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Purification and Properties of Uridine Diphosphate Glucose–Glycogen Glucosyltransferase from Rat Liver*

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ABSTRACT: Soluble uridine diphosphate glucose–glycogen glucosyltransferase has been purified approximately 1500-fold from rat liver by means of a procedure which utilizes reversible thermal inactivation of the enzyme as a means of removing it from the particulate glycogen to which it is bound. The most highly purified preparations were found to be heterogeneous mixtures of protein containing small amounts of phosphorylase, branching enzyme, and glycogen. Further studies of reversible inactivation indicated that it involves either a change in conformation of the enzyme protein or dissociation of the enzyme molecule into smaller, less active (or inactive) units which, in either case, do not bind effectively to glycogen. Glucosyltransferase from muscle did not undergo such a transformation on heating at 37° and differed in other regards from the hepatic enzyme. The structural requirements for stimulation of hepatic glucosyltransferase by hexose phosphate have been defined. Studies of the glucose-6-phosphate stimulation of glucosyltransferase have shown that glucose-6-phosphate decreases the inhibitory effect of uridine diphosphate in a partially competitive manner. From these and other data a more specific hypothesis regarding the mechanism of glucose-6-phosphate stimulation has been formulated.

Leloir and Goldemberg (1960) localized UDP-glucose–glycogen glucosyltransferase (EC 2.4.1.11; Leloir and Cardini, 1957) to the particulate glycogen fraction of liver homogenates, and utilized the association with glycogen as a means for partially purifying the enzyme. They reported that such preparations of glucosyltransferase were stimulated severalfold, and also protected from thermal inactivation, by glucose-6-P. Activity could be eluted from particulate glycogen with glycogen of low molecular weight, lending support to the view of Luck (1961) that the enzyme is bound directly to glycogen molecules rather than to fragments of endoplasmic reticulum. In studies of thermal inactivation of liver glucosyltransferase Steiner (1961) found that partially inactivated preparations could be restored to full activity by incubating them with glucose-6-P and certain salts. Moreover, the less active form of the enzyme was not bound to glycogen particles and could be separated from them by high-speed centrifugation. These observations provided a means for preparing soluble hepatic glucosyltransferase almost free of polysaccharide. In this paper the purification procedure is described. Evidence is presented which indicates that reversible inactivation leads to dissociation of the

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enzyme into a more slowly sedimenting component which is less active (or inactive). Studies of the specificity and mechanism of stimulation of glucosyltransferase by glucose-6-P are also included.

Experimental Procedure

ATP, UTP, UDP, and UDP-glucose were obtained from Pabst Laboratories, Inc. Glucose-6-P and fructose-6-P were obtained from Calbiochem Corp., and galactose-6-P from Mann Chemical Co. 3′,5′-AMP was kindly supplied by Dr. Murray Rabinowitz and 1,5-anhydroglucitol-6-P by Professor Robert K. Crane.

Methods for the preparation of UDP-[^{14}C]glucose and for extraction of glycogen have been described elsewhere (Steiner *et al.*, 1961; Steiner and King, 1964). Mannose-6-P, 2-deoxyglucose-6-P, and glucosamine-6-P were prepared from the free sugars with yeast hexokinase, and were purified by column chromatography (Hurlbert and Potter, 1954). Commercially available fructose-1,6-diP was purified by gradient elution chromatography on a Dowex 1 (formate) column. Sorbitol-6-P was prepared by reduction of glucose-6-P with sodium borohydride (Abdel-Akher *et al.*, 1951), and purified by column chromatography. D-Glucose-6-*O*-sulfate was prepared by direct sulfation of D-glucose with the pyridine–sulfur trioxide reagent according to the procedure of Lloyd (1960). The crude ester sulfate fraction was purified by gradient elution chromatography on a Dowex 1 (formate) column using 1.0 to 5.0 M ammonium acetate, *p*H 6.6, for elution. A single peak was eluted at a concentration of approximately 1.8 M ammonium acetate and the ester crystallized from ethanol as the potassium salt. After hydrolysis in 4 N HCl for 10 hours at 100°, inorganic sulfate (Dodgson, 1961) was found in amounts corresponding to theoretical for the monosulfate derivative. Analysis by both diphenylamine and anthrone yielded the expected amounts of saccharide for the monosulfate derivative. Infrared spectroscopy revealed the presence of absorption bands at 1250 and 820 cm^{-1}, corresponding to the S=O and C—O—S vibrations, respectively, of authentic hexose-6-*O*-sulfate esters (Lloyd and Dodgson, 1961). The compound did not react with glucose oxidase but was oxidized very slowly by glucose-6-P dehydrogenase.

Analytical Procedures. Glycogen was determined with anthrone (Roe, 1955) after digestion in hot KOH (Good *et al.*, 1933). Protein was measured by the method of Lowry *et al.* (1951) after precipitation with 5% trichloroacetic acid to eliminate turbidity from glycogen.

Enzyme Assay Procedures. Glucosyltransferase activity was estimated by measuring the amount of [^{14}C]glucose incorporated into glycogen from UDP-[^{14}C]glucose. The reaction mixture contained 0.05 M potassium glycerophosphate[1] buffer, *p*H 7.4; 10 mg/ml glycogen; 0.0015 M UDP-[^{14}C]glucose (approximately 0.1 μc/ml), 0.01 M potassium glucose-6-P (when added). Enzyme preparation (5–10 μl) was added to 50 μl of reaction mixture. After incubation for 10 minutes at 37° the reaction was stopped by addition of 0.5 ml of 15% KOH and 2 mg of glycogen to serve as carrier. The tubes were heated 5 minutes at 100° and, after cooling, glycogen was precipitated by addition of 1 ml of 95% ethanol. After incubation for at least 1 hour in an ice bath the tubes were centrifuged. The glycogen was dissolved in 0.5 ml of 0.05 N HCl to neutralize residual alkali and precipitated by addition of 1 ml of ethanol. After 1 hour or longer in an ice bath the tubes were centrifuged. The glycogen pellet was dried, dissolved in water, and an aliquot transferred to an aluminum planchet. The samples were dried, and the radioactivity measured in a windowless gas-flow counter (Instrument and Development Products Co., Chicago, Ill.). After appropriate correction for self-absorption, the amount of glucose incorporated into glycogen was calculated. Since incorporation followed first-order kinetics, the results were normalized arbitrarily to the time necessary for incorporation of 10% of the substrate, and were expressed as μmoles per hour per unit of tissue or protein. One unit of activity was chosen to represent the amount of enzyme which catalyzed the incorporation of 1 μmole of glucose into glycogen per hour.

Glycogen phosphorylase activity was measured in a reaction mixture containing 0.02 M sodium glucose-1-P, 1% glycogen, 0.05 M sodium glycerophosphate, and 0.002 M 5′-AMP, *p*H 6.5. The enzyme preparation (50 μl) was added to 0.5 ml of the reaction mixture and incubated for the desired interval at 37°. The reaction was stopped by addition of 1 ml of 4% trichloroacetic acid, and P_i released from glucose-1-P was measured by the method of Gomori (1942).

Branching enzyme activity was detected by incubating enzyme preparations in the same solution used for glucosyltransferase activity until 100% incorporation of labeled glucose into glycogen had occurred. The glycogen was then isolated and degraded with excess crystalline β-amylase (sweet potato), and the amount of label retained in the limit dextrin was used as an indication of branching activity.

Purification of Glucosyltransferase from Rat Liver. Eight female Sprague-Dawley rats weighing 160–180 g were used for each batch of enzyme. The rats were fasted for 3 days, then refed approximately 16 hours before the experiment. After cervical transection, the livers were quickly perfused via the portal vein with cold 0.3 M sucrose containing 0.005 M EDTA, *p*H 7.4, to eliminate blood and cool the liver rapidly. The liver was homogenized in 5 volumes of cold 0.4 M sucrose, 0.01 M EDTA, *p*H 7.4, with a Teflon-glass Potter-Elvehjem device. It was essential to carry out all the succeeding steps at 5°. The homogenate was centrifuged at 800 × *g* to remove cell debris and nuclei. The supernatant from this step, which contained all the activity, was centrifuged at 5000 × *g* to remove mito-

[1] Composition, approximately 40% α- and 60% β-glycerophosphate (Mann Chemical Co.).

chondria and again at 35,000 × *g* for 90 minutes in a Servall centrifuge. The dense particulate glycogen was compacted into a translucent clear pellet at the bottom of the tube, well demarcated from overlying red-brown microsomal material which was carefully rinsed away without disturbing the glycogen pellet. The glycogen was suspended in 40 ml of 0.4 M sucrose, 0.005 M EDTA, *p*H 7.4, by gentle homogenization. The suspension of particulate glycogen was centrifuged 10 minutes at 5000 × *g* to remove residual mitochondria, and the particulate glycogen was resedimented at 100,000 × *g* for 45 minutes in a Spinco Model L centrifuge. Any microsomal material still adhering to the pellet was carefully rinsed away and the particulate glycogen was suspended in 40 ml of 0.4 M sucrose containing 2 mg/ml dialyzed crystalline bovine serum albumin (BSA,[2] Pentex), *p*H 7.2. The suspension was heated for 10 minutes at 37°, iced, and centrifuged at 100,000 × *g* for 45 minutes. The clear, supernatant solution was carefully aspirated from above the glycogen pellet, which was then discarded. Further extraction of the pellet at this stage yielded only small amounts of glucosyltransferase. Sufficient finely ground ammonium sulfate (enzyme grade, Mann Chemical Co.) to achieve 65% saturation was added to the supernatant solution in a small beaker in an ice bath and dissolved by swirling the fluid gently for several minutes. The faintly turbid solution was then allowed to stand overnight at 2° during which time a small flocculent precipitate appeared. The precipitate was collected by centrifugation at 10,000 × *g* for 10 minutes. The supernatant was discarded and the precipitate was dissolved in 1 ml of 0.4 M sucrose, 0.005 M EDTA, 0.05 M diglycine buffer, *p*H 7.4. The resulting solution was clear and faintly yellow in color. Further purification was achieved in some experiments by washing the protein precipitate with a small volume of 30% ethanol in 0.4 M sucrose, 0.005 M EDTA at −12°, which eluted residual albumin and several other proteins, but did not dissolve or inactivate glucosyltransferase. After centrifugation at −8° the pellet was carefully drained free of ethanol-sucrose and dissolved in a small volume of sucrose-EDTA-diglycine buffer. Alternatively the concentrated enzyme solution obtained after ammonium sulfate treatment (protein concentration, 3–5 mg/ml) was refractionated by addition of saturated ammonium sulfate solution. Saturation of 35–40% was sufficient to precipitate glucosyltransferase, eliminating albumin and other more soluble proteins.

Preparation of Glucosyltransferase from Rat Muscle. Approximately 200 g of skeletal muscle was ground in a meat grinder in a cold room and suspended in 200 ml of cold 0.005 M EDTA, *p*H 7.2. After 30 minutes 100 ml of cold 1 M sucrose was added and the mixture was allowed to stand for 10 minutes longer. The mixture was centrifuged at 5000 × *g* for 10 minutes and the pellet was discarded. The supernatant was centrifuged at 35,000 × *g* for 90 minutes. The small translucent pellets were suspended in 5 ml of 0.4 M sucrose, 0.005 M EDTA, yielding a milky suspension which was again centrifuged 10 minutes at 5000 × *g* to eliminate residual mitochondria. Glucosyltransferase activity was enhanced some 50- to 60-fold over that of the initial aqueous extract. Phosphorylase activity was present in amounts equivalent to ten to twenty times glucosyltransferase activity. Only about 1% of the phosphorylase activity was detected in the absence of 5′-AMP indicating the predominance of the *b* form, as would be expected with extraction in the presence of EDTA (Fischer and Krebs, 1958).

Density Gradient Centrifugation. Soluble enzyme sample (100–200 μl) dissolved in 0.25 M sucrose, 0.005 M EDTA, 0.02 M diglycine, *p*H 7.4, was layered on 4.5 ml 10–40% linear sucrose gradients containing 0.02 M diglycine buffer, *p*H 7.4, 0.005 M EDTA, and 2 × 10^4 M UDP or 0.1 M KF and 0.01 M glucose-6-P. The tubes were centrifuged 20 hours at 37,000 rpm in the SW-39 rotor at 2° in a Spinco Model L centrifuge. The bottoms of the tubes were pierced with a needle and fractions were collected in the cold for measurement of protein and enzyme activity as noted in the text. Crystalline rhodanese, sweet potato β-amylase, and bovine serum albumin were used as markers to verify the accuracy of calculated $s_{20,w}$ values (Martin and Ames, 1961).

Gel Electrophoresis. Gel electrophoresis was done by a modification of the method of Goldwasser *et al.* (1962). Polyacrylamide gels (6.4% w/v) were prepared in an aqueous medium containing 0.4 M sucrose and 0.15 M imidazole·HCl, *p*H 8.2. The gels were formed in shallow plastic trays with a 1-mm-wide slit located 2 cm from the cathodal end of the block large enough to contain 20–30 μl of sample. The trays were placed in contact with an ice bath in the refrigerator and connected by six thicknesses of Whatman 3MM paper strips from each end of the gel block to reservoirs containing 130 ml of 0.4 M sucrose, 0.15 M imidazole·HCl, *p*H 8.2. Electrophoresis was carried out for 3 hours at 200 v (5.0 ma per block). The gels were stained with 1% Amido Schwartz (K and K Laboratories, Inc.) in 7.5% acetic acid for 15 minutes, then washed repeatedly with 7.5% acetic acid and stored in 50% ethanol–7.5% acetic acid solution.

Results

Purification of Hepatic Glucosyltransferase. Using the procedure described here we consistently obtained yields of soluble glucosyltransferase amounting to about 10% of the original activity of the homogenate at levels of purification up to 1500-fold. The results are summarized in Table I. For optimum results it was essential to build up the glycogen level in the liver as much as possible. This was accomplished by starving animals for 3 days and refeeding them 12–16 hours before preparing enzyme. Glycogen reached a level of 10–12% at this time after refeeding, and the particulate

[2] Abbreviation used in this work: BSA, bovine serum albumin.

TABLE I: Purification of Glucosyltransferase from Rat Liver.

Fraction	Description	Glycogen Phosphorylase (units/ml)	UDPG Glycogen Glucosyltransferase (units/ml)	(total units)	(units/mg protein)	Relative Purification	Yield (%)
HS	Crude homogenate, after centrifugation at 800 × *g*	30	13.5	4200–4700	0.55	1	100
P-1	Particulate glycogen,[a] washed once	80	70	2400–3000	150–180	300	60
S	Supernatant from heated P-1		55	1300–1700	[b]		33
SAS	Soluble enzyme,[c] after $(NH_4)_2SO_4$ treatment	200–300	800–1200	800–1200	150–300	410	22
E	SAS, pellet washed with 30% ethanol–0.4 M sucrose		450–650	450–650	500–600	1000	12
SE	SAS, reprecipitated with 35% satd $(NH_4)_2SO_4$, washed with 30% ethanol–0.4 M sucrose	40	500–900	250–300	900	1650	6

[a] Glycogen concentration, 60–80 mg/ml. [b] BSA added, 2 mg/ml. [c] Glycogen concentration, 0.25–1.0 mg/ml.

fraction, which constituted over 50% of the total liver glycogen, could be cleanly separated by differential centrifugation. Glucosyltransferase in the washed particulate glycogen fraction (Table I, P-1) was purified about 300-fold (Leloir and Goldemberg, 1960). Glucose-6-P was not added to the medium used to wash the particulate glycogen since its presence, even at low concentration, interfered with subsequent heat inactivation of the enzyme and decreased the yield of soluble enzyme. Also, the particulate glycogen was washed only once with a relatively small volume of sucrose-EDTA medium. Further washing in the absence of glucose-6-P resulted in dissociation of some enzyme from the glycogen into the wash medium.

Both inactivation and reactivation of glucosyltransferase in particulate glycogen preparations were found to be highly dependent not only upon the time and temperature employed (Steiner, 1961) but also upon the concentration of the preparation and the composition of the medium. As shown in Figure 1, dilution resulted in more extensive inactivation and less complete reactivation upon subsequent incubation with 0.2 M KF and 0.01 M glucose-6-P (Steiner, 1961). Under the conditions routinely employed for inactivation, 50–80% of initial activity was lost during 10 minutes' incubation at 37°, but a large proportion of the original activity subsequently could be recovered upon incubation with glucose-6-P and KF. Prolongation of incubation resulted in irreversible loss of activity, owing presumably to denaturation of the inactive form. The presence of sucrose retarded denaturation during incubation at 37°. On the other hand, EDTA retarded inactivation and it was therefore omitted from the medium used to suspend the particulate glycogen during the heating step. Addition of BSA to this medium stabilized the inactive enzyme protein, increasing the recovery of soluble enzyme when the supernatant (Table I, S) was treated with ammonium sulfate (Table I, SAS). The small amount of BSA which was precipitated by ammonium sulfate was estimated by gel electrophoresis

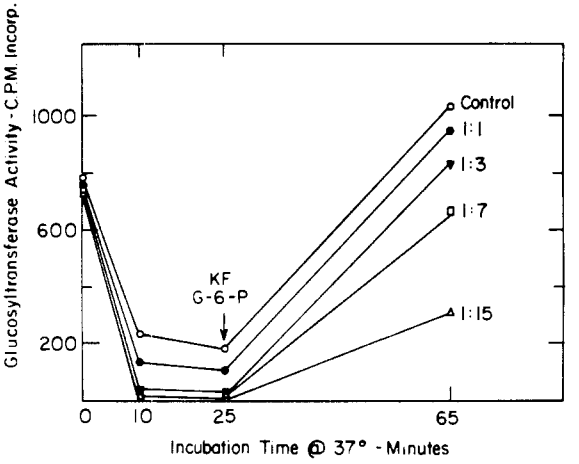

FIGURE 1: Effect of dilution upon inactivation and reactivation of hepatic glucosyltransferase. The enzyme consisted of the washed particulate glycogen fraction prepared as described under Experimental Procedure and diluted in 0.4 M sucrose, 0.005 M EDTA, *p*H 7.4. The preparations were heated for 25 minutes at 37°. Glucose-6-P (0.01 M), and 0.2 M KF were then added and incubation was continued 40 minutes. Aliquots were removed at the times indicated for glucosyltransferase assay in the presence of glucose-6-P as described under Experimental Procedure. Activity measurements were corrected for dilution before graphing.

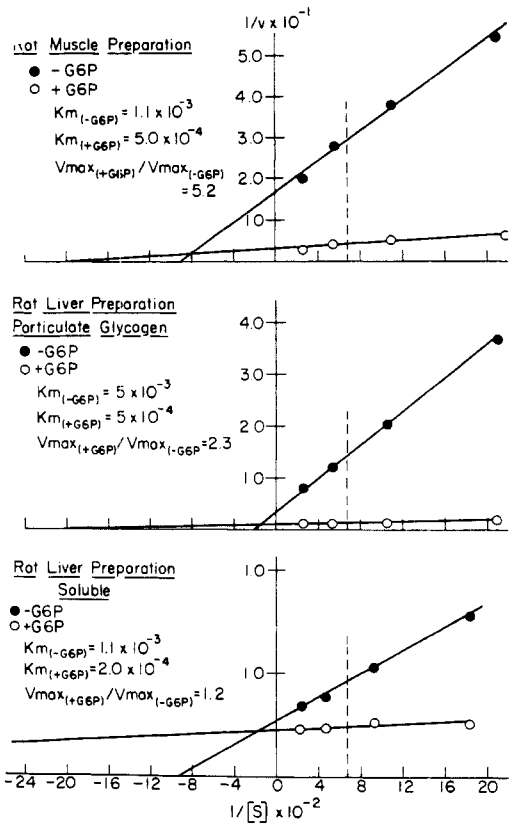

FIGURE 2: The K_m of UDP-glucose and V_{max} in the presence and absence of glucose-6-P of several rat glucosyltransferase preparations. Data plotted by the method of Lineweaver and Burk (1934). The incubation system and isolation procedures are described under Experimental Procedure.

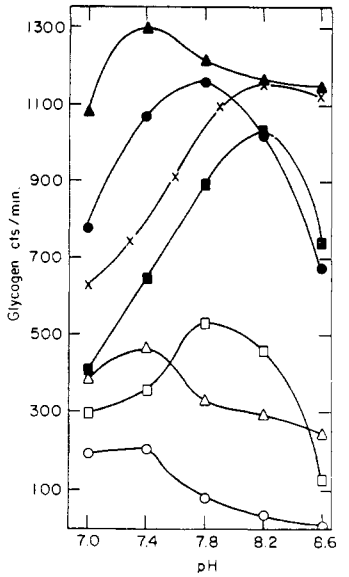

FIGURE 3: The *p*H dependency of the glucosyltransferase reaction determined in various buffer mixtures. The enzyme preparation consisted of the washed particulate glycogen fraction prepared as described under Experimental Procedure. Assay conditions were as described under Experimental Procedure. Open symbols represent results without glucose-6-P added to the reaction mixture; solid symbols represent results with 0.01 M glucose-6-P added to the reaction mixture. —△—, 0.05 M potassium glycerophosphate; —○—, 0.1 M Tris-HCl; —□—, 0.1 M Tris-SO_4; —×—, 0.1 M Tris-maleate.

to account for about 40% of the total protein of the SAS fraction. The dilute S fractions usually underwent as much as a 10-fold increase in activity when incubated with glucose-6-P and KF, but SAS fractions were generally highly active without further treatment, indicating that considerable reactivation occurred either during ammonium sulfate precipitation or immediately upon solution of the enzyme precipitate.

Attempts to fractionate soluble glucosyltransferase preparations with cold ethanol resulted in destruction of enzymatic activity. However, the precipitated protein could be washed with a mixture of 30% ethanol in 0.4 M sucrose before it was dissolved with a significant gain in specific activity (Table I, E). Preparations comparable to fraction E could be obtained by refractionating fraction SAS with ammonium sulfate between 0 and 35% saturation. When the precipitated protein was also washed with cold ethanol-sucrose, material of highest specific activity was obtained (Table I, SE).

Both SAS and E fractions were clear, faintly yellow solutions which contained only small amounts of residual glycogen (~0.05% of P-1, Table I). Most of the soluble preparations showed only about 5% of maximal activity when tested in the absence of glycogen primer. The preparations could be stored frozen at −18° for long periods without much loss of activity. Residual ammonium sulfate appeared to be partly responsible for their stability since preparations that were dialyzed against sucrose-EDTA-diglycine or washed with 30% ethanol-sucrose lost activity more rapidly in the freezer. However, activity could be restored almost completely by incubation with glucose-6-P and KF, indicating slow conversion to the inactive form during frozen storage.

Polyacrylamide gel electrophoresis of SAS preparations revealed the presence of at least five protein bands, the most prominant corresponding to BSA. After refractionation with ammonium sulfate or washing with ethanol-sucrose only two bands remained. Glucosyltransferase activity could not be recovered from any portion of the gel after electrophoresis. Phosphorylase activity was associated with the protein band of lowest anodal mobility.

Soluble glucosyltransferase preparations were free of glucokinase, glucose-6-P phosphatase, ATPase, phosphoglucomutase, hexoseisomerase, and glucose-6-P dehydrogenase. α-Amylase remained firmly bound to the particulate glycogen during incubation and

TABLE II: Inhibition of Glucosyltransferase by *N*-Ethylmaleimide and *p*-Mercuribenzoate.[a]

Enzyme Source	Inhibitor	Inhibitor Concentration (moles/liter)	Glucosyltransferase Activity −Glucose-6-P (% control)	Glucosyltransferase Activity +Glucose-6-P (% control)
Liver, particulate glycogen	*N*-Ethylmaleimide	1.8×10^{-6}	100	100
		1.8×10^{-5}	70	72
		1.8×10^{-4}	<1	<3
	p-Mercuribenzoate	1.8×10^{-6}	70	88
		1.8×10^{-5}	0	<4
		1.8×10^{-4}	0	0
Muscle	*N*-Ethylmaleimide	3.4×10^{-6}	76	81
		3.4×10^{-5}	18	19
		3.4×10^{-4}	<1	<2
	p-Mercuribenzoate	3.4×10^{-6}	90	96
		3.4×10^{-5}	20	37
		3.4×10^{-4}	0	0

[a] Nine parts of enzyme preparation were added to one part of neutral inhibitor solution to give the final inhibitor concentration shown. The mixtures were incubated for 1 hour at 3° and then assayed as described in the methods section. Assay resulted in 10-fold dilution of the inhibitor.

centrifugation and was eliminated. Only glycogen phosphorylase and small amounts of branching-enzyme activity were detected in the soluble preparations. Purification of glucosyltransferase relative to phosphorylase is shown in Table I. The relative proportion of phosphorylase steadily declined during purification. It was possible to separate glucosyltransferase from phosphorylase by means of sucrose gradient centrifugation (see Figure 5). The presence of phosphorylase did not affect the assay of glucosyltransferase activity, provided inorganic phosphate was excluded from reaction mixtures.

The ultraviolet absorption spectrum of soluble preparations was characteristic of protein (260/280 ratio = 1.01). Only small amounts of ribose and phosphorus were found, compatible with the estimate from the ultraviolet data of less than 5% contamination by ribonucleic acid.

Properties of Partially Purified Glucosyltransferase Preparations. Soluble glucosyltransferase preparations were similar in many properties to nonheated, less highly purified enzyme preparations. In general, glucose-6-P produced a less marked increment in activity of soluble preparations, but this was owing in part to the presence of the sulfate anion (Traut and Lipmann, 1963). Dialysis lowered the level of activity in the absence of glucose-6-P.

Figure 2 shows double-reciprocal plots according to the method of Lineweaver and Burk (1934), which permit comparison of V_{max} and apparent K_m of UDP-glucose of several rat glucosyltransferase preparations. The V_{max} of the soluble preparation was not increased so much by glucose-6-P as was V_{max} of the enzyme associated with the particulate glycogen or the muscle enzyme. Also the apparent K_m for UDP-glucose of soluble preparations, both with and without glucose 6-P, was somewhat lower than that of particulate glycogen preparations.

Substances found to inhibit or inactivate hepatic glucosyltransferase were DFP (30% inhibition at 10^{-3} M), $MgCl_2$ (>0.01 M), and KI (>0.1 M). KCl and NaCl (0.1–0.2 M) did not inhibit glucosyltransferase and were almost as effective as KF in stabilizing and reactivating the enzyme in the presence of glucose-6-P. $(NH_4)_2SO_4$ and Na_2SO_4 were less effective than the halides for reactivation. A number of substances did not affect glucosyltransferase, including insulin, citrate, CN^-, palmitate (5×10^{-4} M), imidazole, UTP, ATP, 5′-UMP, 5′-AMP, and 3′,5′-AMP (2×10^{3} M).

Effect of Sulfhydryl Inhibitors. Both *N*-ethylmaleimide and *p*-mercuribenzoate inhibited glucosyltransferase strongly. The data in Table II show that the two inhibitors were equally effective in inhibiting muscle glucosyltransferase, but the *p*-mercuribenzoate was a more powerful inhibitor of liver glucosyltransferase. *N*-Ethylmaleimide inhibited activity measured in the presence or absence of glucose-6-P to the same extent, whereas *p*-mercuribenzoate inhibited activity without glucose-6-P slightly more strongly. The thiol inhibitors thus appeared to interact chiefly with the substrate binding site of the enzymes rather than with the site(s) concerned with glucose-6-P binding. Reduced glutathione or cysteine produced small increments of activity (5–10%) when tested on fresh glucosyltransferase preparations. Preparations that had been stored in the freezer for long periods were sometimes stimulated 50% or more when incubated with reduced glutathione. Reversible inactivation was not affected by either cysteine or GSH.

Effect of pH on Glucosyltransferase Activity. The *p*H dependency of glucosyltransferase was affected markedly by the nature of the buffer system employed

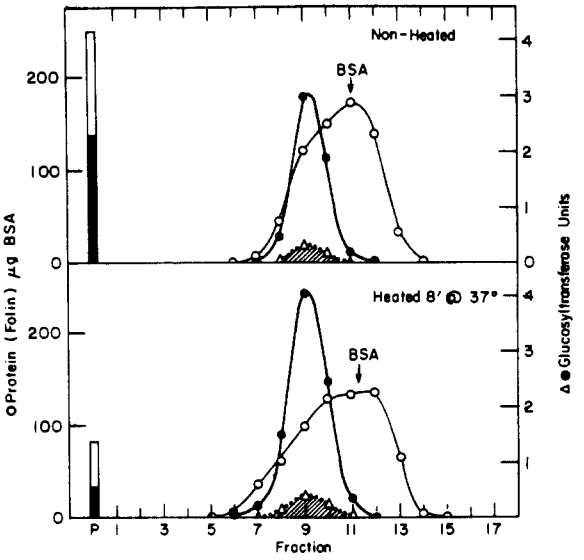

FIGURE 4: Sucrose-gradient centrifugation of soluble glucosyltransferase (SAS, Table I). The enzyme preparation was dialyzed 4 hours against a solution containing 0.25 M sucrose, 0.005 M EDTA, and 0.05 M diglycine, *p*H 7.4. Of this preparation, 150 μl was layered on a 10–40% sucrose gradient, and centrifuged as described under Experimental Procedure. A duplicate aliquot was heated 8 minutes at 37° before centrifugation (lower diagram). Assays for protein and glucosyltransferase were carried out as described under Experimental Procedure. The small cross-hatched area under the peak (open triangles) represents glucosyltransferase activity, measured with glucose-6-P added, before reactivation. The solid circles show the activity measured after incubation of the same fractions for 30 minutes at 37° in a medium containing 0.01 M glucose-6-P and 0.2 M KF. The closed bar, P, depicts the glucosyltransferase activity in the pellet before reactivation; the open bar depicts the increment in activity of this fraction after reactivation with KF and glucose-6-P.

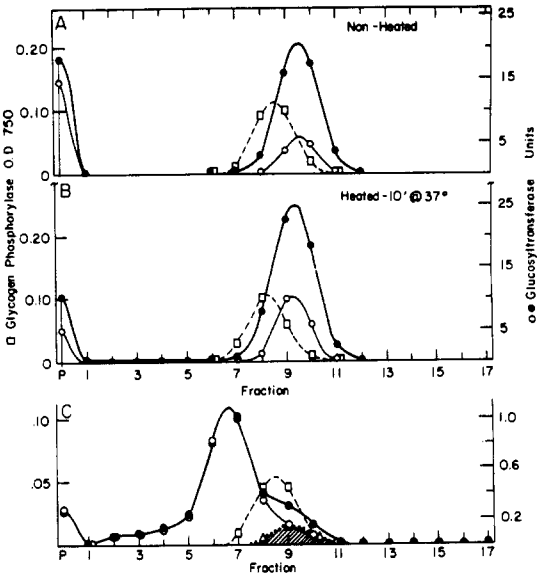

FIGURE 5: Sucrose-gradient centrifugation of soluble glucosyltransferase, before and after reactivation. The experiments shown in diagrams A and B were carried out using a dialyzed soluble preparation as described in the legend for Figure 4, except that this preparation was approximately 5-fold more concentrated with respect to glucosyltransferase activity. The preparation shown in diagram B was incubated for 10 minutes at 37° before centrifugation. Open circles represent glucosyltransferase activity before reactivation; solid circles represent activity after reactivation. Diagram C: the material from the 6–7 S glucosyltransferase peaks of gradients A and B were combined, reactivated by incubating 30 minutes at 37° in the presence of 0.01 M glucose-6-P and 0.2 M KF, concentrated by dialysis against polyethylene glycol (Carbowax, 20 M; Union Carbide Chemicals Co.), and equilibrated with a solution containing 0.25 M sucrose, 0.01 M glucose-6-P, 0.1 M KF, and 0.02 M diglycine, *p*H 7.4. This preparation (150 μl) was layered over a 10–40% sucrose gradient containing 0.1 M KF and 0.01 M glucose-6-P (added to maintain the enzyme in the active form) and centrifuged as described under Experimental Procedure. Fractions were assayed for glucosyltransferase and phosphorylase activity as described under Experimental Procedure. Open circles represent glucosyltransferase activity before incubation of fractions at 30° to promote reactivation. Solid circles represent activity after incubation at 30°. Triangles represent the difference between the two curves.

(Figure 3). The optimum *p*H in Tris-maleate buffer was 8.2 with glucose-6-P present as reported by Leloir and Goldemberg (1960). In Tris-HCl and Tris-SO_4 optima were near *p*H 8.0 in the presence of glucose-6-P, but in its absence were shifted to the region between *p*H 7.4 and 7.8. Similar results have been reported for lamb muscle and yeast glucosyltransferase (Traut and Lipmann, 1963; Algranati and Cabib, 1962). With glycerophosphate buffer a broad maximum at *p*H 7.4 was observed in both the presence and absence of glucose-6-P (Figure 3). Soluble preparations gave comparable results in glycerophosphate buffer. It is noteworthy that the *p*H as well as the nature of the buffer employed for assay can markedly affect the apparent glucose-6-P sensitivity of hepatic glucosyltransferase.

Nature of Reversible Inactivation. The most highly purified soluble preparations of glucosyltransferase declined in activity when heated at 37° in sucrose-EDTA medium in the absence of glucose-6-P or UDP, as observed previously with particulate glycogen preparations (Steiner, 1961). Inactivation resulted in both decreased ability to bind to glycogen and de-

creased susceptibility to glucose-6-P stimulation.[3] Incubation of the inactivated preparations with KF and glucose-6-P restored the initial level of activity, thus supporting the original conclusion that reversible inactivation is due to an alteration of the protein structure which is not the result of an enzymatic process.

Evidence for a change in enzyme structure on inactivation was obtained by means of sucrose-gradient centrifugation of soluble preparations. A modified gradient procedure using a higher sucrose concentration, 10–40%, with 0.0002 M UDP was necessary to prevent complete destruction of activity during prolonged centrifugation. With this combination 60–80% of the initial activity could be recovered in the fractions after centrifugation. Figure 4 shows the result obtained when a dialyzed soluble preparation (fraction SAS) was centrifuged with and without prior incubation for 8 minutes at 37°. The preparation before heating was approximately 50% inactive[4] and was inactivated an additional 50% during the 8 minute incubation. A single symmetrical peak of activity was found in a position corresponding to 6–7 S. Incubation of this fraction at 37° with BSA, KF, and glucose-6-P resulted in a greater than 10-fold increase in activity. Glucosyltransferase activity was also found at the bottom of the tube along with the residual glycogen of the soluble preparation. This fraction consisted mainly of active enzyme.

Heating before centrifugation significantly increased the proportion of the glucosyltransferase activity recovered in the 6–7 S peak and reduced the activity remaining at the bottom of the tube. Glycogen was not required for reactivation of the 6–7 S material. However, essentially no activity was observed when this fraction was assayed without glycogen primer. The 6–7 S peak thus appears to be the inactive form of the enzyme dissociated from glycogen, while the pellet contains active enzyme aggregated with small amounts of glycogen. The peak of Folin reacting material shown in Figure 4 (4–5 S) corresponded to BSA added to the preparation prior to centrifugation. It is of interest that the 6–7 S fraction was stimulated to the same extent by glucose-6-P (before reactivation) as was the dialyzed starting material used in this experiment. Incubation of the 6–7 S fraction with $MgCl_2$ and Na_2SO_3 at 30° (Hizukuri and Larner, 1963, 1964) resulted in significant reactivation but no change in relative sensitivity to glucose-6-P.

In the experiment shown in Figure 5 the concentration of soluble glucosyltransferase added to the gradients was increased 5-fold. Incubation before centrifugation increased the amount of activity recovered in the 6–7 S peak at the expense of the activity in the pellet as observed before (Figure 5A,B). However, the more concentrated 6–7 S material was now proportionately much more active before incubation with KF and glucose-6-P than was the case in the previous experiment (Figure 4). This behavior suggests that the 6–7 S fraction may be a mixture of several forms of glucosyltransferase, either inactive or of intermediate activity, in a mobile equilibrium which is sensitive to enzyme concentration as well as to ionic strength, temperature, and specific configurational stabilizers such as glucose-6-P and UDP.

To observe the effect of reactivation upon sedimentation behavior, 6–7 S fractions were collected from several gradients, incubated with KF and glucose-6-P, concentrated by dialysis against polyethylene glycol, and then equilibrated by dialysis with 0.25 M sucrose containing 0.1 M KF and 0.01 M glucose-6-P to yield a clear colorless preparation of active glucosyltransferase with some phosphorylase activity. This material was centrifuged through 10–40% sucrose gradients containing 0.1 M KF and 0.01 M glucose-6-P. The result is shown in Figure 5C. The peak of glucosyltransferase activity now shifted to the 9–10 S region of the gradient with significant trailing of activity toward heavier regions of the tube and into the pellet. A shoulder was also present on the high side of the peak, which contained small amounts of material which reactivated upon incubation of the fractions at 30°. When the difference between the curves of activity before and after incubation at 30° was plotted, a small peak was evident in the 6–7 S region where the inactive component was previously found. The position of the phosphorylase peak was not changed (7–8 S), indicating that only the sedimentation properties of glucosyltransferase had been altered by treatment with KF and glucose-6-P. Since glycogen was not present, the increase in sedimentation velocity cannot be attributed to polysaccharide binding, but, instead, is suggestive of the association of inactive or less active subunits to form an active polymer.

Structural Requirements for Glucose-6-P Stimulation. Leloir and Goldemberg (1960) found that glucose-6-P, galactose-6-P, and glucosamine-6-P all stimulated hepatic glucosyltransferase, while the corresponding free sugars and a number of other sugar phosphates, including glucose-1-P, were inactive. Their results suggested that the phosphate group in position 6 is essential for activity, but left open the question as to the importance of the pyranose ring and the configuration at carbon 2. We tested mannose-6-P and 2-deoxyglucose-6-P and found them to be inactive, indicating the necessity for an -OH or $-NH_2$ substituent in the proper orientation at the 2 position. 1,5-Anhydroglucitol-6-P was active while sorbitol-6-P, which lacks the ring structure, was inactive. These findings demonstrate the importance of the 5-carbon heterocyclic ring structure, and also indicate that an -OH group on carbon 1 is not essential. These results are in essential agreement with the requirements of dog muscle glucosyltransferase reported by Rosell-Perez and Larner (1964). In contrast to glucose-6-P, glucose-6-*O*-sulfate was completely inactive, suggesting that both negative charges of the phosphate anion also may be important.

[3] The earlier report considered only activity measured with glucose-6-P added to the assay mixture.

[4] Estimated from the increment in activity obtained during incubation in KF and glucose-6-P at 37°.

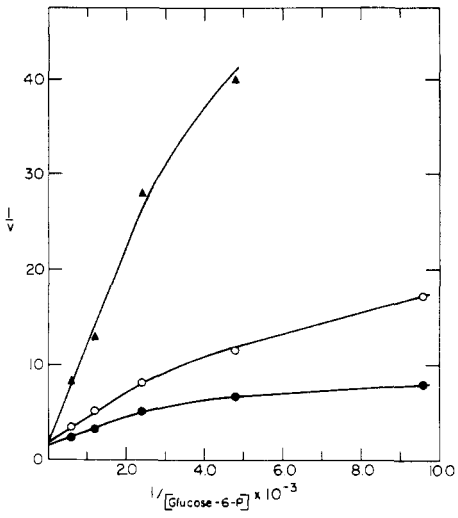

FIGURE 6: Partially competitive interaction between UDP and glucose-6-P. Double-reciprocal plot by the method of Lineweaver and Burk (1934). ●, control without UDP; ○, 1.6×10^{-4} M UDP; ▲, 8.1×10^{-4} M UDP. Enzyme prepared from rat muscle and assay conditions as described under Experimental Procedure. Essentially similar results were obtained with particulate glycogen and soluble enzyme preparations from liver.

Mechanism of Glucose-6-P Stimulation. Many investigators have reported that the apparent K_m for UDP-glucose is lowered and V_{max} is increased in the presence of glucose-6-P as shown in Figure 2. We have found that glucose-6-P also behaves in a partially competitive manner with respect to UDP, the normal product of the reaction and a strong competitive inhibitor with respect to UDP-glucose.[5] When plots were prepared of the reciprocal of initial velocity versus reciprocal glucose-6-P concentration at various concentrations of UDP this competitive relationship could be clearly demonstrated (Figure 6). The behavior was anomalous, however, in that the lines were curved in a concave direction and did not intersect at the ordinate even at very high glucose-6-P concentrations. Similar results were obtained with both liver and muscle enzyme preparations. Since UDP strongly inhibits glucosyltransferase, its dissociation from the enzyme must be regarded as a rate-limiting step in the overall reaction. Decreased binding affinity for UDP in the presence of glucose-6-P would increase V_{max} and lower the apparent K_m for UDP-glucose owing to the competitive relationship between UDP and UDP-glucose. Since UDP and glucose-6-P are unlikely to be competing for the same site(s), the kinetic behavior should not be typically competitive. Instead, competition should be restricted to a finite effect obtainable when all binding sites for glucose-6-P have been saturated (Dixon and Webb, 1964). Analogous instances of partially competitive interaction have been reported by Changeux (1961) and Gerhart and Pardee (1962).

Absence of UDP-Glucose, [^{32}P]UDP Exchange. A possible mechanism to account for the retention of the α configuration at carbon 1 when the glucosyl group is transferred to glycogen might be the formation of a β-glucosyl-enzyme intermediate which can be attacked with inversion by a terminal nonreducing glucose residue of glycogen (Koshland, 1953). The occurrence of such a reaction, if reversible, should be demonstrable by exchange of UDP with UDP-glucose. This possibility was tested by incubating a highly active preparation of soluble glucosyltransferase in a reaction mixture which contained equal amounts of UDP labeled with ^{32}P in the α-phosphate and nonlabeled UDP-glucose. Glycogen primer was omitted so that the overall reaction rate was reduced to about 5% of maximal. After incubation, the reaction mixtures were chromatographed on paper (Paladini and Leloir, 1952) and the UDP-glucose spot was examined for radioactivity. Although approximately 20% of the UDP-glucose had been incorporated into glycogen during the incubation, less than 0.2% of the total [^{32}P]UDP counts were found in the remaining UDP-glucose. Addition of glucose-6-P or glycogen in optimal amounts to the reaction mixture did not alter the results. If a glucosyl-enzyme intermediate is formed, therefore, the reaction must not be readily reversible.

Differences between Liver and Muscle Glucosyltransferase. Glucosyltransferase from muscle appeared to be similar in many properties to the liver enzyme. It was stimulated by glucose-6-P and showed the same partially competitive behavior between UDP and glucose-6-P, and the K_m values for UDP-glucose and for glucose-6-P fell in approximately the same range as for the liver enzyme. However, partially purified muscle preparations differed markedly in thermal stability. On incubation in 0.4 M sucrose, 0.005 M EDTA for periods up to 1 hour at 37°, no decrease in activity measured in the presence or absence of glucose-6-P occurred. After heating 15 minutes at 45° and 53°, activity declined to 54% and 1% of control, respectively, and could not be restored by subsequent incubation with KF and glucose-6-P. Muscle preparations withstood dialysis against sucrose-EDTA for long periods with little loss of activity, in contrast to the liver enzyme at all stages of purification. Although both liver and muscle preparations were inhibited by *N*-ethylmaleimide and *p*-mercuribenzoate (Table II), the liver enzyme was considerably more sensitive to *p*-mercuribenzoate. Incubation of muscle glucosyltransferase with 0.001 M GSH for 1 hour at 37° resulted in a 75% decrease in activity measured in the presence of glucose-6-P. The activity measured without glucose-6-P declined somewhat less, resulting in an apparent increase in the ratios of these two activities from 0.11 to 0.29. This change was somewhat similar to that obtained by incubating liver preparations at

[5] Unpublished data.

37°, but could not be reversed by incubation with KF and glucose-6-P.

The thermal sensitivity of liver glucosyltransferase is demonstrated by Arrhenious plots over the range from 10° to 37° (Figure 7). With the muscle enzyme a linear relationship between log v and reciprocal temperature was obtained both in the presence and absence of glucose-6-P. In the case of the liver enzyme the plot was linear in the presence of glucose-6-P, but in its absence was deflected downward as the temperature rose above 30°. Such deviation usually signifies inactivation of the enzyme protein in the higher temperature range. The energies of activation calculated from these graphs range between 16 and 26 kcal/mole.

It seems likely that the differences in thermal stability and sensitivity to sulfhydryl inhibitors of these two enzymes result from structural differences in the respective proteins. Inactivation of the muscle enzyme during incubation with thiols suggests that disulfide bonds are involved in maintaining the active configuration. If so, this could account for the greater thermal stability of the muscle enzyme.

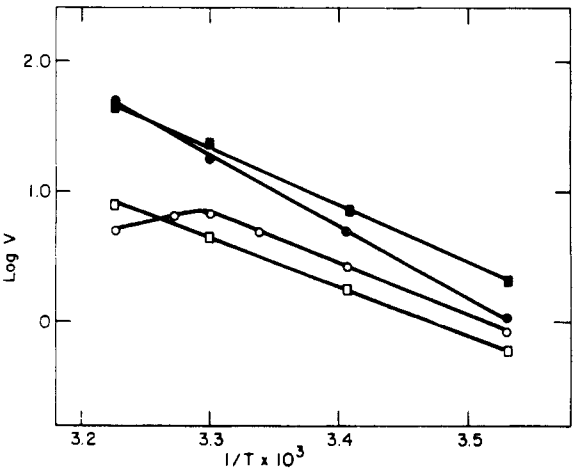

FIGURE 7: Arrhenius plot showing the effect of temperature upon the rate of the glucosyltransferase reaction over the range from 10 to 37°. Circles represent soluble liver glucosyltransferase; squares represent muscle glucosyltransferase. Open symbols represent rate in the absence of glucose-6-P; solid symbols represent rate in the presence of glucose-6-P. Incubation conditions were as described under Experimental Procedure.

Discussion

The success of this method for preparation of soluble glucosyltransferase depends upon the unusual thermal sensitivity of the hepatic enzyme protein, which can be utilized to remove the enzyme from glycogen without grossly altering its properties. Thermal sensitivity is not restricted to the enzyme from rat liver. We have recently prepared glucosyltransferase from mouse, guinea pig, and calf liver, and have found that the enzyme from all three species undergoes reversible inactivation. On the other hand, preparations from guinea pig and mouse muscle are stable during incubation at 37° in the absence of glucose-6-P.[6] Thus the enzymes of mammalian liver and muscle are probably not identical proteins, as in the case of phosphorylase (Stetten and Stetten, 1960), aldolase (Rutter, 1961), and lactic dehydrogenase (Cahn *et al.*, 1962) of liver and muscle.[7]

The most highly purified soluble preparations we have obtained are still heterogeneous mixtures containing at least two additional proteins and small amounts of glycogen. The presence of phosphorylase is not surprising in view of its known association with glycogen and its relative abundance in the liver. However, during incubation of particulate glycogen preparations, phosphorylase activity does not change, and much of it is discarded with the particulate glycogen after centrifugation. It has been reported recently that both phosphorylase and glucosyltransferase undergo redistribution from the particulate glycogen fraction into the supernatant fraction of homogenates when the liver glycogen concentration is markedly reduced *in vivo* by starvation or puromycin administration (Sie *et al.*, 1964; Tata, 1964). Tata studied the relationship between the distribution of phosphorylase and the quantity of glycogen present in homogenates, and concluded that redistribution can be explained in terms of preferential binding of phosphorylase to high molecular weight glycogen. However, Sie *et al.* (1964) reported that the redistribution of glucosyltransferase after hepatic glycogen depletion did not parallel exactly the redistribution of phosphorylase. Such a discrepancy in the behavior of these two enzymes is not surprising in view of our finding that the binding of glucosyltransferase to glycogen is dependent upon maintainance of an active enzyme configuration. The concentration of glucose-6-P, which plays an important role in stabilizing the active enzyme, is significantly reduced in the liver during starvation (Steiner and Williams, 1959). It is probable therefore that glucosyltransferase redistribution depends upon several factors rather than upon glycogen concentration alone. Sie *et al.* (1964) have ignored the fact that far from all of the glycogen of rat liver homogenates appears in the particulate glycogen fraction. In our experience, a considerable proportion of it is found in the supernatant, dependent upon the amount of amylytic activity present, and also upon the nutritional status of the animal. The conclusion that the redistribution of

[6] Unpublished experiments.

[7] It is not unreasonable to suppose that each enzyme as well as its control mechanism(s) are specialized to meet the particular metabolic demands of the tissue in which it occurs. In this connection, insulin has been shown to increase glucosyltransferase activity and glycogen deposition in diabetic rat liver by a mechanism which is sensitive to inhibition by puromycin, ethionine, and actinomycin (Steiner and King, 1964), while the stimulation by insulin of glucose transport and glycogen synthesis in muscle is not affected by these inhibitors (Eboue-Bonis *et al.*, 1963; Carlin and Hechter, 1964; Sorik and Waalas, 1964).

these enzymes represents true dissociation from glycogen should be examined more rigorously.

Larner and co-workers (Rosell-Perez *et al.*, 1962; Friedman and Larner, 1962) have described enzymatic interconversion of two forms of rat muscle glucosyltransferase, only one of which requires glucose-6-P for activity. Our attempts to bring about a similar conversion of hepatic glucosyltransferase by incubating purified preparations with crude extracts or with thiols, ATP, and magnesium, as described by Rosell-Perez *et al.* (1962), have been unsuccessful. Hizukuri and Larner (1963, 1964) have reported recently that incubation of hepatic glucosyltransferase preparations in the presence of magnesium and sodium sulfite resulted in conversion of the enzyme to a form which was no longer activated by glucose-6-P but was still bound to glycogen. The ability of the particulate glycogen fraction to effect this conversion was lost on further purification, and it appeared to be due to a heat-labile particulate factor. It may be that the conditions of incubation used by Hizukuri and Larner (1963, 1964) resulted in selective modification of the enzyme so as to inactivate the glucose-6-P binding site without destroying catalytic activity or ability to bind to glycogen. Further clarification of the nature of this process is required to establish a definite relationship to the transformations of muscle glucosyltransferase.

Three lines of evidence support our interpretation that the reversible inactivation of glucosyltransferase results from a conformational alteration of the enzyme protein rather than an enzymatic process: (a) the very high activation energy of the process (Steiner, 1961); (b) the changes in sedimentation velocity associated with inactivation and reactivation; and (c) the retention of this property even after extensive purification of the enzyme. Our data suggest that this process may involve reversible dissociation of the enzyme protein into less active or inactive subunits. Since these structural transitions can markedly influence the apparent activity, the effects of various incubation procedures on hepatic glucosyltransferase activity should be interpreted with caution.

The partially competitive interaction of glucose-6-P and UDP (Figure 6) can account for the observed changes in V_{max} and apparent K_m of UDPG produced by glucose-6-P. However, it is equally possible that glucose-6-P modifies the structure of the active center so as both to enhance the affinity for UDPG and at the same time decrease the affinity for UDP. A plausible hypothesis to explain such a highly selective effect might be that the additional negative charge acquired by UDP after transfer of the glucosyl group to glycogen may be instrumental in its dissociation from the active center. It is of interest in this regard that the optimum *p*H of glucosyltransferase in the presence of glucose-6-P is shifted to a more alkaline region, where the second hydrogen of the phosphoric acid residues of both UDP and glucose-6-P are fully dissociated. Lamb muscle and yeast glucosyltransferase have similar *p*H curves (Traut and Lipmann, 1963; Algranati and Cabib, 1962). It may also be pertinent that glucose-6-*O*-sulfate, which lacks a second ionizable acidic group, is completely inactive in stimulating glucosyltransferase, although it satisfies the other structural criteria. The assumption that glucose-6-P acts as an allosteric effector by producing a conformational change in the enzyme protein (Monod *et al.*, 1963) does not, of course, preclude the possibility that the binding site for glucose-6-P lies near enough to the active center to permit direct electrostatic interaction between its negatively charged phosphate group and the terminal phosphate of UDP.

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The Abnormal Carboxyl Groups of Ribonuclease. I. Preparation and Properties of Methylated Ribonuclease*

Clarence A. Broomfield,† John P. Riehm, and Harold A. Scheraga

ABSTRACT: Previous work has indicated that ribonuclease A contains at least two carboxyl groups which do not titrate normally, and it has been postulated that these abnormal carboxyl groups are associated with the abnormal tyrosine residues known to be present in the molecule. The present work is an attempt to locate in the amino acid sequence those carboxyl groups which are abnormal, by esterifying the "exposed" carboxyl groups under conditions where the "buried" residues do not react. It has been found that a product could be obtained which contained three free carboxyl groups. This product possessed essentially no enzymatic activity; the activity was largely regained by saponification under mild conditions. At least two of the abnormal tyrosine residues were not normalized in the methylated derivative, as indicated by the spectrophotometric titration curve, although there is an apparent loosening of the structure.

In the acid range, the methylated protein underwent low-temperature transitions and thermally induced transitions which were similar to those of ribonuclease. That the folded structure of the derivative is similar to that of the native protein is shown by the difference spectrum of the methylated protein versus ribonuclease at neutral *p*H and low temperature. These results, together with the location in the sequence of the three free carboxyl groups (the subject of the following paper), provide information about the role of specific side-chain interactions in maintaining the folded structure of ribonuclease.

In order to fit the experimental titration curve of ribonuclease A to a theoretical curve, it is necessary to assume that the intrinsic ionization constants, $pK°$, of some of the carboxyl groups are abnormally low. Tanford and Hauenstein (1956) assumed that five of the ten side-chain carboxyl groups have a $pK°$ of 4.0, while the remaining five groups have a more normal $pK°$ of 4.70. To explain ultraviolet difference spectral data and conformational changes at low *p*H, Hermans and Scheraga (1961) have assumed that one carboxyl group has a $pK°$ of 2.5, another a $pK°$ of 3.65, and the remainder a $pK°$ of 4.6. The latter authors have also postulated the existence of interactions involving carboxyl groups and "buried" tyrosyl side chains (Shugar, 1952, Tanford *et al.*, 1955) in a nonpolar region.

Since the sequence of amino acids in ribonuclease A is known, the identification of the abnormal carboxyl groups as well as the buried tyrosine residues would provide valuable information about the conformation

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