryl phosphate, fructose 1-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, glucose 6-phosphate, P_i, NAD^+, NADH, $NADP^+$, NADPH, phosphoenolpyruvate, 6-phosphogluconate, 2-phosphoglycerate, 3-phosphoglycerate, pyridoxal 5′-phosphate, pyruvate, ribose 5-phosphate. Of all the compounds tested, only

Table 3. *Granulose-negative mutant strains of Cl. pasteurianum*

Granulose-negative mutants were produced and isolated as described in the Methods section. Revertant strains were selected as I_2-staining colonies obtained on plating the products of mutagenesis of cultures of granulose-negative 'parent' strains. Ability (+), or inability (−), to synthesize granulose, was determined both by I_2-staining and by assay of the glucose content of acid-hydrolysed washed suspensions of the organism (see the Methods section). The total absence (−) or presence (+) in normal specific activity of the two key enzymes of granulose biosynthesis was assessed by using crude extracts of organisms harvested at the late-exponential phase of batch growth (as in Fig. 2).

Strain	Ability to synthesize granulose	Possession of ADP-glucose pyrophosphorylase	Possession of Granulose synthase
9486			
Wild-type	+	+	+
MR 30	−	+	−
MR 30 revertant	+	+	+
MR 31	−	−	+
MR 31 revertant	+	+	+
6013			
Wild-type	+	+	+
MR 505	−	+	−
MR 505 revertant	+	+	+

ADP was found to inhibit the enzyme; it acted as a competitive inhibitor of ATP utilization with a K_i of 19mM.

Properties of the granulose synthase

Nucleotide sugar specificity. Even in crude cell extracts, UDP-glucose was unable to substitute for ADP-glucose as substrate for granulose synthase (when both nucleotide sugars were supplied at a final concentration of 0.25mM).

Requirement for a glucan primer. Since the granulose synthase obtained from wild-type *Cl. pasteurianum* was invariably bound to particles of granulose (see the Methods section) it was not possible to use such a preparation for studies of the requirement of the enzyme for a primer. Therefore the granulose-negative mutant strain MR-31 (lacking ADP-glucose pyrophosphorylase) was exploited as a ready source of primer-free granulose synthase. The activity of this enzyme was measured both in the presence and absence of amylopectin (2.5mg/ml) by the procedure described in the Methods section. After the reaction had been stopped, amylopectin was added to those mixtures from which it had formerly been omitted, so that co-precipitation of any newly synthesized

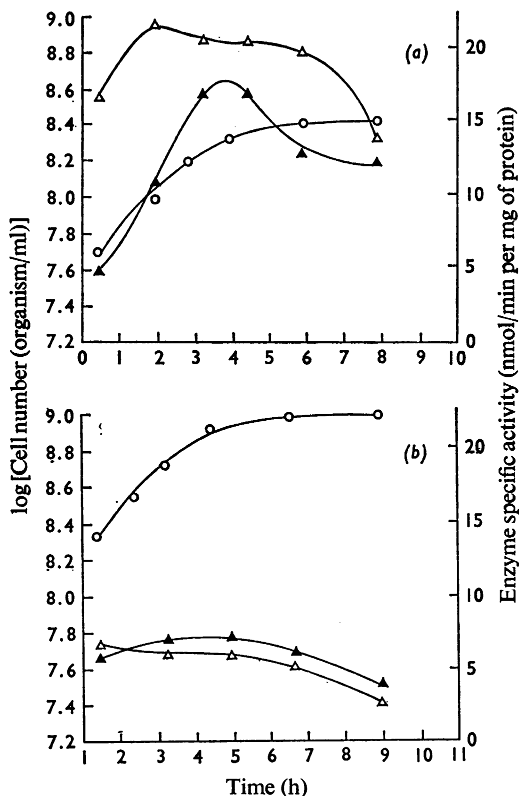


Fig. 2. *Specific activities of key enzymes of granulose biosynthesis during growth of wild-type strains of Clostridium pasteurianum*

(*a*) *Cl. pasteurianum* 6013; (*b*) *Cl. pasteurianum* 9486. The organisms were grown in batch culture as in Fig. 1. Cell number (○) and the specific activities of ADP-glucose pyrophosphorylase (▲) and of granulose synthase (△) were measured as described in the Methods section.

Table 4. *Nucleotide triphosphate specificity of the ADP-glucose pyrophosphorylase of Cl. pasteurianum*

The ADP-glucose pyrophosphorylase activity of a crude cell extract of *Cl. pasteurianum* and of a partially purified preparation (see the Methods section) was assayed by the procedure described in the Methods section. ATP was replaced by an equimolar concentration of an alternative nucleotide triphosphate. The resultant activities of the enzyme are expressed as a percentage of that activity demonstrated with ATP.

Nucleotide triphosphate (10mM)	Enzyme activity (%)	
	Crude extract	Partially purified preparation
ATP	100.0	100.0
UTP	47.5	0.6
GTP	4.2	2.4
CTP	2.1	—

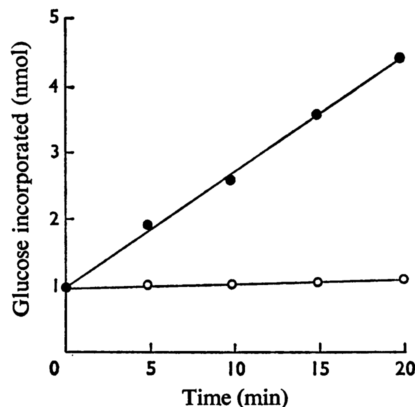


Fig. 3. *Dependence of activity of granulose synthase on provision of a polyglucan primer*

Granulose synthase activity in a crude extract of the ADP-glucose pyrophosphorylase-less (granulose-negative) mutant *Cl. pasteurianum* MR 31 was assayed in the presence (●) or absence (○) of amylopectin (2.5mg/ml), by using the radioactive procedure (see the Methods section) to measure incorporation into granulose of [^{14}C]glucose from ADP-[^{14}C]glucose.

polyglucan could be effected. Negligible synthesis of [^{14}C]polyglucan from ADP-[^{14}C]glucose was in fact achieved in the absence of primer (Fig. 3).

pH optimum. The enzyme was maximally active over a relatively broad range of pH values (pH6.5–8.5).

Effects of changes in substrate concentration. The primary plot of enzyme activity against ADP-glucose concentration was hyperbolic (with no trace of sigmoidicity). The double-reciprocal plot of $1/v$ versus $1/[S]$ was linear, and the apparent K_m for ADP-glucose was about 20μM.

Effects of various metabolites. None of the compounds tested (above) as possible effectors of ADP-glucose pyrophosphorylase enhanced the activity of granulose synthase when added (at 3mM) to crude cell extracts or to partially purified preparations of the enzyme (see the Methods section), nor had ATP or glucose 1-phosphate any effect on the activity of the enzyme. On the other hand ADP, AMP and pyridoxal 5′-phosphate were all competitively inhibitory with apparent K_i values of 0.2, 3.5 and 1.2mM respectively.

Discussion

From the nature of the enzymic lesions in granulose-negative mutant strains of *Cl. pasteurianum*, we conclude unequivocally that the polyglucan is produced from ADP-glucose. The similar bacterial synthesis of glycogen is regulated by a sophisticated

combination of coarse and fine controls, with production of the biosynthetic enzymes being subject to repression/derepression and the activity of the ADP-glucose pyrophosphate being modulated by positive and negative allosteric effectors, whose concentrations reflect the flow of metabolites through the major pathway of carbon metabolism, and/or the adenylate energy-charge status of the organism (Preiss, 1969; Preiss *et al.*, 1973; Dawes & Senior, 1973).

In *Cl. pasteurianum* 6013 massive deposition of granulose coincided with a threefold increase in the specific activity of ADP-glucose pyrophosphorylase. In strain 9486, in which granulose accumulation was neither so rapid nor so extensive (Fig. 1), this derepression of synthesis of ADP-glucose pyrophosphorylase was not observed and granulose synthase was present at a consistently lower specific activity (Fig. 2). The most surprising finding was that the ADP-glucose pyrophosphorylase of *Cl. pasteurianum* was not activated by metabolites which are positive allosteric effectors of the analogous enzymes of bacterial glycogen synthesis. Further, only ADP significantly inhibited the enzyme from *Cl. pasteurianum*, and that (*a*) competitively with glucose 1-phosphate and (*b*) only at relatively high concentrations (K_i of 19mM compared with a K_m of 2.5mM for ATP and 0.07mM for glucose 1-phosphate). Otto *et al.* (1972) who reported derepression of synthesis of ADP-glucose pyrophosphorylase during growth of *Cl. pasteurianum* W-5, and who could similarly discover no activator of the enzyme, reported it to be inhibited by pyridoxal 5′-phosphate and NADH. We did not observe significant inhibition of the enzyme by these compounds.

The granulose synthase of *Cl. pasteurianum* resembles the glycogen synthases of other prokaryotes (Preiss *et al.*, 1973; Elbein & Mitchell, 1973). It effectively used only ADP-glucose as glucosyl donor, its activity was dependent on the provision of a suitable α-1,4-polyglucan primer, and there was no evidence of complex effector interactions or of interconversion of different forms of the enzyme (as is the case with regulatory, eukaryotic glycogen synthases; Ryman & Whelan, 1971). Product inhibition of granulose synthase by ADP ($K_i = 0.2$mM) could be of physiological significance in *Cl. pasteurianum*, as it has proved to be in glycogen synthesis in other bacteria (Greenberg & Preiss, 1965; Elbein & Mitchell, 1973; Dietzler & Strominger, 1973). However, the consequences *in vivo* of the susceptibility of granulose synthase to inhibition by AMP ($K_i = 3.5$mM) and pyridoxal 5′-phosphate, are less obvious. The intimate association of granulose synthase with the product polymer is of interest, since Laishley *et al.* (1973) have suggested that the native granules are bounded by a membranous coat.

Since ATP is utilized as a primary substrate, and ADP is an inhibitor both of granulose synthase and (less effectively) of ADP-glucose pyrophosphorylase, the rate of granulose synthesis in *Cl. pasteurianum* must reflect the adenylate energy charge (Atkinson & Walton, 1967) and the availability of glucose 1-phosphate. It is tempting to interpret the apparent lack of any more refined control of granulose synthesis as evidence of the primitive nature of the system, in *Clostridium*. In this light, the ADP-glucose pyrophosphorylase of *Cl. pasteurianum* would seem to be a primordial enzyme, the evolutionary progenitor of those forms of the enzyme whose sensitive response to allosteric controls is a feature of glycogen production in many bacteria.

The possible role of branching or other modifying accessory enzymes which may be concerned with granulose fabrication, was not investigated in the present study. It has been reported that the granulose of *Cl. pasteurianum* is an essentially unbranched glucan with linear sequences of α[1→4]-linked glucose residues alternating with sequences of α[1→6]-linked units (Laishley *et al.*, 1974). Whether this means that the granule-bound granulose synthase should consist of at least two distinct transglucosylases (with α[1→4] and α[1→6] specificities respectively) has yet to be determined.

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