Purification and Properties of Rabbit-Liver Glycogen Synthase†

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ABSTRACT: Glycogen synthase *b* was purified from rabbit liver by a procedure involving isolation of the glycogen–enzyme complex, DEAE-cellulose chromatography, and affinity chromatography. The purified enzyme had a specific activity of 25 μmol of glucose transferred from UDPglucose into glycogen per min per mg of protein at 30 °C in the presence of 10 mM glucose 6-P, and appeared to be homogeneous by the criterion of polyacrylamide disc gel electrophoresis. The *b* form was convertible into the *a* form by a rabbit-liver protein phosphatase. A subunit size of 85 000 was determined by electrophoresis in sodium dodecyl sulfate and molecular weights of 183 000 ± 20 000 and 170 000 ± 21 000 were determined for the *a* and *b* forms of the enzyme, respectively. On conversion of the *a* into the *b* form, 1.13 mol of phosphate was incorporated per 85 000 g of protein. The degree of phosphorylation and loss of glycogen synthase *a* activity paralleled each other.

Glycogen synthase (UDPglucose[1]: glycogen 4-α-glucosyltransferase, EC 2.4.1.11), the rate limiting enzyme of glycogen synthesis, was first described by Leloir and Cardini (1957). In most tissues the enzyme is found in two forms, a phosphorylated, physiologically inactive form called glycogen synthase *b* (or D) which requires glucose 6-P for activity, and a dephosphorylated, active form called glycogen synthase *a* (or I) which is active in the absence as well as the presence of glucose 6-P. The two forms are interconverted by phosphorylation and dephosphorylation reactions which are catalyzed by cAMP-dependent protein kinase and a phosphatase, respectively (Larner and Villar-Palasi, 1971). On the other hand, the other two key enzymes of glycogen metabolism, phosphorylase (1,4-α-D-glucan:orthophosphate α-glucosyltransferase, EC 2.4.1.1) and phosphorylase kinase (ATP:phosphorylase phosphotransferase, EC 2.7.1.37), each of which also exists in two interconvertible forms, are activated by phosphorylation (Krebs and Fischer, 1956; DeLange et al., 1968) and inactivated by dephosphorylation reactions (Wosilait and Sutherland, 1956; Riley et al., 1968).

While the molecular basis for the interconversion and regulation of these enzymes, particularly the phosphorylation process, has been intensively studied in muscle, relatively little is known about the regulation of the corresponding enzymes of liver glycogen metabolism at the molecular level. We have begun such a study and this paper describes a procedure for the isolation of rabbit-liver glycogen synthase *b* to homogeneity, plus some characteristics of the enzyme. When we began this study, no procedure for the purification of liver glycogen synthase to homogeneity had been described. Since then the trout- and rat-liver enzymes have been isolated (Lin et al., 1972; Lin and Segal, 1973; McVerry and Kim, 1974). Both the *a* and *b* forms of rabbit muscle glycogen synthase have also been prepared (Soderling et al., 1970; Smith et al., 1971; Brown and Larner, 1971), and glycogen synthase has been isolated from pig kidney (Issa and Mendicino, 1973) and pig adipose tissue (Miller et al., 1975). A comparison with these other glycogen synthases is made.

Experimental Section

Materials. Chemicals and biochemicals were obtained as follows: UDPglucose, glucose 6-P, glucose 1-P, cAMP, ATP, cytidine 5′-diphosphate, succinic anhydride, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, β-glycerophosphate, imidazole (Grade I), bovine serum albumin, ovalbumin, beef-liver catalase, α-amylase (type I-A, DFP treated), oyster glycogen (type II), and rabbit-liver glycogen (type III) from Sigma Chemical Co.; amylose [degree of polymerization (DP) ca. 260] from Nutritional Biochemicals Corporation; cyanogen bromide and 1,6-diaminohexane from Eastman, DE-52 from Reeve Angel; Sepharose 4B and Sephadex G-50 (fine) from Pharmacia Fine Chemicals; picrylsulfonic acid from Aldrich Chemical Co., Inc.; UDP-[U-^{14}C]glucose from New England Nuclear; [2-^{14}C]cytidine 5′-diphosphate from Schwarz BioResearch; [γ-^{32}P]ATP from Amersham/Searle. Imidazole was recrystallized from acetone–toluene. Rabbit-liver glycogen solutions were treated with Bio-Rad mixed-bed resin AG 501-X8. Shellfish glycogen was purified by trichloroacetic acid and ethanol precipitation prior to treatment with the mixed-bed resin AG 501-X8. R-Phycoerythrin and R-phycocyanin were isolated from *Rhodymenia palmata* and *Porphyra laciniata* respectively, as by O'Carra (1965). Homogeneous rabbit-liver phosphorylase phosphatase (Brandt et al., 1975) was a gift from Dr. E. Y. C. Lee. Rabbit-liver phosphorylase *a* was isolated as described below and, when required, was converted into the *b* form with rabbit-liver phosphorylase phosphatase (Brandt et al., 1975). Rabbit-skeletal muscle cAMP-dependent protein kinase (peak 2 fraction) purified up to the DEAE-cellulose chromatography step by a modification of the procedure of Miyamoto et al. (1969) was a gift from Dr. R.-J. Ho. With type II-A histone as substrate, this kinase preparation catalyzed the incorporation of 15 pmol of P_i per min per μg of protein at pH 7.0 and 30 °C.

Enzyme Assays. Glycogen synthase was assayed as by Thomas et al. (1968). The enzyme was diluted to the desired activity in 50 mM Tris-HCl, 5 mM EDTA, 2 mM dithiothreitol, 100 mM NaF, 0.1% bovine serum albumin, and 0.1% rabbit-liver glycogen, pH 7.8. Reactions were

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[1] Abbreviations used are: UDPglucose, uridine 5′-diphosphate glucose; glucose 6-P, glucose 6-phosphate; glucose 1-P, α-glucose 1-phosphate; cAMP, cyclic adenosine 3′,5′-phosphate.

started by adding 25 μl of the diluted enzyme to 50 μl of substrate solution containing 7.5 mM UDP-[^{14}C]glucose (40 000 cpm/μmol), 15 mM glucose 6-P, 15 mg/ml of rabbit-liver glycogen, 50 mM Tris-HCl, and 5 mM EDTA, pH 7.8. When assaying for glycogen synthase *a*, the glucose 6-P was replaced by 15 mM Na_2SO_4 (Thomas et al., 1973). After incubation for 10 min at 30 °C, 50-μl samples were spotted on to Whatman 31 ET paper discs, washed with 66% ethanol, dried, and counted (Thomas et al., 1968). One unit of activity is the amount of enzyme which catalyzes the incorporation of 1 μmol of glucose from UDPglucose into glycogen per min under the above conditions.

Phosphorylase was assayed as by Hedrick and Fischer (1965). Liver phosphorylase *b* was also assayed by this method, but Na_2SO_4 (0.7 M) was included in the assay mixture (Appleman et al., 1966). One unit of activity is the amount of enzyme which releases 1 μmol of P_i/min from glucose 1-P.

Electrophoresis. Polyacrylamide disc gel electrophoresis under nondenaturing conditions was carried out as by Schiefer et al. (1973) with the exceptions that sucrose was omitted from the buffers, and 1 mM glucose 6-P was included in the upper reservoir buffer (Soderling et al., 1970). Protein bands were detected by staining with Coomassie blue (Chrombach et al., 1967). Glycogen synthase activity was detected by two methods.

Method A. The gels were incubated for 16 h at 25 °C, in 25 mM sodium citrate, 25 mM Tris, 5 mM EDTA, 2 mM dithiothreitol, 2 mM UDPglucose, 5 mM glucose 6-P, and 0.01% amylose primer, pH 7.8. The gels were then rinsed with distilled water and placed in an iodine solution (0.2% iodine, 2% potassium iodide, 0.01 N HCl) for 2 h. The polysaccharide synthesized by the glycogen synthase was detected as a blue band.

Method B. The gels were sliced into 2-mm segments, each of which was incubated with the standard glycogen synthase *b* assay mixture at 30 °C for 20 min. Fifty-microliter samples were then spotted onto filter papers, washed, and counted.

Polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate was carried out as by Dunker and Rueckert (1969) using 5% polyacrylamide gels. The reference polypeptides for determination of subunit molecular weight were bovine serum albumin dimer and monomer, rabbit-muscle phosphorylase *a* and ovalbumin. Bovine serum albumin monomer was the internal standard.

Sucrose Density Gradient Centrifugation. Sucrose density gradient centrifugation was carried out as by Martin and Ames (1961). Gradients (5–20% sucrose) were run in a SW 50L rotor in a Spinco Model L centrifuge (39 000 rpm for 16 h at 4 °C). The gradients were prepared in 50 mM imidazole chloride, 5 mM EDTA, and 2 mM dithiothreitol, pH 7.4, in the presence and absence of 5 mM glucose 6-P. Fractions (~30) of 3 drops each (about 0.17 ml) were collected from the bottom of the tubes. The sedimentation coefficients of the protein markers, liver phosphorylase (*a* and *b* forms), catalase, R-phycocyanin, and R-phycoerythrin were taken to be 8.4S, 11.3S, 6.2S, and 12S respectively, and the molecular weights were taken to be 185 000, 250 000, 138 000, and 290 000, respectively (Wolf et al., 1970; Tanford and Lovrien, 1962; Ericksson-Quensel, 1938).

Preparation of the Affinity Support. Cytidine 5′-diphosphate–succinylaminohexyl-Sepharose-4B was prepared following the general procedure of Cuatrecasas (1970). Cyanogen bromide (12.5 g) was dissolved in 250 ml of water. Washed, packed Sepharose 4B (50 ml) was added to this solution, and the reaction was allowed to proceed for 6 min. The pH was maintained at pH 10.5–11.0 with 8 N NaOH. The activated gel was washed rapidly on a sintered glass funnel with 1 l. of ice-cold water and was immediately added to 50 ml of ice-cold water containing 2 mM of 1,6-diaminohexane per ml, and then this mixture was titrated to pH 10.0 with 6 N HCl. The mixture was stirred gently for 16 h at 4 °C. The substituted gel was then washed with 4 l. of water. The aminohexyl-Sepharose was then suspended in 50 ml of water and succinic anhydride (5 g) was added in small increments. After each addition the pH was adjusted to pH 6.5–7.0 with 20% NaOH. After the last addition the reaction mixture was stirred for a further 30 min. After this time the trinitrobenzenesulfonate test (Cuatrecasas, 1970) showed that all the amino groups had reacted, and the succinylaminohexyl-Sepharose was then washed with 4 l. of water. The succinylaminohexyl-Sepharose was then added to 50 ml of water containing 500 mg of ^{14}C-labeled cytidine 5′-diphosphate (4000 cpm/μmol) at 25 °C. To this stirred suspension was added dropwise a freshly made solution, containing 1 g of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide in 3 ml of water, over a 5-min period. The pH was maintained at pH 4.7 with 0.1 N NaOH during this time and for 1 h after that. The reaction was then allowed to continue with gentle stirring at 25 °C for 16 h. The gel was washed with 4 l. of 1 M NaCl and then with about 20 l. of water over a 2-day period. The degree of ligand substitution was determined by scintillation counting to be 0.26 μmol per ml of packed gel. When not in use the gel was washed with 10 volumes of 1 M NaCl followed by 10 volumes of water and stored at 4 °C in the presence of 0.02% sodium azide. The gel was washed free of sodium azide before use.

Purification of Glycogen Synthase b. All procedures were carried out at 0–4 °C unless stated otherwise. The standard buffer used was 50 mM β-glycerophosphate, 5 mM EDTA, and 2 mM dithiothreitol, pH 7.6. Protein determinations were carried out by the method of Lowry et al. (1951) following trichloroacetic acid precipitation. Bovine serum albumin was used as the standard.

Isolation of Glycogen Pellets. Livers from fed rabbits were homogenized in about 250-g quantities in 2.5 volumes of standard buffer containing 100 mM NaF for 30 s in a Waring Blendor at low speed. The homogenate was centrifuged at 20 000*g* for 30 min, and the supernatant was filtered through glass wool to remove lipid material. The supernatant was then centrifuged at 40 000*g* for 2 h. The supernatant and microsomal material overlaying the glycogen pellets were decanted after inverting the centrifuge tubes twice to dislodge the microsomal material. The glycogen pellets could be stored at −20 °C without loss of the glycogen synthase activity. When glycogen pellets from 2 kg of liver had been obtained by this procedure, they were thawed, combined, and resuspended in standard buffer containing 0.1% shellfish glycogen, by gentle homogenization with a Teflon–glass homogenizer, and brought to a total volume of 500 ml with the resuspending buffer. This suspension was then centrifuged at 100 000*g* for 90 min. The supernatant and the red fluffy layer of microsomal material overlaying the glycogen pellets were siphoned off and the glycogen pellets were resuspended in the standard buffer as before.

DEAE-Cellulose Chromatography. The resuspended material was centrifuged at 5000*g* for 10 min to remove particulate material which would otherwise block the DEAE-cel-

Table I: Purification of Glycogen Synthase *b* from Rabbit Liver.

Step	Vol (ml)	Total Enzyme Activity (Units)	Specific Activity (Units/mg)	Yield (%)	Purification
1. Crude extract (2 kg liver)	6000	2640	0.014	100	1
2. First glycogen pellet	500	1230	0.36	47	26
3. Second glycogen pellet	550	885	1.87	34	134
4. DEAE-cellulose	176	475	5.4	18	360
5. Affinity column	22	286	25	11	1790

lulose column. The supernatant was then applied to a DEAE-cellulose column (2.5 × 40 cm) which was equilibrated with the standard buffer. After the application of the sample, the column was washed with the equilibrating buffer containing 0.25% shellfish glycogen (about 300 ml). The column was then washed with 0.05 M NaCl in the same glycogen-containing buffer (about 1 l.) to remove residual phosphorylase and branching enzyme (not shown) activities. The glycogen synthase was then eluted by washing the column with 0.2 M NaCl in the glycogen-containing buffer. The results of typical runs are shown in Figure 1. Over 50% of the glycogen synthase activity applied to the column was recovered. When the shellfish glycogen was omitted from the eluting buffers, less than 40% of the applied activity was recovered.

The eluted glycogen synthase was dialyzed against the standard buffer for 4 h and the solution was then concentrated to about 100 ml by placing dry Sephadex G-200 outside the dialysis bag. The enzyme was further concentrated by centrifugation of the solution at 150 000*g* for 3 h. Most of the supernatant was discarded and the reddish-colored glycogen pellets containing over 90% of the glycogen synthase activity were resuspended in a final volume of 10 ml with standard buffer.

Affinity Chromatography. The glycogen-containing glycogen synthase preparation was passed through a cytidine 5′-diphosphate–succinylaminohexyl-Sepharose 4B column (1.5 × 5 cm) equilibrated with the standard buffer containing 0.1% shellfish glycogen, at 25 °C. In the presence of glycogen, the glycogen synthase is not retained by the column and washes through. This step removes protein which would otherwise contaminate the final glycogen synthase preparation. No glycogen synthase was lost by this procedure. The glycogen synthase preparation was then made 5 mM with respect to NaCl and incubated with pancreatic α-amylase (2 mg) for 2 h at 25 °C. Then the enzyme preparation was dialyzed for 16 h at 4 °C against the standard buffer containing 10% sucrose. After dialysis the enzyme solution was centrifuged at 40 000*g* for 30 min to remove an insoluble reddish precipitate. After warming to room temperature (25 °C), the enzyme solution was then applied to a second affinity column (1.5 × 12 cm) equilibrated with the dialysis buffer. After application of the sample, the column was washed with the equilibrating buffer until the absorbance at 280 nm fell to a basal level. A linear gradient consisting of 60 ml of the equilibrating buffer and 60 ml of 1 M NaCl in the same buffer was then applied to the column. The result of a typical run is shown in Figure 2. The glycogen synthase was eluted as a single peak at approximately 0.2 M NaCl. About 60% of the activity applied to the column was recovered. The active fractions were pooled, dialyzed against the standard buffer containing 10% sucrose, and then quick-frozen in small portions (0.5 ml) in a dry ice–acetone bath and stored at −70 °C.

Preparation of Glycogen Synthase a. The isolated glycogen synthase *b* was converted into the *a* form by purified rabbit-liver phosphorylase phosphatase, which has also been shown to be glycogen synthase phosphatase (Killilea et al., 1976). The conversion was carried out for 2 h at 25 °C in reaction mixtures of 320 μl containing 50 mM imidazole chloride, 1 mM EDTA, 5 mM dithiothreitol, 10% sucrose, pH 7.4, 140 μg of glycogen synthase *b*, and 0.34 μg of phosphorylase phosphatase. The activity ratio (glycogen synthase glucose 6-P independent activity:activity in presence of glucose 6-P) of the converted enzyme was 0.95. The glycogen synthase *b* had negligible activity in the absence of glucose 6-P. The activities of equivalent amounts of the *a* and *b* forms were the same in the presence of glucose 6-P.

Conversion of Glycogen Synthase a into b. The reaction mixture at pH 7.4 contained 50 mM imidazole chloride, 1 mM EDTA, 5 mM dithiothreitol, 10% sucrose, 50 mM NaF, 0.2 mM [γ-^{32}P]ATP, 2 mM magnesium acetate, 2 μM cAMP, 240 μg of glycogen synthase *a*, and 1.4 μg of rabbit-muscle cAMP-dependent protein kinase in a final volume of 1 ml. [The preparation of synthase *a* contained protein phosphatase (see above). The inclusion of 50 mM NaF inhibited the phosphatase, thereby avoiding interference with the rephosphorylation of the synthase.] The reaction was carried out at 25 °C and was initiated by the addition of the [γ-^{32}P]ATP and magnesium acetate. The rate of phosphorylation was followed by spotting 50-μl portions of the reaction mixture onto a sheet of Whatman ET 31 paper (47 × 30 cm), the origin of which was first treated with 10% trichloroacetic acid and allowed to dry immediately prior to the experiment. After all the aliquots were spotted, the chromatography sheet was irrigated by descending chromatography for 16 h with a 5% trichloroacetic acid solution (2 l.). Areas of the paper (6 cm^2) containing each of the samples were then cut out, placed in Aquasol (New England Nuclear), and counted. The rate of loss of glycogen synthase *a* activity was followed by diluting 20-μl samples 1:20 in 50 mM Tris-HCl, 5 mM EDTA, 0.1% bovine serum albumin, 0.1% glycogen, 2 mM dithiothreitol, and 100 mM NaF, pH 7.8, and assaying for glycogen synthase *a* activity.

Results

Purification of Rabbit-Liver Glycogen Synthase b. A typical overall purification scheme is shown in Table I. The isolated enzyme had a specific activity of 25 IU per mg of protein at 30 °C at pH 7.8 in the presence of 10 mM glucose 6-P. The enzyme was purified approximately 1800-fold from the crude extract with about an 11% recovery of activity. In the crude rabbit-liver extracts most of the glycogen synthase (about 80%) was in the phosphorylated, *b* form, and sodium fluoride was included in the homogenizing buffer to inhibit endogenous phosphoprotein phosphatase activity. In the next two steps advantage was taken of the affinity of the glycogen synthase for the endogenous glycogen, and the enzyme–glycogen complex was isolated by high-speed centrifugation. Phosphorylase (mainly phosphorylase *a*)

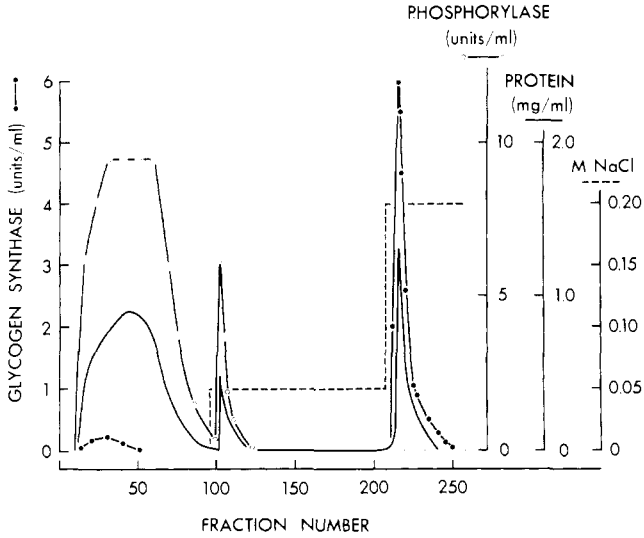

FIGURE 1: Chromatography of rabbit-liver glycogen synthase and phosphorylase on DEAE-cellulose. (●) Glycogen synthase *b* activity; (○) phosphorylase *a* activity; (—) protein concentration determined on 50-μl samples as by Lowry et al. (1951); (- - -) NaCl (M). The column size was 2.5 × 40 cm. The flow rate was 40 ml/h and 11-ml fractions were collected.

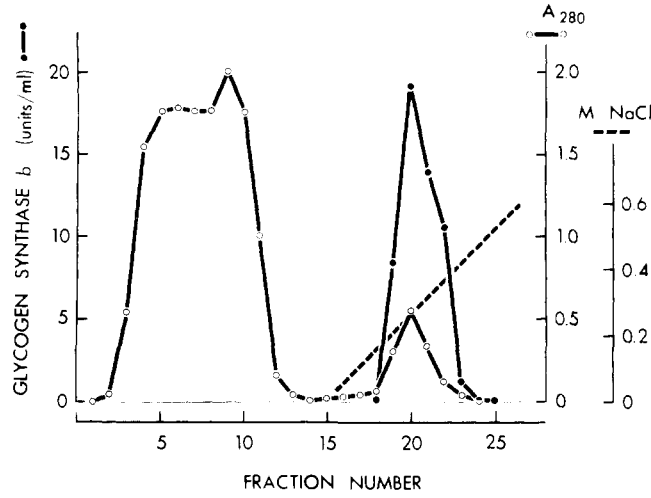

FIGURE 2: Chromatography of rabbit-liver glycogen synthase *b* on a cytidine 5′-diphosphate-succinylaminohexyl-Sepharose 4B column (1.5 × 12.0 cm) at 25 °C. (●) Glycogen synthase *b* activity; (○) A_{280}; (- - -) NaCl gradient. The flow rate was 30 ml/h and 5.5-ml fractions were collected.

also sedimented with the glycogen and was separated from the glycogen synthase by the DEAE-cellulose chromatographic step. Under the conditions employed, the phosphorylase was not retarded on this column (Figure 1) and in a separate experiment was further purified to homogeneity by the DEAE-cellulose chromatography procedure of Wolf et al. (1970). In the DEAE-cellulose step employed in the purification of glycogen synthase, glycogen was included in the eluting buffer since it was observed that the presence of glycogen not only improved the recovery of the enzyme from the column but also provided a convenient method of concentrating the enzyme by high-speed centrifugation after the chromatography. Efforts to concentrate solutions containing the glycogen synthase by ammonium sulfate precipitation or retention behind Amicon membranes were unsuccessful and resulted in inactivation of the enzyme.

The final step in the purification of the glycogen synthase involved affinity chromatography on a cytidine 5′-diphosphate-succinylaminohexyl-Sepharose-4B support. The glycogen synthase preparation was first incubated with α-amylase to digest the glycogen since the glycogen prevented retention of the synthase by the affinity support. Indeed the synthase could be eluted from the affinity column by including glycogen (0.2%) in the eluting buffer. This observation that the enzyme could be eluted by its substrate strongly indicates that a true affinity procedure was operating. However, under these conditions the glycogen synthase was eluted slowly and the final preparation was contaminated by the glycogen. Thus elution of the glycogen synthase by a salt gradient in the presence of sucrose was preferred (Figure 2).

Glycogen synthase could not be assayed in the fractions that emerged from the column prior to the application of the salt gradient. This was because of the α-amylase present in the fractions. The column was, however, tested with purified muscle glycogen synthase, prepared as by Soderling et al. (1970), and this enzyme was completely retained by the column.

Cytidine 5′-diphosphate was chosen as the ligand for the affinity support since this compound is an analogue of uridine 5′-diphosphate, a product and competitive inhibitor (Steiner et al., 1965; McVerry and Kim, 1974) of the glycogen synthase reaction. Cytidine 5′-diphosphate contains a 4-amino group in place of the 4-hydroxy group found on the pyrimidine ring of uridine 5′-diphosphate, and it was assumed that this 4-amino group would be the site of the carbodiimide-catalyzed covalent attachment of the ligand to the carboxyl group of the succinylaminohexyl-Sepharose (Cuatrecasas, 1970). However, more recent studies (Barry and O'Carra, 1973) on the carbodiimide-catalyzed attachment of NAD^+ and related compounds to succinylated Sepharose derivatives have shown that in these cases attachment takes place through the secondary hydroxyl groups of the ribose ring rather than the 6-amino group of the adenine ring. Thus the derivative prepared here might also be attached through the ribosyl moiety of the cytidine 5′-diphosphate. Indeed, evidence against covalent attachment through the 4-amino group was obtained from spectral studies. Both the free and matrix-bound cytidine 5′-diphosphate had the same absorption maximum. If the 4-amino group of the pyrimidine ring was involved in the linkage to the spacer arm, a shift in the absorption maximum would have been expected (Mosbach et al., 1972; Barry and O'Carra, 1973). Thus, using the procedure described here it should be possible to attach uridine 5′-diphosphate to the succinylaminohexyl-Sepharose and use this rather than the cytidine 5′-diphosphate derivative for the purification of glycogen synthase. Other affinity supports have been described for the purification of glycogen synthases from other tissues. Miller et al. (1975) have described the use of an affinity column having glucosamine 6-phosphate as the ligand in the purification of the enzyme from pig subcutaneous adipose tissue, and Barker et al. (1972) described the use of a UDP-hexanolamino-agarose support for the extensive purification of rabbit-muscle glycogen synthase. In the latter study the spacer arm–ligand complex was first synthesized and purified before coupling to the Sepharose 4B support (Barker et al., 1972). The advantage of the affinity support used in the present investigation is that the less laborious approach of constructing the spacer arm–ligand complex in steps on the gel matrix was employed.

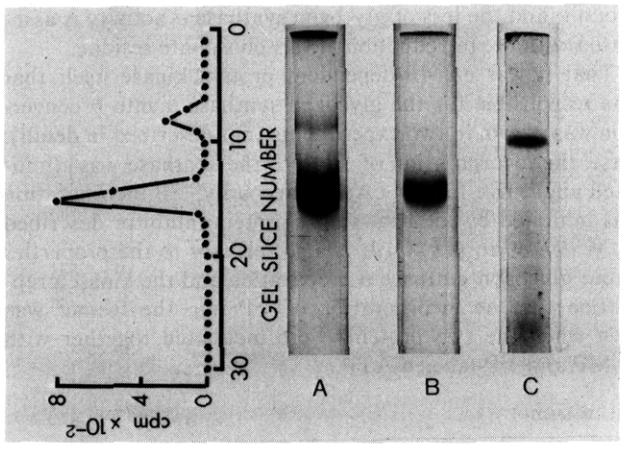

FIGURE 3: Polyacrylamide disc gel electrophoresis of purified rabbit-liver glycogen synthase *b*. For gels A and B, electrophoresis of the native enzyme (40 μg of protein per gel) was carried out at pH 7.3 in 6% acrylamide. Gel A: protein stain. Gel B: activity stain (a minor band of activity was seen corresponding to the minor protein band in A but was too faint to appear on the photograph). A third gel was sliced into 2-mm segments which were assayed for activity. The activity profile obtained is shown in the diagram on the left. Gel C: 10 μg of the enzyme was electrophoresed in 5% acrylamide in the presence of sodium dodecyl sulfate. Details are in the text.

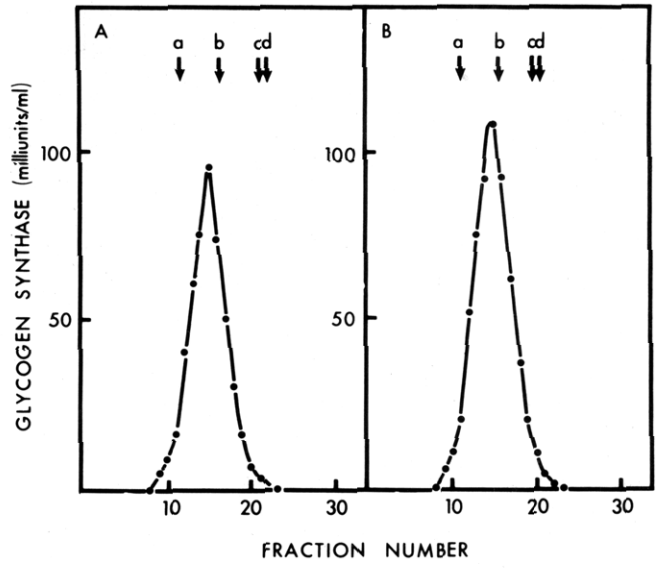

FIGURE 4: Sucrose density gradient centrifugation of purified rabbit-liver glycogen synthase. (Panels A and B) Glycogen synthase *b* and *a*, respectively, run in the presence of glucose 6-P (5 mM). Arrows marked a, b, c, and d indicate the positions of the peaks of the protein markers R-phycocyanin, liver phosphorylase (*a* form in panel A; *b* form in panel B), catalase, and R-phycoerythrin, respectively. The recovery of glycogen synthase activity was greater than 80% in each case. Direction of sedimentation is from left to right.

Enzyme Stability. In the absence of glycogen, complete loss of the glycogen synthase *b* activity was observed when the enzyme was incubated at 25 °C for 4 h in Tris or glycylglycine buffers, pH 7.6. Under the same conditions no loss of activity was observed in β-glycerophosphate and imidazole buffers. Maximal stability of the enzyme was observed in the β-glycerophosphate buffer and the enzyme has been stored in this buffer at −70 °C for up to 1 year without loss of activity. Freezing of the enzyme in the imidazole buffer led to precipitation and loss of activity. However, β-glycerophosphate was found to be a potent inhibitor of the glycogen synthase *b* activity and most of the studies were carried out on samples of the enzyme equilibrated with imidazole buffer by passage through a small Sephadex G-50 (fine) column.

Purity of the Synthase. Polyacrylamide disc gel electrophoresis of the isolated glycogen synthase *b* under nondenaturing conditions resolves the preparation into a major and a minor protein band and a small amount of protein did not enter the gel (Figure 3). From other gels, run at the same time, it was found that these major and minor protein bands were both enzymically active. This was noted by an activity stain and by slicing a gel into segments and assaying each slice (Figure 3). The slower migrating band is presumably an aggregate of the faster migrating species. When electrophoresis was carried out on denatured enzyme in the presence of sodium dodecyl sulfate, all the protein entered the gel and only one protein band could be detected (Figure 3). Thus by the criterion of polyacrylamide disc gel electrophoresis, glycogen synthase *b*, isolated by the procedure described above, is homogeneous. Complexing of plant glycogen or starch synthases with branching enzymes has been noted by Schiefer et al. (1973). These complexes comigrate in gel electrophoresis. In the case of the liver enzyme, however, the branching enzyme activity was separated in the DEAE-cellulose chromatography step. This was confirmed in the electrophoretic studies since in gel B (Figure 3) the polysaccharide synthesized by the glycogen synthase stained blue with iodine.

Conversion of Glycogen Synthase b into a. Incubation of glycogen synthase *b* with a liver protein phosphatase preparation led to the formation of an enzyme form displaying the characteristics by which glycogen synthase *a* is defined; that is, the activity was almost wholly independent of glucose 6-P, addition of which increased the activity only by 5%. [The activity of synthase *a* was measured in presence of sodium sulfate, as by Thomas et al. (1973). The synthase *a* was activated 35% by sulfate. Synthase *b*, measured in presence of glucose 6-P, was not activated by sulfate. The same behavior was already noted by Thomas et al. (1973) for bovine-heart and rabbit-skeletal-muscle glycogen synthases.] The protein phosphatase was a homogeneous preparation from rabbit liver, which, we have shown elsewhere (Killilea et al., 1976), exerts coordinate control over liver glycogen synthase *b* and phosphorylase *a* by acting on both of these phosphoproteins.

Subunit Size and Molecular Weight. The subunit size of the isolated enzyme was determined by electrophoresis in the presence of sodium dodecyl sulfate in 5% acrylamide. As noted above, only one protein band was discernible (Figure 3) and a polypeptide size of 85 000 was determined. The physical properties of both the *a* and *b* forms of the glycogen synthase were examined by sucrose density gradient centrifugation. The enzyme sedimented as a single symmetrical peak in each case (Figure 4). Using liver phosphorylase, catalase, R-phycocyanin, and R-phycoerythrin as protein markers, the sedimentation coefficient and molecular weight of each enzyme type were calculated according to the procedure of Martin and Ames (1961). Sedimentation coefficients of 8.32 ± 0.3S and 8.0 ± 0.3S, corresponding to molecular weights of 183 000 ± 20 000 and 170 000 ± 21 000, were calculated for the *a* and *b* forms of the enzyme, respectively. In these experiments glucose 6-P (5 mM) was included in each of the ultracentrifugation runs. Essentially similar results were obtained for glycogen synthase *b* from runs in which this effector was omitted. Under

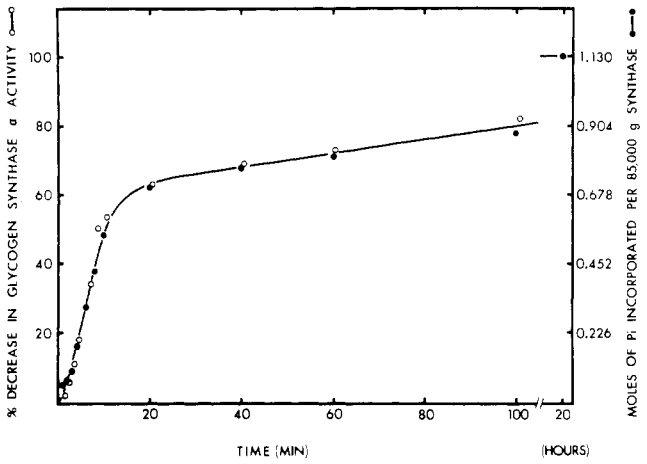

FIGURE 5: Phosphorylation of rabbit-liver glycogen synthase *a* with rabbit-muscle cAMP-dependent protein kinase. (●) Phosphorylation; (○) % decrease in glycogen synthase *a* activity. Conditions were as described in the text.

these conditions, the enzyme sedimented also as a single symmetrical peak for which a sedimentation coefficient of 8.32 ± 0.3S, corresponding to a molecular weight of 182 000 ± 33 000, was calculated from the experimental data. On the basis of a subunit size of 85 000, these results demonstrate that both the *a* and *b* forms of rabbit-liver glycogen synthase behave as dimers during sucrose density gradient centrifugation.

Conversion of Glycogen Synthase a into b. In order to determine the relationship between phosphorylation and change in enzymic activity in the conversion of glycogen synthase *a* into *b*, glycogen synthase *a* was incubated with a rabbit-muscle cAMP-dependent protein kinase preparation in the presence of [γ-^{32}P]ATP. The time course of phosphorylation of glycogen synthase is shown in Figure 5. The rate of phosphorylation was rapid up to a loss of 60% of synthase *a* activity and then dropped markedly. The maximal extent of phosphorylation determined after a 20-h incubation amounted to 1.13 mol of ^{32}P per 85 000 molecular weight subunit, or 1.33 mol of ^{32}P per 100 000 g of protein. In a control experiment in which glycogen synthase *a* was omitted, no incorporation of ^{32}P into trichloroacetic acid insoluble material was observed over the 100-min time period. This result would rule out the possibility of the time-dependent formation of a trichloroacetic acid-insoluble complex of [γ-^{32}P]ATP and magnesium and fluoride ions, which has been observed to occur under certain assay conditions (Greenaway, 1972), and also self-phosphorylation by the protein kinase preparation. During the time course of phosphorylation, the loss of glucose 6-P independent activity was also determined. When the loss in glycogen synthase *a* activity was plotted on a scale such that 100% loss of activity equaled the 1.13 mol of ^{32}P incorporation per 85 000 molecular weight subunit, the loss of glycogen synthase *a* activity was found to parallel exactly the percentage phosphorylation of the enzyme up to 100 min of incubation (Figure 5). At the 20-h time point, no glucose 6-P independent activity could be detected, but the glucose 6-P dependent activity had decreased to 25% of the initial glucose 6-P dependent activity, indicating that considerable loss of the enzyme activity had taken place over the extended time period. The results indicate that the conversion of glycogen synthase *a* into *b*, by the cAMP-dependent protein kinase preparation, involves the incorporation of about one P_i group per enzyme subunit, and the loss of glycogen synthase *a* activity is associated with the introduction of this phosphate residue.

That it was cAMP-dependent protein kinase itself that was responsible for the glycogen synthase *a* into *b* conversion was shown in two experiments (not described in detail). First, the incorporation of ^{32}P into the synthase was stimulated about five-fold by cAMP. Secondly, ^{32}P incorporation was inhibited by the heat-stable protein inhibitor described by Walsh et al. (1971). It is also relevant to the properties of our glycogen synthase *b* preparation and the kinase preparation that no incorporation of ^{32}P into the former was seen when the two proteins were incubated together with cAMP and ^{32}P-labeled ATP.

Discussion

The specific activity of the homogeneous rabbit-liver glycogen synthase *b*, isolated by the procedure reported above, was of the same order as those reported for the rabbit-muscle enzyme (Brown and Larner, 1971) and the rat-liver enzyme isolated by Lin and Segal (1973) allowing for the higher assay temperature used in the latter case, and was higher than those reported for the trout-liver enzyme (Lin et al., 1972) and the rat-liver enzyme isolated by McVerry and Kim (1974), when the higher assay temperature used in these cases is considered.

The subunit molecular weight of the rabbit-liver glycogen synthase *b* was determined to be 85 000 by polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate. This value is identical with the subunit size reported for both the rabbit-muscle enzyme (Takeda et al., 1975) and the rat-liver enzyme isolated by Lin and Segal (1973) and is intermediate between the values of 92 500, 90 000, and 77 000–80 000 reported for the pig kidney (Issa and Mendicino, 1973), pig adipose tissue (Miller et al., 1975), and the rat-liver enzyme isolated by McVerry and Kim (1974), respectively. While there is a general correspondence in the reports of the subunit size of glycogen synthase, a variety of molecular weights, corresponding to dimers, trimers, and tetramers, have been reported for the native enzyme. Molecular weights of 400 000 and 250 000 have been reported for rabbit-muscle glycogen synthases *a* and *b*, respectively (Soderling et al., 1970; Brown and Larner, 1971), and 370 000 for both forms of the pig-kidney enzyme (Issa and Mendicino, 1973). The trout-liver enzyme was resolved by sucrose density gradient centrifugation into four active peaks, and a molecular weight of 274 000–312 000 was calculated for the heaviest peak (Lin et al., 1972). A molecular weight of 250 000 was determined for rat-liver glycogen synthase *b* by Lin and Segal (1973) while McVerry and Kim (1972) reported a value of 140 000 for glycogen synthase *b* from the same source and a complex series of aggregates having molecular weights of 180 000, 310 000, and 470 000 for the *a* form of the rat-liver enzyme. Later McVerry and Kim (1974) reported that, in the presence of glucose 6-P, both forms of the rat-liver enzyme had a molecular weight of 310 000. In the present investigation sucrose density gradient centrifugation studies revealed that glucose 6-P had no effect on the sedimentation coefficient of rabbit-liver glycogen synthase *b*, and molecular weights of 170 000 ± 21 000 and 183 000 ± 20 000 were calculated for the *b* and *a* forms of the enzyme, respectively, corresponding to the active enzyme being a dimer.

We observed that about 1 mol of phosphate was incorporated per 85 000 g of protein when the rabbit-liver glycogen

synthase *a* was converted into *b* by cAMP-dependent protein kinase from muscle, and that the rates of phosphorylation and loss of glycogen synthase *a* activity paralleled one another; that is, the introduction of a single phosphate residue per subunit was responsible for the conversion of glycogen synthase *a* into *b*. This result is consistent with similar studies carried out on the conversion of rabbit-muscle glycogen synthase *a* into *b* (Soderling et al., 1970; Nimmo and Cohen, 1974). On the other hand, chemical analyses have shown that rat-liver glycogen synthase *b* contains as many as 12 mol of phosphate per 85 000 g (Lin and Segal, 1973). Similar analyses of rabbit-muscle glycogen synthase *b* had indicated 6 mol of phosphate per 85 000 g (Smith et al., 1971), but this value was later re-estimated to be about 3 mol of phosphate per 85 000 g (Takeda et al., 1975).

Our results are not necessarily at variance with the idea that liver synthase *b* contains more than one phosphate group per subunit. What we isolate is a homogeneous phosphoenzyme (*b*), which we convert into a dephospho form (*a*) using a homogeneous protein phosphatase. One phosphate group per subunit is removed in the process. The phosphate can be re-incorporated using cAMP-dependent beef-heart or rabbit-muscle protein kinase (Killilea et al., 1976) while, as noted above, no ^{32}P incorporation occurs when the *b* form of the enzyme is offered to the kinase as substrate. Therefore our *b* form of synthase may be said to contain $n + 1$ atoms of phosphorus per subunit where n may or may not be zero. Nevertheless, the conversion of the enzyme into a form wholly dependent on glucose 6-P for activity is entirely dependent on the introduction of that one definite phosphate group.

It is relevant that recent work on the enzymic phosphorylation of rabbit-muscle glycogen synthase by Nimmo and Cohen (1974) and Soderling (1975) has also revealed multiple phosphorylation sites, the phosphorylation of some of which are not associated with changes in enzymic activity. Nimmo and Cohen (1974) have reported that skeletal muscle contains a previously undescribed cAMP-independent protein kinase. This new form of kinase, in contrast to the cAMP-dependent protein kinase (Walsh et al., 1971), was found to label different site(s) on the glycogen synthase without affecting the glycogen synthase *a* activity (Nimmo and Cohen, 1974). Soderling (1975) has also reported that multiple sites on the muscle enzyme could be phosphorylated, at least 5 mol of phosphate being incorporated per subunit, and that phosphorylation of only two of these was associated with the loss of glucose 6-P independent activity. It may be noted that some of the phosphorylation reactions described by Soderling (1975) were not inhibited by the cAMP-dependent protein kinase inhibitor, indicating that his enzyme preparations possibly contained the cAMP-independent protein kinase described by Nimmo and Cohen (1974). The possible physiological significance of the multiple phosphorylation sites in glycogen synthase is presently unknown, but multisite phosphorylation of phosphorylase kinase has been postulated as a means of increasing the potential for regulation of the enzyme function (Cohen, 1974).

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