Enzymes Regulating Glycogen Metabolism in Swine Subcutaneous Adipose Tissue. II. Glycogen Synthase†

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ABSTRACT: Glycogen synthase from swine adipose tissue was purified to apparent homogeneity using ethanol precipitation, DEAE chromatography, and affinity chromatography utilizing glucosamine 6-phosphate as the ligand. The purified enzyme migrated as a single protein component during electrophoresis on polyacrylamide gels at pH 7.3 although some protein failed to enter the running gel. Enzyme incubated with sodium dodecyl sulfate (SDS) migrated as one component (mol wt ~90,000) on SDS–polyacrylamide gel electrophoresis. The enzyme was relatively unstable at all stages of the purification procedure, but stability was increased in the presence of glucose 6-phosphate, UDPG, or glycerol. The isoelectric point of the purified enzyme and of enzyme activity in crude homogenates was pH 4.8. The sedimentation coefficient of the enzyme in crude homogenates was 8.5 S. The pH–activity profile showed an optimum at pH 7.8 in the absence of glucose 6-phosphate but no definable optimum between pH 7.0 and 9.2 in its presence. The K_m of glycogen synthase I for UDPG was 250 μM in the absence and 37 μM in the presence of glucose 6-phosphate; the K_a for glucose 6-phosphate was 18 μM. The K_m of glycogen synthase D for UDPG was 130 μM in the presence of glucose 6-phosphate; the K_a for glucose 6-phosphate was 1 mM. The anions sulfate and phosphate activated the enzyme when assays were performed in the absence of glucose 6-phosphate. Fluoride produced activation of enzyme assayed either in the presence or in the absence of glucose 6-phosphate.

The synthesis of glycogen in adipocytes, as in other tissues, is regulated in part by changes in the activity of glycogen synthase (UDP-glucose:glycogen 4-α-glucosyltransferase, EC 2.4.1.11) (Wiley and Leville, 1970). Two different forms of the enzyme are present in most tissues, and these forms are interconverted by phosphorylation and dephosphorylation (Larner and Villar-Palasi, 1971). The phosphorylated form of the enzyme is dependent upon the allosteric activator glucose 6-phosphate for activity and is thought to be relatively inactive in vivo. The nonphosphorylated form of the enzyme does not require a small molecule activator and is considered to be the active form of the enzyme. Interconversion of the two forms of adipose tissue glycogen synthase in response to hormonal treatment of adipose tissue has been demonstrated (Jungas, 1966; Khoo et al., 1973). Specifically, adipose tissue glycogen synthase is converted to the glucose 6-phosphate dependent form in adipocytes exposed to lipolytic hormones (e.g., epinephrine) and to the glucose 6-phosphate independent form in adipocytes exposed to insulin (Khoo et al., 1973).

The enzymatic interconversion of the two forms of glycogen synthase and differences in their susceptibility to metabolic effectors have been extensively investigated in skeletal muscle, heart, and liver (Larner and Villar-Palasi, 1971; Thomas and Larner, 1973). The mechanism of glycogen synthase inactivation and activation in adipose tissue has not been studied in detail but it seems likely that, as in other tissues, the processes are catalyzed by cyclic AMP dependent protein kinase (Corbin et al., 1972, 1973; Soderling et al., 1973) and glycogen synthase phosphatase (Kato and Bishop, 1972), respectively. The mechanism for insulin-mediated activation of glycogen synthase has not been delineated; however, it is clear that this may involve a decrease in the rate of phosphorylation, an increase in the rate of dephosphorylation, or both.

Glycogen synthase from skeletal muscle (Soderling et al., 1970; Brown and Larner, 1971), heart (Thomas and Larner, 1973), liver (Lin and Segal, 1973), and kidney (Issa and Mendicino, 1973) has been purified to homogeneity and characterized. The enzyme from adipose tissue has not previously been purified, although some kinetic properties of the enzyme from adipose tissue crude homogenates have been reported (Barash et al., 1973). In order to study the hormonal regulation of glycogen synthase activity in adipose tissue it is necessary to obtain and characterize substrate quantities of the enzyme essentially free of its interconverting enzymes. Prompted by this consideration, this study was undertaken to devise a method for the isolation of adipose tissue glycogen synthase.

Experimental Section

Materials and Methods. Chemicals were obtained as follows: uridine diphospho[^{14}C]glucose, Amersham/Searle Corp.; D-[1-^{14}C]glucosamine 6-phosphate and Omnifluor, New England Nuclear Corp.; glucose 6-phosphate, glucosamine 6-phosphate, oyster glycogen, uridine diphosphoglucose, creatine phosphokinase, yeast glucose-6-phosphate dehydrogenase, and hog pancreatic α amylase, Sigma Chemical Co.; 3,3′-diaminodipropylamine, 1-cyclohexyl-3-(2-mor-

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pholinoethyl)carbodiimide metho-*p*-toluenesulfonate, and *N*-hydroxysuccinamide, Aldrich Chemical Co.; 4% agarose (Sepharose 4B) and Ficoll, Pharmacia Fine Chemicals; Ampholine, LKB Instruments, Inc.; Aquaside, Calbiochem; NCS[1] tissue solubilizer, Amersham/Searle Corp.

Swine subcutaneous adipose tissue was obtained and stored as previously described (Miller et al., 1975).

Assays. The glycogen synthase assay procedure was patterned after that described by Villar-Palasi et al. (1966) based on measurement of the rate of incorporation of [^{14}C]glucose from UDP-[^{14}C]glucose into glycogen. Standard assay mixtures (0.2 ml) contained 10 mg/ml of oyster glycogen, 2.5 m*M* UDP-[^{14}C]glucose (0.05 μCi/μmol), 10 m*M* glucose 6-phosphate, 5 m*M* EDTA, 50 m*M* Tris-HCl (pH 7.8), and sufficient enzyme to catalyze the incorporation of 0.025 to 0.100 μmol of [^{14}C]glucose into glycogen during the assays. Assays (30°) were linear with time for at least 90 min or until at least 20% of the substrate (UDPG) had been consumed. Assays were terminated (usually after 15 or 30 min) by addition of 2 ml of 70% ethanol containing 0.1 *M* ammonium acetate. Samples were centrifuged (2000*g*, 5 min, −5°). The well-drained glycogen–protein pellets were redissolved in 0.2 ml of water and reprecipitated with 2 ml of the ethanol–ammonium acetate solution, and the samples were recentrifuged. Finally, the well-drained glycogen–protein pellets were dissolved in 0.7 ml of water and decanted into scintillation vials. Sample tubes were washed with a second 0.7-ml aliquot of water which was added to the scintillation vials. Radioactivity was assayed in a Packard scintillation spectrometer after addition of 10 ml of Omnifluor scintillation mixture containing Triton X-100–toluene (1:2). Assay results are similar to those obtained using the filter paper method of Thomas et al. (1968) but results are more reproducible and blank activities (assays stopped at zero time) are lower. One unit of glycogen synthase activity is defined as the amount of enzyme catalyzing the incorporation of 1 nmol of [^{14}C]glucose into glycogen per minute under the standard assay conditions. The glycogen synthase *activity ratio* is defined as the ratio between activity determined in the absence and that determined in the presence of 10 m*M* glucose 6-phosphate.

Glycogen phosphorylase, phosphorylase phosphatase (Miller et al., 1975), and glucose-6-phosphate dehydrogenase (Miller and Stadtman, 1972) were assayed as previously described. Protein concentration was determined by the method of Lowry et al. (1951).

Polyacrylamide disc gel electrophoresis at pH 7.3 was performed as described by Hedrick and Smith (1968). Polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate was performed according to the procedures of Weber and Osborn (1969) with the exception that 25 m*M* *N*-tris(hydroxymethyl)methyl-2-aminothanesulfonic acid–40 m*M* Tris (pH 7.1) was substituted for sodium phosphate buffer both for gel polymerization and in the electrode buffers. This modification reduced by 75% the time required and by 80% the current required for electrophoresis (J. Baker, personal communication). Bovine serum albumin, ovalbumin, and muscle phosphorylase *b* were used as standards for molecular weight estimation. Glycogen determinations were performed using anthrone (Seifter et al., 1950).

[1] Abbreviations used are: NCS, Nuclear-Chicago solubilizer; SDS, sodium dodecyl sulfate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

Sucrose Density Gradient Sedimentation and Isoelectric Focusing. Sucrose density gradient sedimentation was performed by the method of Martin and Ames (1961). Isoelectric focusing was performed according to the procedures described in the instruction manual supplied by LKB Instruments Inc.

Affinity Chromatography. Succinylaminodipropylaminoagarose was prepared as described by Cuatrecasas (1970); the *N*-hydroxysuccinamide ester of succinyldiaminodipropylaminoagarose was prepared as described by Cuatrecasas and Parikh (1972) using the water-soluble carbodiimide 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate. For coupling of glucosamine 6-phosphate to the activated agarose derivative, the freshly prepared *N*-hydroxysuccinamide ester of succinylaminodipropylaminoagarose was added to a solution of 50 m*M* [^{14}C]glucosamine 6-phosphate (1.2 μCi/mmol) in 0.1 *M* $NaHCO_3$ (pH 7.9). After mixing for 10 to 12 hr (4°) the gel was washed with 1 vol of 1 *M* glycine at room temperature followed by 10 vol of 1 *M* NaCl and at least 20 vol of water until no ^{14}C could be detected in the wash. The amount of covalently bound [^{14}C]glucosamine 6-phosphate was estimated by scintillation counting of a slurry of the gel. Results were similar whether or not the gel was preincubated in NCS tissue solubilizer. The values obtained (2–3 μmol/ml packed bed volume) are conservative estimates of the glucosamine 6-phosphate covalently bound to the agarose. When other matrices were substituted for the succinylaminodipropylamine moiety similar procedures were used. The affinity gel prepared as described above was regenerated after each use by washing the gel with one bed volume of 8 *M* urea and then 10 bed volumes of water. This regeneration procedure did not alter the concentration of covalently bound glucosamine 6-phosphate.

Results

Purification Procedure. Glycogen synthase was purified to homogeneity from swine subcutaneous adipose tissue using an ethanol precipitation step, DEAE-cellulose chromatography, and glucosamine 6-phosphate affinity chromatography. Unless otherwise indicated, all procedures were performed at 4° in buffer containing 20 m*M* Tris-HCl (pH 7.8), 5 m*M* EDTA, and 15 m*M* 2-mercaptoethanol.

STEP 1: CRUDE HOMOGENATE. Frozen swine subcutaneous adipose tissue was suspended in the buffer in a ratio of 2 g of tissue for each 3 ml of buffer. The suspension was homogenized in a Waring Blendor (1–2 min), filtered through cheesecloth, and centrifuged (30,000*g* for 30 min). The floating lipid was discarded and the opalescent infranatant fluid was filtered through glass wool in preparation for the next step. The pellet, which contained less than 10% of the glycogen synthase present in the infranatant fraction, was discarded.

STEP 2: ETHANOL PRECIPITATION. The filtered infranatant solution from step 1 was adjusted to 0.1 *M* KCl with the solid salt and to 0.5 mg/ml of glycogen with a concentrated solution of oyster glycogen. Absolute ethanol at −60° was slowly added to the crude enzyme preparation at 0° with mixing to achieve a final concentration of 25% ethanol (v/v). After equilibration for 15 min at −5° in a salt–ice bath the mixture was centrifuged (30,000*g* for 40 min). The supernatant fraction was discarded and the pellet was homogenized (glass–Teflon homogenizer) in a volume of buffer equal to one-fifth of the volume of the infranatant solution from step 1. The turbid homogenate was partially

clarified by centrifugation (30,000*g* for 15 min). The opalescent supernatant solution usually contained more than 75% of the activity recovered in the infranatant solution of step 1. The insoluble material was discarded.

The addition of exogenous oyster glycogen was omitted in some cases without altering the yield in the ethanol step. Rarely, however, yields fell below 50% when exogenous glycogen was omitted. This may reflect a low tissue content of glycogen in some animals.

STEP 3: DEAE-CELLULOSE ADSORPTION. DEAE-cellulose (Whatman DE 52) (equal in packed bed volume to the volume of enzyme sample to be chromatographed) previously equilibrated with buffer was suspended in the enzyme preparation from step 2. After equilibration for 15 min with occasional mixing, the suspension was filtered through a sintered glass funnel and the moist DEAE-cellulose was washed with two bed volumes of buffer containing 70 m*M* KCl. With this procedure, most of the phosphorylase appeared in the filtrates while nearly all of the glycogen synthase and phosphorylase phosphatase were retained by the DEAE-cellulose. Glycogen synthase and phosphorylase phosphatase were eluted with approximately 2.5 bed volumes of buffer containing 0.3 *M* KCl.

The glycogen synthase in the pooled 0.3 *M* KCl fractions was very unstable. More than 50% of the activity was lost after 18 hr at 4°. When DEAE-cellulose column chromatography and KCl linear gradient elution (0–1.0 *M* KCl) were substituted for the batch procedure less than 50% of the glycogen synthase activity applied to the column was detected in the column effluent. In contrast, the batch procedure regularly resulted in recovery of more than 70% of the activity from the preceding step. This difference between column and batch chromatography procedures probably reflects the marked instability of the enzyme and the relatively longer time required to perform the gradient elution (5–10 hr) as compared to the short time for batch elutions (less than 2 hr).

STEP 4: SECOND ETHANOL PRECIPITATION. Oyster glycogen (0.5 mg/ml) was added to the 0.3 *M* KCl eluate from step 3. The procedures of step 2 were then repeated with the exception that the well-drained pellets were suspended in $\frac{1}{30}$ the volume of the infranatant fraction of step 1. This step results in little if any purification of glycogen synthase but serves to concentrate the enzyme and to reduce the ionic strength of the enzyme solution.

Steps 1 through 4 can be completed in 8 to 10 hr using 1 kg of adipose tissue as starting material.

STEP 5: GLUCOSAMINE 6-PHOSPHATE AFFINITY CHROMATOGRAPHY. The enzyme preparation from the preceding step was applied to an affinity column composed of the specific glycogen synthase activator glucose 6-phosphate (as glucosamine 6-phosphate) covalently linked through an organic matrix (succinyldiaminodipropylamine) to 4% agarose (see Experimental Section). The column (2.5 × 18.5 cm) was previously equilibrated with buffer at 4° and after sample application it was washed with at least four bed volumes of the same buffer (Figure 1). The column was then moved to room temperature and elution of virtually homogeneous glycogen synthase (Figure 1) was accomplished by washing the column with buffer containing 10 m*M* glucose 6-phosphate. Sample application and column washing required approximately 18 hr. Elution of activity required approximately 4 hr.

In the example shown in Figure 1, approximately 6000 units of glycogen synthase was applied to the column. Approximately 1000 units (16%) appeared in the breakthrough fractions and 2400 units (40%) was eluted in fractions containing glucose 6-phosphate. These values are based on simultaneous assays of eluted fractions and of an aliquot of the sample originally applied to column (stored at 4°). Forty-four percent of the activity applied to the column was not recovered. It was not eluted either at higher glucose 6-phosphate concentrations (up to 50 m*M*) or with 0.5 *M* KCl (not shown). The reason for the loss in enzyme activity appears to be related to exaggerated instability of glycogen synthase in the sample applied to the column. During the 14 hr between application of sample on the affinity column and elution of the purified glycogen synthase there was a 30–50% loss in the activity of glycogen synthase in the aliquot not subjected to chromatography.

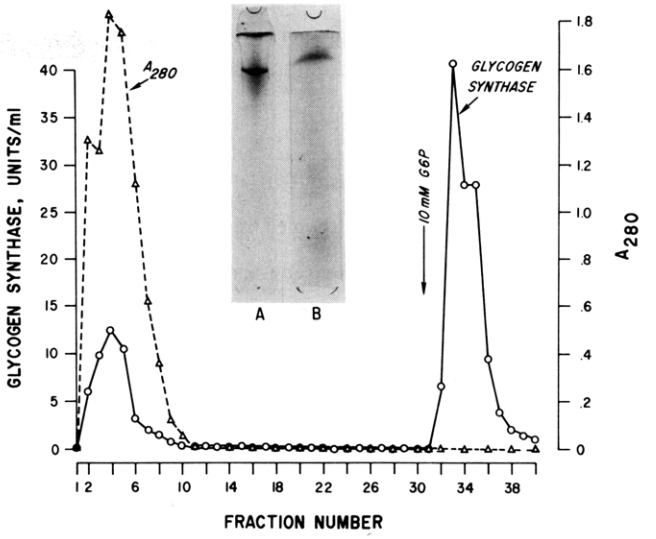

FIGURE 1: Affinity chromatography of adipose tissue glycogen synthase. Column was 2.5 × 18.5 cm. Flow rate was 10 ml/cm^2 per hr. Fractions were 20 ml. The buffer contained 20 m*M* Tris-HCl, 5 m*M* EDTA, and 15 m*M* 2-mercaptoethanol (pH 7.8). The sample was 32 ml of partially purified glycogen synthase from the second ethanol precipitation (see Purification Procedure). Sample was applied and the column was washed at 4° with the buffer. After collection of fraction number 30, the column was eluted at room temperature (21°) with buffer containing 10 m*M* glucose 6-phosphate, 20 m*M* Tris-HCl, 5 m*M* EDTA, and 15 m*M* 2-mercaptoethanol (pH 7.8). The affinity column ligand was glucosamine 6-phosphate covalently linked to succinylaminodipropylaminoagarose (see Experimental Section). Insert: polyacrylamide gel electrophoresis (Experimental Section) at pH 7.3 in the presence of 1 m*M* glucose 6-phosphate (A) and in the presence of 0.1% sodium dodecyl sulfate (B). Acrylamide concentration was 6% in A and 10% in B. Sample for both A and B: approximately 35 μg of glycogen synthase obtained after Ficoll concentration of enzyme eluted from the affinity column with glucose 6-phosphate.

The glycogen synthase containing fractions eluted with 10 m*M* glucose 6-phosphate were pooled and concentrated either (1) by ethanol precipitation in the presence of added 0.1 *M* KCl and 0.5 mg/ml of glycogen (as in step 2 of the Purification Procedure) or (2) by surrounding dialysis tubing containing the enzyme with either Ficoll or Aquacide. In either case, after centrifugation, about 60% of the enzyme activity was recovered and the specific activity of the enzyme was unchanged. Exhaustive dialysis of the concentrated enzyme against buffer was required to remove all traces of glucose 6-phosphate and, as in the case of storage at 4°, this procedure resulted in further, apparently irreversible, losses in activity (30–50%). Purified enzyme in the presence of 20 m*M* Tris-HCl (pH 7.8)–5 m*M* EDTA [frozen in liquid nitrogen and stored at −80°] retained constant

Table I: Representative Purification of Glycogen Synthase from Swine Subcutaneous Adipose Tissue.

Step	Vol (ml)	Total Enzyme Act. (Units[a])	Yield (%)	Total Protein (mg)	Sp Act. (Units/mg)	Purification
1. Crude homogenate (30,000*g* infranatant fraction)	960	10,990	100	2540	4.3	1
2. First ethanol precipitation	200	9,550	87	360	27	6
3. DEAE-cellulose adsorption (pooled fractions)	480	8,490	77	163	52	12
4. Second ethanol precipitation	31	7,050	64	120	59	14
5. Affinity chromatography (pooled fractions)	149	2,220	20	0.92	1850	433

[a] Standard assay (Experimental Section) performed in the presence of glucose 6-phosphate. One unit is defined as the amount of enzyme catalyzing the incorporation of 1 nmol of [^{14}C]glucose into glycogen per min.

activity for at least 2 months.

Table I summarizes the results of a typical purification. The procedure resulted in a 430-fold enrichment of glycogen synthase activity over that in the 30,000*g* infranatant fraction of the crude homogenate.

Homogeneity of the Purified Enzyme. Glycogen synthase from the pooled affinity column fractions, concentrated with Ficoll, migrated as a single component during polyacrylamide disc gel electrophoresis at pH 7.3 (see Experimental Section) performed in the presence of 1 m*M* glucose 6-phosphate (Soderling et al., 1970) (Figure 1, insert A). However, a significant portion of the protein applied to the gel remained at the interface between the stacking gel and the running gel. When the same enzyme preparation was disaggregated, reduced, and alkylated (Weber and Osborn, 1969), it migrated as a single component during polyacrylamide gel electrophoresis performed in the presence of 0.1% sodium dodecyl sulfate (Figure 1, insert B). There was no detectable protein that failed to enter the running gel. This result suggests that the protein not entering the disc gels run at pH 7.3 (Figure 1, insert A) represents aggregated enzyme and/or enzyme associated with glycogen, lipid, or both. The apparent molecular weight of the enzyme after reduction and disaggregation in SDS was approximately 90,000, similar to that reported for glycogen synthase from skeletal muscle (Larner and Villar-Palasi, 1971) and liver (Lin and Segal, 1973).

The purified enzyme had no detectable glycogen phosphorylase activity; however, it did show some phosphorylase phosphatase activity (specific activity <2.0% of that present in the crude homogenate).

Stability of Partially Purified Enzyme. The stability of the glycogen synthase was studied using enzyme from step 4 of the Purification Procedure. In an effort to prevent or retard apparent irreversible inactivation of the enzyme, it was incubated (4°) in the presence of several compounds (Table II). Of the compounds studied, glucose 6-phosphate, UDPG, and glycerol significantly retarded inactivation. In addition, UDP, a product of the glycogen synthase catalyzed reaction and an inhibitor of enzyme activity, also significantly retarded glycogen synthase inactivation. Dithiothreitol did retard rapid loss in activity for up to 3 days, while neither 2-mercaptoethanol nor K_2SO_4 was effective in stabilizing enzyme activity. These three compounds have been reported to stabilize the glycogen synthase from skeletal muscle (Thomas et al., 1973). Although there was no apparent effect of glycogen or EDTA upon enzyme stability, these compounds cannot be properly evaluated since the enzyme preparations studied contained some glycogen (added in step 4 of the Purification Procedure) and some EDTA was transferred with the enzyme (Table II).

Table II: Stability of Swine Adipose Tissue Glycogen Synthase at 4°.[a]

Compound Added	Relative Specific Act. after 30 min	1 Day	3 Days	10 Days
None	100	65	51	21
KCl, 100 m*M*	100	86	65	29
NaCl, 100 m*M*	88	86	56	26
KF, 50 m*M*	89	84	55	26
EDTA, 10 m*M*	97	96	68	27
$MgCl_2$, 10 m*M*	88	87	53	16
K_2SO_4, 10 m*M*	78	82	65	43
$(NH_4)_2SO_4$, 50% saturated	49	48	35	18
UDPG, 10 m*M*	108	108	95	61
UDP, 8 m*M*	58	69	59	44
2-Mercaptoethanol, 50 m*M*	97		51	9
Dithiothreitol, 10 m*M*	105	94	66	18
Glucose 6-phosphate, 10 m*M*	100	103	98	59
Glucose, 200 m*M*	78	74	45	18
Sucrose, 200 m*M*	107	86	56	17
Glycerol, 20%	97	107	81	50
Glycogen, 10 mg/ml	98	88	58	31
Bovine serum albumin, 1 mg/ml	99	86	53	21

[a] Each sample, stored at 4°, contained 48 m*M* Tris-HCl (pH 7.8), 2 m*M* EDTA, the indicated compound at the indicated concentration, and approximately 1.5 mg/ml of adipose tissue glycogen synthase obtained after the second ethanol precipitation (step 4, Purification Procedure). Enzyme used was prepared in the absence of 2-mercaptoethanol. The concentrations of EDTA and glycogen indicated are in addition to that transferred with the enzyme. Standard assays were used (Experimental Section). A relative specific activity of 100 corresponds to 93 nmol of glucose incorporated into glycogen per minute per milliliter of sample. At the indicated times, aliquots of the samples were diluted eightfold into assay mixtures for determination of activity. Corrections were made for UDPG transferred to assay mixtures.

Isoelectric pH and Sedimentation Coefficient. The isoelectric pH of glycogen synthase in crude homogenates was 4.8 as determined by isoelectric focusing. The isoelectric pH of purified glycogen synthase (Figure 2) was identical with that of the enzyme in crude homogenates. The sedimentation coefficients of glycogen synthase and glycogen phosphorylase in crude homogenates were 8.5 and 7.3 S, respectively (Figure 3). Yeast glucose-6-phosphate dehydrogenase ($s_{20,w}$ = 6.1 S; mol wt 101,600) was used as reference standard (Yue et al., 1969). The value obtained for adipose tissue glycogen synthase is significantly lower than that obtained for the enzyme from rabbit skeletal muscle (Larner and Villar-Palasi, 1971) or swine kidney (Issa and Mendicino, 1973). The activity of purified glycogen synthase was lost during sucrose gradient sedimentation. Therefore, the sedimentation coefficient of the purified enzyme was not determined.

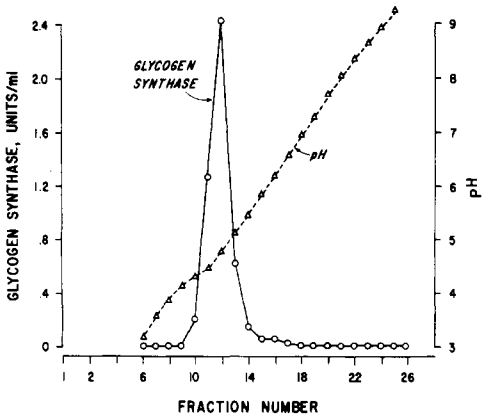

FIGURE 2: Isoelectric focusing of purified adipose tissue glycogen synthase. Sample: 54 units of glycogen synthase from the affinity column pooled fractions (see Purification Procedure): Ampholine, 1%; pH range, 3-10; column volume, 110 ml (LKB Instruments, Inc., Type LKB 81); fractions, 4 ml. Electrofocusing was carried out for 48 hr; final voltage, 900 V; final amperage, 1.8 mA; recovery of applied activity, 37%. Prior to assay, fractions were adjusted to pH 7.0 with either Tris base or HCl: (△) pH; (○) enzyme activity.

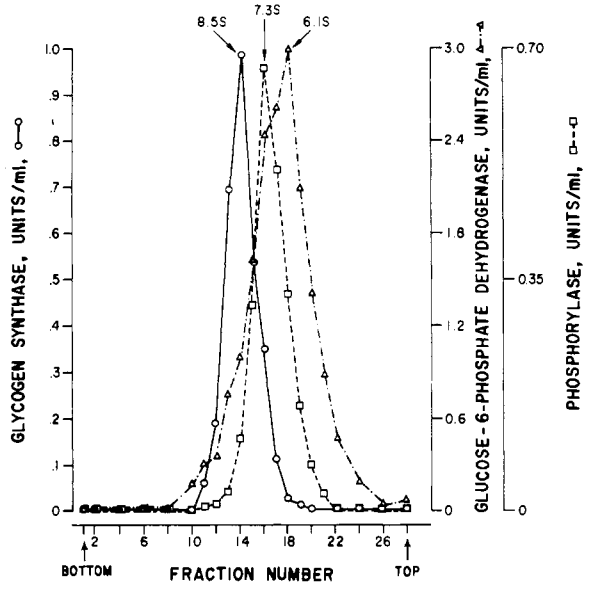

FIGURE 3: Sucrose density gradient sedimentation analysis of glycogen synthase and glycogen phosphorylase in crude adipose tissue homogenates. Sample and gradients (5-20% sucrose) were prepared in buffer containing 20 m*M* Tris-HCl, 5 m*M* EDTA, and 50 m*M* 2-mercaptoethanol (pH 7.8). The sample (0.1 ml) layered on the gradient was centrifuged in an L3-50 Beckman ultracentrifuge at 40,000 rpm (4°) in an SW 50.1 rotor for 12 hr. The sample contained 0.1 ml of crude adipose tissue homogenate and approximately 35 μg of yeast glucose-6-phosphate dehydrogenase (Sigma Chemical Co., type XV from Bakers yeast); units for glycogen synthase and phosphorylase, nanomoles per minute; units for glucose-6-phosphate dehydrogenase, micromoles per minute.

Kinetic Parameters. The activity-pH profile for enzyme eluted from the affinity column, having an activity ratio of 1.0, and freed of glucose 6-phosphate using glucose-6-phosphate dehydrogenase and $NADP^+$, is shown in Figure 4. The optimum pH for activity in the absence of glucose 6-phosphate was 7.8. In the presence of glucose 6-phosphate, however, there was no discrete optimum observed between pH 5 and 9. Instead there was a marked increase in activity over the pH range 5 to 6 and a more gradual increase in activity with increasing pH between 6 and 9. Similar activity-pH profiles were observed for enzyme with an activity ratio

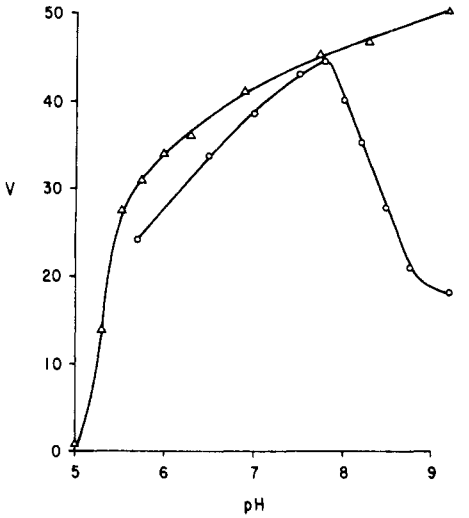

FIGURE 4: pH-activity profile of swine adipose tissue glycogen synthase determined in the absence (○) or in the presence (△) of 10 m*M* glucose 6-phosphate. Two assay mixtures were mixed to achieve the indicated pH values. One contained 50 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) and 50 m*M* 2-(*N*-morpholino)-ethanesulfonic acid (Mes) (pH 4.9), and the other contained 50 m*M* Tris base (pH 9.5). In addition to the buffers, each assay mixture contained 5 m*M* EDTA, 10 mg/ml of oyster glycogen, and either 10 m*M* glucose 6-phosphate (△) or no glucose 6-phosphate (○). Reaction mixtures contained assay mixture (0.2 ml) at the indicated pH, and enzyme (0.02 ml) eluted from the affinity column (step 5, Purification Procedure) (Table I) and then freed of glucose 6-phosphate by dialysis and incubation in the presence of 0.5 m*M* $NADP^+$ and 10 units/ml of glucose-6-phosphate dehydrogenase. Enzyme used for the assays had an activity ratio of 1.0.

of 0.3 obtained after the first ethanol precipitation (step 2, Purification Procedure). However, in the presence of 10 m*M* glucose 6-phosphate this preparation showed no dependence of activity upon pH between pH 6 and 9.5.

Uridine diphosphoglucose and glucose 6-phosphate saturation kinetics for glycogen synthase were hyperbolic. For enzyme eluted from the affinity column (activity ratio of 1.0) and freed of glucose 6-phosphate with $NADP^+$ and glucose-6-phosphate dehydrogenase, the K_m for UDP-glucose was 250 μM in the absence of glucose 6-phosphate and 37 μM in the presence of 10 m*M* glucose 6-phosphate. For the same enzyme preparation the apparent K_a for glucose 6-phosphate was 18 μM when the UDP-glucose concentration was 250 μM. For enzyme obtained after the first ethanol precipitation (step 2, Purification Procedure), having an activity ratio of <0.1, the K_m for UDP-glucose was 130 μM in the presence of 10 m*M* glucose 6-phosphate and the K_a for glucose 6-phosphate was 1 m*M* in the presence of 1 m*M* UDP-glucose.

Effects of Salts. Both Na_2SO_4 and K_2SO_4 had biphasic effects upon glycogen synthase assayed in the absence of glucose 6-phosphate. Figure 5 (left panel) shows the effects of K_2SO_4 upon activity. The effects of Na_2SO_4 (not shown) were nearly identical with those of K_2SO_4. At concentrations below 0.125 *M* both salts activated the enzyme. Maximal activation (45%) occurred at 0.04 *M*. At concentrations between 0.125 and 0.700 *M* there was a progressive decrease in activity. In the presence of glucose 6-phosphate only inhibition was observed over the entire range of Na_2SO_4 and K_2SO_4 studied (0-0.7 *M*).

Like the sulfate salts, sodium phosphate (Figure 5, right panel) and potassium phosphate (data not shown) had biphasic effects upon glycogen synthase assayed in the ab-

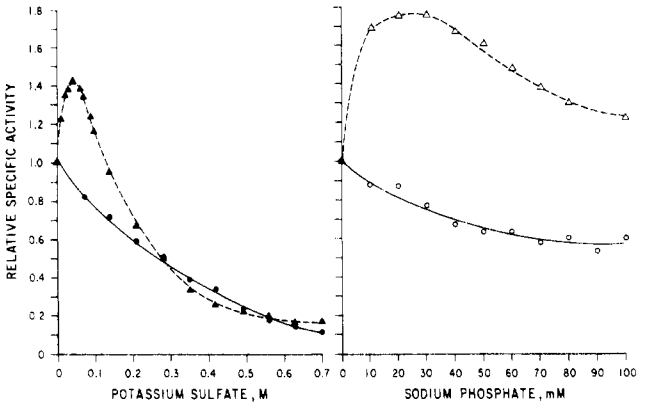

FIGURE 5: The effects of potassium sulfate (left panel) and of sodium phosphate (right panel) on glycogen synthase activity in the absence (△, ▲) and in the presence (○, ●) of 10 m*M* glucose 6-phosphate. Assay mixtures (0.2 ml) contained 50 m*M* Tris-HCl (pH 7.8), 5 m*M* EDTA, 10 mg/ml of oyster glycogen, K_2SO_4 or sodium phosphate at the indicated concentration, enzyme, and either 10 m*M* glucose 6-phosphate or no glucose 6-phosphate. Absolute enzyme activities in the absence of added salts were 28 and 95 units/ml, respectively, in the absence and in the presence of glucose 6-phosphate. Enzyme used (0.02 ml) was from the first ethanol precipitation and contained less than 2 nmol of glucose 6-phosphate per ml. The activity ratio of the enzyme preparation used for these experiments was 0.3.

sence of glucose 6-phosphate. Maximal activation (~75%) occurred between 20 and 30 m*M*. When similar studies were performed in the presence of 10 m*M* glucose 6-phosphate there was inhibition at all concentrations studied (0–100 m*M*).

Both KCl and NaCl inhibited glycogen synthase activity over the entire range of concentrations between 0 and 1.0 *M*. In the absence of glucose 6-phosphate, 50% inhibition occurred at 300 m*M* NaCl or KCl. In the presence of 10 m*M* glucose 6-phosphate, 1 *M* KCl or NaCl produced less than 25% inhibition of activity. In contrast to the effect of the chloride salts, both NaF and KF at 0.2 *M* produced activation of glycogen synthase in the absence and in the presence of 10 m*M* glucose 6-phosphate, 80 and 25%, respectively (data not shown). These results indicate that the effects of sulfate and phosphate cannot be explained on the basis of ionic strength alone.

Interconversion of Synthase I and Synthase D. The activity ratio of glycogen synthase ranged from 0.05 to 0.20 in crude homogenates, from 0.05 to 0.50 after the second ethanol precipitation step (step 4, Purification Procedure), and from 0.5 to 1.0 after affinity chromatography (step 5, Purification Procedure) and Ficoll concentration of the purified enzyme. Since no attempt was made to inhibit endogenous glycogen synthase phosphatase during the purification procedure it seems likely that the increasing activity ratio observed in preparations of increasing purity reflects the action of endogenous glycogen synthase activating activity (presumably glycogen synthase phosphatase).

Affinity Chromatography of Glycogen Synthase. Purified rabbit skeletal muscle glycogen synthase prepared according to the procedure of Soderling et al. (1970) was almost quantitatively retained when 120 μg of the enzyme was applied to a column (1 ml bed volume) of glucosamine 6-phosphate-succinyldiaminodipropylaminoagarose. Less than 10% of the applied activity was found in washes with 3 to 4 bed volumes of buffer (50 m*M* Tris (pH 7.8), 5 m*M* EDTA, and 15 m*M* 2-mercaptoethanol). Elution of more than 80% of the applied activity resulted when the column was washed with the same buffer containing 10 m*M* glucose 6-phosphate. This observation led to studies designed to determine the reason for incomplete binding of adipose tissue glycogen synthase to the affinity column.

In preliminary studies of affinity chromatography of glycogen synthase, it became apparent that either there were materials interfering with affinity binding or that the synthase must be heterogeneous. Thus, enzyme activity in crude homogenates was not retained at all by the affinity column. Only 20 to 40% of the enzyme activity obtained after the first ethanol precipitation step was retained and eluted with glucose 6-phosphate. Enzyme obtained after the second ethanol precipitation (Purification Procedure) was also retained by the affinity column but only to about the same extent as that obtained after the first ethanol precipitation step. The specificity of the affinity binding was not absolute. When the DEAE-cellulose chromatography step was omitted, the protein eluting from the affinity column with glycogen synthase migrated as two components during polyacrylamide gel electrophoresis. However, when the DEAE chromatography was performed prior to the affinity chromatography, glycogen synthase eluting from the affinity column with glucose 6-phosphate migrated as a single component on polyacrylamide gel electrophoresis (Figure 2, inset). The percentage binding to the affinity column after the DEAE step remained about the same.

The fraction of adipose tissue glycogen synthase binding to the affinity column (20–40%) was not significantly influenced by (1) the temperature at which the sample was applied, (2) the rate of sample application below 10 ml/cm^2 per hr, (3) the fraction of enzyme dependent upon glucose 6-phosphate for activity, (4) the ratio of sample size to column bed volume within limits (described below), or (5) the glycogen content of the sample.

The fraction of adipose tissue glycogen synthase (obtained after the second ethanol precipitation step of the Purification Procedure) retained by the affinity column was not influenced significantly if sample application was made at 4 or at 20°. Prior mixing of the sample with a slurry of the glucosamine 6-phosphate-agarose for up to 1 hr did not increase the fraction of the enzyme bound to the gel when compared with that bound by the usual sample application (10 ml/cm^2 per hr) to a column of similar bed volume.

The fraction of glycogen synthase in the D form did not seem to influence the fraction of the enzyme binding to the affinity column. In the case of purified rabbit skeletal muscle glycogen synthase, there was almost quantitative binding of the enzyme by the affinity column when the activity ratio was 0.43. The activity ratio of adipose tissue glycogen synthase obtained after step 4 (Purification Procedure) ranged from 0.05 to 0.50 (no attempt was made to inhibit glycogen synthase phosphatase during the purification procedure). Nevertheless, only 20 to 30% of the activity was retained by the affinity column. In addition, in one preparation with 40% of the enzyme in the I form, the enzyme retained by the column had the same percentage of synthase I activity as the enzyme not retained.

The application of a constant amount of adipose tissue glycogen synthase obtained after the second ethanol precipitation (step 4, Purification Procedure) to affinity columns of different bed volumes resulted in binding of about the same absolute amount of enzyme (Table III). This suggests that incomplete binding of the enzyme was not due to a limitation in the number of available binding sites on the affinity column.

Table III: Binding of Adipose Tissue Glycogen Synthase to the Affinity Column.[a]

Column Bed Vol (ml)	Fraction of Applied Act. Eluted in the Absence of Glucose 6-Phosphate	Fraction of Applied Act. Eluted with 20 m*M* Glucose 6-Phosphate
5	0.76	0.18
10	0.68	0.20
15	0.61	0.18

[a]Columns were packed with 4% agarose covalently linked to glucosamine 6-phosphate by means of an organic matrix (Experimental Section). The concentration of glucosamine 6-phosphate bound to the gel was approximately 2.9 μmol/ml of packed bed volume. The sample consisted of 2.7 ml of adipose tissue glycogen synthase from step 4 of the Purification Procedure. The columns were washed with 40 ml of buffer at 4° prior to elution of activity with glucose 6-phosphate. Columns were eluted with buffer (30 ml) containing 20 m*M* glucose 6-phosphate at 21°; buffer, 20 m*M* Tris, 5 m*M* EDTA, and 15 m*M* 2-mercaptoethanol (pH 7.8); column diameter, 1.5 cm; flow rate, approximately 20 ml/hr.

The possibility that glycogen present in the enzyme interferes with binding to the affinity column was suggested by results obtained with preparations of muscle glycogen synthase. The preparation used was the resuspended pellet containing glycogen and proteins obtained after the centrifugation step at 30,000 rpm in the phosphorylase kinase preparation of De Lange et al. (1968). This glycogen synthase preparation failed to bind to the affinity column prior to α-amylase digestion. Following hog pancreatic α-amylase digestion adequate to reduce the glycogen content of the sample by at least 70%, about 30% of the skeletal muscle enzyme was retained and could be recovered from the affinity column only by elution with buffer containing 10 m*M* glucose 6-phosphate. In contrast, addition of oyster glycogen (5 mg/ml) to purified muscle glycogen synthase prepared by the method of Soderling et al. (1970) did not significantly alter binding of the purified enzyme to the affinity column. Incubation of adipose tissue glycogen synthase (from step 4 of the Purification Procedure) with hog pancreatic α-amylase did not change affinity column recoveries although enzyme glycogen content was reduced by at least 80%.

When the matrix binding glucosamine 6-phosphate was omitted and glucosamine 6-phosphate was coupled directly to the cyanogen bromide activated agarose, or when glycine, or β-alanine, was substituted for glucosamine 6-phosphate, or succinylaminoethylamine, or succinylaminohexylamine was substituted for succinyldiaminodipropylamine, there was no binding of adipose tissue glycogen synthase to the derivatized agarose. When the length of the matrix was doubled, binding of the enzyme was significantly reduced.

Discussion

Glycogen synthase from rabbit skeletal muscle (Soderling et al., 1970; Brown and Larner, 1971), bovine heart (Thomas and Larner, 1973), rat liver (Lin and Segal, 1973), and swine kidney (Issa and Mendicino, 1973) has been purified to homogeneity using conventional techniques for protein isolation. We have succeeded in purifying swine adipose tissue glycogen synthase more than 400-fold using glucosamine 6-phosphate affinity chromatography after preliminary purification by ethanol precipitation and DEAE-cellulose adsorption. The studies suggest that adipose tissue glycogen synthase has hydrodynamic properties ($s_{20,w}$ = 8.5 S) significantly different from those of the enzyme from other tissues. The sulfate and phosphate activation of swine adipose tissue glycogen synthase I is similar to the effects of these anions reported for glycogen synthase I from liver and muscle (Mersmann and Segal, 1967; Rosell-Perez and Villar-Palasi, 1964). In contrast, Barash et al. (1973) reported that rat adipose tissue glycogen synthase, both the D and I forms, was inhibited by 5 m*M* inorganic phosphate, 5 m*M* Na_2SO_4, or 20 m*M* KF. These results suggest that there may be significant differences between the rat and the swine adipose tissue enzymes.

In studies of the purification of galactosyltransferase (UDP-galactose:*N*-acetylglucosamine galactosyltransferase), Baker et al. (1972) discussed affinity chromatography of glucosyltransferases using agarose immobilized ligands resembling (1) the nucleotide portion of the substrate and (2) the acceptor substrate. Galactosyltransferase from bovine milk whey was purified to constant specific activity using affinity chromatography on UDP-hexanolaminoagarose followed by affinity chromatography on either *N*-acetylglucosamine-hexanolaminoagarose or α-lactalbumin-agarose. In the same study it was shown that rabbit muscle glycogen synthase could be significantly purified using UDP-hexanolaminoagarose affinity chromatography. The starting material for affinity chromatography was the amylase-digested glycogen-protein fractions prepared as described by Soderling et al. (1970). Although recoveries are not given, it appears from the published elution profile that skeletal muscle glycogen synthase is retained on UDP-hexanolaminoagarose as it is on glucosamine 6-phosphate-succinyldiaminodipropylaminoagarose. Elution of the enzyme from the UDP-agarose column was performed using buffer containing glycogen (Baker et al., 1972). It is noteworthy that the enzyme was not eluted from the UDP-agarose column by buffer containing UDP. In contrast, skeletal muscle glycogen synthase bound to the glucosamine 6-phosphate affinity column is readily eluted by the specific ligand, glucose 6-phosphate.

The studies of the binding of adipose tissue glycogen synthase to the glucosamine 6-phosphate affinity column strongly suggest that there are at least two populations of glycogen synthase molecules only one of which is retained by the affinity column. The explanation for this heterogeneity remains to be determined. The possibility remains that extremely small amounts of enzyme-associated glycogen, unavailable to α-amylase digestion, are at least in part responsible for the incomplete binding of enzyme by the affinity column. Enzyme-associated lipid may also play a role. The possibility that the adipose tissue enzyme sample contains a protein which competes with glycogen synthase for glucose 6-phosphate binding seems unlikely because of the large number of binding sites on the affinity gel (2–3 μmol/ml) and also because increasing the number of binding sites did not increase the absolute amount of enzyme bound to the gel (Table III). Finally, in view of the recent work of Huang and Cabib (1974), the possibility that proteolysis converts some of the native enzyme to a form with altered affinity for glucose 6-phosphate must be considered.

The specific activity of purified swine adipose tissue glycogen synthase determined in the presence of glucose 6-phosphate was 1.86 μmol/min per mg (Table I). Under similar assay conditions the specific activity of purified glycogen synthase from other tissues was significantly greater: rabbit skeletal muscle, 4.7 (Soderling et al., 1970) and 15.1 (Brown and Larner, 1971); bovine heart, 1.0–4.7 (Thomas

and Larner, 1973); swine kidney, 9.1 (Issa and Mendicino, 1973); and rat liver, 35.4 (Lin and Segal, 1973). The purification required to achieve apparent homogeneity varied from 470-fold for the enzyme from rabbit skeletal muscle (Soderling et al., 1970) to more than 10,000-fold for glycogen synthase from swine kidney (Issa and Mendicino, 1973). In view of the considerable variation in the specific activities reported for purified glycogen synthase from other tissues, it is difficult to assign significance to the fact that specific activity of the adipose tissue appears to be lower than that for the enzyme from other sources. The possibility that the enzyme is not completely free of contaminant proteins must be considered. The possibility that inactivated glycogen synthase copurifies with active molecules may also partly explain the relatively low specific activity.

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