The Role of Primer in Glycogen Biosynthesis in *Aerobacter aerogenes**

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ABSTRACT: During the logarithmic growth phase *Aerobacter aerogenes* A3(S1) produces an active glycogen synthetase but accumulates very little endogenous glycogen. Cell extracts show an absolute primer requirement for incorporation of glucose from adenosine diphosphate glucose into glycogen. Amylopectin, two different bacterial glycogens, and the corresponding β-amylase limit dextrins (each of which has distinct structural features) serve as primers for glycogen synthesis. The structure of the product is similar to *A. aerogenes* A3(S1) glycogen formed by cultures during a nitrogen-limited stationary growth phase, regardless of the primer used. Apparent primer K_M values for unfractionated cell extracts, expressed as concentration of nonreducing terminals, show a progressive decrease with increasing chain length of the primer. Upon partial purification of the glycogen synthetase by pelleting the activity at 105,000*g*, the apparent primer K_M values are increased three- to eightfold and the correlation of K_M with chain length of the primer is lost. The original kinetic parameters are restored when the 105,000*g* supernatant is recombined with the pellet. The data suggest that the cell extract and the partially purified pellet may utilize different acceptor sites on the glycogen primers. Preliminary evidence indicates that both enzyme preparations glucosylate less than half of the primer nonreducing terminals.

Within a single organism D-glucose and its derivatives occur in a number of distinct polymeric carbohydrates. In *Aerobacter* strains D-glucose, or the D-glucosamine and D-glucuronic acid derived from it, is found in capsules (Wilkinson *et al.*, 1955), lipopolysaccharides (Sutherland and Wilkinson, 1966), glycogen (Strange *et al.*, 1961; Segel *et al.*, 1964), and cell wall components (Schocher *et al.*, 1962). Although the activated form of glucose which serves as precursor for each of these polymers has not been established, it has been demonstrated by Greenberg and Preiss (1964) that ADP[1]-glucose is the precursor for glycogen in *Aerobacter aerogenes* as well as in a variety of other organisms.

During the course of our studies on the structure of *A. aerogenes* capsular polysaccharides (Sandford and Conrad, 1966; Conrad *et al.*, 1966; Gahan *et al.*, 1967), methods for microanalysis of polysaccharide structure have been developed which now permit an examination of the relationship between primer[2] and product in glycogen biosynthesis. Furthermore, one of our strains, A3(S1), when grown on a glucose–mineral salts medium, contains an active glycogen-synthesizing system but does not accumulate glycogen during logarithmic growth. This organism, therefore, has little or no endogenous primer in cell extracts. This has permitted an examination of the effect of several structurally characterized primers on glycogen biosynthesis in unfractionated cell extracts as well as in a partially purified system. It has been found that the kinetic properties of the biosynthetic system are markedly altered on purification. This suggests that the *in vivo* glycogen-synthesizing system may not be adequately described by summation of the properties of purified nucleoside diphosphate glucose:glycogen transglucosylases and branching enzymes.

Methods. [^{14}C]ADP-glucose was obtained from International Chemical and Nuclear Corp. and diluted with unlabeled ADP-glucose (Calbiochem) to 0.1 mc/mmole for use in the enzyme assay. [^{3}H]Methyl iodide from New England Nuclear Corp. was diluted with unlabeled methyl iodide to 0.2 mc/mmole for methylation analysis of primers. Crystalline sweet potato β-amylase was obtained from Worthington Biochemical Corp. and assayed before each use as described by Bernfeld (1955). Glycogen-type glucans

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[1] Abbreviations used: ADP, adenosine diphosphate; UDP, uridine diphosphate; DTT, dithiothreitol; $\overline{CL}$, average chain length; $\overline{ECL}$, average external chain length; $\overline{ICL}$, average internal chain length; NRT, nonreducing terminal; NRT K_M, the apparent K_M of the primer, expressed in terms of molar concentration of nonreducing terminals; AG K_M, the apparent K_M of the primer, expressed in terms of molar concentration of anhydroglucose residues.

[2] The terms "primer" and "acceptor" are used interchangeably to describe a glycogen-type substrate which must be present in assay mixtures to obtain glycogen synthesis. Direct addition of glucose from ADP-glucose to the primer has not been unequivocably established.

from *Mycobacterium* species (Lee, 1966) were gifts from Dr. C. E. Ballou. Total carbohydrate and D-glucose were determined as described previously (Gahan *et al.*, 1967).

Preparation of Primers. Amylopectin (waxy maize, amylose free) was purchased from Calbiochem and used without further purification. Bacterial glycogens were isolated from cells of *A. aerogenes* strains A3(S1)-(ATCC 12658) and NCTC 243. The growth media were (1) a tryptone–glucose medium (Strange *et al.*, 1961) and (2) the glucose–mineral salts medium described by Wilkinson *et al.* (1955), modified by reducing the ammonium sulfate concentration to 0.045% so that nitrogen was made growth limiting. Carboys containing 14 l. of medium were inoculated with 600 ml of an 8-hr shake culture and the cells were grown at 30° with aeration and vigorous mechanical stirring. Two hours after entering the stationary growth phase the cells were harvested in a Sharples supercentrifuge. Most of the capsular and slime polysaccharides were dissociated from the cells by the vigorous stirring during growth. Residual surface polysaccharide was removed by homogenizing the packed cells in five volumes of 0.01 M potassium phosphate buffer (pH 7.0) for 45 sec in a Waring Blendor and centrifuging the cells at 30,000*g*. The tryptone–glucose medium gave approximately 12 g of washed cell paste/l.; the nitrogen-limited medium gave 2.3 g/l.

The freshly harvested cells were disrupted by sonication and glycogen was recovered as described by Strange *et al.* (1961). The crude product was purified by passage through a column of DEAE-cellulose in 0.01 M potassium phosphate buffer (pH 7.0). Impurities were absorbed on the column while glycogen emerged in the break-through volume. The effluent solution was dialyzed and concentrated to a small volume from which glycogen was precipitated with two volumes of ethanol. The precipitate was washed with ethanol and ether and dried *in vacuo* at 60°.

β-Amylase limit dextrins of the amylopectin and the two bacterial glycogens were prepared by exhaustive treatment of polysaccharide solutions (0.5% in 0.015 M acetate buffer, pH 5.0) with crystalline sweet potato β-amylase (50 μg/10 mg of polysaccharide) until no further increase in reducing value could be observed. After dialysis the β-dextrins were recovered as above. Aqueous stock solutions of primers (5 mg/ml) for use in kinetic studies were stored in the freezer.

[^{3}H]Glycogen was kindly prepared for us by Dr. R. F. Nystrom (Radiocarbon Laboratory, University of Illinois) using the Wilzbach technique (Wilzbach, 1957). NCTC 243 glycogen (200 mg, mixed with 300 mg of Darco G-60) was exposed to $^1/_{20\text{th}}$ atm of tritium gas (1 c, carrier free) at room temperature for 14 days. The product was suspended in water, centrifuged to remove the carbon, and dialyzed exhaustively for 4 days to remove degradation products and exchangeable ^{3}H. The [^{3}H]glycogen was recovered from solution as above; yield, 24 mg (12%) of a product with a specific activity of 55 μc/mmole of anhydroglucose.

Characterization of Primers and Biosynthethic Glycogens. Methods for characterization of glycogen-type polymers in terms of their iodine-staining properties, chain-length parameters, and per cent conversion to maltose by β-amylase (β-amylolysis) have been reviewed by Manners (1957) and are restated here only as they apply in this work. Chain-length parameters describe the number of glucose residues in a defined segment of the branched polymer. An average chain length ($\overline{CL}$) is defined as the sum of the average external chain length ($\overline{ECL}$) from a branch point to a non-reducing terminal (NRT), the average internal chain length between two branch points ($\overline{ICL}$), and the glucose residue forming the branch point which joins an $\overline{ECL}$ and an $\overline{ICL}$.

$$\overline{CL} = \overline{ECL} + \overline{ICL} + 1 \qquad (1)$$

$\overline{ECL}$ and $\overline{ICL}$ vary from one type of glycogen to another but for all β-dextrins the $\overline{ECL}$ is reduced to 2.5 glucose residues (Manners, 1962). The $\overline{ICL}$ of a glycogen is not altered by β-amylolysis and is therefore the same for any glycogen and its β-dextrin. From methylation analysis $\overline{CL}$ for any glycogen or β-dextrin can be derived from the relationship

$$\overline{CL} = \left(\frac{\text{trimethylglucose}}{\text{tetramethylglucose}}\right) + 2 \qquad (2)$$

Since for a β-dextrin $\overline{ECL}$ = 2.5 and $\overline{CL}$ can be determined by methylation analysis, $\overline{ICL}$ for both the β-dextrin and its parent glycogen can be calculated from eq 1. Using the $\overline{ICL}$ thus determined and the $\overline{CL}$ for the parent glycogen, derived from methylation data, the $\overline{ECL}$ of the parent glycogen can be calculated from eq 1.

Methylation Analysis. All chain-length data presented here are based on methylation analysis of the polymers before and after exhaustive β-amylolysis. Three types of samples are analyzed for chain-length parameters: (1) the original primer molecules, (2) that portion of the product glycogen derived from ADP–glucose, exclusive of the acceptor portion of the product, and (3) that portion of the product glycogen derived from the acceptor, exclusive of the glucose transferred from ADP–glucose. The methylation analysis is facilitated by labeling that part of the glycogen sample under consideration with ^{3}H or ^{14}C. This permits quantitation of the amounts of di-, tri-, and tetramethylglucoses by radiochromatography as described below.

The unlabeled primer glycogens are labeled in the methylation reaction by using [^{3}H]methyl iodide (0.2 μc/mmole) as the alkylating reagent. For analysis of the two separate portions of the enzymatically synthesized glycogen each portion is labeled separately and the methylation is performed with unlabeled methyl iodide. Products labeled in the portion derived from ADP–glucose are enzymatically synthesized from unlabeled primer and [^{14}C]ADP–glucose. The product with the label in the portion derived from primer is

synthesized from the [^{3}H]glycogen described above and unlabeled ADP–glucose.

Methylation analyses were performed on glycogen samples (1–50 mg) by a modification (Sandford and Conrad, 1966) of the method of Hakomori (1964) which uses methylsulfinyl anion and methyl iodide in dimethyl sulfoxide solutions of polysaccharides. The stoichiometric conversion of neutral polysaccharides to fully substituted derivatives by this method, as required for the validity of this approach for chain-length determination (Manners, 1957), has been demonstrated for glycogen and laminarin (Sandford, 1967) and for a galactan derived from the A3(S1) lipopolysaccharide (H. E. Conrad and J. D. Epley, unpublished results). Aliquots of the mixtures of labeled methyl sugars recovered from hydrolysates of the methylated glycogens were analyzed by chromatography on 0.75-in. wide strips of Whatman No. 1 paper in water-saturated methyl ethyl ketone. The developed strips were cut into 0.75-in. segments which were immersed in 5 ml of 99% ethanol in scintillation vials to solubilize the methyl sugars. After 30 min, 10 ml of a scintillation fluid containing 7.5 g of 2,5-diphenyloxazole/l. of toluene was added and samples were counted to ±3% error in a Beckman CPM 200 scintillation counter using optimal settings for the isotope in question. Uniform counting efficiencies (85% for ^{14}C, 22% for ^{3}H) were obtained for all extracted segments of the chromatograms (determined by external standardization). Thus, when the portion of primer or product in question was uniformly labeled, the ratios of counts per minute in the trimethylglucose region of the chromatogram to those in the tetramethylglucose region gave a direct indication of the molar ratios. When [^{3}H]methyl iodide was used in the analysis of unlabeled glycogens, molar ratios were obtained by multiplying the ratio of trimethylglucose to tetramethylglucose counts per minute by four-thirds to correct for the different number of [^{3}H]methoxyls on the tri- and tetramethylglucoses.

β-Amylolysis. Per cent β-amylolysis for the primers used in this work was determined during preparation of the β-dextrins described above. The value was calculated from the amount of maltose released (Bernfeld, 1953) and the total number of maltose equivalents present, determined by total carbohydrate analysis using maltose as a standard. The labeled glycogen products were similarly treated with β-amylase. In the latter cases the incubation mixtures were analyzed at 1-hr intervals by spotting aliquots on a 0.75-in. wide strip of Whatman No. 1 paper and developing the chromatogram in ethanol–1 M ammonium acetate, pH 4.0 (7:3). Segments (0.75 in.) of the strip were counted without elution in a scintillation fluid containing 5 g of diphenyloxazole/l. of toluene. Per cent β-amylolysis was calculated from the number of counts in the maltose region of the chromatogram and the total counts applied to the chromatogram.

Iodine-Staining Properties. Solutions of primer in 0.1 M Tris-HCl buffer (pH 7.4) contained, in weight per volume per cent: primer, 0.01; potassium iodide, 0.2; and I_2, 0.02. The solutions were placed in a 1-cm cuvet and scanned from 400 to 600 mμ in a Cary Model 15 recording spectrophotometer. $E_{1\,cm}^{mM}$ values were calculated from the observed absorbancy at λ_{max} and glycogen concentration was expressed as millimoles of anhydroglucose per liter.

Enzyme Preparation. A. aerogenes strains A3(S1) and NCTC 243 were maintained on Difco stock agar slants and subcultured every 6 weeks. For enzyme production the organisms were grown in shake flasks at 30° on the glucose–mineral salts medium described by Wilkinson *et al.* (1955). When the culture reached the top of the logarithmic growth phase it was homogenized for 45 sec in a Waring Blendor to dissociate the capsule. The cells were then harvested and washed with 0.01 M potassium phosphate buffer (pH 7.0). The washed cell paste was suspended in 0.05 M Tris-HCl buffer (pH 7.5) containing 0.001 M dithiothreitol (Tris–DTT buffer) using 4 ml of buffer/g of cell paste. Cells were disrupted at 0° using a Bronson sonic oscillator (Model S110) operated at maximum output for 5 min/100 ml of cell suspension. The broken cell suspension was frozen in several small vials. Just prior to use a vial was thawed and its contents were centrifuged at 30,000*g* for 15 min. The supernatant, designated "native" enzyme, contained approximately 20 mg of protein/ml and lost 50% of its activity in 4 days at 4°.

The "pellet" enzyme was prepared by centrifugation of native enzyme at 105,000*g* for 2 hr in the SW39L head of a Spinco L2-65 ultracentrifuge. The pellet, which contained approximately 25% of the native enzyme protein, was resuspended in cold Tris–DTT buffer and stored at 0°. The enzyme is completely destroyed upon freezing and thawing and has a half-life of 72 hr at 4°. The activity is somewhat stabilized in the presence of 0.5% glycogen.

Enzyme Assay. Assays are carried out at room temperature in 5 × 50 mm test tubes containing in μmoles/0.1 ml: Tris-HCl buffer (pH 7.4), 4; $MgCl_2$, 2.5; [^{14}C]ADP–glucose, 0.5 (approximately 50,000 cpm when counted as below); and primer, 0.35 (as anhydroglucose). Reaction is initiated by addition of 0.3 mg of native or 0.1 mg of pellet enzyme. At intervals 10-μl aliquots are transferred to a 1 × 1.25 in. square of Whatman 3MM paper using disposable micropipets (Drummond Microcaps, Kensington Scientific Corp.). The paper square is immediately dipped into acetone to stop the reaction and unreacted [^{14}C]-ADP–glucose is removed from the dried papers by two 30-min washes in 50% ethanol containing 0.1% ammonium hydroxide followed by a final 10-min wash in acetone. The papers are air dried and counted without elution to ±3% error in a scintillation fluid containing 5 g of diphenyloxazole/l. of toluene. The ^{14}C-counting efficiency in the assay is routinely 65%.

Enzyme Kinetic Studies. The effects of ADP–glucose and primer concentrations on rates of glycogen synthesis were determined using the rate of [^{14}C]glucose incorporation between 7 and 15 min in the standard assay. Since primer is not consumed in the usual sense,

the rates at varying primer levels were essentially linear for at least 15 min in the presence of saturating levels of ADP-glucose (5×10^{-3} M). Molar concentrations of primers are expressed in two ways: (1) in terms of the amount anhydroglucose (AG) present in the assay, and (2) in terms of the level of nonreducing terminal (NRT) glucose present. The latter value is obtained by dividing the former by the chain length of the primer.

Since the native enzyme system gave sigmoid primer saturation curves, apparent K_M values were taken as the primer concentration at one-half the maximum observed velocity. K_M values for the pellet system were calculated from Lineweaver–Burk plots.

Isolation of Enzymatically Synthesized Glycogens. Glycogen products for structural analysis were prepared by scaling up the usual assay mixture tenfold and reducing the primer concentration to 1.5 times the K_M for the particular primer and enzyme system under study (see Table II). Following a 1-hr incubation period the total reaction mixture was heated at 100° in 0.05 N acetic acid for 1 hr to dissociate the glycogen from protein which interferred in the methylation analysis. This treatment caused no alteration of the chain-length parameters of an unincubated glycogen control; therefore, any hydrolytic degradation was considered to be very minor. The soluble fraction following the acetic acid treatment contained 85–95% of the radioactivity initially present in the reaction mixture except in the case of some of the pellet system products where yields were as low as 70%. Unreacted ADP-glucose and acetic acid were removed by dialysis and the glycogen solution was concentrated to dryness and taken up in 1 ml of water. An aliquot (0.1 ml) was analyzed for per cent β-amylolysis and the remainder was subjected to methylation analysis. By this procedure 50–80% of the total labeled glycogen was recovered from the native enzyme incubations; approximately 20% was recovered from pellet incubations.

Results

The catalysis of glucose transfer from [^{14}C]ADP-glucose into glycogen by cell extracts of *A. aerogenes* strains A3(S1) and NCTC 243 is shown in Figure 1. Although the two extracts synthesize glycogen at similar rates they differ markedly in primer requirement. The A3(S1) extract has very low activity unless primer is added; the 243 extract alone gives a rapid rate of glycogen synthesis which is stimulated by exogenous glycogen. The primer dependency of the A3(S1) extracts persists after pelleting the glycogen synthetase at 105,000*g* for 2 hr, a treatment which increases the specific activity three- to fourfold and which would be expected to pellet endogenous glycogen in these extracts (Holme *et al.*, 1958).

The suggestion from these experiments that these two strains differ in their capacity to store glycogen was confirmed by attempts to isolate glycogen from the cells obtained when the organisms were grown on a tryptone–glucose medium shown previously to give

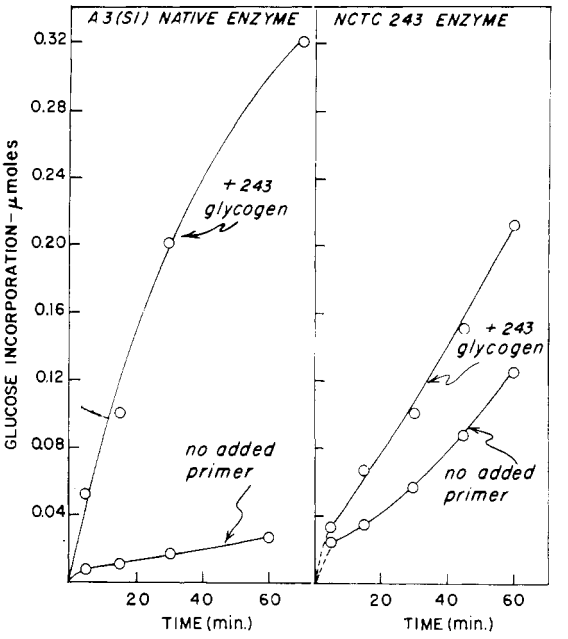

FIGURE 1: Primer dependency for glycogen synthesis catalyzed by *A. aerogenes* cell extracts. Assays were performed with no added primer and in the presence of 0.35 μmole of primer anhydroglucose/0.1-ml assay volume, as described in Methods. The primer used was 243 glycogen.

maximal glycogen accumulation in *A. aerogenes* (Strange *et al.*, 1961). As shown in Table I, strain 243 accumulates glycogen both on this medium and on a defined, nitrogen-limited medium, but strain A3(S1) could be induced to store glycogen only under the nitrogen-limiting growth conditions (compare Segal *et al.*, 1964). The purified glucose polymers used in this work are identified as glycogen by their glucose content, their approximately 50% conversion to maltose by β-amylase, and their methylation analyses which yield 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,3,6-tri-*O*-methyl-D-glucose, and 2,3-di-*O*-methyl-D-glucose in appropriate ratios. The methyl sugars were identified by paper chromatographic comparison with standards.

The availability of an organism having a highly active glycogen synthetase but little or no endogenous glycogen has permitted a study of the effect of primer structure on the kinetics of glycogen synthesis in native (cell extract) and pellet (105,000*g* pellet) enzyme. All experiments described below were performed with the glycogen-synthesizing system obtained from A3(S1) cells grown under conditions such that their extracts showed essentially complete dependency on added glycogen for glucose incorporation.

Structure of Glycogen Acceptors. The primers used were: (1) a waxy maize amylopectin ("amylose free," Calbiochem), (2) strain 243 glycogen, isolated from cells grown on tryptone–glucose medium, (3) strain A3(S1) glycogen from cells harvested 2 hr into the stationary growth phase on nitrogen-limited medium,

TABLE I: Glycogen Production by *A. aerogenes* Strains.

Growth Medium	*A. aerogenes* Strain			
	A3(S1)		NCTC 243	
	Glycogen Yield[a] (g/100 g of dry cells)	Glucose[b] (% of carbohydrate)	Glycogen Yield[a] (g/100 g of dry cells)	Glucose[b] (% of carbohydrate)
Tryptone–glucose[c]	0.01	100	3.0	96
Glucose–salts;[d] N limited	2.0	100	2.0	

[a] Cells harvested 2 hr after onset of stationary growth phase. [b] D-Glucose was determined by the glucostat procedure after hydrolysis of glycogen for 6 hr in 1 N H_2SO_4 at 100°. Total carbohydrate was determined on the hydrolysate by the phenol–H_2SO_4 method and expressed as glucose equivalents. [c] Strange *et al.* (1961). [d] This medium is that described by Wilkinson *et al.* (1955), modified by reducing the ammonium sulfate concentration to 0.045%.

TABLE II: Characterization of Primer Molecules.

Primer	β-Amylolysis (%)	$\overline{CL}$[a]	$\overline{ECL}$[a]	$\overline{ICL}$[a]	I_2-Staining Properties	
					λ_{max} (mμ)	E[b] (cm²/μmole)
Amylopectin	65	18.3	14.3	3.0	525	1.00
243 glycogen	46	12.5	8.3	3.2	470	0.34
A3(S1) glycogen	64	12.4	10.5	0.9	470	0.29
Amylopectin β-dextrin	0	6.5	2.5	3.0	510	0.42
243 β-dextrin	0	6.7	2.5	3.2	460	0.05
A3(S1) β-dextrin	0	4.4	2.5	0.9		

[a] $\overline{CL}$, $\overline{ECL}$, and $\overline{ICL}$ refer to average values for chain length, external chain length, and internal chain length, respectively. Values are determined by methylation analysis. [b] The extinction coefficient is calculated for a 1 mM solution of glycogen, as anhydroglucose, in an I_2–KI solution using a 1-cm light path, as described in Methods.

and (4-6) the β-amylase limit dextrins of (1-3). The average chain-length parameters and iodine-staining properties of these acceptors given in Table II show that in this group each of these polysaccharides is structurally unique. Thus, even though the β-dextrins of amylopectin and 243 glycogen are almost identical in their chain-length parameters, they give quite distinct iodine-staining reactions (compare Manners and Wright, 1962; Kjölberg *et al.*, 1963). The A3(S1) glycogen is structurally the most unusual of the primers tested having such a low $\overline{ICL}$ that its very tightly branched, structurally compact β-dextrin fails to react with iodine.

Characterization of the Enzyme Systems. The native glycogen-synthesizing system of A3(S1) is an 18,000*g* supernatant from sonicated cells. When the native preparation is centrifuged for 2 hr at 105,000*g*, approximately 70% of the original activity is recovered in the pellet and the specific activity is increased three- to fourfold. A comparison of the capacity of the native and pellet systems to catalyze glycogen synthesis has shown a number of similarities but also some striking differences.

Both preparations have the following properties in common. They exhibit an absolute dependency on a glycogen-type primer for activity; inactive primers include an acid-hydrolyzed, nondialyzable amylose fraction, dextran, several glucose-rich polysaccharide fractions prepared from 243 lipopolysaccharide, and several samples of glucans from *Mycobacterium* species (Lee, 1966). As glucosyl donor, ADP-glucose is approximately 50 times as active as UDP-glucose as observed previously for bacterial glycogen synthetases (Greenberg and Preiss, 1964). The K_M for ADP-glucose in the presence of saturating levels of 243 glycogen is 1.2×10^{-3} M. Variations in this K_M with primer structure and concentration or with other components of the assay have not been determined. α-D-Glucose 1-phosphate at 5×10^{-3} M does not replace ADP-glucose as the glucosyl donor for glycogen synthesis and has no effect on the rate of glycosyl transfer from ADP-glucose. If, however, ATP and α-D-glucose 1-

phosphate are added together to the assay in place of ADP-glucose, glycogen synthesis occurs at the rate obtained with ADP-glucose. It appears, therefore, that these extracts contain an active ADP-glucose pyrophosphorylase. D-Glucose 6-phosphate at 5×10^{-3} M has no effect on the V_{max} (Rosell-Perez and Larner, 1962) or the primer K_M (Vardanis, 1967) observed for 243 glycogen. The enzymatically synthesized products yielded only glucose on hydrolysis and were incompletely degraded by β-amylase yielding a nondialyzable β-dextrin and a radioactive product with an R_F identical with that of maltose. Methylation analysis of the products gave the expected methyl sugars as indicated by chromatographic comparison with standards.

Marked differences in the two enzyme systems are observed, however, when they are compared on the basis of their interaction with the acceptors, as shown in the acceptor saturation curves in Figure 2. For these curves acceptor concentrations are expressed as molar amounts of nonreducing terminals added to the assay system since it has been assumed that the terminal glucose residues in the primer molecule act as acceptors for the transferred glucosyl residues. With the native enzyme system sigmoid saturation curves are obtained with all active primers with the possible exception of A3(S1) glycogen. This suggests that the primers may serve not only as glucosyl acceptors but also as activators of the enzyme system. The different acceptors appear to fall into groups with respect to their capacity to saturate the enzyme system. Amylopectin is superior; the bacterial glycogens are intermediate; the β-dextrins of amylopectin and 243 glycogen are the poorest; and the A3(S1) β-dextrin is completely inactive.

With the pellet system, on the other hand, the activating effect of the acceptors has been lost as evidenced by the hyperbolic saturation curves, and, while the grouping of the acceptors with respect to their enzyme saturating capacity is the same as for the native system, the acceptor K_M's are three- to eightfold higher for the pellet. Perhaps even more striking is the fact that the A3(S1) β-dextrin, which is completely inactive in the native system over the range $0.2\text{–}200 \times 10^{-5}$ NRT's, is an active acceptor for the pellet and gives the highest V_{max} of all the primers tested. In the native system this polysaccharide is, in fact, an inhibitor of glycogen synthesis, reducing the glucose incorporation rate to approximately 50% when added in equimolar concentration (anhydroglucose) with 243 glycogen.

Comparison of Kinetic Parameters in Native and Pellet Systems. The kinetic parameters derived from the data in Figure 2 are given in Table III. Apparent Michaelis constants are recorded both as concentrations of NRT's (NRT K_M; see Leloir, 1964) and as concentrations of anhydroglucose units (AG K_M; see Villar-Palasi *et al.*, 1966). For the native system the V_{max}'s and the AG K_M's are very similar for all acceptors. The NRT K_M's, on the other hand, vary over a sixfold range and show a progressive increase as $\overline{CL}$ decreases. It may be noted that for each NRT

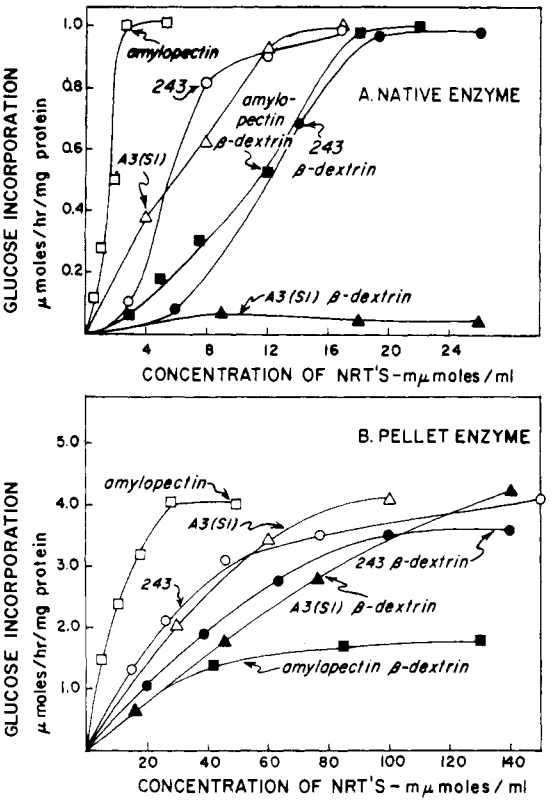

FIGURE 2: Primer saturation curves for glycogen synthesis catalyzed by (A) native and (B) pellet enzyme systems from *A. aerogenes* A3(S1). Rates of glycogen synthesis and primer levels in the assay, expressed as concentrations of nonreducing terminals, are plotted on different scales for the two enzyme systems. Open and filled symbols (*e.g.*, ○ and ●) represent the original glycogen primer and its β-dextrin, respectively.

there is one $\overline{CL}$; thus, the concentration of NRT's is equal to that of $\overline{CL}$'s. This striking correlation of NRT K_M with $\overline{CL}$ suggests that extended chains of 1,4-linked glucose residues give the best activation of the system, and/or that such stretches (perhaps by reduced steric restrictions) present to the enzyme system more efficient glucosyl-acceptor sites.

The values for the pellet system differ from those of the native system with respect to both magnitude and structural correlations. For amylopectin and the glycogens V_{max}'s are identical but for the β-dextrins there is a progressive increase in V_{max} with decreasing $\overline{ICL}$ (see Table II). The correlation of NRT K_M with $\overline{CL}$ found for the native system has disappeared and the AG K_M's are no longer similar. There is, however, a correlation of AG K_M with the molecular weights and/or the structural compactness of the acceptors. Although absolute values for molecular weight have not been determined, the β-dextrins have approximately one-half the molecular weight of the glycogen from which they are derived (Table II). They are also more compact structures than the parent glycogens since

TABLE III: Kinetic Parameters for Primers of Glycogen Synthetase.

Primer	$\overline{CL}$	Enzyme System: Native[a] NRT K_M[b] (M × 10^5)	Native[a] AG K_M[c] (M × 10^5)	Native[a] V_{max} (μmoles/hr mg)	Pellet[a] NRT K_M[b] (M × 10^5)	Pellet[a] AG K_M[c] (M × 10^5)	Pellet[a] V_{max} (μmoles/hr mg)
Amylopectin	18.3	0.2	3.7	1.2	1.5	27.4	4.1
243 glycogen	12.5	0.6	7.3	1.3	3.5	43.8	4.2
A3(S1) glycogen	12.4	0.6	7.4	1.3	3.5	42.5	4.1
Amylopectin β-dextrin	6.5	1.2	7.8	1.4	1.7	11.1	1.8
243 β-dextrin	6.7	1.3	8.7	1.3	3.9	26.2	3.6
A3(S1) β-dextrin	4.4		Inactive		6.0	24.6	4.5

[a] Values for native enzyme estimated from saturation curves (Figure 2) with K_M taken as the substrate concentration at one-half the observed V_{max}. Values for the pellet enzyme calculated from Lineweaver–Burk plots. [b] NRT K_M is the apparent K_M in terms of the molar concentration of nonreducing terminals. [c] AG K_M is the apparent K_M in terms of the molar concentration of anhydroglucose.

the extended external chains are markedly reduced in length. It is seen that halving the molecular weight of the acceptor and increasing its compactness in all cases gives a corresponding decrease in AG K_M. A similar correlation is not found for the native system.

Reconstitution of the Native System. Reconstitution of the native system by combining the 105,000*g* pellet and supernatant is shown in Figures 3 and 4. The enzyme preparations are assayed at three levels of 243

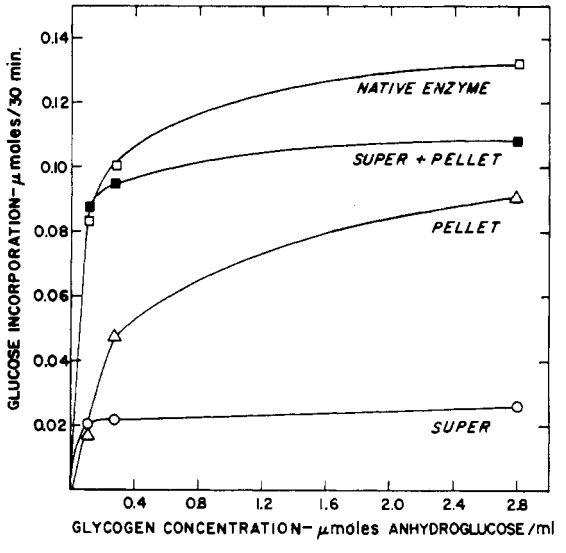

FIGURE 3: Reconstitution of native enzyme system upon recombination of 105,000*g* supernatant and pellet. The amount of protein in each assay was an follows: native enzyme (□), 0.29 mg; pellet (△), 0.07 mg; supernatant (○), 0.22 mg; and supernatant plus pellet (■), 0.29 mg. The primer used was 243 glycogen.

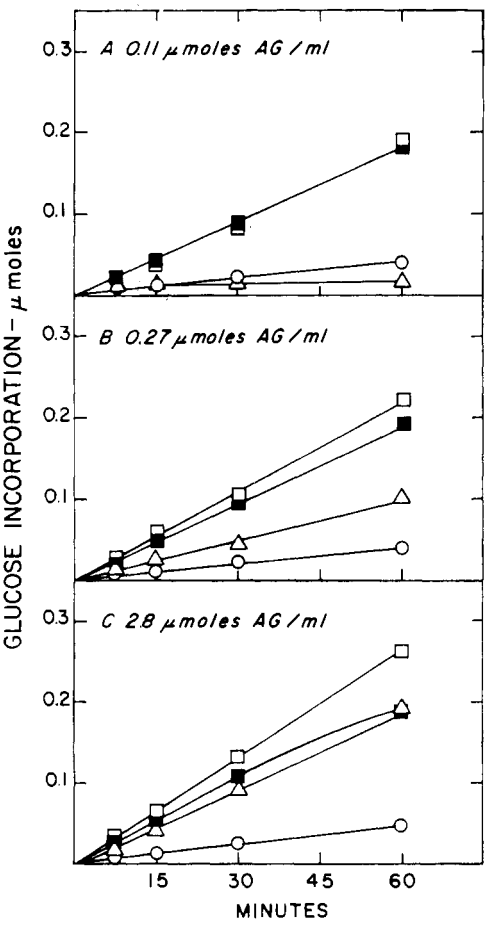

FIGURE 4: Reconstitution of native enzyme system upon recombination of 105,000*g* supernatant and pellet. Magnitude and linearity of rates as a function of glycogen concentration. Symbols and conditions are the same as described for Figure 3.

TABLE IV: Characterization of the Portion of the Enzymatically Synthesized Glycogen Derived from ADP–Glucose.[a]

Enzyme	Primer	Glucoses Added		β-Amylolysis (%)	$\overline{CL}$[b]	$\overline{ECL}$[b]	$\overline{ICL}$[b]
		Per AG	Per NRT				
Native	Amylopectin	14	253	67	18.5	14.9	2.6
Native	243 glycogen	15	188	75	17.8	15.9	0.9
Native	A3(S1) glycogen	19	230	68	15.9	13.3	1.6
Native	Amylopectin β-dextrin	18	117	67	17.5	14.2	2.3
Native	243 β-dextrin	15	100	71	18.6	15.7	1.9
Pellet	243 glycogen	2.5	31	77			
Pellet	243 glycogen	1.1	14		11.3		
Pellet	243 β-dextrin	1.6	11	66			

[a] Products were isolated from 1-hr incubations in which the assay was scaled up tenfold. Primers were added at a concentration of 1.5 times their K_M (Table III). [b] See footnote *a*, Table II.

glycogen chosen such that the lowest level does not saturate either native or pellet, the intermediate level almost saturates the native but not the pellet system, and the highest level saturates both systems. The amounts of pellet and supernatant protein used in these assays are those amounts of each derived from the native enzyme preparation such that when they are added together to the assay, the recombination contains the same amount and complement of protein as the native enzyme assay. From Figure 3 it can be seen that the supernatant and the reconstituted systems are saturated at the same low glycogen concentration which saturates the native system, whereas the pellet requires a higher glycogen level for saturation, as observed previously (Figure 2). Since only three glycogen concentrations were tested in this experiment, it is not clear whether the sigmoid features of the saturation curve at low glycogen concentrations were also reconstituted. Figure 4 shows that the rates of glucose incorporation in this experiment are linear for 1 hr for all assays except that of the pellet at the lowest glycogen level. Also it is seen that at the lowest glycogen level the rate for the reconstituted system is far in excess of the summation of the rates for pellet and supernatant, whereas at the highest glycogen level the pellet alone is almost as active as the reconstituted system. Thus, two fractions are implicated in the rates described here. Glucose 6-phosphate at 5×10^{-3} M does not replace the supernatant fraction in the reconstitution (Vardanis, 1967). Dialysis of the native enzyme causes a shift to higher primer K_M similar to that obtained on pelleting the activity, but boiled extracts do not restore the native primer kinetics.

Characterization of the Enzymatically Synthesized Glycogen. The synthetic products formed using the different primers have been analyzed to determine whether the primer structure has any affect on the structure of the synthesized product. By synthesizing glycogen using, on the one hand, unlabeled acceptor and [^{14}C]ADP–glucose and, on the other, ^{3}H acceptor and unlabeled ADP–glucose, it has been possible to characterize separately the structural features of (1) the glucose residues that are incorporated into glycogen from ADP–glucose and (2) those residues in the original acceptor after it has been substituted. In each case the isotopically labeled portion of the product has been examined by methylation analysis (using unlabeled methyl iodide) and by β-amylolysis. In all of these experiments the assay system was scaled up tenfold and acceptor was added at a concentration of 1.5 times its apparent K_M. The data in Table IV show that the portion of the glycogen product derived from ADP–glucose, irrespective of the primer or the enzyme system used, is highly branched and resembles structurally the A3(S1) glycogen (see Table II). In the native system there is almost net (but not *de novo*) synthesis with up to 20-fold multiplication of the original acceptor glycogen. The amount of glucose transferred to acceptor by the pellet system is much less and is probably limited by the solubility of the product (see below).

The substitution of the acceptor molecule during glycogen synthesis is indicated in Table V. The most significant observation is that many of the NRT's are not recipients of glucose from ADP–glucose. If all NRT's were covered in this experiment, β-amylase would cleave very little from the highly branched product and methylation analysis would yield no [^{3}H]-tetramethylglucose. The data in Table V show that β-amylase removes 20% of the ^{3}H from the product of the native enzyme and 32% of the ^{3}H from the product of the pellet enzyme (or 54 and 86%, respectively, of the original β-amylase susceptible fraction of the acceptor), indicating that a large proportion of the NRT's of the acceptor was not glucosylated in the reaction. This result is confirmed by methylation analysis which show that more than half of the original NRT's of the acceptor molecules remain as NRT's in the products from both native and pellet systems. The result is especially notable for the native system

TABLE V: Characterization of the Portion of the Enzymatically Synthesized Glycogen Derived from the Primer.

Glycogen Analyzed[a]	Glycogen Characterization: Glucoses Added Per AG	Glucoses Added Per NRT	β-Amylolysis (%)[c]	$Me_3:Me_4$ Ratio[c]	NRT's Glucosylated[b] (%)
[^{3}H]Glycogen			37 ± 7	6.3 ± 0.4	
1, incubated with native enzyme			30 ± 7	3.9 ± 0.3	
1, incubated with native enzyme + ADP-glucose	14	115	20 ± 7	9.0 ± 1	27–51
1, incubated with pellet enzyme			27 ± 7	6.0 ± 0.4	
1, incubated with pellet enzyme + ADP-glucose	1	8	32 ± 5	9.1 ± 1	28–31

[a] Products were isolated from 1-hr incubations in which the assay, using unlabeled ADP-glucose, was scaled up tenfold. [^{3}H]Glycogen was added at 1.5 times its K_M. The amount of glucose added to primer was determined in a parallel assay with [^{14}C]ADP-glucose. [b] % NRT's glucosylated = $(Me_3:Me_{4\,final} - Me_3:Me_{4\,initial}/1 + Me_3:Me_{4\,final}) \times 100$. The derivation of this relationship is based on the fact that for each primer NRT glucosylated in glycogen synthesis a glucose residue of the primer which would yield tetramethylglucose in a methylation analysis is converted to a residue which will yield trimethylglucose. Thus, the final $Me_3:Me_4$ ratio is equal to the sum of the initial $Me_3:Me_4$ ratio and the fraction of NRT's glucosylated divided by 1 minus the fraction of NRT's glucosylated. [c] Standard deviations are calculated for sample counts per minute minus background counts per minute. Values are based on the assumption that the [^{3}H]glycogen is uniformly labeled, although this has not been verified.

where 14 glucoses from ADP-glucose were incorporated/primer glucose (determined in a parallel control experiment using [^{14}C]ADP-glucose).

A range of values for per cent NRT's covered is given in Table V for the following reasons. In the control experiment where the [^{3}H]glycogen is incubated with the enzymes in the absence of ADP-glucose, the glycogen is apparently degraded (or possibly branched, though the $\overline{ECL}$ would appear too small for this) as indicated by a decrease in the per cent β-amylolysis and in the $Me_3:Me_4$ ratio. Since the calculated per cent NRT's covered in glycogen synthesis increases with the difference between $Me_3:Me_4$ ratios for the original and the substituted acceptor (see footnote *b*, Table V), a lowering of the initial ratio as a result of degradation of the primer will increase the apparent per cent of NRT's covered. Thus, the higher values for per cent NRT's covered given in Table V are obtained using the $Me_3:Me_4$ ratio of the degraded primer (lines 2 and 4) as the initial value. Since synthesis proceeds more than 50 times faster than degradation (see below), the lower values given in Table V, calculated using the $Me_3:Me_4$ ratio of the undegraded primer, would appear to describe more appropriately the value for per cent NRT's which are glucosylated by the enzyme systems.

For this experiment the [^{3}H]glycogen was prepared by tritiation of a sample of 243 glycogen by the Wilzbach technique, a procedure which caused considerable degradation of the glycogen and which gave only a 12% yield of high molecular weight glycogen after exhaustive dialysis. The possibility exists that this treatment caused modification of a number of the NRT's in such a way that they are in fact no longer glucose residues and hence not reactive in the synthetase reaction. However, using the glucose content of the primer determined by the phenol–sulfuric acid method, the [^{3}H]glycogen had a primer activity (AG $K_M = 9.5 \times 10^{-5}$ M) almost identical with that of the 243 glycogen. If a significant number of the glucoses were altered in the Wilzbach tritiation so that they no longer offered the specificity required for acceptor sites, the alteration would have to have the subtlety of isomerization of glucose to other hexoses which would behave like glucose both in the β-amylolysis and in the methylation analyses. β-Amylase attack on glycogen at such modified terminals in the substituted primer seems unlikely. Since the conclusion that many NRT's are not substituted is based on comparison of the substituted and unsubstituted primer by chemical or enzymatic analyses directed more or less specifically toward D-glucose, the data appear to describe the normal fate of primer in this enzyme reaction.

The analyses of the ^{3}H primer are based on the assumption that the ^{3}H is uniformly distributed in the molecule, but this has not been demonstrated. Although all samples were counted to less than ±5% error, the [^{3}H]glycogen was not as highly labeled as desirable for the analyses. The absolute values for per cent NRT's covered must therefore be considered tentative. Significantly, both methods of analyzing the product indicate that no more than half of the NRT's are substituted in the enzymatic synthesis of glycogen. The only obvious alternative to this conclusion (that a large fraction of the [^{3}H]glycogen is inactive as acceptor) is ruled out by the solubility data below.

TABLE VI: Solubility of the Fractions of Glycogen Derived from Primer and from ADP-Glucose Following Enzymatic Synthesis of Glycogen.

System[a]			Solubility of Product[b]				ADPG-Derived Glucose/Primer-Derived Glucose in Product (μmoles/μmole)	
			From [^{3}H]Glycogen		From [^{14}C]ADPG			
Enzyme	[^{3}H]-Glycogen (μmoles of AG/ml)	Addition	Soluble (%)	Insoluble (%)	Soluble (%)	Insoluble (%)	Soluble	Insoluble
Native	0.3	None	79	21				
Pellet	3.5	None	72	28				
Native	0.3	ADP-glucose	25	75				
Native	0.3	[^{14}C]ADP-glucose			13	87	6.5	14
Pellet	3.5	[^{14}C]ADP-glucose	10	90	8	92	1.0	1.3

[a] Standard assay as described in Methods with the variations in addition of primer and ADP-glucose indicated. [b] Following a 1-hr incubation glycogen was precipitated from the assay mixture by addition of two volumes of ethanol. The precipitate was washed twice with 67% ethanol to remove unreacted ADP-glucose and then extracted twice with 0.3 ml of water to obtain the soluble fraction. The residue from water extraction is the insoluble fraction of the product. Aliquots were counted as described in Methods to determine the amount of product in each fraction.

Solubility of Product. During incubation of enzyme with ADP-glucose and acceptor there is a progressive increase in turbidity as the reaction proceeds, apparently due to the increasing concentration of a poorly soluble glycogen product (Brown and Cori, 1961; Brown *et al.*, 1965). This is observed in assays with both enzyme systems. Since all products have quite similar structures with fairly large $\overline{ECL}$'s (Table IV) they would be expected to have relatively low limits of solubility which are similar regardless of the primer or enzyme system used. As synthesis proceeds to the point where the product begins to aggregate, further transfer of glucose is not possible because precipitation of primer and/or enzyme from solution causes the reaction to cease. The amount of glucose that can be transferred to acceptor would therefore be limited by the concentration of acceptor required to obtain V_{max}. The difference in apparent glycogen K_M's for the native and pellet systems thus limits the reaction to a transfer of 15–20 glucoses/primer glucose in the native system and two to four glucoses per primer glucose in the pellet system. In both systems glycogen synthesis ceases when the glycogen concentration reaches approximately 1 mg/ml.[3]

Since the synthesized glycogen is a poorly soluble product, the extent to which the [^{3}H]glycogen is rendered insoluble by glucose addition has been taken as a measure of that fraction of acceptor which actually participates as a glucose acceptor in the reactions. The experiments described in Table VI were carried out by precipitating glycogen from the assay mixture with ethanol and, after washing the precipitate with 66% ethanol to remove unreacted ADP-glucose, extracting the precipitate several times with water. When ADP-glucose is omitted from the incubation (lines 1 and 2, Table VI), approximately 75% of the primer can be solubilized by this treatment. A test of the insoluble portion from these experiments shows that when it is resuspended and swollen in buffer, it is as active in the assay as the original acceptor. When ADP-glucose is included in the incubation, both the transferred glucose and the acceptor are to a large extent insolubilized. When only one to two glucoses are added per acceptor glucose (line 5), both ^{3}H and ^{14}C can be counted in the same sample, but when 13 glucoses are transferred (lines 3 and 4) it is necessary to perform a second experiment with unlabeled ADP-glucose in order to count the ^{3}H to ±5% error. The data show that glucosylation of less than half of the NRT's is not due to a large fraction of inactive acceptor in the tritiated glycogen. Thus, by the solubility criterion, at least 75% of the [^{3}H]glycogen reacts in the native system and at least 90% in the pellet system. The percentages of reacted acceptor are probably even higher since, even in the soluble fraction of the product, the acceptor is associated with transferred glucose. The implication from the results in Tables V and VI that in the normal course of glycogen synthesis only a fraction of the NRT's acts as glucosyl acceptors is consistent with the recent observations of Brown *et al.* (1965).

Glycogen Synthesis with Limiting Acceptor. Preliminary experiments show that when subsaturating levels of 243 glycogen (less than 0.5 K_M) are used in the

[3] This solubility explanation of reaction termination is based on observations of the assay and on the difficulties encountered in recovering the products. An alternative explanation for reaction termination is the inhibition by accumulating ADP which reaches similar concentrations in all assays.

incubation with the native system, an autocatalytic rate of glucose incorporation into glycogen is observed following a 3–4-hr lag period. The reaction ceases after incorporation of 75 glucoses/primer glucose but before reaching the saturating glycogen concentration. If no primer is added there is no apparent glycogen synthesis during a 6-hr incubation period.

Glycogen Degradation during Synthesis. By reducing the [^{14}C]ADP-glucose in the assay to a level which is completely consumed in the early stages of reaction and then following the rate of disappearance of the [^{14}C]glycogen product by the usual assay procedure, the effect of degradative enzymes on glycogen biosynthesis has been estimated. For the native system degradation takes place at $^{1}/_{120\text{th}}$ the synthesis rate; for the pellet system, $^{1}/_{55\text{th}}$. While this procedure does not indicate the nature of the glycogen degrading enzymes in these preparations, it does give an indication of the relative rates of synthesis and degradation under the assay conditions. The rate of degradation is very low compared to the glucose incorporation rates in both native and pellet systems and is not sufficient to block the autocatalytic synthesis of glycogen at very low primer concentrations described above.

Discussion

Glycogen synthesis in microorganisms is dependent upon (1) the activity of enzymes for ADP-glucose synthesis and glucosyl transfer to polymer (Preiss and Greenberg, 1965; Greenberg and Preiss, 1964; Preiss *et al.*, 1966); (2) the availability of carbon and energy in excess of that needed for essential growth processes (Madsen, 1963; Segel *et al.*, 1964; Sigal *et al.*, 1964); and (3) presumably the presence of primer glycogen to serve as an acceptor for the transferred glucose. Extracts of *A. aerogenes* A3(S1) cells harvested in the logarithmic growth phase contain both glycogen synthetase and ADP-glucose pyrophosphorylase. Furthermore, during growth the organism is able to generate sufficient excess carbon and energy for polysaccharide biosynthesis as evidenced by its capacity to synthesize large amounts of an extracellular slime polysaccharide both during logarithmic growth (Duguid and Wilkinson, 1953) and, more notably, during carbon-limited chemostat growth (Sandford and Conrad, 1966). Thus, unless A3(S1) synthesizes glycogen and slime polysaccharide from different pools of carbon and energy, it appears that, under conditions where very little glycogen can be detected in the cell, the organism has all of the necessary components for glycogen synthesis with the possible exception of primer. If these cells are, in fact, completely devoid of glycogen at some stage of logarithmic growth, they must possess the capacity for *de novo* biosynthesis of glycogen since, upon entering a nitrogen-limited stationary phase, glycogen begins to accumulate. In any event, it is interesting to note that the synthesis of glycogen is strictly controlled in the cell (in part at least at the ADP-glucose pyrophosphorylase level; Preiss *et al.*, 1966) under conditions where there is apparently very little restriction on extracellular polysaccharide biosynthesis.

Although it is difficult to evaluate the nature of a specific enzymatic process, such as glycogen synthesis, in the presence of the full cellular complement of enzymes and cofactors, several significant observations have arisen from these studies. The most important of these is the shift in kinetic properties of the enzyme–primer interactions when the glycogen synthetase is pelleted at 105,000*g*. A similar shift in apparent K_M for the primer is obtained upon dialysis of the native system but it has not been possible to restore the native enzyme behavior by adding boiled extracts to the dialyzed enzyme. The mildness of the centrifugation (or dialysis) treatment giving rise to this change, and the ready reconstitution of the native enzyme–primer kinetics suggest that the shift does not result from loss of a homotropic effector site as often observed in the purification of allosteric enzymes. Furthermore, the K_M shift for the pellet apparently cannot be explained by separation of glycogen synthetase from branching enzyme since the pelleted enzyme system catalyzes glycogen synthesis at three to four times the rate (specific activity) of the native system, and, even though 14 glucoses are transferred to glycogen/NRT in the reaction, the chain-length parameters of the product are not consistent with simple chain lengthening of the external chains of the primer. Indeed, if only one-third of the NRT's were substituted and if no branching had occurred, the method of analysis used would show a $\overline{CL}$ of 41 [$Me_3:Me_4 = (3 \times 14) - 1$].

An alternate explanation for the K_M shift is that the primary glucose acceptor sites differ for the native and pellet systems. According to the present concept of glycogen biosynthesis, external chains are uniformly extended from the NRT's during glycogen synthesis. Our preliminary results indicate that a reexamination of this concept is in order, especially for *in vivo* systems. Our attempts to evaluate the relative degree to which NRT's act as glucosyl acceptors in the native and pellet systems have not shown a clear difference. In both cases approximately one-third of the primer NRT's were substituted but, for the pellet system, only 8 glucoses were transferred to primer/NRT while for the native system 115 glucoses were transferred/NRT. To assess the extent to which the K_M shift can be attributed to different acceptor sites on the glycogen primer, these experiments must be repeated with a better characterized uniformly labeled primer of higher specific activity under parallel conditions of primer enlargement in the two systems.

The native glycogen-synthesizing system described here may be compared to both glycogen phosphorylase (Brown and Cori, 1961; Manners, 1962) and polynucleotide phosphorylase (Grunberg-Manago, 1961, 1962). In early observations both of the latter enzymes showed primer requirements for polymer synthesis. It was later shown that under appropriate conditions both glycogen phosphorylase (Illingworth *et al.*, 1961) and polynucleotide phosphorylase (Grunberg-Manago,

1961) can catalyze *de novo* polymer synthesis from their respective monomer donors. In each case there is a time lag during which oligomers are formed and enlarged to such a size that they serve to "prime" the reaction to its maximum rate. Time lags are shortened and eventually eliminated as increasing levels of exogenous primer are incorporated into the assay mixture.

In all work to date on glycogen synthetases a primer has been required for glycogen synthesis and it has been assumed that the glucose residues from UDP–glucose or ADP–glucose are added to the NRT's of the primer. The direct demonstration here that there is substitution of some of the NRT's confirms in part these assumptions. If, however, at any given stage in glycogen biosynthesis only 30–50% of the existing NRT's become glucosylated, the final product might be quite asymmetric, in contrast to the present concepts of glycogen structure (Manners, 1957). The A3(S1) glycogen synthetase is not activated by preincubation with glycogen and thus differs from glycogen phosphorylase (Wang *et al.*, 1965; Metzger *et al.*, 1967). However, the shape of the primer saturation curve for the native enzyme system raises the possibility that glycogen, once formed *in vivo*, may activate the enzyme. Although it has not been possible to obtain *de novo* synthesis of glycogen, the autocatalytic increase in rate of glucose incorporation into polymer at very low primer concentrations is similar to the effect noted with glycogen and polynucleotide phosphorylases. Therefore, it is perhaps reasonable to anticipate that appropriate conditions may be found under which the enzyme system will be able to synthesize glycogen from ADP–glucose alone. Present indications are that in the intact cell *de novo* glycogen biosynthesis does occur.

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References

Bernfeld, P. (1955), *Methods Enzymol. 1*, 149.

Brown, D. H., and Cori, C. F. (1961), *Enzymes 5*, 220.

Brown, D. H., Illingworth, B., and Kornfeld, R. (1965), *Biochemistry 4*, 486.

Conrad, H. E., Bamburg, J. R., Epley, J. D., and Kindt, T. J. (1966), *Biochemistry 5*, 2808.

Duguid, J. P., and Wilkinson, J. F. (1953), *J. Gen. Microbiol. 9*, 174.

Gahan, L. C., Sandford, P. A., and Conrad, H. E. (1967), *Biochemistry 6*, 2755.

Greenberg, E., and Preiss, J. (1964), *J. Biol. Chem. 239*, 4314.

Grunberg-Manago, M. (1961), *Enzymes 5*, 257.

Grunberg-Manago, M. (1962), *Ann. Rev. Biochem. 31*, 301.

Hakomori, S. (1964), *J. Biochem. (Toyko) 55*, 205.

Holme, T., Laurent, T., and Palmstierna, H. (1958), *Acta Chem. Scand. 22*, 1959.

Illingworth, B., Brown, D. H., and Cori, C. F. (1961), *Proc. Natl. Acad. Sci. U. S. 47*, 469.

Kjölberg, O., Manners, D. J., and Wright, A. (1963), *Comp. Biochem. Physiol. 8*, 353.

Lee, Y. C. (1966), *J. Biol. Chem. 241*, 1899.

Leloir, L. F. (1964), *Proc. 6th Intern. Congr. Biochem., New York, 33*, 15.

Madsen, N. B. (1963), *Can. J. Biochem. Physiol. 41*, 561.

Manners, D. J. (1957), *Advan. Carbohydrate Chem. 12*, 261.

Manners, D. J. (1962), *Advan. Carbohydrate Chem. 17*, 371.

Manners, D. J., and Wright, A. (1962), *J. Chem. Soc.*, 4592.

Metzger, B., Helmreich, E., and Glaser, L. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 994.

Preiss, J., and Greenberg, E. (1965), *Biochemistry 4*, 2328.

Preiss, J., Shen, L., Greenberg, E., and Gentner, N. (1966), *Biochemistry 5*, 1933.

Rosell-Perez, M., and Larner, J. (1962), *Biochemistry 1*, 769.

Sandford, P. A. (1967), Ph.D. Thesis, University of Illinois, Urbana, Ill.

Sandford, P. A., and Conrad, H. E. (1966), *Biochemistry 5*, 1508.

Schocher, A. J., Bailey, S. T., and Watson, R. W. (1962), *Can. J. Microbiol. 8*, 89.

Segel, I. H., Cattaneo, J., Sigal, N. (1964), *Proc. Intern. CNRS Symp. Mechanism Reg. Cellular Act. Bacteria, Paris*, 337.

Sigal, N., Cattaneo, J., and Segel, I. H. (1964), *Arch. Biochem. Biophys. 108*, 40.

Strange, R. E., Dark, F. A., and Ness, A. G. (1961), *J. Gen. Microbiol. 25*, 61.

Sutherland, I., and Wilkinson, J. F. (1966), *Biochim. Biophys. Acta 117*, 261.

Vardanis, A. (1967), *J. Biol. Chem. 242*, 2312.

Villar-Palasi, C., Rosell-Perez, M., Hizukuri, S., Huijing, F., and Larner, J. (1966), *Methods Enzymol. 8*, 381.

Wang, J. H., Shonka, M. L., and Graves, D. J. (1965), *Biochemistry 4*, 2296.

Wilkinson, J. F., Dudman, W. F., and Aspinall, G. O. (1955), *Biochem. J. 59*, 446.

Wilzbach, K. E. (1957), *J. Am. Chem. Soc. 79*, 1013.