Purification and Properties of Glycogen Synthetase from Trout Liver†

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ABSTRACT: Glycogen synthetase *b* (UDPG:glycogen α-1,4-glucosyl transferase, EC 2.4.1.11) from trout liver has been purified to an essentially homogeneous state by a procedure including differential centrifugation, DEAE-cellulose chromatography, and calcium phosphate gel adsorption. On polyacrylamide gel electrophoresis, either in the presence or absence of glucose 6-phosphate (G-6-P), a single protein band having glycogen synthetase activity was detected. The purified enzyme converts 26 μmoles of uridine diphosphate glucose (UDPG) to products per min per mg of protein under the conditions of the assay at 37°. G-6-P is a "V effector" in this system. Double-reciprocal plots of both the G-6-P and UDPG saturation data exhibited transitions to lines with greater slopes at higher concentrations of the respective ligand. Mg^{2+} and Ca^{2+} stimulated the activity, while P_i, sulfate, and sulfite inhibited. ATP produced no significant inhibition. The synthetase activity of certain fractions could be increased by incubation with appropriate ligands. Storage of the purified enzyme in the cold led to partial inactivation which could also be reversed by ligands. These observations, plus the kinetic characteristics of the system, suggest the existence of an additional form or forms of the enzyme. On sucrose density gradient centrifugation four active components were detected. An approximate molecular weight of 274,000–312,000 was calculated for the heaviest. UDPG and UDP shifted the distribution toward the heavier components, while G-6-P or ATP produced the opposite effect.

The glycogen synthetase reaction is a key site of control of glycogen synthesis in the liver (Hornbrook *et al.*, 1965, 1966) and is linked to control of glycogen utilization as well, through a reciprocal relationship with phosphorylase (De Wulf *et al.*, 1970; Stalmans *et al.*, 1971). Regulation of flux through the system depends upon the interconversion of active (*a*) and inactive (*b*) forms (Mersmann and Segal, 1967), mediated by a phosphokinase (*a* to *b* reaction) and a phosphatase (*b* to *a* reaction). The phosphokinase, recently identified as cAMP[1]-dependent protein kinase (Soderling *et al.*, 1970), is responsive to hormones which affect cAMP levels. The phosphatase is modulated either directly or indirectly by glucocorticoids (Mersmann and Segal, 1969), glycogen (De Wulf *et al.*, 1970), insulin (for discussion, see Hers *et al.* (1970)), and glucose (De Wulf *et al.*, 1970; Stalmans *et al.*, 1970; Gruhner and Segal, 1970).

The metabolically relevant distinctions between the *a* and *b* forms are the greater affinity of the former for its substrate, UDPG, and activator, G-6-P (Mersmann and Segal, 1967), and lesser affinity for the inhibitor, ATP (Gold, 1970).

A recent review of the glycogen synthetase system has appeared (Larner and Villar-Palasi, 1971).

In this paper we report the preparation of glycogen synthetase *b* from trout liver and some of its kinetic and physical characteristics. The final preparation is free of polysaccharide and shows essentially a single protein band on gel electrophoresis corresponding to the location of synthetase activity.

Materials

Rainbow trout with liver weights between 5 and 10 g were obtained from the State Hatchery, Caledonia, N. Y. The fish were transported in ice water from the hatchery to the laboratory. The livers, after rinsing with 0.14 M NaCl, were sealed in plastic bags and stored at −86°. No significant loss of glycogen synthetase activity was observed after 2-months storage. UDPG, uniformly labeled in the glucose moiety with ^{14}C and of specific activity greater than 200 mCi/μmole, was purchased from New England Nuclear, Boston, Mass. The radioactive substrate was diluted to 6000 cpm/μl with distilled water before use. DEAE-cellulose (DE-52), cellulose powder (CF-2), and chromatography paper (31ET) were from Whatman. Rabbit muscle pyruvic kinase, beef heart lactic dehydrogenase, yeast alcohol dehydrogenase, beef liver catalase, and all other chemicals were obtained from Sigma Chemical Co.

Methods

Enzyme Assays. Glycogen synthetase was assayed by both optical and radioactivity methods.

The optical method involves the coupling of UDP formed to NADH oxidation via the pyruvic kinase and lactic dehydrogenase reactions as previously described (Sanada and Segal, 1971), with 6 mM G-6-P present for assay of *a* + *b* activity (omitted for assay of the *a* form). A unit of enzyme is the amount catalyzing the formation of 1 μmole of UDP/min under the conditions of the assay.

The radioactivity assay was essentially by the procedure of Thomas *et al.* (1968), involving the use of [^{14}C]UDPG uniformly labeled in the glucose moiety and the separation of glycogen for counting at the end of the reaction period. Concentrations of components were as in the optical assay with the addition of 12,000 cpm of [^{14}C]UDPG in a final volume of 0.1 ml. Reactions were at 30° for 5 min.

Alcohol dehydrogenase was assayed optically at pH 8.5 in

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[1] Abbreviations used are: cAMP, cyclic adenosine 3′,5′-phosphate; UDPG, UDP-glucose; G-6-P, glucose 6-phosphate.

the presence of ethanol and NAD. Catalase was assayed by measurement of the first-order decline in H_2O_2 absorbance at 240 nm.

Other Determinations. Protein was measured by the method of Lowry *et al.* (1951), and glycogen by the method of Hassid and Abraham (1957).

Results

Purification of Glycogen Synthetase. Livers were thawed, rinsed in 0.14 M NaCl, and homogenized in a Servall Omnimixer with 5 ml/g of a solution containing 0.25 M sucrose, 0.1 M glycylglycine (pH 7.4), 0.1 M NaF, 10 mM 2-mercaptoethanol, 5 mM EDTA, and 0.1% rabbit liver glycogen (Sigma type III). The homogenate was centrifuged at 20,000*g* for 15 min, and the supernatant layer was filtered through glass wool (crude extract). About 90% of the total enzyme activity in the original homogenate was recovered in the supernatant fraction. This was then centrifuged at 80,000*g* for 2 hr in a Spinco L-2, and the supernatant layer and microsomes were removed by decantation and discarded. The glycogen pellet, tightly packed at the bottom of the tubes, was rinsed once with a solution of 0.1 M glycylglycine (pH 7.4), 10 mM 2-mercaptoethanol, and 1 mM EDTA in 25% (v/v) glycerol, then homogenized in a glass homogenizer with a volume of this buffer equal to approximately one-tenth that of the crude extract. The recovery at this step was between 30 and 50% with purification generally over 100-fold.

The preswollen DEAE-cellulose was mixed with cellulose powder in a 1:1 ratio (dry weight) to improve the flow rate, equilibrated with the glycerol buffer, and packed in a column under gravity. A column with bed dimensions of 1.7 × 25 cm was used, sufficient to adsorb at least 150 units of the enzyme. After loading the enzyme, the column was washed with the same buffer, which removed the glycogen, followed by the buffer with 0.15 M NaCl added, until the $A_{280\,nm}$ fell to zero. The NaCl concentration was raised to 0.25 M which eluted glycogen synthetase. A typical elution profile is shown in Figure 1.

The active fractions were pooled and concentrated in a collodion bag (Brinkmann Instruments). Simultaneously with concentration, the enzyme was dialyzed against a solution containing 10 mM KPO_4 (pH 7.3), 10 mM 2-mercaptoethanol, and 1 mM EDTA in 25% glycerol (v/v). The dialyzing buffer was changed after the concentration was completed and the dialysis was continued for 3 hr. This was followed by a dialysis against the same buffer but without EDTA for another 3 hr. At this step the enzyme purification was usually 200- to 300-fold, and the recovery was about 13% relative to the crude extract.

Calcium phosphate gel was employed as the final step in the purification. The gel was washed with the final dialysis buffer (above), suspended in a small volume of the same buffer and added to the enzyme solution with stirring. Ten milligrams dry weight of calcium phosphate per milligram of protein was used. The mixture was left in the cold room for 15 min. The gel with adsorbed protein was then collected by centrifugation at 3000*g* for 10 min, and was washed twice with 5% ammonium sulfate in 25% glycerol (v/v) containing 10 mM 2-mercaptoethanol. The volume of each washing was equal to that of the original enzyme solution. These washings removed virtually all the remaining inactive protein as judged by gel electrophoresis. The washed gel was suspended in the phosphate–mercaptoethanol–glycerol buffer and mixed with cellulose powder (two times the wet weight of the gel) suspended in a small volume of the same buffer. The mixture was packed in a small column and glycogen synthetase was eluted with three bed volumes of a solution of 50 mM KPO_4 (pH 8.0) and 10 mM 2-mercaptoethanol in 25% glycerol (v/v). The enzyme solution was then concentrated in a collodion bag and dialyzed against a solution of 50 mM glycylglycine (pH 7.4), 10 mM $MgCl_2$, and 10 mM 2-mercaptoethanol in 25% glycerol (v/v) for 4 hr with one change of the buffer. The final recovery was about 5% and purification was about 1000-fold. On polyacrylamide gel electrophoresis of the purified enzyme, with or without G-6-P, a single protein band was detected containing all the glycogen synthetase activity (Figure 2). On prolonged staining a small amount of dye binding material without synthetase activity close to the cathode was also observed (dotted line in illustration). A typical purification protocol is shown in Table I.

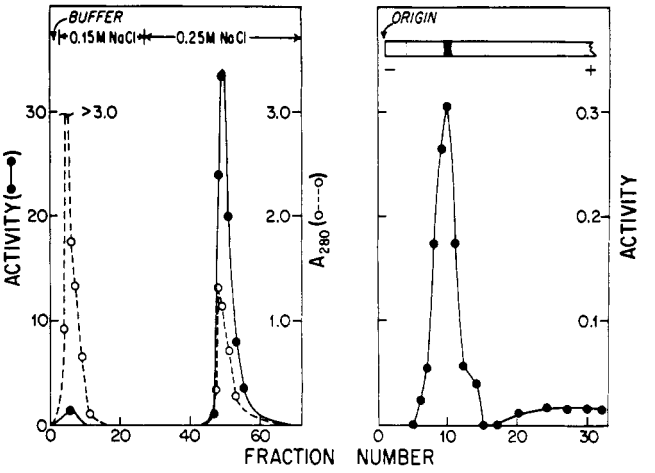

FIGURE 1 (left): DEAE-cellulose chromatography of glycogen synthetase. Activity ($a + b$) is expressed as absorbancy changes produced in the optical assay per milliliter of enzyme solution. Flow rate was about 18 ml/hr. Fractions of 9 ml were collected with the 0.15 M NaCl buffer and 3 ml with the 0.25 M NaCl buffer.

FIGURE 2 (right): Gel electrophoresis of purified glycogen synthetase. Gels were prepared from 6% acrylamide in 30 mM Tris-glycine (pH 8.6). Electrophoresis was at 6° in a vertical apparatus. After a 20-min preelectrophoresis at 60 mA, 18 μg of enzyme was added to the well and the electrophoresis was continued for 2 hr. After removal of a strip of gel for enzyme localization the remainder was stained for protein with 0.1% Coomassie Brilliant Blue in methanol–acetic acid–water (5:5:1). The strip for enzyme assay was cut into 2-mm segments which were placed in tubes containing the components for assay of $a + b$ activity with bacteriological dextrin in place of glycogen. The mixtures were shaken for 10 hr at room temperature, heat denatured, and the UDP formed was assayed optically. Activity is expressed as total optical density change produced per segment. The stained gel is illustrated at the top.

Kinetics. Under the conditions of the assays activity was proportional to enzyme concentration and was linear with time for at least 40 min.

Stimulation of the enzyme by G-6-P is shown in Figure 3. The kinetics were complex, with a break in the double-reciprocal plot to a curve with a greater slope at activator concentrations above 1 mM. The points at lower concentrations extrapolated to an apparent K_a of 0.4 mM and those at higher concentrations to a value of 1.4 mM.

The results with varying UDPG concentrations at several fixed G-6-P concentrations are shown in Figure 4. With this enzyme G-6-P appeared to be a V effector, as has been reported to be the case with the *b* form of the muscle enzyme (Rosell-Pérez *et al.*, 1962; Rosell-Pérez and Larner, 1964a,b).

TABLE I: Glycogen Synthetase Purification.

Fraction	Vol (ml)	Glycogen Synthetase				Protein (mg/ml)	Glycogen (mg/ml)
		Total Units[a]	Sp Act.	% *a* Form	Recov (%)		
Crude extract	460	154	0.02	18.0	100	16.2	4.2
Glycogen pellet	40	63.5	3.95	0.7	41	0.40	27
DEAE-cellulose eluate (concentrated)	5.5	20.0	6.89	0.0	13	0.54	0.009
Calcium phosphate gel eluate (concentrated)	0.8	7.5	26.1	0.0	4.9	0.36	Undetectable

[a] Activity was measured by the optical method.

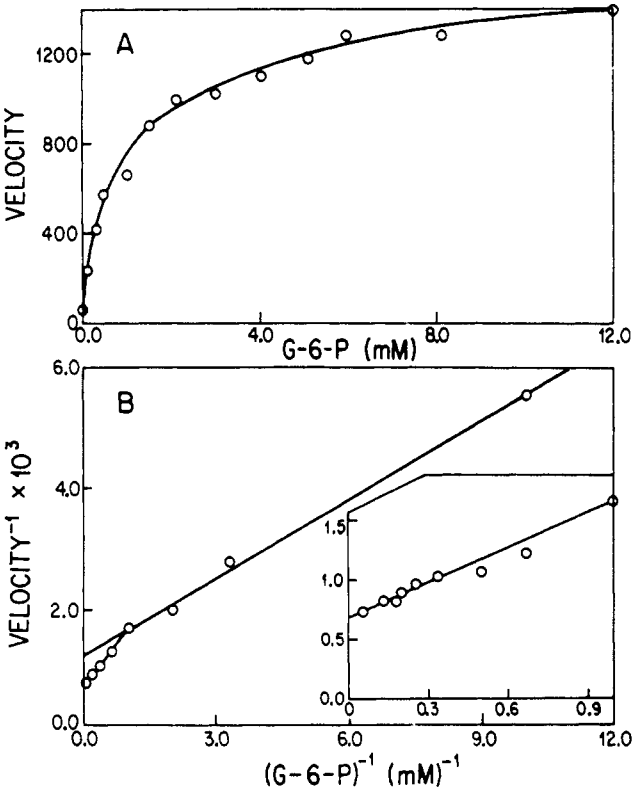

FIGURE 3: Kinetics of G-6-P stimulation of glycogen synthetase. The enzyme was a calcium phosphate gel eluate prepared as described under purification. Velocity is expressed as cpm incorporated into glycogen in the standard radioactivity assay. The inset in the double-reciprocal plot (B) shows the data at the higher G-6-P concentrations on an expanded scale.

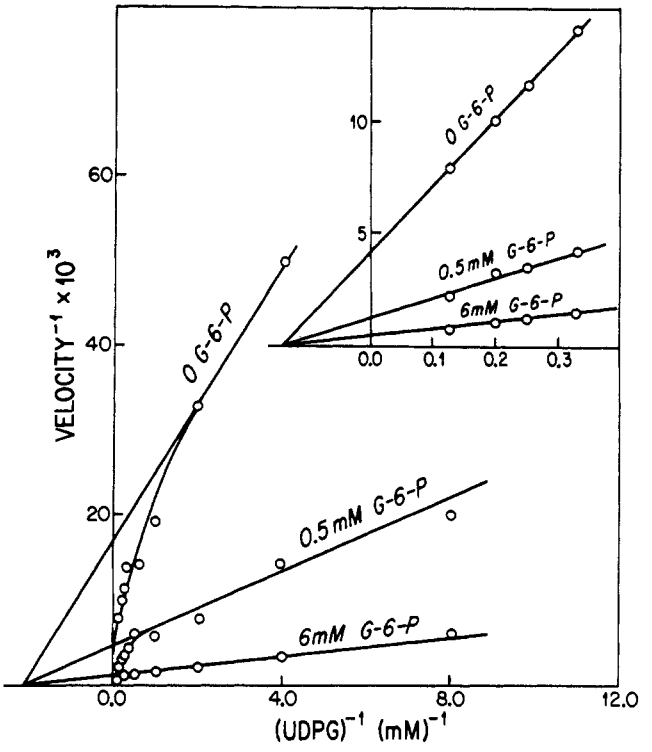

FIGURE 4: Kinetics of substrate saturation of glycogen synthetase. Enzyme, velocity units, and assay method were as in Figure 3. The inset shows the data at the higher UDPG concentrations on an expanded scale.

(With the corresponding rat (Mersmann and Segal, 1967) and tadpole (Sevall and Kim, 1970) liver enzymes G-6-P is a K effector.) As in the case with G-6-P (Figure 3), the double-reciprocal plots of UDPG dependence also exhibited a break with a greater slope at concentrations above 3 mM. Extrapolation of the points at the lower concentrations gave an apparent K_m of 0.5 mM and those at the higher concentrations gave 7.2 mM.

Incubation at 20° led to *b* to *a* conversion with the crude extract (Figure 5) and to some extent with the glycogen pellet, but not with more purified fractions.

Synthetase activity was inhibited by phosphate and sulfate (Figure 6), as well as sulfite, and was stimulated by Mg^{2+} and Ca^{2+} (Figure 6). Similar results of anions (De Wulf *et al.*, 1968) and Mg^{2+} (Rosell-Pérez *et al.*, 1962; Rosell-Pérez and Larner, 1964b; Gold, 1968) have been reported with enzyme preparations from other species. ATP produced no significant inhibition, unlike its effect with the rat (Gold, 1970) and mouse (De Wulf *et al.*, 1968) liver enzymes.

Enzyme Activation. Glycogen synthetase activity of certain fractions could be increased by the addition of ligands. Incubation of the crude (20,000*g*) extract with $MgCl_2$ resulted in an increase in total activity.[2] NaF inhibited the activation (Figure 7). Centrifugal fractionation of the synthetase activity

[2] In all the experiments reported in this section, which were performed with livers which had been stored at −20°, no significant *b* to *a* conversion took place even in the absence of F^-. The latter occurred only with crude extracts from fresh livers.

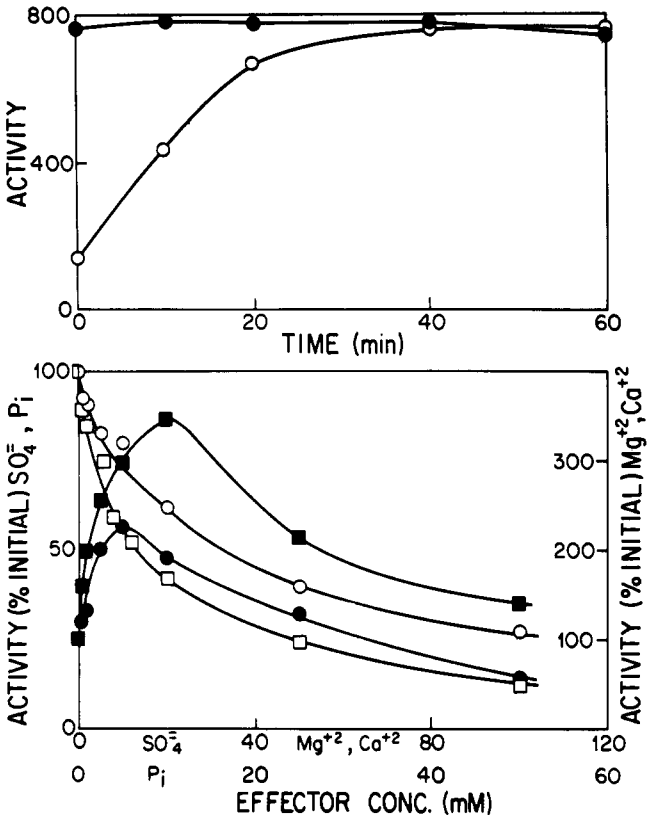

FIGURE 5 (upper): Glycogen synthetase *b* to *a* conversion in a crude extract. Crude extract was prepared as described under purification except NaF was omitted. Incubation was at 20°. Aliquots were removed at the times shown for assay with (●) and without (○) G-6-P. Activity is expressed as in Figure 3.

FIGURE 6 (lower): Effect of ions on glycogen synthetase activity. Enzyme and assay method (*a* + *b*) were as in Figure 3. (□) KH_2PO_4, (○) $(NH_4)_2SO_4$ (neutralized), (■) $MgCl_2$, and (●) $CaCl_2$.

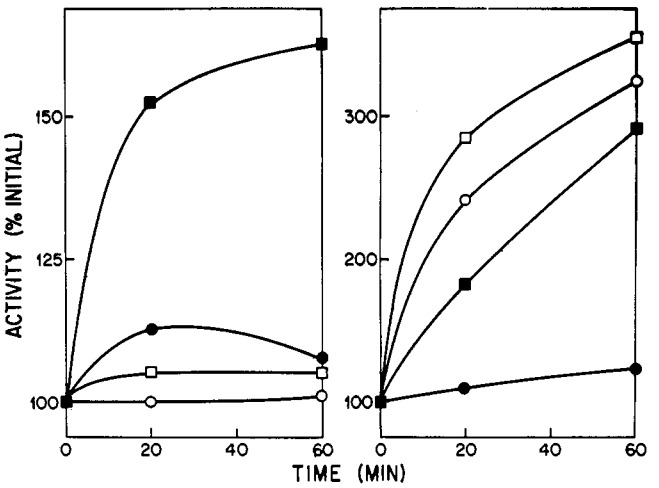

FIGURE 7 (left): Activation by Mg of glycogen synthetase activity in the crude extract. Crude extract was prepared as described in Figure 5. Incubation was at 20°. Aliquots were removed at the times indicated for assay of *a* + *b* activity by the optical method. (●) No additions, (■) 10 mM $MgCl_2$, (○) 0.1 M NaF, and (□) 10 mM $MgCl_2$ and 0.1 M NaF.

FIGURE 8 (right): Activation by ligands of glycogen synthetase dissociated from glycogen by DEAE-cellulose chromatography. The concentrated DEAE-cellulose eluate was prepared as described under purification. The procedure was as in Figure 7. (●) No addition, (■) 10 mM $MgCl_2$, (○) 4 mM UDPG, and (□) 6 mM G-6-P.

in the crude extract revealed that the activatable material was entirely in the soluble fraction. Activatable material also appeared in soluble enzyme preparations which had been dissociated from glycogen by DEAE-cellulose chromatography (Figure 8). In addition to the Mg^{2+} effect, it can be seen that an even more rapid activation by other ligands also occurred. Glycogen (5 mg/ml) produced no effect. No activation by $MgCl_2$ was observed with the final homogeneous preparation, presumably because the Mg-activatable species had already been activated, or its formation prevented, by dialysis against the Mg-containing buffer during concentration of the calcium phosphate gel eluate. However, some further activation of this preparation could be achieved by incubation with other ligands.

Stability and Reactivatibility of Stored Preparations. The decline in activity of preparations stored at −20° is shown in Figure 9. Glycogen and, to a lesser extent, UDPG had some protective effect. Most or all of the inactivation suffered during 1 week of storage at −20° could be reversed by incubation at 20° in the presence of Mg^{2+} or UDPG (Figure 10). After 3-weeks storage, however, only a relatively small fraction of the lost activity could be recovered by this process.

Density Gradient Centrifugation Experiments. Centrifugation in a sucrose gradient was employed to determine the approximate molecular weight of the purified synthetase and the effect of ligands on its sedimentation characteristics. Four separate peaks of activity could be distinguished (Figure 11A) whose proportion, as well as the total yield of activity, varied with the nature of the ligands present. In the presence of G-6-P (Figure 11B) or ATP (Figure 12B) the lighter species predominated, while UDPG (Figure 11C) or UDP (Figure 12C) shifted the distribution toward the heavier forms, with greater stabilization of the activity. The recovery was markedly reduced with decreased EDTA and increased with higher EDTA concentrations (100% at 5 mM). The pres-

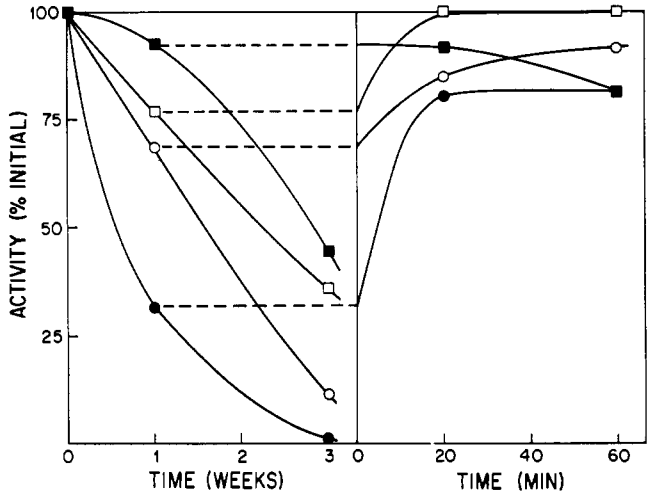

FIGURE 9 (left): Stability of glycogen synthetase in the presence of various ligands. The preparations were stored at −20° in the presence of 10 mM $MgCl_2$ (●), 5 mg/ml of glycogen (■), 1.2 mM UDPG (○), or 10 mM $MgCl_2$ plus 5 mg/ml of glycogen (□), and assayed as in Figure 7 at the times shown.

FIGURE 10 (right): Reactivation of partially inactivated preparations of glycogen synthetase. Preparations stored for 1 week as described in Figure 9 were incubated at 20°. Assay was as in Figure 7. Symbols are as in Figure 9.

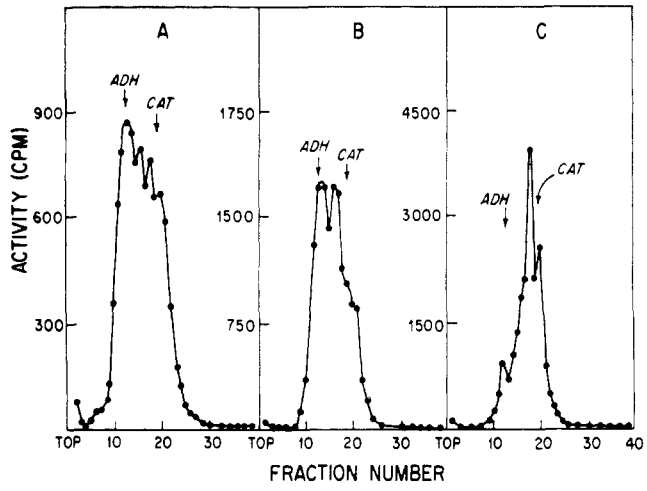

FIGURE 11: Effect of G-6-P and UDPG on the sedimentation characteristics of glycogen synthetase. The enzyme was a calcium phosphate gel eluate prepared as described under purification. Before centrifugation 25 μg of alcohol dehydrogenase and 100 μg of catalase were added and the mixture was dialyzed against a solution of 50 mM glycyclglycine (pH 7.4), 20 mM 2-mercaptoethanol, 2 mM EDTA, and 2% sucrose (curve A). In curve B 4 mM G-6-P and in curve C 4 mM UDPG were also present. The dialyzed enzyme solution (100 μl) was layered over 4.6 ml of a linear gradient of 5–20% sucrose which contained all the components present in the respective enzyme solutions. Centrifugation was at 38,000 rpm for 8 hr at 3°. Seven-drop fractions (40) were collected and assayed for synthetase activity (a + b) by the radioactivity method, as well as for alcohol dehydrogenase and catalase activity. The recovery of synthetase activity was 28, 45, and 84% in tubes A, B, and C, respectively. Arrows marked ADH and CAT indicate the positions of the alcohol dehydrogenase and catalase peaks.

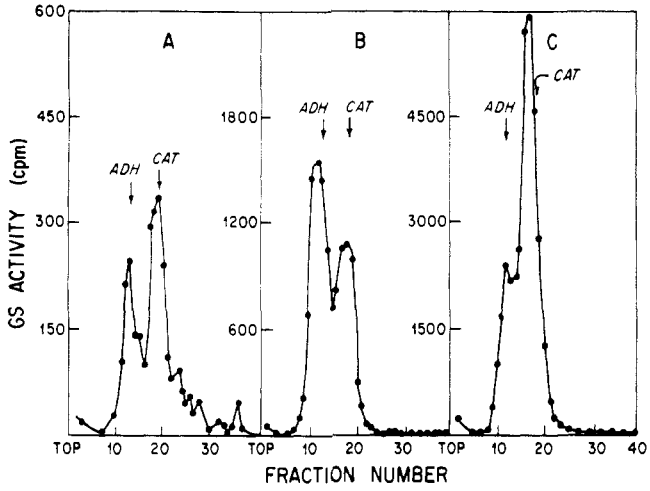

FIGURE 12: Effect of ATP and UDP on the sedimentation characteristics of glycogen synthetase. The procedure was as described in Figure 11 except the EDTA concentration was 1 mM. In curve B 4 mM ATP and in curve C 4 mM UDP were also present. The recovery of synthetase activity was 4, 17, and 50% in tubes A, B, and C, respectively.

ence of Mg^{2+} also reduced the activity recovered, which was almost entirely in the heaviest peak.

Average values from ten separate experiments of $s^{0.725}_{20,w}$ for the four peaks, calculated according to the method of Martin and Ames (1961), were 7.1 ± 0.1 S (SEM), 8.8 ± 0.1 S, 10.6 ± 0.1 S, and 12.0 ± 0.1 S, respectively. Calculations of the approximate molecular weight of the heaviest peak gave figures of 274,000 relative to catalase and 312,000 relative to alcohol dehydrogenase (Martin and Ames, 1961). The relative s values of the heaviest and lightest peaks corresponded to a ratio of molecular weights of 2.2.

Discussion

Glycogen synthetase has now been obtained in a highly purified state from rabbit muscle (Soderling *et al.*, 1970; Brown and Larner, 1971) and from trout liver as reported here.[3] The specific activity of the preparation of Brown and Larner was of the order of that obtained with the liver enzyme, allowing for the lower temperature of assay in the former case. Sevall and Kim (1970) have reported a preparation from tadpole liver of similar specific activity. No indication was given of the degree of homogeneity of their preparation, which contained substantial amounts of glycogen.

It is worthy of note that only about half the synthetase activity in trout liver homogenates was sedimented with the glycogen pellet (an even lower figure has been reported by Ingraham (1970)), while almost all the activity in rat liver homogenates remains bound to glycogen during centrifugation (Mersmann and Segal, 1969). Furthermore, we were unable to dissociate the rat enzyme from glycogen by DEAE-cellulose chromatography, as was accomplished with the trout enzyme (see Purification Procedure). Both of these observations indicate a greater affinity between rat liver synthetase and glycogen than with the trout system.

The activity remaining in the particulate-free supernatant, as well as that in other non-glycogen-associated fractions obtained during the purification procedures, particularly cold aged preparations, could be increased by incubation at 20° with Mg^{2+} and other ligands. These observations suggest the existence of a form or forms of the enzyme which are inactive under the conditions of assay for a + b activity. This is further supported by the bimodal nature of the UDPG and G-6-P saturation data. With both ligands there was a break in the double-reciprocal plots at higher concentrations to a new line extrapolating to higher K values.

Hidalgo and Rosell-Pérez (1971) have observed a similar fluoride-inhibited increase in total synthetase activity on preincubation of kidney extracts and have interpreted the results to indicate the presence of a superphosphorylated inactive form. Bimodal kinetics have also been reported with yeast (Rothman-Denes and Cabib, 1971) and chloroma tumor (Assaf and Yunis, 1971) synthetase preparations. As proposed by the latter workers, these observations are consistent with a form of synthetase with lower affinity for activator and substrate than the a and b forms, which thus requires exceptionally high levels of G-6-P to elicit activity.

The notion of a progression of phosphorylated forms with decreasing ligand affinity is intriguing. However, the manifestation of the inactive to active interconversion in highly purified preparations, where the presence of a phosphatase is unlikely, argues against this concept. The fluoride inhibition could reflect its ability to complex Mg^{2+}, which appears to be required for the activation, rather than an effect on a putative phosphatase. In this view, the inactive form or forms would differ from the active forms conformationally rather than in degree of phosphorylation.

Ultracentrifugational patterns similar to those obtained

[3] Homogeneous preparations have also been obtained from rat liver (D. C. Lin and H. L. Segal, manuscript in preparation).

here, including ligand-induced shifts, have also been reported with synthetase preparations from other species and tissues (Steiner *et al.*, 1965; Staneloni and Piras, 1971; Sanada and Segal, 1971). The molecular weight of the muscle enzyme (*a* form) has been reported to be about 400,000, with four subunits of 90,000–100,000 each (Soderling *et al.*, 1970). Staneloni and Piras (1971), working with partially purified preparations from muscle containing both *a* and *b* forms, have reported findings consistent with these values, observing species of approximately 195,000, 270,000, and 370,000 in density gradient centrifugation experiments. On the other hand, Brown and Larner (1971) obtained a value of 250,000 with a muscle synthetase *b* preparation. Calculations of approximate molecular weights by the density gradient method (Martin and Ames, 1961) yielded a value of about 270,000 for the heaviest of two observed forms of rat liver synthetase *b* (Sanada and Segal, 1971), compared to 290,000 reported here for the heaviest of four forms observed with the trout liver enzyme.

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