An Enzyme System for *de Novo* Biosynthesis of Glycogen in *Aerobacter aerogenes**

Linda C. Gahan† and H. E. Conrad

ABSTRACT: A glycogen synthetase fraction and an activator protein, which together catalyze *de novo* glycogen synthesis from adenosine diphosphate glucose, have been purified 400- and 40-fold, respectively, from extracts of late log cells of *Aerobacter aerogenes* A3(S1) and have been shown to be devoid of glycogen. In the presence of adenosine diphosphate glucose and magnesium chloride the purified glycogen synthetase catalyzes a slow rate of glycogen synthesis which is stimulated several hundredfold by supernatant activator protein. Similar stimulations of the glycogen synthetase activity are obtained with glycogen, bovine serum albumin, Triton X-100, several *A. aerogenes* lipopolysaccharides, and the lipid A fraction of the A3(S1) lipopolysaccharide. The diversity of physical and chemical properties of the compounds which stimulate glycogen synthetase suggests that they are all activators of glycogen synthetase rather than acceptors for the glucosyl residues transferred from adenosine diphosphate glucose. As glycogen synthetase loses activity on standing it can be activated only by glycogen and by bovine serum albumin and thus becomes more like a typical glycogen-dependent glycogen synthetase. The mechanism of the *de novo* synthesis has been studied by pulse-labeling experiments. These show that, of the glucose residues incorporated into glycogen in the early stages of the reaction, approximately 50% remain in the external chains of the product recovered after a four- to sixfold increase in the amount of glycogen synthesized. This observation indicates that the *de novo* process differs from the usual mechanism for glycogen biosynthesis and suggests a mechanism like that found for O-antigen biosynthesis (Robbins, P. W., Bray, D., Dankert, M., and Wright, A. (1967), *Science 158*, 1536) in which repeating oligosaccharide units are polymerized from the reducing end of the growing polysaccharide chain.

Glycogen synthetases, regardless of their source, have shown an absolute requirement for a glycogen-type glucan to accept glucose from the system-specific nucleoside diphosphate glucose (Leloir and Cardini, 1962; Greenberg and Preiss, 1965; Preiss and Greenberg, 1965). In previous work (Kindt and Conrad, 1967) it was shown that extracts of late log cells of *Aerobacter aerogenes* A3(S1) would synthesize glycogen from ADP-glucose only when exogenous glycogen was added, in spite of the fact that up to 2 g of glycogen/100 g of dry cells could be extracted from stationary-phase cultures of this organism. The tentative conclusion based on these observations was that late-log cells, in contrast to stationary-phase cells, were devoid of glycogen. This has been demonstrated to be the case (Koeltzow *et al.*, 1968) but the question has remained, "How is glycogen synthesis activated when the stationary growth phase begins?"

In the earlier work several unique features of the *A. aerogenes* glycogen synthetase were reported. Unfractionated extracts and a particulate preparation (fourfold purified) obtained from the crude extract by centrifugation at 105,000*g* were studied. Both preparations catalyzed glucosylation of less than half of the primer nonreducing terminals and formed highly branched glycogens. Apparent primer K_M values for unfractionated cell extracts, expressed as concentration of nonreducing terminals, showed a progressive increase with decreasing chain length of the primer. Upon pelleting the activity the primer K_M values were increased three- to eightfold and the correlation of K_M with chain length of primer was lost. The kinetic parameters observed for initial cell extracts were restored upon recombination of the 105,000*g* supernatant and pellet. These observations implicated at least two fractions in the glycogen-synthesizing system of *A. aerogenes*, with the activities for glucosyl transfer and branch formation both in the pellet fraction.

The purification and partial characterization of these two fractions are described here. The glucosyl transferase and branching activities are recovered together in a 400-fold-purified GS[1] fraction. This fraction actively synthesizes glycogen only when SAP is added. Evidence

* From the Division of Biochemistry, University of Illinois, Urbana, Illinois 61801. *Received June 27, 1968.* Supported by a grant from the U. S. Public Health Service, National Institute of Allergy and Infectious Diseases (AI 05696).

† Holder of a National Institutes of Health predoctoral traineeship on U. S. Public Health Service Training Grant GM-321.

[1] The abbreviations used that are not given in *Biochemistry 5*, 1445 (1966), are: BSA, bovine serum albumin; GS, glycogen synthetase; SAP, supernatant activator protein; DTT, dithiothreitol; $\overline{ECL}$, the average number of glucose residues in an external chain of glycogen; $\overline{ICL}$, the average number of glucose residues in an internal chain of glycogen; $\overline{CL}$, the average number of glucoses in a chain length of glycogen ($\overline{CL} = \overline{ECL} + \overline{ICL} + 1$); LPS, lipopolysaccharide; PS, the polysaccharide portion of a lipopolysaccharide; lipid A, the lipid portion of a lipopolysaccharide.

is presented to show that neither of the purified fractions contains glycogen. The supernatant fraction can be replaced by BSA, lipopolysaccharide, lipid A, Triton X-100, or glycogen. In all cases a highly branched product is formed. Pulsing experiments show that approximately 50% of the glucose residues incorporated into glycogen in the early stages of synthesis remain in the nonreducing periphery of the molecule as glycogen synthesis proceeds. The data suggest that the *de novo* process may proceed by a mechanism similar to that described for O-antigen biosynthesis (Robbins *et al.*, 1967; Osborn and Weiner, 1968) rather than by the classical glucosyl transferase–branching enzyme mechanism.

Methods

[^{14}C]ADP–glucose was obtained from New England Nuclear Corp. and diluted with unlabeled ADP–glucose (Calbiochem) to 0.1 mCi/mmole for use in the enzyme assay. Crystalline sweet potato β-amylase (400 units/mg of protein) was obtained from Worthington Biochemical Corp. and crystalline swine pancreas α-amylase (500 units/mg of protein) from Sigma Chemical Co. One unit of these enzymes, assayed as described by Bernfeld (1955), releases from amylopectin 1 μmole of maltose equiv/min at 25°. Crystalline bovine pancreas trypsin (40 units/mg of protein; 1 unit hydrolyzes 1 μmole of *p*-tosyl-L-arginine methyl ester/min at 25°) was obtained from Calbiochem and assayed by the method of Hummel (1959). Crystalline soybean trypsin inhibitor and crystalline pancreatic ribonuclease were purchased from Worthington Biochemical Corp.; DTT, Triton X-100, and *p*-tosyl-L-arginine methyl ester from Calbiochem; and crystalline pancreatic deoxyribonuclease from Sigma Chemical Co.

Paper chromatograms were run in 1-butanol–pyridine–water (6:4:3) and standards were detected by spraying with aniline acid phthalate reagent (Partridge, 1949). Radioactive compounds on chromatograms were quantitated by cutting the chromatograms into segments and counting the segments as before (Kindt and Conrad, 1967).

The glycogen used throughout this work was isolated from stationary-phase *A. aerogenes* A3(S1) cells as for the previous work (Kindt and Conrad, 1967). LPS from several strains of *A. aerogenes* and the lipid A and polysaccharide fractions from the A3(S1) LPS were prepared as described by Koeltzow *et al.* (1968). The characterization of the biosynthetic glycogen by methylation analysis and β-amylolysis and the use of chain length parameters to define the glycogen structure have been described (Kindt and Conrad, 1967).

Assay of GS. Assays were carried out at room temperature in 5 × 15 mm test tubes containing, per 50 μl: Tris-HCl (pH 7.4), 2 μmoles; magnesium chloride, 1.25 μmoles; [^{14}C]ADP–glucose, 0.25 μmole (25,000 cpm when counted as below); BSA, 100 μg; glycogen, 25 μg; and enzyme. Reaction was initiated by adding GS (0.05–0.4 unit) or, in some cases, ADP-glucose. At 7.5, 15, 30, and 45 min 10-μl aliquots were removed with an Eppendorf pipet (Brinkmann Instruments, Inc.) and spotted on a strip of Whatman No. 4 paper, and the strip was dipped into acetone to stop the reaction. The paper strip was 1 in. wide and 3 in. long and was marked off in 0.5-in. segments numbered 1–6. Successive aliquots from the assay were streaked on segments 2–5. Segment 6 was cut to a drip tip and segment 1 was attached to a thick paper wick (four 1 × 2 in. segments of Whatman No. 3MM paper stapled together) with a paper clip. The unreacted substrate was eluted from the paper strip with 50% ethanol in 0.1% aqueous ammonium hydroxide for 1 hr, then rinsed with acetone and air dried. Segments 2–5 were cut out of the strip and counted to ±3% error in a liquid scintillation counter as before (Kindt and Conrad, 1967). Rates of glycogen synthesis are calculated from the counting data as micromoles of glucose incorporated into the 50% ethanol-insoluble product. A unit of GS catalyzes incorporation of 1 μmole of glucose into glycogen per hr under these assay conditions.

Assay of SAP. SAP was assayed as above, using a reaction mixture containing, per 50 μl: Tris-HCl (pH 7.4), 2 μmoles; magnesium chloride, 1.25 μmoles; [^{14}C]ADP–glucose, 0.25 μmole (25,000 cpm); BSA, 100 μg; 176,000*g* pellet fraction containing GS activity (step 2, Table II), 50 μg; and SAP, 0.05–0.25 unit. One unit of SAP induces an increase in the rate of glycogen synthesis of 1 μmole of glucose incorporation/hr over that obtained with the same assay system in the absence of SAP.

Protein was determined by the method of Lowry *et al.* (1951). Specific activities are expressed as units per milligram of protein.

Purification of Components of the Glycogen-Synthesizing System. A. aerogenes A3(S1) (ATCC 12658) was grown at 30° on a glucose–mineral salts medium, harvested in the late logarithmic growth phase, and washed as before (Kindt and Conrad, 1967). Frozen cell paste (14 g) was broken in a Hughes press and suspended in 34 ml of 0.05 M Tris-HCl buffer (pH 7.4) containing 0.001 M DTT (Tris-DTT buffer). All subsequent operations were carried out at 0–5°. Deoxyribonuclease (0.5 mg) and ribonuclease (0.5 mg) were added to depolymerize the nucleic acids. After 45 min at 0° the liquified mixture was centrifuged at 32,000*g* for 15 min and the pellet was washed with 20 ml of Tris-DTT buffer and recentrifuged. The combined supernatants were centrifuged for 150 min at 176,000*g* in a 65 rotor of a Spinco L2-65 ultracentrifuge to pellet most of the GS activity and leave the SAP activity in the 176,000*g* supernatant. The supernatant was used for further purification of the SAP; GS activity was purified from the pellet fraction.

Further Purification of SAP. To 54 ml of the 176,000*g* supernatant 10.6 g of ammonium sulfate was added with stirring to give 33% saturation. After 20 min the resulting precipitate was collected by centrifugation at 32,000*g* for 10 min, suspended in 4 ml of Tris-DTT buffer, and dialyzed against this buffer overnight. The dialyzed fraction was chromatographed on a 1.3 × 10 cm DEAE-cellulose column equilibrated with Tris-DTT buffer, using as eluate a linear gradient made up of 110 ml each of the Tris-DTT buffer and 0.5 M sodium chloride containing 0.001 M DTT. The column was de-

TABLE I: Purification of Supernatant Activator Protein.[a]

Fraction	Vol (ml)	Protein (mg)	Activity		
			Units[b]	Recov (%)	Sp Act. (units/mg)
1. 176,000*g* supernatant	54.0	836.0	4010	100	4.8
2. $AmSO_4$ precipitate, 0–33% saturation	5.9	82.5	3080	77	37.4
3. DEAE-cellulose eluate, 0.20–0.28 M NaCl	39.6	17.3	2930	73	170.0
4. Concentrate of DEAE-cellulose eluate	8.0	16.2	2900	72	179.0

[a] Starting material was 14 g of cell paste. [b] One unit of SAP induces the incorporation of 1 μmole of D-glucose/hr from ADP-glucose into glycogen in an assay system containing, per 50 μl: Tris-HCl (pH 7.4), 2 μmoles; $MgCl_2$, 1.25 μmoles; [^{14}C]ADP-glucose, 0.25 μmole (25,000 cpm); BSA, 100 μg; GS, 0.3 unit; and SAP, 0.05–0.25 unit.

veloped at a flow rate of 0.15 ml/min and 3-ml fractions were collected and assayed for SAP. To concentrate the SAP the combined peak fractions of activity, emerging between 0.20 and 0.28 M sodium chloride, were dialyzed for 6 hr against Tris-DTT buffer and adsorbed on a 1.3 × 5 cm DEAE-cellulose column equilibrated with Tris-DTT buffer. The activity was washed directly through the column with 30 ml of 0.4 M sodium chloride containing 0.001 M DTT and stored at 4° in the elution solvent.

Further Purification of Glycogen Synthetase. The 176,000*g* pellet from above was allowed to stand 14 days at 4° and was then suspended in 13 ml of cold saline and added to 133 ml of 0.025 M Tris-HCl buffer (pH 7.4) containing 0.001 M DTT and 0.23 ml of Triton X-100. After standing for 1 hr at 0° the solution was centrifuged at 100,000*g* for 1 hr and the supernatant, containing the solubilized GS, was separated carefully with a pipet from the pellet. To the solubilized protein was added 24 g of solid ammonium sulfate (to give 30% saturation) and, after stirring for 20 min, the suspension was centrifuged at 32,000*g* for 10 min to bring the precipitated protein to the surface of the centrifugate. The underlying solution was carefully withdrawn with a Pasteur pipet and the precipitate was dissolved from the sides of the centrifuge tubes in 8 ml of 0.025 M Tris-HCl (pH 7.4) containing 0.001 M DTT, dialyzed overnight against the same buffer, and applied to a 1.3 × 10 cm DEAE-cellulose column equilibrated with the above buffer containing 0.15% (w/v) Triton X-100 (Tris-DTT–Triton buffer). The column was eluted with a linear gradient consisting of 120 ml each of Tris-DTT–Triton buffer and 1 M sodium chloride containing 0.001 M DTT and 0.15% Triton X-100. The column was eluted at a flow rate of 0.5 ml/min and 3.5-ml fractions were collected and assayed for GS and protein. The active fractions, recovered between 0.35 and 0.47 M sodium chloride, were either stored at 4° in the eluting buffer or concentrated by dialyzing the pooled fractions against Tris-DTT–Triton buffer, adsorbing the protein on a 1.3 × 5 cm DEAE-cellulose column in the same buffer, and washing the activity from the column with 30 ml of 0.5 M sodium chloride containing 0.001 M DTT and 0.15% Triton X-100. The concentrated fractions were pooled and stored at 4° with gradual loss of activity over a 2-week period.

Results

General Description of Glycogen-Synthesizing System. The glycogen-synthesizing system of *A. aerogenes* consists of two fractions which are separable by ultracentrifugation at 176,000*g* for 2.5 hr. The GS recovered in the pellet contains all of the catalysts necessary to synthesize a highly branched glycogen from ADP-glucose but gives very low rates of glycogen synthesis unless activated by the supernatant fraction. This requirement for SAP may be replaced in part by BSA, but the BSA-activated GS is stimulated to still higher rates of glycogen synthesis by SAP, *i.e.*, the BSA and the SAP activation of GS are more or less additive. For assay during purification the SAP activity is determined by measuring the extent to which it will stimulate the activity of GS in the presence of BSA. BSA is included in the SAP assay since in its absence mixtures of the crude GS and SAP from the ultracentrifugation give nonlinear rates of glycogen synthesis. When the two fractions are further purified, however, they will give linear rates of glycogen synthesis when assayed together. Maximum activation of GS is obtained with a mixture of BSA and glycogen or with a mixture of BSA and SAP. Since the former activation mixture is made up of the most stable and reproducible components, it is used in the assay for purification of GS. In the results presented below, *units* of GS activity refer to that activity obtained with *both* BSA and glycogen in the GS assay. Where rates are given, they refer to the rate observed in the experiment described.

Purification of SAP. The purification of SAP is shown in Table I. Initial activities are measured in the 176,000*g* supernatant which contains most of the SAP activity and approximately two-thirds of the protein present in cell extracts. A 37-fold purification is obtained with a 72% recovery of units. In development of the DEAE-cellulose chromatography step it was observed that more shallow salt gradients than used in the purification

TABLE II: Purification of Glycogen Synthetase System.[a]

Fraction	Vol (ml)	Protein (mg)	Activity		
			Units[b]	Recov (%)	Sp Act. (units/mg)
1. Cell extract, 32,000*g* supernatant	57.0	1175.0	3660	100	3.1
2. Pellet, 176,000*g*	144.0	429.0	2400	66	5.6
3. Triton-solubilized pellet, 100,000*g* supernatant	138.0	236.0	2060	56	8.7
4. $AmSO_4$ precipitate, 0–33% saturation	5.4	37.8	2000	55	43.0
5. DEAE-cellulose eluate, 0.35–0.47 M NaCl	36.0	1.2	1720	47	1390.0
6. Concentrate of DEAE-cellulose eluate	10.8	1.4	1680	46	1200.0

[a] Starting material was 14 g of cell paste. [b] One unit catalyzes the incorporation of 1 μmole of D-glucose/hr from ADP-glucose into glycogen in an assay system containing, per 50 μl: Tris-HCl (pH 7.4), 2 μmoles; $MgCl_2$, 1.25 μmoles; [^{14}C]ADP-glucose, 0.25 μmole (25,000 cpm); BSA, 100 μg; glycogen, 25 μg; and GS, 0.05–0.40 unit.

procedure finally adopted gave a marked smearing of the SAP activity over a number of column fractions. The concentrated SAP is devoid of GS activity and can be stored for at least 6 months at 4° without loss of activity.

Purification of GS. The purification of GS is shown in Table II. For fractionation of the 176,000*g* pellet, the GS is solubilized by extraction of the pellet with 0.15% Triton X-100 in 0.05 M Tris-HCl buffer (pH 7.4). It was found that this concentration of detergent extracts 100% of the GS activity, provided the pelleted protein is allowed to age for 2 weeks at 4°. GS activity is slowly lost after longer periods of aging but is incompletely extracted when the pellet is aged less than 2 weeks. Detergents other than Triton X-100 (*e.g.*, sodium dodecyl sulfate, sodium cholate) either are less effective in extraction of activity or inhibit glycogen synthesis. Triton X-100 is needed not only to solubilize the activity but to keep it in solution. Dialyzed solutions of solubilized enzyme become cloudy and lose activity rapidly.[2] When detergent is not included in the buffer used for elution in the DEAE-cellulose purification step, much of the protein and activity precipitates at the top of the column and only 15–20% of the activity is recovered and is smeared over a number of fractions. In contrast, when the buffer containing Triton X-100 is used, the activity emerges from the column in a very sharp peak which follows most of the protein. The purification gives a 400-fold over-all increase in specific activity and yields a product which loses its activity rapidly on freezing and thawing but more slowly when stored at 4°.

TABLE III: Requirements for Glycogen Synthesis.

Assay System	Glucose Incorp (μmole/hr)
Complete[a]	0.34
−GS	0.00
−SAP	0.25
−BSA	0.27
−BSA and SAP	0.00
−SAP, + glycogen; 25 μg	0.34
$-MgCl_2$	0.05

[a] The complete system contained, in 50 μl: Tris-HCl (pH 7.4), 2 μmoles; $MgCl_2$, 1.25 μmoles; [^{14}C]ADP-glucose, 0.25 μmole (25,000 cpm); BSA, 100 μg; SAP, 2.25 units (259 units/mg, see Table I); and 0.34 unit of GS (680 units/mg).

Requirements for Glycogen Synthesis. The data in Table III show that glycogen synthesis by the purified system requires GS, $MgCl_2$, and an activator or a combination of activators. As indicated above both SAP and BSA serve as activators but alone neither one induces the maximum activity of the GS. Table III shows that glycogen plus BSA will replace SAP plus BSA in inducing the maximal activity of GS. It is because of this maximum stimulation obtained with BSA plus glycogen that both activators are used in the assay system for GS purification.

The Activity of GS in the Absence of Activators. The rates of glycogen synthesis when increasing levels of purified GS are assayed in the absence of activator are shown in Figure 1. At the usual levels of GS which give high linear rates when activators are present (the three lowest levels in Figure 1) glycogen synthesis proceeds only after a lag period which is gradually eliminated as the level of GS is increased. Only at high levels of GS

[2] Triton X-100 has a molecular weight of 635 and exists in aqueous solution as a micelle with approximately 100 molecules/micelle (Dwiggins *et al.*, 1960). The micelle–monomer equilibrium permits the dialysis of the detergent. Its loss from aqueous solution on dialysis has been demonstrated by a decrease both in absorbancy of the solution at 280 mμ and in the weight of detergent recoverable.

TABLE IV: Activation of Glycogen Synthetase.[a]

Activator	Amt (μg/50 μl)	Glucose Incorp (μmole/hr)
None		0.00
SAP[b]	4.5	0.23
Glycogen	5.0	0.22
BSA	6.0	0.16
Triton X-100	50.0	0.17
Boiled SAP	8.7	0.00
Boiled glycogen	5.0	0.22
Boiled GS	0.5	0.00
Boiled BSA	6.0	0.00
Lipopolysaccharides[c]		
NCTC 9652	25.0	0.00
NCTC 9656	25.0	0.00
NCTC 9644	25.0	0.00
NCTC 9504	25.0	0.06
NCTC 418	25.0	0.15
NCTC 243	25.0	0.15
A3(S1)	25.0	0.13
A3(S1) PS	200.0	0.00
A3(S1) lipid A	100.0	0.14

[a] Assay with 0.3 unit of GS (505 units/mg) as described in Table II, but without BSA and glycogen except where indicated. [b] Specific activity of SAP was 259 units/mg. [c] All lipopolysaccharides tested were from *A. aerogenes* strains.

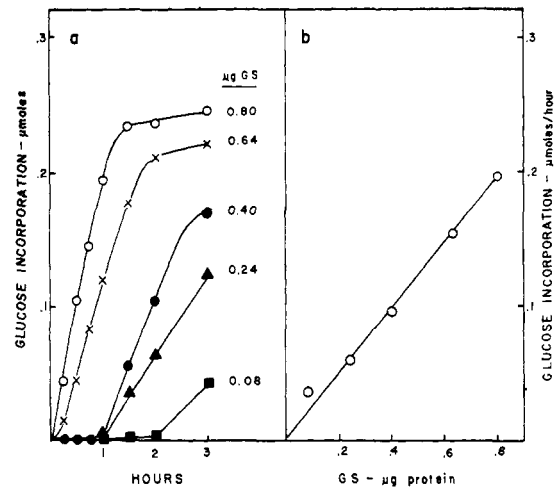

FIGURE 1: Glycogen synthesis catalyzed by purified GS. Assays were performed as in Table II, but without glycogen or BSA. Specific activity of GS (step 5, Table II), 715 units/mg. (a) The lag in glycogen synthesis as a function of GS level in assay. (b) Linearity with GS of maximum rate attained after lag.

does glycogen synthesis proceed without a lag. As shown in Figure 1b, the maximum rate of synthesis attained after the lag is a linear function of the amount of GS in the assay. These maximum rates are only one-third of the rates obtained in the presence of BSA and glycogen. In the 45-min period used in the normal assay the three lowest levels of GS shown in Figure 1a would be considered completely inactive in the absence of activators.

Activity of Glycogen Synthetase in the Presence of Activators. A number of substances, when added to the assay system with GS, eliminate the lag at the low GS levels and increase the maximum rate of glucose incorporation attainable at all GS levels. The relative effects of a variety of activators on the rate of glycogen synthesis are shown in Table IV. All of these activators induce linear rates of glycogen synthesis from the time of initiation of the assay. In addition to the activation by SAP, BSA, and glycogen described above, Triton X-100 and the lipopolysaccharides from some (but not all) strains of *A. aerogenes* activate the GS. Since the 45-min assay period was used in these experiments any onset of activity that might have occurred after a lag period was not recorded. Several points merit special comment. The effect of glycogen in the assay is identical with that of the nonglycogen activators. The possibility therefore arises that glycogen functions primarily as an activator rather than as a glucosyl acceptor. The fact that when SAP, GS, or BSA are boiled they do not serve as activators is evidence that these preparations do not contain significant levels of glycogen though it is possible that the denaturation–aggregation process renders any glycogen that may be present inaccessible to the enzyme system. A second observation of note is that when the A3(S1) LPS is separated into its PS and lipid A components, the activator activity is found only in the lipid A fraction. It is because of the diversity of physical and chemical properties among the group of substances which stimulate GS that these compounds are considered to be activators rather than glucosyl acceptors. Further evidence to indicate that SAP is not a glucosyl acceptor is that upon ultracentrifugation at 176,000*g* of an assay mixture in which SAP was used to activate [^{14}C]glycogen synthesis, none of the ^{14}C in the product is recovered in the supernatant with SAP.

Figure 2 shows the linear relationship between the rate of glycogen synthesis and the amount of GS added to assay systems containing saturating levels of activators or combinations of activators. When no activator is present only the highest levels of GS assayed give measurable initial rates of glycogen synthesis (*cf.* Figure 1). In the presence of activators the rates of glycogen synthesis are directly proportional to the amount of GS in the assay. The partially additive activation effects of BSA and glycogen or BSA and SAP and the lack of additive effects with glycogen and SAP suggest that glycogen and SAP act at the same site on GS while BSA acts at a different site. In experiments not shown here it has been found that when glycogen and SAP are both present at subsaturating levels in the assay their activation effects are additive.

The saturation of GS with various activators is shown in Figure 3. On a weight basis, glycogen and SAP are most active. Since no attempt has been made to bring SAP to a high level of purity for these studies, it is anticipated that, on a weight basis, SAP will prove to be

TABLE V: Change in GS Activity with Different Activators during Storage.[a]

Conditions of Storage	GS Activity (% of original)				
	SAP	Triton X-100	Lipid A	BSA	Glycogen
Freshly prepared, step 5, Table II	100	100	100	100	100
3 days, 4°	100	100	95	95	98
7 days, 4°	47	55	47	82	95
15 days, 4°	2	2	2	20	20
20 hr, −10° (frozen)	0	33	36	86	90

[a] Purified GS (1510 units/mg) stored either at 4° or at −10°. The preparation stored at 4° was in the buffer used to elute the activity from DEAE-cellulose (step 5, Table II). The preparation stored at −10° had been concentrated as in step 6, Table II, and then made 50% saturated with ammonium sulfate. All assays contained 0.5 μg of GS. Activator levels used were: 6.8 μg of SAP (259 units/mg); 50 μg of Triton X-100, lipid A, or BSA; and 25 μg of glycogen.

much superior to glycogen as an activator for GS. No data have been obtained concerning the molar concentrations for optimum activator activity.

Effect of Aging of GS on Activation. When the purified GS stands at 4° or is frozen at −10° with ammonium sulfate in the buffer used to elute it from DEAE-cellulose, it loses activity and the relative activities of different activators change as shown in Table V. Most significant is the fact that the activity obtained with SAP, the natural activator of the system, is lost quite rapidly while that with glycogen and with BSA is retained for the longest period. Thus, the *de novo* system for glycogen biosynthesis reverts to a system similar to those previously described (Leloir and Cardini, 1962; Greenberg and Preiss, 1965; Preiss and Greenberg, 1965) in which glycogen is required in the assay and BSA stimulates the rate of glycogen synthesis. In the earlier work with this system (Kindt and Conrad, 1967) this reversion apparently took place when cell extracts were prepared by sonication, since such ex-

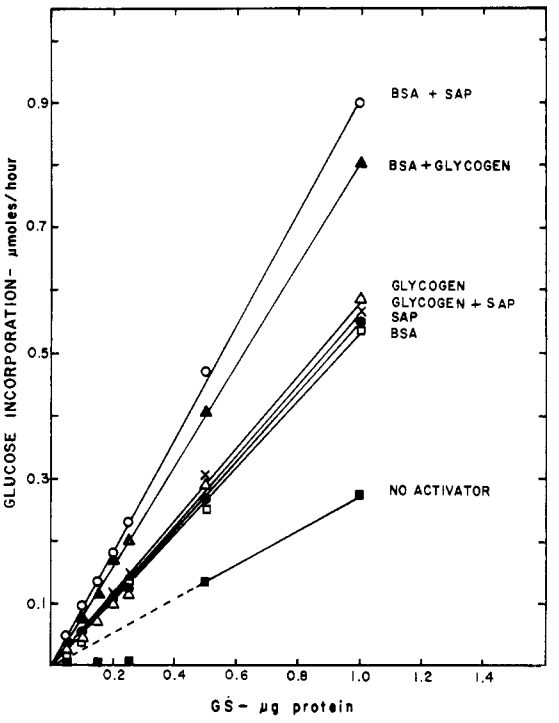

FIGURE 2: Linearity of initial rates of glycogen synthesis with GS level when saturating levels of activators are present alone, or in combination. Per 50 μl of assay volume the activator levels used were: BSA, 100 μg; SAP (259 units/mg), 8.7 μg; and glycogen, 25 μg. Rates of glycogen synthesis were linear over the 45-min assay period except at the low GS levels when no activator was present.

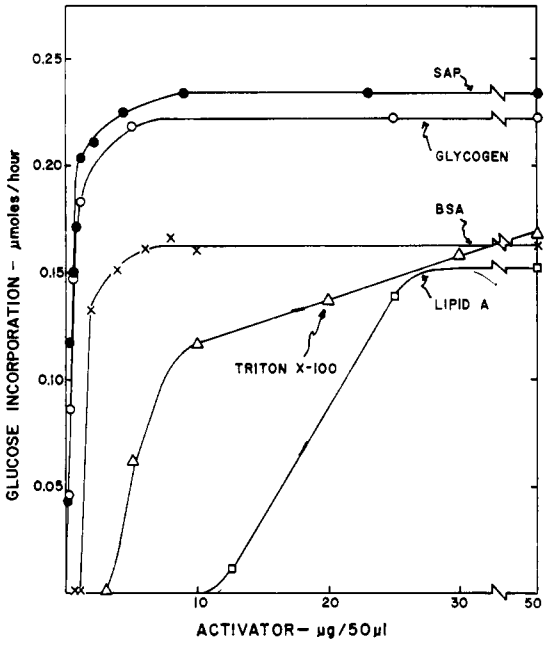

FIGURE 3: Saturation of GS with activators. Assays were performed with 0.30 unit of GS (507 units/mg) as in Table II but with replacement of the BSA and glycogen with the various activators at the levels indicated. Enzyme was dialyzed *vs.* Tris-DTT buffer prior to assay. For SAP the amounts added are given in micrograms of protein; for other activators amounts are in micrograms dry weight.

TABLE VI: Properties of Supernatant Activator Protein.

Additions to Assay[a]	Glucose Incorp (μmoles/hr)
Experiment 1	
a. None	0.26
b. SAP and α-amylase	0.37
c. SAP and α-amylase, preincubated[b]	0.35
d. Glycogen and α-amylase	0.50
e. Glycogen and α-amylase, preincubated[b]	0.28
Experiment 2[c]	
a. Trypsin and trypsin inhibitor	0.52
b. SAP, trypsin, and trypsin inhibitor	1.44
c. SAP and trypsin, preincubated trypsin inhibitor	0.49

[a] Assays as described in Table II, but without glycogen and BSA, except where indicated. The GS preparation used had a specific activity of 411 units/mg and, due to the Triton X-100 in the buffer in which it was stored, gave a measurable rate of glycogen synthesis in the absence of added glycogen or SAP. For experiment 1, 0.76 unit of GS was used; for expt 2, 1.51 units. When added, 25 μg of glycogen or 2.25 units of SAP (259 units/mg) was present in the assays. [b] The indicated assay mixtures were preincubated with 0.0005 unit of α-amylase for 2 hr at room temperature. The ADP–glucose and GS were omitted from the preincubations and were added simultaneously to the assay mixtures to initiate the assays. [c] Trypsin and trypsin inhibitor were added to the assays in the following manner. A 2.5-μl aliquot of a trypsin solution (5 mg/ml, 40 units/mg) was mixed with 0.5 μl of 1 M $CaCl_2$ and 50 μl of buffer (expt 2a) or a solution (step 3, Table I) of SAP (expt 2b,c). To 10 μl of this mixture was added 2.5 μl of a solution of trypsin inhibitor (1 mg/ml, 40 units/mg). After a 5-min incubation period 10 μl of the latter solution was added to the assay mixture and the assay was performed as usual. For the preincubation experiment (2c) trypsin and SAP were incubated for 18 hr at room temperature before adding trypsin inhibitor. Under the preincubation conditions SAP was completely stable when no trypsin was present.

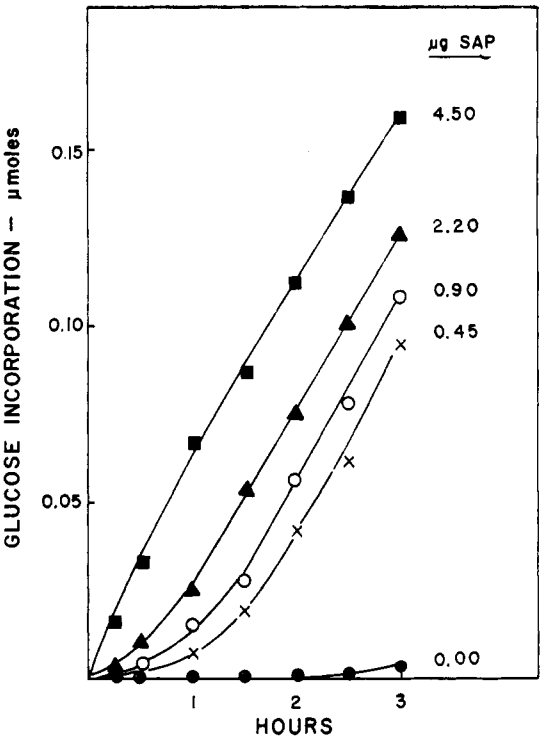

FIGURE 4: Activation of aged GS with SAP. Assays were performed with an aged preparation of purified GS as described in Table II, but with substitution of SAP (259 units/mg) for BSA and glycogen. The freshly prepared GS (1200 units/mg) was aged at 4° for 2 weeks in the eluting buffer used in the DEAE-cellulose chromatography step of purification (step 5, Table II) and 0.31 unit was used in these assays. Final specific activity (with glycogen plus BSA), 330 units/mg.

tracts were almost completely dependent upon glycogen for activity (BSA was not tested as an activator).

The effect of increasing concentrations of SAP on aged GS is shown in Figure 4. Whereas with freshly prepared GS 0.45 μg of SAP protein induces almost maximal GS activity (Figure 3), this amount of SAP gives very little activity with aged GS in the regular assay period but does induce the aged GS to synthesize glycogen after a 1-hr lag. The lag period is reduced and finally eliminated as more SAP is added. The maximum activity attainable with SAP after GS is aged 2 weeks is about 20% of its activity when freshly prepared.

Properties of Supernatant Activator Protein. The purified SAP is a heat-labile, nondialyzable protein which contains no glycogen. It was demonstrated previously (Koeltzow *et al.*, 1968) that *A. aerogenes* A3(S1) cells grown under the conditions used here contain less than 7 μg of glycogen/100 g of cell paste. Data in Table IV have shown that the purified SAP loses all activating activity when boiled under conditions where glycogen is completely stable. The absence of glycogen in purified SAP is confirmed by the data in expt 1 of Table VI which show that an α-amylase treatment, which destroys the activating activity of glycogen, has no effect on the activity of SAP. The experiment was performed by preincubation of the activators for 2 hr with a level of α-amylase sufficient to destroy glycogen but too low to interfere markedly with the assay.

Experiment 2 in Table VI shows that preincubation of SAP with trypsin completely destroys its activating activity. In this experiment trypsin inhibitor, added at the beginning of each assay to prevent destruction of the GS by trypsin, had no effect on the glycogen synthesis rate. When SAP was preincubated in the absence of trypsin, it was completely stable.

Absence of Glycogen in the Glycogen Synthetase.

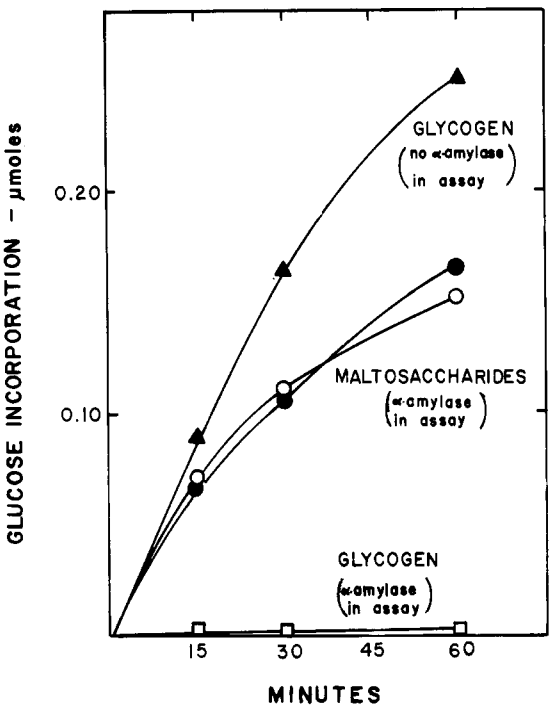

FIGURE 5: Effect of α-amylase on glycogen synthesis. Assays were run as in Figure 1, with 0.95 unit of GS (1500 units/mg) and no activator. Where indicated, α-amylase (500 units/mg) was added at a level of 0.1 μg/50 μl. All assay components except [^{14}C]ADP-glucose were mixed and reactions were initiated by addition of [^{14}C]ADP-glucose. For the analyses indicated by open symbols (□,○), α-amylase and ADP-glucose were added simultaneously. For the experiment indicated by closed circles, α-amylase was incubated with the assay mixture at room temperature for 15 min before initiation of reaction. Maltosaccharides were determined by spotting aliquots from the assays on 1-in. wide strips of Whatman No. 1 paper and chromatographing in 1-butanol–pyridine–water (6:4:3) for 40 hr to separate maltotriose and maltose from glycogen and unreacted ADP-glucose. The micromoles of glucose recovered in maltosaccharides was calculated from the total counts per minute in the maltose–maltotriose region of the chromatograms.

The purified GS is not sufficiently stable at room temperature for the preincubation period required for the α-amylase destruction of glycogen as described above. Therefore, to test for the possible presence of glycogen in GS the following approach was used. If, in fact, glycogen is formed *de novo* from ADP-glucose, then α-amylase, added to the assay, should not interfere with the transfer of the first several glucose residues to the primary acceptor of the system but should cleave maltosaccharides from the growing glycogen molecule as it becomes large enough to serve as an α-amylase substrate. Thus, in the presence of α-amylase the GS should catalyze the conversion of ADP-glucose into maltosaccharides. The data from such an experiment are shown in Figure 5. The assay mixtures are prepared with all components but [^{14}C]ADP-glucose, and α-amylase is added to two assays but not to a third. Assays are started by the addition of ADP-glucose and the results obtained in the presence and absence of α-amylase are compared. Glycogen synthesis proceeds at a normal rate when no α-amylase is added, but in the presence of α-amylase no glycogen accumulates. The rate of incorporation of glucose into maltosaccharides, identified as maltose and maltotriose by paper chromatographic comparison with standards, approaches the rate of incorporation of [^{14}C]glucose into glycogen by the system. The rates of maltosaccharide formation are identical whether ADP-glucose is added at the time of addition of α-amylase or when the assay mixtures is incubated 15 min with α-amylase before addition of ADP-glucose. The difference in rates of conversion of ADP-glucose into glycogen and to maltosaccharides suggests that the incorporation of the first several glucose residues into a glycogen molecule may proceed somewhat slower than the rate of extension of the chains.

When ADP-glucose is preincubated with α-amylase and then heated to destroy the α-amylase it serves as a substrate for glycogen synthesis and gives the same rate as unincubated ADP-glucose in an assay system containing purified GS and BSA. Thus, all components of the glycogen-synthesizing system from *A. aerogenes* have been shown to be free of glycogen.

TABLE VII: Activities of GS and SAP at Different Growth Stages.

Growth Stage[a]	Sp Act. (units/mg) GS[b]	SAP[c]
Early log	0.36	3.60
Mid log	2.58	3.31
Late log	7.04	3.65
Stationary	5.91	4.17

[a] The A_{660} values at the time of harvest were: early log, 0.40; mid log, 1.00; late log, 2.25; stationary (3 hr after late log), 2.35. [b] Assay as described in Table II, but without glycogen. Enzyme preparations assayed were at the step 2 stage of purification. [c] Assay as described in Table I. Enzyme preparations assayed were at the step 1 stage of purification.

Changes in Glycogen Synthesis Activities during Growth. The specific activities of GS and SAP in cells at different stages of cell growth are shown in Table VII. The SAP activity is quite constant throughout growth, but the GS in late log cells shows a 20-fold increase in specific activity over that found for early log cells. Thus, the onset of glycogen synthesis and accumulation when the cells enter the stationary growth phase (Kindt and Conrad, 1967; Koeltzow *et al.*, 1968) cannot be attributed to a rapid rise in SAP at the end of the logarithmic growth phase. Instead, glycogen accumulation appears to be controlled at the level of ADP-glucose pyrophosphorylase (Preiss *et al.*, 1966) and glycogen synthetase levels (Govons *et al.*, 1968).

Structure of the Synthesized Glycogen. Structures of the products formed by the purified GS alone and by the GS when it is activated by BSA, Triton, glycogen, or SAP are all very similar to the structures obtained

TABLE VIII: Direction of Glycogen Synthesis.

Activator[a]	ADP-glucose in Chase[b]	Glucose in Glycogen (μmoles)[c] At Start of Chase	Glucose in Glycogen (μmoles)[c] At Time of β-Amylolysis	β-Amylolysis[d] (% of ^{14}C)
None	^{14}C	0.026	0.140	42
	Unlabeled	0.026	0.150	44
BSA	^{14}C	0.067	0.333	49
	Unlabeled	0.067	0.244	54
Glycogen	^{14}C	0.068	0.486	46
	Unlabeled	0.068	0.330	27

[a] Assay as in Table II, but without glycogen (25 μg) or BSA (100 μg) except where indicated. Assays contained 1.52 units of GS (760 units/mg). [b] Assays initiated with 0.25 μmole of [^{14}C]ADP-glucose (600,000 cpm/μmole) and chased with 1.0 μmole of unlabeled ADP-glucose. The chase was added to each assay after 5 min in 2 μl of water and reaction was continued for 55 min. [c] Aliquots (5 μl) were taken at intervals for assay and micromoles of glucose incorporated into glycogen were calculated taking into account the specific activity of the [^{14}C]ADP-glucose in the particular assay and the dilution factor due to the aliquot added for the chase. [d] At the end of the period the remaining sample (42 μl) was adjusted to pH 4.8 with 2 μl of 0.1 N HCl and 0.3 μl of 1.6 M sodium acetate buffer (pH 4.8). A 10-μl aliquot was removed for measurement of the amount of ^{14}C in the glycogen in the usual way and 1 μl of β-amylase solution (1 mg/ml) was added to the remaining volume. After 30 min a 10-μl aliquot was taken to determine the amount of ^{14}C in the β-dextrin by the usual assay. Maltose, but not β-dextrin, is washed from the assay paper with the 50% ethanol–0.1% NH_4OH.

with the initial cell extracts (Kindt and Conrad, 1967). The ^{14}C in these products is converted quantitatively to glucose by acid hydrolysis and to maltosaccharides by α-amylase. β-Amylase solubilizes 50–60% of the glycogen and yields maltose (identified by R_F). The average chain length, determined by methylation analysis, was 10, somewhat lower than found previously for the system before purification.

Direction of Glycogen Synthesis. Since the glycogen-synthesizing system described here catalyzes glycogen formation in the absence of the nonreducing terminals of a primer, the direction of growth of glycogen during synthesis can be tested readily by pulse-labeling experiments. According to the current view of glycogen synthesis, illustrated in Figure 6, external chains of glycogen are extended at the nonreducing terminals by successive additions of glucose until they become long enough to serve as substrates for branching enzyme. The action of branching enzyme is to transfer an outer segment of the glycogen molecule from its position in the external chain to a C-6 hydroxyl on a residue in the external chain close to the previous branch point. Those glucoses added early in the reaction should become buried further and further in the internal part of the molecule as the synthesis proceeds. The buried glucosyl residues thus become inaccessible to β-amylase and, following exhaustive β-amylolysis, should remain in the β-dextrin. To determine whether the usual mechanism of glycogen synthesis operates in the *de novo* system, synthesis was initiated with relatively high specific activity [^{14}C]ADP-glucose (0.54 mCi/mmole) and after a short period (5 min) unlabeled ADP-glucose was added to give a fivefold dilution of the [^{14}C]ADP-glucose. The reaction was allowed to proceed until the amount of glycogen formed was four to six times that present when the cold ADP-glucose was added. As a control a parallel assay was run in which the chase was carried out with [^{14}C]-ADP-glucose having the same specific activity as that used to initiate the reaction. After termination of the reactions, the per cent β-amylolysis of the ^{14}C in the final product was determined. The data presented in Table VIII show that, in the absence of glycogen activator, and when BSA is used as an activator, the same fraction of label is cleaved from the molecule whether labeled or unlabeled ADP-glucose is used for the chase. Thus, the per cent of the label that can be cleaved from the product by β-amylase appears to become fixed early in the reaction and to remain unchanged with further synthesis of the molecule. This result is not consistent with that expected for the glucosyl transferase–branching enzyme pathway described above. When the activator is glycogen, it is seen that a significant fraction of the [^{14}C]glucose added early in the reaction does become buried by the further reaction. Even in this case, however, the glucosyl transferase–branching enzyme mechanism would not predict that such a large fraction of the [^{14}C]glucose would still be accessible to β-amylase after the chase.

Several explanations of these preliminary data are possible. For example, the system may initiate the synthesis of many new molecules of glycogen throughout the reaction period rather than continuing to add glucose residues to those few glycogen molecules formed early in the assay. Alternatively, a pathway similar to that described for the synthesis of branched O-antigens (Osborn and Weiner, 1968) by successive additions of oligosaccharides to the reducing terminals of the growing molecule (Robbins *et al.*, 1967) would account for

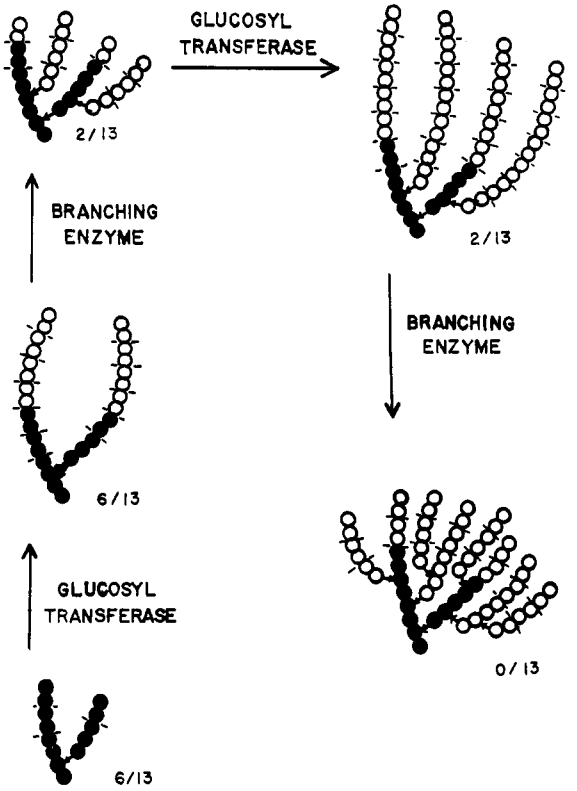

FIGURE 6: The glucosyl transferase–branching enzyme mechanism for the growth of the A3(S1) glycogen molecule ($\overline{ICL}$ = 1) in a pulse-labeling experiment. The closed circles represent [^{14}C]glucosyl residues incorporated into glycogen in the early phases of reaction. The open circles are unlabeled glucosyl resides incorporated after the chase. Adjacent circles are joined by 1,4 linkages; the 1,6 linkages at branch points are indicated by arrows. Growth proceeds by succesive transfers of glucosyl residues to the nonreducing terminals by glucosyl transferase (Manners, 1962) until the external chains are sufficiently long to be acted upon by branching enzyme (Larner, 1953; Verhue and Hers, 1966). The action of β-amylase in removal of successive maltose residues from the nonreducing end is shown by the dashed lines. For each structure the fraction of the total number of [^{14}C]glucose residues that will be removed by β-amylase is indicated.

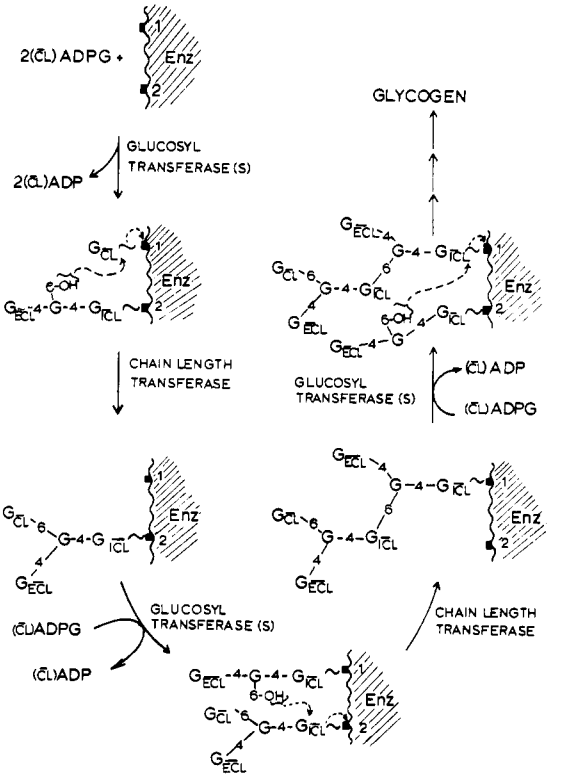

FIGURE 7: Mechanism proposed for *de novo* synthesis of glycogen from the reducing end of the molecule (see Robbins *et al.*, 1967). The glucosyl transferase system is proposed to catalyze formation of the activated "repeating units" of glycogen by transfer of the number of glucosyl residues in $\overline{CL}$ from ADP–glucose to each of the sites (1 and 2) on the enzyme complex. One of the linear α-1,4-linked oligosaccharides thus formed is then divided into $\overline{ECL}$ and $\overline{ICL}$ by the attack of the C-6 hydroxyl group of its potential branching residue on the activated reducing terminal of the linear oligosaccharide attached to the adjacent site on the enzyme. If the attacking residue is always a part of the newly formed $\overline{CL}$, the glycogen molecule will grow from the reducing end. Note that in the first enzyme–$\overline{CL}$ complex the chain lengths at sites 1 and 2 are identical but are designated differently ($\overline{CL}$ = $\overline{ECL}$ + $\overline{ICL}$ + 1). The initial oligosaccharide on site 1 is designated $G_{\overline{CL}}$ throughout the scheme and, as seen, remains in the external nonreducing region in the final product. The symbols used are: $G_{\overline{CL}}$, a linear α-1,4-linked sequence containing the number of glucosyl residues in a $\overline{CL}$; $G_{\overline{ECL}}$, a linear α-1,4-linked sequence containing the number of glucosyl residues in an $\overline{ECL}$; $G_{\overline{ICL}}$, a linear α-1,4-linked sequence of glucosyl residues in an $\overline{ICL}$. The numbers 4 and 6 indicate the positions of linkage on the glucosyl residue to the right of the number.

these data. The latter mechanism, illustrated in Figure 7, is discussed below.

Discussion

The purified GS from *A. aerogenes* catalyzes the synthesis of a highly branched glycogen in the presence of SAP. The absence of glycogen from the purified cell components and from all other components of the assay system has been demonstrated. Thus, the system synthesizes glycogen directly and solely from ADP–glucose. Although a specific heat-labile activator protein has been purified from the *A. aerogenes* extracts, any one of a group of physically and chemically diverse compounds will also serve as activators. Because of the nonglycogen nature of the compounds which stimulate the activity of GS and their diverse properties, they appear to serve as activators rather than as glucosyl acceptors. This conclusion is supported in the case of SAP by the demonstration that SAP does not become labeled during the *de novo* glycogen synthesis. With the exception of glycogen, all of the activators have a more or less hydrophobic character. The hydrophobic properties of BSA and its capacity to adsorb fatty acids have been reported (Laurence, 1952). SAP, the natural activator of the synthetase, although a soluble protein, shows some tendency to chromatograph with smearing on DEAE-cellulose columns, a behavior that could be attributed to hydrophobic surface characteristics.

Since the synthetic glycogen is highly branched, the

synthetase fraction must contain at least two activities, one for making α-1,4 linkages and one for α-1,6 linkages. The effect of the activator may be simply to induce among the components of the synthetase fraction the appropriate conformational relationship needed for activity. Whatever mechanisms are involved in the activation of GS, the effect is complex since no single activator gives maximal stimulation.

The replacement of the activator by glycogen requires special comment. According to the classical view, glycogen synthetase requires glycogen as a cosubstrate which accepts glucose from the appropriate activated glucosyl donor. Since, in the system described here, glycogen has the same effect on the synthetase as found for the nonglycogen activators, one must ask whether glycogen serves as an activator, a glucosyl acceptor, or both. In the initial studies of this system it was found that in the glycogen-activated glycogen biosynthesis, approximately 30% of the nonreducing terminals of glycogen are glucosylated, indicating that glycogen primer serves at least in part as an acceptor. On the other hand, sigmoid glycogen saturation curves were observed for the unfractionated cell extracts, suggesting an activation activity for glycogen. Thus, glycogen may play the dual role of activator and acceptor in glycogen synthesis. The observation that glycogen and SAP act on GS in a similar manner explains the earlier observation (Kindt and Conrad, 1967) that when SAP is separated from GS by ultracentrifugation, higher levels of glycogen are required to saturate GS.

The [^{14}C]glucosyl residues incorporated into the external chains of the polymer in the initial stages of incubation remain in external chains after an additional incorporation of five to six times as much glucose from ADP-glucose into the product. If it is assumed that the glucoses incorporated in the later stages of synthesis are covalently joined to the glycogen formed initially, the data suggest that the *de novo* synthesis proceeds by addition of glucose residues at the reducing terminals of the glycogen molecule. A mechanism similar to that demonstrated by Robbins *et al.* (1967) for biosynthesis of the O-antigenic side chain of the LPS of *Salmonella anatum* could account for these data. In the latter system a trisaccharide repeating unit, activated through its reducing terminal, is presynthesized on a carrier lipid and then transferred to the reducing end of the growing polysaccharide chain to give a linear polymer. The branched-chain O-antigen of *Salmonella typhimurium* is apparently synthesized by the same mechanism (Osborn and Weiner, 1968). Here a presynthesized linear tetrasaccharide is attached to the growing polymer through the third glycosyl residue from its reducing end to yield a polysaccharide having a one-unit branch on every third glycosyl residue.

An analogous mechanism for *de novo* glycogen synthesis is shown in Figure 7. In this proposal, $\overline{\text{CL}}$, which has been a useful parameter in describing glycogen structure, is suggested to be a functional intermediate in biosynthesis and a "repeating unit" in the glycogen structure. Synthesis is initiated by formation of two $\overline{\text{CL}}$'s (linear α-1,4-linked oligosaccharides made up of 10–15 glucosyl residues). The initial branch linkage is formed by catalysis of an attack by a C-6 hydroxyl in one chain on the activated reducing group of the other. The branching reaction thus divides a $\overline{\text{CL}}$ into $\overline{\text{ECL}}$, $\overline{\text{ICL}}$, and a branching residue, and fixes, at the earliest stage of glycogen synthesis, the fraction of initially incorporated glucose that will be found in the external chain. Subsequent steps in the polymerization of chain lengths would involve attacks of C-6 hydroxyls in the newly formed $\overline{\text{CL}}$'s on the activated reducing terminal of the growing chain. The average distance of the attacking glucosyl residue on the newly formed $\overline{\text{CL}}$ from the point of attachment of the $\overline{\text{CL}}$ to the carrier would determine the $\overline{\text{ICL}}$ of the final polymer. In the *A. aerogenes* system, where the $\overline{\text{ICL}}$ is 1 (Kindt and Conrad, 1967), the second glucose from the activated reducing terminal would bear the attacking hydroxyl.

Glycogen, when present in the assay, might compete with such a mechanism in two ways: its nonreducing terminals could compete with the nonreducing terminals of the growing chain length for the glucosyl transferase(s), or its C-6 hydroxyls might compete with those on the newly synthesized chain length for the chain length transferase. As a result of such competition with the *de novo* process, glucoses incorporated early in the reaction might become buried and inaccessible to β-amylase after the chase. The indication that glycogen may act as an activator as well as an acceptor opens the possibility that products formed when glycogen is present are a mixture of *de novo* glycogen and original primer glycogen which may or may not be substituted. Definitive experimental procedures for distinguishing such a mixture from a multiglucosylated glycogen primer have not been used for characterization of the products of *in vitro* glycogen synthesis and, indeed, are difficult to conceive and execute.

No data have been obtained on the nature of the primary glucosyl acceptor sites for the *de novo* process. In Figure 7 these sites are depicted as a part of the GS complex, but the possibility remains that ADP-glucose, either free or bound, may serve as a glucosyl acceptor as well as a glucosyl donor. In the biosynthesis of the *Salmonella* O-antigen a phosphorylated polyisoprenoid has been shown to be the carrier of the repeating unit (Wright *et al.*, 1967). A similar or identical lipid carrier participates in cell wall biosynthesis (Higashi *et al.*, 1967) and in synthesis of a mannan by *Micrococcus lysodeikticus* (Scher *et al.*, 1968). All attempts to date to demonstrate such a carrier in glycogen biosynthesis have been unsuccessful.

Several important features of the proposed mechanism may be pointed out. First, it would readily account for the observed irreversibility of the branching reaction (Hassid and Neufeld, 1962). Also, by this mechanism growth of glycogen would take place at the least hindered site of the molecule. The steric crowding at the nonreducing terminals of a glycogen molecule, apparent in Figure 6, has been discussed by Madsen and Cori (1958). While this crowding may not markedly hinder the interaction of a glucosyl transferase with a glycogen substrate, the steric problem becomes more

critical for a branching enzyme which must carry a segment of the external chain to a site very close to a branch point in the most hindered part of the molecule. The smaller the $\overline{ICL}$ of a glycogen is, the greater this latter problem becomes.

An alternative explanation for the pulse labeling data is that the growth of these molecules formed early in the reaction is terminated early, perhaps due to steric hindrance at the nonreducing terminals, and that the system therefore initiates synthesis of new glycogen molecules throughout the assay period. Definitive experiments to determine the relative molecular size of the biosynthesized glycogen present as synthesis proceeds are required to establish the mechanism of glycogen synthesis in this system.

The glycogen synthetase system described here is not unlike those described previously in the literature. Only by careful preparation of cell extracts and by retaining the system in detergent at all stages of purification is the capacity for *de novo* synthesis preserved. The most highly purified preparations gradually lose their capacity to be activated by the nonglycogen primers but retain much of their glycogen-activated activity. The aged enzyme thus becomes a typical glycogen synthetase with properties like those of the mammalian and bacterial systems described in the literature.

Acknowledgment

The authors wish to express their appreciation to Dr. I. C. Gunsalus for helpful dicussions during the preparation of this manuscript. They also wish to acknowledge the valuable technical assistance of Mr. William Waud.

References

Bernfeld, P. (1955), *Methods Enzymol. 1*, 149.
Dwiggins, C. W., Bolen, R. J., and Dunning, H. N. (1960), *J. Phys. Chem. 64*, 1175.
Govons, S., Sabraw, A. and Preiss, J. (1968), *Federation Proc. 27*, 289.
Greenberg, E. and Preiss, J. (1965), *J. Biol. Chem. 240*, 2341.
Hassid, W. Z., and Neufeld, E. F. (1962), *Enzymes 6*, 292.
Higashi, Y., Strominger, J., and Sweeley, C. C. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 1878.
Hummel, B. C. W. (1959), *Can. J. Biochem. Physiol. 37*, 1393.
Kindt, T. J., and Conrad, H. E. (1967), *Biochemistry 6*, 3718.
Koeltzow, D. E., Epley, J. E., and Conrad, H. E. (1968), *Biochemistry 7*, 2920.
Larner, J. (1953), *J. Biol. Chem. 202*, 491.
Laurence, D. J. R. (1952), *Biochem. J. 51*, 168.
Leloir, L. F., and Cardini, C. E. (1962), *Enzymes 4*, 317.
Lowry, O. H., Rosebrough, N. J., Farr, A. C., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Madsen, N. B., and Cori, C. F. (1958), *J. Biol. Chem. 233* 1251.
Manners, D. J. (1962), *Advan. Carbohydrate Chem. 17*, 371.
Osborn, M. J., and Weiner, I. M. (1968), *J. Biol. Chem. 243*, 2631.
Partridge, S. M. (1949), *Nature 164*, 443.
Preiss, J., and Greenberg, E. (1965), *Biochemistry 4*, 2328.
Preiss, J., Shen, L., Greenberg, E., and Gentner, N. (1966), *Biochemistry 5*, 1833.
Robbins, P. W., Bray, D., Dankert, M., and Wright, A. (1967), *Science 158*, 1536.
Scher, M., Lennarz, W. J., and Sweeley, C. C. (1968), *Proc. Natl. Acad. Sci. U. S. 59*, 1313.
Verhue, W., and Hers, H. G. (1966), *Biochem. J. 99*, 222.
Wright, A., Dankert, M., Fennessey, P. and Robbins, P. W. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 1798.