dimer in which the two sites alternate in a catalytically active form, *i.e.*, the "flip-flop" model (Lazdunski *et al.*, 1970), or as an example of conventional cooperativity (Simpson and Vallee, 1970). The presently observed activation is more consistent with the latter view, for it permits activator to remain bound to one subunit while stimulating multiple turnovers at the other site. The enzyme can bind ligands at both sites simultaneously; catalytic activity at one site is not abolished by binding competitive inhibitor at the other site (Halford, 1971). The "flip-flop" model requires, for stimulation of 4-nitrophenyl phosphate hydrolysis at one site, prior association of activator with, and subsequent dissociation from, the other site. These additional steps would be required each time the enzyme turns over. It is difficult to conceive how doubling the number of association–dissociation steps could lead to a faster overall rate for the reaction.

Recently, Bloch and Schlesinger (1973) have reported the presence in purified preparations of *E. coli* alkaline phosphatase of up to 2 mol of endogeneous phosphate which directly affects the amplitude of the burst transient at acid pH and which should affect the apparent dissociation constant for added phosphate. Accordingly, they suggest that previously cited evidence indicating subunit cooperativity may instead be due to endogenous phosphate. In models involving independence of subunits, subtle kinetic anomalies have been accounted for (Reid and Wilson, 1971; Halford *et al.*, 1972; Halford, 1972) by a mechanism involving isomerization of the enzyme between two forms, only one of which binds substrate strongly. Stimulation of activity of PP_i analogs at low substrate concentration as reported here may be explained in terms of such models by a mechanism in which the analogs shift the equilibrium of the isomerization in favor of the form which has the higher affinity for substrate.

References

Bloch, W., and Schlesinger, M. J. (1973), *J. Biol. Chem. 248,* 5794.
Eaton, R. H., and Moss, D. W. (1967), *Biochem. J. 102,* 917.
Fernley, H. N., and Walker, P. G. (1967), *Biochem. J. 104,* 1011.
Halford, S. E. (1971), *Biochem. J. 125,* 319.
Halford, S. E. (1972), *Biochem. J. 126,* 727.
Halford, S. E., Lennette, D. A., and Schlesinger, M. J. (1972), *J. Biol. Chem. 247,* 2095.
Kelly, S. J., Feldman, F., Sperow, J. W., and Butler, L. G. (1973), *Biochemistry 12,* 3338.
Lazdunski, M., Petitclerc, C., Chappelet, D., and Lazdunski, C. (1970), *Eur. J. Biochem. 20,* 124.
Reid, T. W., and Wilson, I. B. (1971), *Biochemistry 10,* 380.
Sillen, L. G., and Martell, A. E. (1964), *in* Stability Constants of Metal-Ion Complexes, London, The Chemical Society.
Simpson, R. T., and Vallee, B. L. (1968), *Biochemistry 7,* 4343.
Simpson, R. T., and Vallee, B. L. (1970), *Biochemistry 9,* 953.

Purification and Kinetic Mechanism of Rat Liver Glycogen Synthase†

Patrick H. McVerry‡ and Ki-Han Kim*

ABSTRACT: Glycogen synthase has been purified from rat liver by a rapid and unique procedure involving the reversible precipitation of the glycogen-free enzyme in the cold. The final enzyme was purified 1400-fold over crude tissue extract and has a specific activity of 22 μmol of glucose incorporated into glycogen min^{-1} mg of $protein^{-1}$ at 37° in the presence of 20 mM glucose-6-P. The purified synthase appeared homogeneous when subjected to polyacrylamide gel electrophoresis. Synthase subunit molecular weight was determined by electrophoresis in sodium dodecyl sulfate to be 77,000–80,000. Analysis of substrate and inhibition kinetics indicate the synthase reaction involves the formation of a ternary complex of enzyme and substrates with random binding of substrates. No significant exchange between UDP and UDPglucose was detected in the absence of glycogen. 1,5-Gluconolactone was found to be an inhibitor of the reaction suggesting an intermediate with the glucosyl moiety in a half-chair conformation exists during the reaction sequence.

Uridine diphosphate glucose:glycogen α-1,4-glucosyltransferase (EC 2.4.1.11), the rate-limiting enzyme for glycogen synthesis, is present in most tissues in two forms which are interconvertible by phosphorylation and dephosphorylation reactions (Larner and Villar-Palasi, 1971). The phosphorylated enzyme, which is dependent on glucose-6-P[1] for activity (synthase D) is thought to have little activity under physiological conditions, so that dephosphorylation to a glucose-6-P independent form (synthase I) turns on glycogen synthesis *in vivo* (Mersmann and Segal, 1967). These interconversions are mediated by a protein kinase and phosphatase which in turn may be influenced by various hormonal (Blatt and Kim, 1971) and metabolic states (Kato and Bishop, 1972). Synthase activity in rat liver also displays a diurnal rhythm due to control of its synthesis (McVerry and Kim, 1972a) and is regulated by cellu-

† From the Department of Biochemistry, Purdue University, Lafayette, Indiana 47907. *Received February 14, 1974.* This investigation was supported in part by the U. S. Health Service Grant AM 12865 from the National Institutes of Arthritis and Metabolic Diseases. Journal paper No. 5372 of the Purdue Agricultural Experimental Station.

‡ Present address: Department of Microbiology, University of Connecticut Health Center, Farmington, Connecticut 06032.

[1] Abbreviations used are: UDP, uridine 5′-diphosphate; glucose-6-P, glucose 6-phosphate.

lar metabolite concentrations (Magner and Kim, 1973; Gold, 1970). Thus it appears that a coordinated interaction of intra- and extracellular mechanisms affords a sensitive control of glycogen synthesis. The effects of these mechanisms at the molecular level, however, remain to be elucidated.

Only recently have homogeneous preparations of glycogen synthase been available for physical and kinetic studies. Skeletal muscle synthases I and D have been prepared (Brown and Larner, 1971; Schlender and Larner, 1973; Soderling *et al.*, 1970) and kidney synthase D has been isolated (Issa and Mendicino, 1973). The purification of hepatic synthase has been hampered by its high affinity for glycogen and instability in the absence of the polysaccharide. Sanada and Segal (1971) prepared a soluble synthase D form from livers of starved-adrenalectomized rats and Steiner *et al.* (1965) solubilized the enzyme from glycogen by reversible heat inactivation. The apparent molecular weights of muscle synthases D and I are 250,000 and 400,000, respectively, with subunits of about 92,500 (Brown and Larner, 1971; Soderling *et al.*, 1970; Smith *et al.*, 1971). We have shown liver synthase D to sediment at about 140,000 and the I form to be a heterogeneous mixture of higher molecular weight species (McVerry and Kim, 1972b).

In this paper we describe a rapid and novel method for the preparation of glycogen synthase from rat liver. Advantage has been taken of the tendency of the enzyme to aggregate in the cold after release from glycogen. The subunit molecular weight has been determined and appears to differ from the muscle enzyme. Also included is a study of the reaction kinetics which indicates that the reaction proceeds through a sequential mechanism. While this work was in progress Lin and Segal (1973) reported the purification of hepatic synthase D by another method.

Materials and Methods

Chemicals. Glucose-6-P, UDPglucose, shellfish glycogen, *p*-hydroxymercuribenzoate, and UDP were purchased from Sigma Chemical Co. Radioactive UDP-[U-^{14}C]glucose was obtained from Schwarz/Mann, Inc., and [5-^{3}H]UDP was from Amersham/Searle.

Assay of Glycogen Synthase. The standard assay system contained 2 mM UDP-[^{14}C]glucose (10,000 cpm/assay), 4 mg/ml of glycogen, 20 mM glucose-6-P, 1 mM dithiothreitol, 40 mM glycylglycine at pH 7.4, and 0.005–0.010 unit of synthase in a 0.25-ml volume. The reaction was run at 37° for 10 min after the addition of UDPglucose and terminated with 1 ml of 4% trichloroacetic acid in 80% ethyl alcohol containing 0.5 mg/ml of lithium bromide. The samples were then treated as previously described (Magner and Kim, 1973). One unit of activity is defined as the incorporation of 1 μmol of glucose into glycogen in 1 min under these conditions.

Analytical Methods. For protein determinations, samples containing 0.05–0.25 mg/ml of protein were precipitated by heating for 5 min at 100° in 10% trichloroacetic acid and collected by centrifugation. The precipitates were washed once with 10% trichloroacetic acid, and dissolved in a small volume of 0.1 *N* sodium hydroxide, and the protein content was determined by the method of Lowry *et al.* (1951). Crystalline bovine serum albumin, used as standard, was dissolved in the same solution as the test sample. This procedure removes glycogen and other interfering substances present in most buffers.

Glycogen in purified enzyme samples was too low to measure using the usual methods. Therefore, an estimate of the amount of residual glycogen was made by using it as a substrate for synthase. Standard assays were performed with UDP-[^{14}C]glucose with increased specific activity and a series of known concentrations of glycogen added. After termination of the reaction carrier glycogen was added to each sample. A standard curve, constructed using the differences between radioactivity incorporated in the absence and presence of added glycogen, was linear at the low concentrations of glycogen used.

Disc gel electrophoresis was performed as described by Soderling *et al.* (1970); 5% gels were used and electrophoresis was run at room temperature because of increased synthase aggregation in the cold. Electrophoresis in sodium dodecyl sulfate was accomplished by published methods (Weber *et al.*, 1972) using 7.5 and 3.3% polyacrylamide gels. Sodium dodecyl sulfate was recrystallized from ethyl alcohol before use. Molecular weight standards were phosphorylase *b*, lactate dehydrogenase, aldolase (all from Sigma), and bovine serum albumin (Nutritional Biochemicals). To obtain high molecular weight markers aldolase subunits were cross-linked with dimethyl suberimidate (a gift of Dr. G. Kohlhaw) as described by Kohlhaw and Boatman (1971), and run on 3.3% gels.

Results

Preparation of the Liver Glycogen Fraction. The following procedure results in a good yield of synthase activity and a glycogen fraction which is essentially free of nonspecifically bound proteins. Female Wistar rats were used, weighing 250–300 g, and fed *ad libitum* on a standard laboratory diet. Animals were sacrificed by a blow to the head and the livers removed immediately and rinsed well in cold 0.25 M sucrose–10 mM Tris (pH 7.4). About 175 g of tissue was used for a single preparation and was taken between 10:00 a.m. and noon, the diurnal peak for synthase activity (McVerry and Kim, 1972a). All subsequent steps were performed at 0–4°.

The tissue was homogenized in 350 ml of Tris-phosphate buffer containing 50 mM Tris–1 mM EDTA–10 mM sodium phosphate at pH 7.4 using a Virtis apparatus at ¾ full speed for 20 sec. The homogenate was centrifuged at 11,000*g* for 15 min. The supernatant was filtered through glass wool and diluted to 500 ml with Tris-phosphate buffer. The glycogen fraction was then precipitated by adding 230 ml of 95% ethyl alcohol cooled to −70° in a Dry Ice–alcohol bath. After stirring slowly in an ice–salt bath for 15 min, the mixture was centrifuged at 2300*g* for 10 min. The pellet was resuspended in 150 ml of Tris-phosphate buffer using the Virtis homogenizer at low speed.

Approximately 20 ml of the thick suspension was then layered over 10-ml volumes of 2.1 M sucrose (in Tris-phosphate buffer) in 30 ml of cellulose nitrate tubes and centrifuged at 105,000*g* for 2.5 hr. As reported by Luck (1961), glycogen and associated protein sediment into the dense sucrose while other protein cannot enter the layer and forms a pellet at the interface. This is easily aspirated off along with the supernatant. The pinkish sucrose layers were then filtered through cheesecloth and the tubes rinsed with Tris-phosphate buffer. A small amount of glycogen which pellets was collected and resuspended by homogenization in the buffer. This was pooled together with the sucrose layers and rinsings and buffer added to give about a 200 ml volume.

The glycogen fraction was then washed once by recentrifugation at 105,000*g* for 2 hr. The well-packed, colorless glycogen pellets were rinsed, pooled and resuspended in 35 ml of glycerol buffer containing 25% (w/v) glycerol, 50 mM Tris-chloride, and 1 mM EDTA at pH 7.4. Resuspension was accomplished with a Teflon–glass homogenizer operated at low speed to avoid excessive shearing of glycogen particles. At this stage, the enzyme could be stored frozen (−15°) for extended

TABLE I: Purification of Liver Glycogen Synthase.[a]

Fraction	Vol (ml)	Protein (mg/ml)	Total Activity (units)	Specific Activity (units/mg)	Recovery (%)
1. Liver extract	500	22	180	0.016	100
2. 30% alcohol precipitate	250	35	192	0.022	106
3. Glycogen-enzyme pellet	38	0.54	79	3.8	44
4. 30% ammonium sulfate precipitate	19	0.42	51	6.4	28
5. Final washed enzyme	19	0.07	30	22	17

[a] Glycogen synthase was prepared as described in the text. At each major step in the purification a portion was assayed under standard assay conditions. When assaying fractions 1 and 2 the samples were first desalted on small Sephadex G-50 columns equilibrated with the glycerol buffer described in the text.

periods of time with little loss of total (glucose-6-P dependent) activity. However, synthase I appeared to be unstable and rapidly converted to a form dependent on glucose-6-P. It should also be noted that accurate determination of enzyme activity at this stage required less than 0.002 ml of sample because of the presence in the preparation of an inhibitor of the reaction.

Isolation of Synthase from the Glycogen Fraction. In the presence of glucose-6-P, glycogen synthase can be reversibly inactivated by a number of thiol reagents (Ernest and Kim, 1973). One result of the thiol inactivation is that the enzyme no longer binds to glycogen and thus can be removed from it by centrifugation (Ernest and Kim, 1974). Early attempts to further purify glycogen free synthase after reactivation with reduced sulfhydryl reagent were frustrated by its tendency to irreversibly form large precipitable aggregates, especially in the cold. If glycogen was added back to the enzyme it did not aggregate in the cold and purification could be accomplished using adsorption to calcium phosphate gel and DEAE-cellulose chromatography in the presence of the polysaccharide.[2] Upon searching for a stabilizing agent we found that in the presence of high concentrations of maltose, cold precipitation of the glycogen free enzyme was completely reversible upon warming and resulted in no loss of enzymatic activity. This provided a rapid method of separation from extraneous proteins.

The following procedure has been found to yield an apparently homogeneous preparation of synthase with minimal formation of irreversibly aggregated protein. For every 10 ml of the above glycogen fraction, 2.5 ml of 0.1 M glucose-6-P was added and the pH was adjusted to 8.5 with 1M Tris after warming to room temperature. Then 0.14 ml of 4.0 mM *p*-hydroxymercuribenzoate was added slowly with stirring. This amount was found to be sufficient to release the glycogen-bound enzyme and dissociate synthase into inactive subunits (Ernest and Kim, 1974) which were found not to be subject to cold induced precipitation. While stirring continued for 10 min, 1.2 g of solid maltose hydrate was dissolved into the preparation. This was found to increase the yield of active enzyme in later steps. The inactivated, released enzyme was then separated from glycogen by centrifugation at 198,000*g* for 90 min and 0°.

The supernatant was carefully transferred to a beaker and warmed to room temperature before reactivating the enzyme by adding 0.01 ml of 2-mercaptoethanol and incubating at 37° for 20 min. The enzyme was then concentrated by dissolving in 3.6 g of solid ammonium sulfate at room temperature and letting stand on ice for 60 min followed by centrifugation at 11,000*g* for 20 min. The precipitate from this step was readily soluble in 2 ml of warm (37°) 1.0 M maltose buffer, but not in cold buffer. Maltose buffer was prepared by dissolving sufficient maltose in 50 mM Tris–1 mM EDTA (pH 7.4) and filtering after decoloring the somewhat yellow solution with 0.5 g of activated charcoal/100 ml. Cold induced aggregation of the enzyme occurred upon cooling of the synthase–maltose solution. After standing overnight at 4° a flocculent, white precipitate appeared which was predominately glycogen synthase. The enzyme was collected after the addition of ½ volume of 50 mM glucose-6-P (which increased recovery), cooling at −5° in an ice–salt bath for 15 min, and centrifuging at 11,000*g* for 20 min. The pellet was dissolved in warm 1.0 M maltose buffer and then washed by adding the glucose-6-P, cooling, and centrifuging. The final pellet was dissolved in a small volume of warm 1.0 M maltose buffer. To help stabilize the activity, concentrated ammonium sulfate and dithiothreitol were added to give 5% (w/v) and 1 mM, respectively. This preparation was stable for about a week at 4° and for at least 3 months at −15°. Final yields varied from 10 to 20% of the activity in the tissue homogenate and the 1400-fold purified enzyme had a specific activity of 22 units/mg under standard assay conditions (see Table I). The amount of glycogen retained in the final preparation was estimated to be about 0.07 mg/mg of protein. Reaction in the absence of added glycogen was only 2% of that at saturating concentrations.

Gel Electrophoresis. Glycogen synthase, purified by the above method, is homogeneous by the criterion of polyacrylamide gel electrophoresis (Figure 1). A single protein staining band appears on the gels. A small amount of still aggregated protein did not enter the gel. However, when electrophoresis was performed in the presence of sodium dodecyl sulfate, a slow moving minor component was observed. Because this component is present with enzyme prepared by both the maltose and a calcium phosphate gel/DEAE-cellulose method (not shown) and migrates on 3.3% gels with a molecular weight exactly double that of the major band, we believe that it may represent synthase dimers resistant to denaturation. This same phenomenon has been shown to occur with purified liver acetyl CoA carboxylase by Inoue and Lowenstein (1972).

Subunit Molecular Weight. The electrophoretic mobility of synthase subunit in 0.1% sodium dodecyl sulfate was compared with standard proteins coelectrophoresed on separate gels. The subunit size was determined from linear plots of the distances of migration relative to the tracking dye *vs.* the logarithm of the molecular weights of the standards on 7.5 and 3.3% gels. Aldolase subunits, cross-linked with dimethyl suberimidate,

[2] Unpublished results.

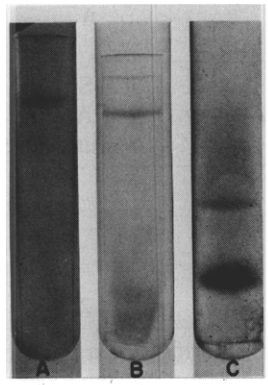


FIGURE 1: Polyacrylamide gel electrophoresis of purified glycogen synthase: (A) 10 μg of protein were electrophoresed at room temperature in 0.035 M asparagine-Tris, 0.005 M cysteine, and 0.005 M glucose-6-P at pH 7.4 using 5% gels; (B) 18 μg and (C) 21 μg of protein were electrophoresed in 0.1% sodium dodecyl sulfate and 0.1% 2-mercaptoethanol on 7.5 and 3.3% gels, respectively. Gels were cut at the position of the tracking dye. Procedures are described under Methods.

provided high molecular weight standards. The molecular weight of the hepatic synthase subunits is estimated to be 77,000–80,000.

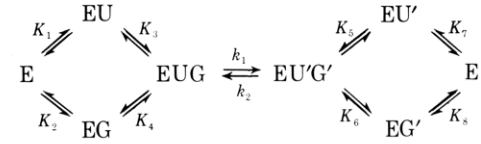
Kinetic Analysis of the Synthase Reaction. For all kinetic experiments, maltose and ammonium sulfate were removed from purified synthase by gel filtration on a 1 × 5 cm column of Sephadex G-50 (coarse) equilibrated with 25% glycerol, 50 mM Tris, and 1 mM EDTA at pH 7.4. The desalted enzyme was immediately made 1 mM in dithiothreitol and, depending on the experiment, glucose-6-P or glycogen added to stabilize the enzyme. All work was done at room temperature. Kinetic plots were drawn visually since only qualitative interpretations were required.

Glycogen is both a substrate and product of the synthase reaction. Rate equations for reactions in which there is a functional equivalence between one substrate and product have been worked out by Chao *et al.* (1969). Depending on the mechanism, predicted substrate kinetics depart strikingly from usual four-component systems and should be observed for all homopolysaccharide synthetases and phosphorylases. For glycogen synthase in particular, for ping-pong bi-bi mechanism or ordered bi-bi mechanisms in which UDPglucose binds first glycogen should produce a substrate inhibition effect. Furthermore, the equation for the ping-pong bi-bi predicts the slopes of reciprocal plots of reaction rate *vs.* UDPglucose concentration should increase with increasing concentrations of glycogen. For other basic mechanisms the equations are kinetically indistinguishable from those of usual four-component reactions.

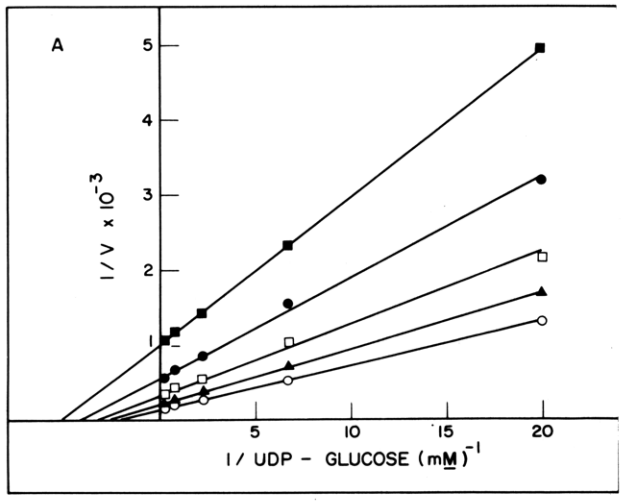
Double reciprocal plots of glycogen synthase activity *vs.* substrate concentrations are shown in Figure 2. With UDPglucose as variable substrate a set of linear plots intersecting at a point in the third quadrant is obtained with slopes decreasing with increasing amounts of glycogen. With glycogen as variable substrate, the plots are nonlinear and indicate an apparent negative cooperativity effect with respect to polysaccharide. The possible explanations for this effect are discussed below.

These data could be linearized by plotting $1/v$ against $1/(\text{glycogen})^n$ where n (=0.55) is the Hill coefficient determined from a Hill plot of the data in Figure 2B. From the crossover point of the linear plots a dissociation constant of 0.03 mg/ml was estimated. When synthase was assayed at glycogen concentrations up to 1000 times this value no substrate inhibition was observed (not shown). It can be concluded, then, that substrate kinetics do not support a ping-pong bi-bi or UDPglucose first-ordered bi-bi mechanisms for the glycogen synthase reaction.

UDP Inhibition. Product inhibition patterns can distinguish between certain sequential mechanisms (Cleland, 1963b). Glycogen cannot experimentally be used as a product inhibitor. The possibility that product inhibition patterns with UDP for the synthase system differed from the usual four-component systems was tested by constructing rate equations to predict the types of inhibition expected. Interestingly, expected patterns are the same for all but the random bi-bi mechanisms, in which all product inhibitions are normally competitive. For the synthetase reaction the mechanism can be described as



where E, U, U′, G, and *K's* are, respectively, enzyme, UDPglucose, UDP, glycogen, and dissociation constants. G′ represents glycogen binding as a product, and k_1 and k_2 are first-



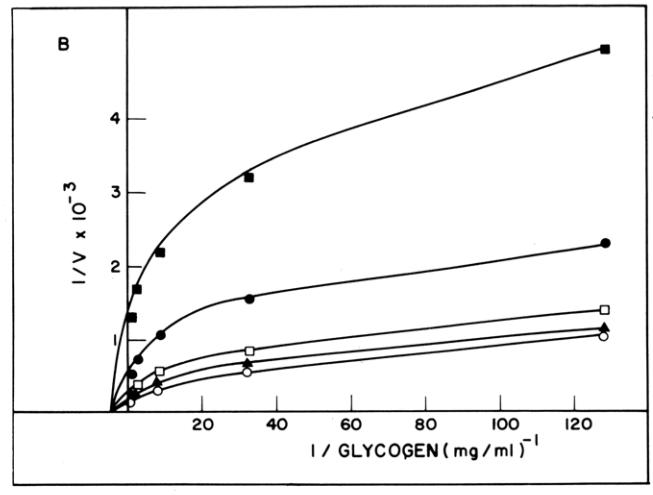


FIGURE 2: Analysis of substrate kinetics of glycogen synthase: (A, left) double reciprocal plot at variable UDPglucose concentrations and 0.0078 (■), 0.031 (●), 0.125 (□), 0.50 (▲), and 2.0 (○) mg/ml of glycogen. (B, right) double reciprocal plot at variable glycogen concentrations and 0.05 (■), 0.15 (●), 0.45 (□), 1.35 (▲), and 4.0 (0) mM UDPglucose. Initial velocity is expressed as μmoles of glucose incorporated per minute. The procedure is described in the text.

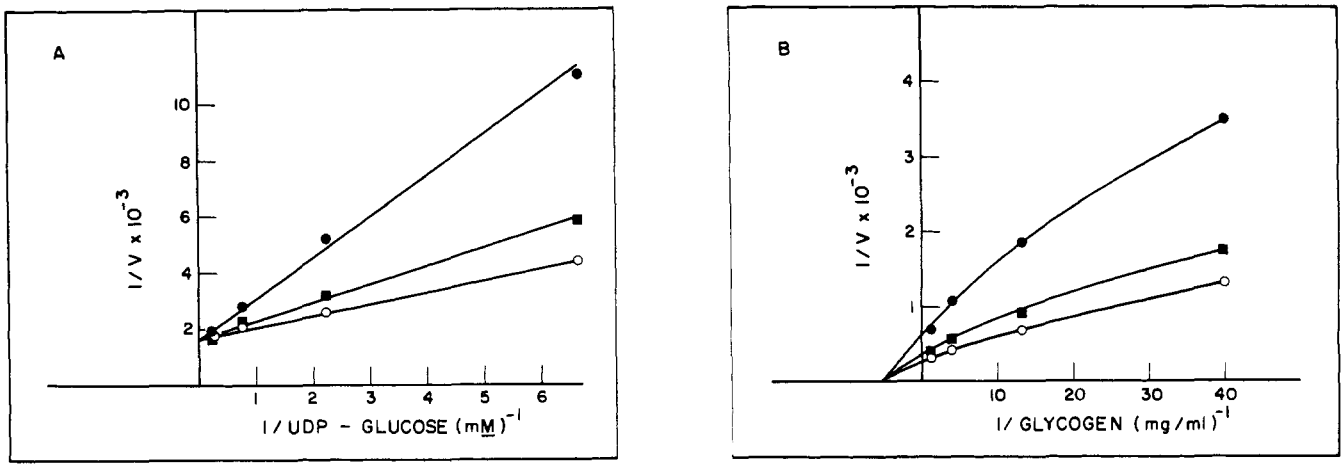


FIGURE 3: Kinetics of UDP inhibition of glycogen synthase: (A, left) double reciprocal plot at 4.0 mg/ml of glycogen, variable UDPglucose concentrations and 0 (○), 0.1 (■), and 0.5 (●) mM UDP; (B, right) double reciprocal plot at 0.5 mM UDPglucose, variable glycogen concentrations, and 0 (○), 0.2 (■), and 0.75 (●) mM UDP. The procedure is described in the text.

order rate constants. The reciprocal form of the rate equation as derived from the complete rate equation of Cleland (1963a), in terms of initial velocity, v, total enzyme, E_t, and reactant concentrations is

$$E_t/v[[U] - [U']k_2/k_1] = 1/[G][K_2K_3/k_1 + K_4[U]/k_1 + K_6[U']/k_1] + [K_3 + K_5]/k_1 + [U]/k_1 + [U']/k_1$$

This expression predicts that the intercept and slope of a plot of v^{-1} *vs.* $[G]^{-1}$ will be functions of the concentration of UDP which thus would appear to inhibit noncompetitively. On the other hand, UDP would still inhibit competitively with respect to UDPglucose.

As shown in Figure 3A and B, UDP inhibitions are consistent with the above random sequential mechanism. Excluded by these results are a glycogen first-ordered sequential or ping-pong mechanisms for which UDP would inhibit noncompetitively against UDPglucose. When the data in Figure 3B are linearized as described above the same qualitative results are obtained (not shown).

UDPglucose, UDP Exchange. If the proposed mechanism is correct no exchange of label between [^{3}H]UDP and UDPglucose should occur in the absence of glycogen. To test this we prepared an enzyme sample free of glycogen. Briefly, 6 ml of liver glycogen fraction was inactivated as usual but in the absence of maltose. The 198,000g supernatant was treated with 10 units of pancreatic α-amylase for 20 min at 37° and pH 7.0 (adjusted with 1 M acetic acid). Amylase was removed on a Sephadex G-75 column equilibrated with 25% glycerol, 50 mM Tris, 1 mM EDTA, and 5 mM glucose-6-P at pH 7.4. Maltose and 2-mercaptoethanol were added to reactivate glycogen synthase. Immediately before the exchange experiment maltose was removed on Sephadex G-50 as usual. Although the yield of activity from this procedure is poor, no glycogen could be detected in the final enzyme preparation. The conditions for the exchange experiment and its results are shown in Table II. UDP and UDPglucose were separated by the paper chromatographic method of Palladini and LeLoir (1952). There was no significant incorporation of label into UDPglucose (entry 1) compared to the control (entry 3) in which exchange was not possible. Some exchange was observed in the presence of glycogen but is low reflecting the unfavorable reverse reaction. Therefore, no stable glucosyl-enzyme intermediate exists in the absence of the polysaccharide as predicted by the proposed sequential mechanism.

Inhibition by 1,5-Gluconolactone. The potent inhibition of glycosidases (Leaback, 1968) and polysaccharide phosphorylases (Tu *et al.*, 1971) by 1,5-gluconolactones has been suggested to be due to the high degree of similarity between the inhibitors and the glycosyl moiety in the transition state of the reaction. The lactone exists in a half-chair conformation resembling the oxonium ion intermediate originally proposed by Mayer and Larner (1959) for amylase catalysis and which may be a common transition state for all glycosyl transferase reactions involving the C-1 carbon. Thus it is of interest that 1,5-gluconolactone also inhibits the glycogen synthase reaction quite effectively (Figure 4). This suggests that at some step in the reaction sequence the transferred glucosyl group exists in a conformation with significant planar character about this C-1 carbon. Abstraction of the UDP moiety seems the easiest pathway to such an intermediate, especially since attack by the C-4 oxygens of glycogen on the same side of the glucosyl ring would explain retention of its α configuration. Since the intermediate

TABLE II: The Absence of the UDPglucose, UDP Half-Reaction with Glycogen Synthase.[a]

Conditions	% of Activity as UDPglucose
1. Minus glycogen	0.04
2. Plus 0.4 mg/ml glycogen	0.18
3. Minus glycogen, minus UDPglucose	0.02

[a] A glycogen-free preparation described in the text was incubated with 2 mM UDPglucose and 2 mM [^{3}H]UDP (2.5×10^5 cpm/μmol). Reactions took place at 37° for 30 min in 0.5 ml with 20 mM glucose-6-P, 1 mM dithiothreitol, 10% glycerol, and 50 mM glycine (pH 7.4). Under these conditions, but with 2 mM UDP-[^{14}C]glucose, 0.4 mg/ml of glycogen, and no UDP, 12% of labeled glucose was incorporated into glycogen. No incorporation was detected in the absence of added glycogen. The amount of radioactivity found in UDP-glucose is given as per cent of total activity of UDP plus UDPglucose eluted from the paper chromatogram.

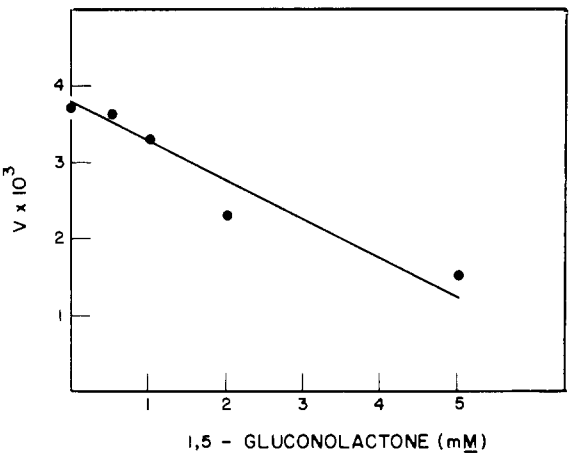


FIGURE 4: Inhibition of synthase activity by 1,5-gluconolactone. Reactions were run as described under Methods with 0.4 mg/ml of glycogen and 0.5 mM UDPglucose.

cannot be detected kinetically, however, this picture seems somewhat oversimplified.

Discussion

The procedure reported here has yielded a glycogen synthase preparation from rat liver which is purified 1400-fold over a crude tissue extract. The purified enzyme, which appeared homogeneous on polyacrylamide gel electrophoresis, has a specific activity of 22 units/mg of protein at 37° and pH 7.4 in the presence of saturating glucose-6-P. The procedure is a rapid and unique method, involving no column chromatographic steps, and is based on the insolubility of the glycogen-free enzyme in the cold.

Glycogen synthase was solubilized from a partially purified liver glycogen fraction by *p*-hydroxymercuribenzoate inactivation of the enzyme which is accompanied by the loss of glucose-6-P dependent binding to glycogen. Steiner *et al.* (1965) solubilized synthase by reversible heat inactivation of the enzyme but the relationship between the two methods is not clear. Recently, Lin and Segal (1973) reported the release of synthase by digestion of the glycogen upon extended incubation in the presence of endogenous glycolytic enzymes. Further purification was then achieved using fractional adsorption to calcium phosphate gel. It appears that the method reported here would be more rapid and easier for preparing larger quantities of synthase. As reported by others (Brown and Larner, 1971; Issa and Mendicino, 1973; Lin and Segal 1973) glycogen-free synthase tends to precipitate in the cold; the yeast enzyme is also cold labile but does not aggregate (Huang and Cabib, 1973). We found that this aggregation could be made reversible in the presence of high concentrations of maltose, thus providing a means to separate the enzyme from more soluble proteins. While maltose presumably replaces glycogen in stabilizing the enzyme activity the mechanism of cold precipitation is unknown. A reversible cold inactivation and aggregation of glycogen phosphorylase *b* has been reported by Graves *et al.* (1965), who suggested hydrophobic interactions might be an important factor. It is thus of interest that both synthase and phosphorylase are tightly bound to hydrocarbons covalently linked to Sepharose (Shaltiel and Er-el, 1973; Er-el *et al.*, 1972). There have also been reports of insoluble, stoichiometric complexes between small amounts of glycogen and proteins such as phosphorylase, α-amylase, and phosphorylase *b* kinase (Selinger and Schramm, 1963; DeLange *et al.*, 1968). The fact that purified synthase contains trace amounts of residual glycogen may indicate a common phenomenon.

The subunit unit molecular weight of liver glycogen synthase was estimated to be 77,000–80,000 by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. This is smaller than the 92,500 subunit reported for muscle or kidney synthase but consistent with the estimate of 85,000 made by Lin and Segal (1973) for the heptatic enzyme. Recently, Rosenkranz and Larner (1973) have shown that muscle and liver synthases yield nonidentical chymotryptic peptides and others have suggested that the two are isozymes (Sato *et al.*, 1972). Therefore differences in subunit molecular size are not surprising. Sanada and Segal (1971) have determined the molecular weight of native liver synthase D to be 258,000–284,000 in the presence of glucose-6-P. We have found similar values for both I and D forms (310,000) in the presence of glucose-6-P (unpublished results) while in the absence of activator synthase D dissociates and I aggregates (McVerry and Kim, 1972b). Therefore, the active form of the enzyme appears to be a tetramer, although the influence of glycogen on its quaternary structure has not yet been studied.

The substrate kinetics and product inhibition patterns of the synthase reaction are consistent with a reaction mechanism involving the formation of a ternary complex of substrates and enzyme with no obligatory order of reactant binding. Recently, Brown and Larner (1971) reported the substrate kinetics of muscle synthase D. Double reciprocal plots indicated ping-pong kinetics where a stable enzyme–substrate intermediate exists during the reaction sequence. The mechanism we propose does not exclude the possibility of a glycosyl-enzyme intermediate, but predicts that, if it exists, its product, UDP, is retained in the active site throughout the reaction sequence. If UDP dissociated prior to the transfer of a glucosyl moiety to glycogen, UDP inhibition should not be competitive with respect to UDPglucose (Cleland, 1963b), as we have shown. Also an exchange between UDP and UDPglucose should be observable in the absence of glycogen. Kornfeld and Brown (1962) and Steiner *et al.* (1965) reported the lack of this exchange except in the presence of added glycogen. These early synthase preparations contained significant amounts of glycogen which might have influenced the degree of any exchange. Therefore, we prepared a special amylase-treated enzyme to test for the half-reaction in the absence of glycogen. No significant exchange was observed, indicating a free glucosyl-enzyme complex does not exist. This is in accordance with our conclusion from kinetic analyses. The absence of exchange could still be explained if glycogen acts synergistically to form or stabilize a glucosyl-enzyme intermediate. This can only be tested with a glycogen-like molecule that cannot serve as a glucosyl acceptor. Obviously more information is required before the exact nature of the transition state can be ascertained.

Although there are a number of possibilities, none of which seriously affects our kinetic analysis, the explanation for the nonlinear kinetics exhibited with glycogen as substrate remains to be established. Of considerable interest is the possibility that the apparent negative cooperativity may be physiologically significant and result from interactions between enzyme subunits. As mentioned by Cleland (1970) this type of regulation would allow an enzyme to always operate in a region of proportional control, *i.e.*, never saturated with substrate under normal conditions. This may be particularly important for liver synthase since at times large amounts of glycogen must be stored in this tissue. Another speculation is that, under the conditions of the assay, glycogen molecules might bind to the enzyme in such a way that catalysis cannot proceed, and compete with glycogen

binding in the correct orientation. Glycogen would then appear to be an inhibitor present in constant proportion to the variable substrate, which, as shown by Cleland *et al.* (1973), results in the observed curvature in certain cases.

In view of the large class of glycosyl transferases utilizing nucleotide diphosphate sugars it will be of interest to determine if they share a common catalytic mechanism. Furthermore, allosteric regulation of glycogen synthetase activity should ultimately be explainable as induced alterations of certain steps in its mechanism. In fact, Steiner *et al.* (1965) postulated that glucose-6-P might activate synthase by releasing the enzyme from product inhibition by UDP. It will be of interest especially to determine if steps in the reaction are affected by the state of phosphorylation of the enzyme. A chemical interaction between the phorphorylated site and reaction mechanism has not yet been shown for any enzyme subject to this type of control.

References

Blatt, L. N., and Kim, K. H. (1971), *J. Biol. Chem. 246,* 7256–7264.

Brown, N. E., and Larner, J. (1971), *Biochim. Biophys. Acta 242,* 69–80.

Chao, J., Johnson, G. F., and Graves, D. J. (1969), *Biochemistry 8,* 1459–1466.

Cleland, W. W. (1963a), *Biochim. Biophys. Acta 67,* 104–137.

Cleland, W. W. (1963b), *Biochim. Biophys. Acta 67,* 188–196.

Cleland, W. W. (1970), *Enzymes, 3rd Ed. 1,* 1–65.

Cleland, W. W., Gross, M., and Folk, J. E. (1973), *J. Biol. Chem. 248,* 6541–6542.

DeLange, R. L., Kemp, R. G., Riley, W. D., Cooper, R. A., and Krebs, E. G. (1968), *J. Biol Chem. 243,* 2200–2208.

Er-el, A., Zaidenzaig, Y., and Shaltiel, S. (1972), *Biochem. Biophys. Res. Commun. 49,* 383–390.

Ernest, M. J., and Kim, K. H. (1973), *J. Biol. Chem. 248,* 1550–1555.

Ernest, M. J., and Kim, K. H. (1974), *J. Biol Chem.* (in press).

Gold, A. H. (1970), *Biochemistry 9,* 946–952.

Graves, D. J., Sealock, R. W., and Wang, J. H. (1965), *Biochemistry 4,* 290–296.

Huang, K. P., and Cabib, E. (1973), *Biochim. Biophys. Acta 302,* 240–248.

Inoue, H., and Lowenstein, J. M. (1972), *J. Biol. Chem. 247,* 4825–4832.

Issa, H. A., and Mendicino, J. (1973), *J. Biol. Chem. 248,* 685–696.

Kato, K., and Bishop, J. S. (1972), *J. Biol. Chem. 247,* 7420–7429.

Kohlhaw, G., and Boatman, G. (1971), *Biochem. Biophys. Res. Commun. 43,* 741–746.

Kornfeld, R., and Brown, D. H. (1962), *J. Biol. Chem. 237,* 1772–1777.

Larner, J., and Villar-Palasi, C. (1971), *Curr. Top. Cell. Regul. 3,* 195–232.

Leaback, D. H. (1968), *Biochem. Biophys. Res. Commun., 32,* 1025–1030.

Lin, D. C., and Segal, H. L. (1973), *J. Biol. Chem. 248,* 7007–7011.

Lowry, O. H., Rosenburg, N. J., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265–275.

Luck, D. J. (1961), *J. Biophys. Biochem. Cytol. 10,* 195–209.

Magner, L. N., and Kim, K. H. (1973), *J. Biol. Chem. 248,* 2790–2795.

Mayer, F. C., and Larner, J. (1959), *J. Amer. Chem. Soc. 81,* 188–193.

McVerry, P. H., and Kim, K. H. (1972a), *Biochem. Biophys. Res. Commun. 46,* 1242–1246.

McVerry, P. H., and Kim, K. H. (1972b), *Biochem. Biophys. Res. Commun. 48,* 1636–1640.

Mersmann, H. J., and Segal, H. L. (1967), *Proc. Nat. Acad. Sci. U. S. 58,* 1688–1695.

Palladini, A. C., and LeLoir, L. (1952), *Biochem, J. 51,* 427–430.

Rosenkranz, A., and Larner, J. (1973), *Biochim. Biophys. Acta 315,* 317–325.

Sanada, Y., and Segal, H. L. (1971), *Biochem. Biophys. Res. Commun. 45,* 1159–1168.

Sato, K., Abe, N., and Tsuiki, S. (1972), *Biochim. Biophys. Acta 268,* 646–653.

Schlender, K. K., and Larner, J. (1973), *Biochim. Biophys. Acta 293,* 73–83.

Selinger, Z., and Schramm, M. (1963), *Biochem. Biophys. Res. Commun. 12,* 208–214.

Shaltiel, S., and Er-el, Z. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 778–781.

Smith, C. H., Brown, N. E., and Larner, J. (1971), *Biochim. Biophys. Acta 242,* 81–88.

Soderling, T. R., Hickenbottom, J. P., Reimann, E. M., Hunkeler, F. L., Walsh, D. A., and Krebs, E. G. (1970), *J. Biol. Chem. 245,* 6317–6328.

Steiner, D. F., Younger, L., and King, J. (1965), *Biochemistry 4,* 740–750.

Tu, J. I., Jacobson, G. R., and Graves, D. J. (1971), *Biochemistry 10,* 1229–1236.

Weber, K., Pringle, J. R., and Osborn, M. (1972), *Methods Enzymol. 26,* 3–27.