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# MOLECULAR CHARACTERISTICS OF THE TOTALLY DEPENDENT AND INDEPENDENT FORMS OF GLYCOGEN SYNTHASE OF RABBIT SKELETAL MUSCLE

## I. PREPARATION AND CHARACTERISTICS OF THE TOTALLY GLUCOSE 6-PHOSPHATE DEPENDENT FORM

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SUMMARY

Directions are detailed for preparing the totally glucose-6-*P* dependent (D) form of glycogen synthase (UDPG:glycogen α-4-glucosyltransferase, EC 2.4.1.11) from rabbit skeletal muscle from which the glycogen has been removed. The conversion is carried out in the cold (3°) for several days in order to achieve full phosphorylation. Glycogen is removed by digestion with α-amylase. The enzyme is then isolated by gel filtration over Sepharose 4-B and Sephadex G-200 columns. The molecular weight by sedimentation diffusion is determined as 250 000. The enzyme is totally dependent on glucose-6-*P* and on added glycogen for activity. The residual carbohydrate content is about 10 μg/mg protein. The two substrate kinetics (glycogen, UDPG) was examined. In reciprocal plots parallel lines were obtained with either substrate, indicating a ping pong mechanism of catalysis.

## INTRODUCTION

The enzyme UDPG glycogen synthase (UDPG:glycogen α-4-glucosyltransferase, EC 2.4.1.11) exists in two protein forms which are interconverted by phosphorylation and dephosphorylation[1,2] catalyzed by a kinase and phosphatase. The differential response of the two forms to metabolic effectors underlie physiologic regulation of glycogen formation[3-6]. Utilizing the glucose 6-phosphate dependence of activity, the two forms can be separately assayed and their interconversion conveniently followed[7]. Techniques for preparation of the enzyme, enriched in the

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glucose 6-phosphate dependent (D), or independent (I) form have been previously described[8].

More recently a method for preparing the totally independent form has also been described[9]. In the present paper, the preparation of the totally glucose-6-*P* dependent form is described, together with a procedure for removing essentially all of the glycogen and achieving homogeneity. The molecular weight and some kinetic properties of this totally dependent D form have been determined. In the succeeding paper, the phosphate content is compared with that of the totally independent I form. Other chemical data and a subunit molecular weight of these two forms is also presented.

## MATERIALS AND METHODS

UDP-glucose, glucose-6-*P*, ATP, 3′,5′-cyclic adenylic acid and Tris were purchased from the Sigma Chemical Co. [$^{14}$C]UDP-glucose was a gift from Drs. J. A. Thomas and K. K. Schlender. Rabbit liver glycogen was purchased from the Nutritional Biochemical Co. DEAE-cellulose, type 20, was obtained from the Brown Co. and [$^{32}$P]phosphate (polyphosphate and pyrophosphate free) was obtained from Tracerlab. Sephadex G-200, Sepharose 4-B and Ficoll were purchased from Pharmacia Fine Chemicals; Amberlite MB-3 was obtained from Rohm and Haas. Glyceraldehyde-3-*P* dehydrogenase, phosphoglycerate kinase and 3-phosphoglyceric acid were obtained from the Boehringer-Mannheim Corp.

### *Assay of glycogen synthase activity*

The assay was essentially that described by THOMAS *et al.*[10].

### *Column treatment of rabbit liver glycogen*

Commercially prepared rabbit liver glycogen was treated with mixed ion exchange resin (Amberlite MB-3) before it was used in the test mixture[11].

### *Protein determination*

Protein was determined by two methods: the Lowry method[12], and the spectrophotometric method[13].

### *Determination of carbohydrate*

Carbohydrate was determined by the phenol–sulfuric acid method of DUBOIS *et al.*[14].

### *Purification of human salivary α-amylase*

The method of purification was essentially that of BERNFELD[15]. The preparation was carried through the second $(NH_4)_2SO_4$ precipitation. The activity of the enzyme was determined using soluble starch dissolved in 50 mM Tris–HCl (pH 6.8), 5 mM EDTA, as substrate. The amount of reducing activity released was measured using the Nelson–Somogyi method[16]. The enzyme activity was equivalent to 98 mg of reducing equivalents per mg protein per min.

## RESULTS

### *Purification of D form of glycogen synthase from rabbit muscle*

*Preparation of the extract.* White male rabbits (approx. 6 lbs) were injected with 2 ml of a 7% Seconal solution in 0.9% NaCl into a marginal ear vein, sacrificed and thoroughly bled. Animals were skinned, the hind legs and back muscles removed and trimmed of fat, and connective tissues. The muscle was weighed and 2.5 vol. (w/v) of 50 mM Tris–5 mM EDTA buffer (pH 8.2) containing 100 mM KF was added and the muscle homogenized in a large capacity Waring Blender at medium speed for 1.5 min. The homogenate was then centrifuged at 9500 rev./min (14 600 × *g*) in the Sorvall Model RC-2 for 50 min. The Sorvall supernatant was then filtered through glass wool.

*Ethanol precipitation.* To the filtered extract which had been placed in an ice-salt bath at −10° was added ethanol (−65°) with constant stirring to a final concentration of 30%. The temperature of the mixture was not allowed to exceed 5° while the ethanol was added. The mixture was then centrifuged for 30 min at −10° and 14 600 × *g*. The supernatant was discarded and after the precipitate had been well drained it was taken up in 1/5 the volume of 50 mM Tris–5 mM EDTA buffer (pH 8.2) containing 100 mM KF.

*Spinco centrifugation.* The resuspended precipitate was then centrifuged for 2.5 h at 78 000 × *g* (30 000 rev./min) in the 30 rotor of the Spinco Model L-2. The supernatant was discarded and the precipitate was stored at −65°. The preparation was stable for at least 6 months under these conditions.

*Solubilization of the enzyme.* The Spinco precipitates obtained from 1500 g of muscle were resuspended in 700 ml of 50 mM Tris–5 mM EDTA–50 mM mercaptoethanol buffer (pH 7.8) and incubated at 30° for 1 h. The incubated material was then centrifuged for 1 h at 54 000 × *g* (25 000 rev./min in the 30 rotor) in the Spinco Model L-2 to remove insoluble material.

*DEAE-cellulose column chromatography.* The 54 000 × *g* supernatant was then applied to a 600-ml bed volume DEAE column (5 cm × 30 cm) which had been well washed with 3 bed vol. of fresh Tris–EDTA–mercaptoethanol buffer (pH 7.8). The column was then washed with 1 bed vol. of Tris–EDTA–mercaptoethanol buffer; 7 bed vol. of Tris–EDTA–mercaptoethanol buffer containing 100 mM NaCl which removed glycogen phosphorylase; finally the enzyme was eluted with Tris–EDTA–mercaptoethanol buffer containing 300 mM NaCl. This salt concentration was necessary to ensure that glycogen synthase I kinase was also eluted from the column along with the glycogen synthase.

*Ethanol precipitation of the enzyme.* The enzymes were concentrated and freed of NaCl by precipitation with ethanol at −10° in the presence of added glycogen. Rabbit liver glycogen was added (1 mg/ml of solution) and then absolute ethanol with constant stirring to a final concentration of 30% (−10°) as above. Glycogen synthase precipitates at 15% ethanol, but it was found necessary to increase the ethanol concentration to precipitate the synthase I kinase. The precipitate was collected by centrifugation for 15 min at 15 000 rev./min (27 000 × *g*) in the Sorvall at −10°. The precipitate was well drained and dissolved in 1/20 the volume of the column fraction of Tris–EDTA–mercaptoethanol buffer (pH 8.2).

*Conversion to the dependent form.* ATP, $MgCl_2$, 3′,5′-cyclic adenylate were added

to a final concentration of 10, 8 and 0.01 mM, respectively. Incubation was carried out for 3–4 days at 3° and yielded a preparation which was 98–99% D. When the conversion was run at higher temperatures (30°) for shorter times, the conversion was not as complete.

*Second and third ethanol precipitation.* The conversion reaction was terminated by the addition of two volumes of Tris–EDTA–mercaptoethanol buffer (pH 7.8) and then ethanol was added at −10° to a final concentration of 15%. After standing 5 min, the precipitate was collected by centrifuging at 15 000 rev./min (27 000 × *g*) for 15 min. The precipitate was resuspended in the same volume of Tris–EDTA–mercaptoethanol buffer and precipitated a second time with ethanol to 15% as above. The precipitate was taken up in 10–20 ml of Tris–EDTA–mercaptoethanol buffer (pH 7.8) and stored at −65°F. This method yielded enzyme which had been purified 600-700 fold with a 15–20% recovery of activity (Table I).

TABLE I

PURIFICATION OF RABBIT MUSCLE GLYCOGEN SYNTHASE—LARGE SCALE

1500 g of rabbit skeletal muscle was treated as described in the text. Transferase was assayed by the standard method and protein was determined by the methof of LOWRY *et al.*[12].

| *Fraction* | *Activity (units/ml)* | *Specific activity (units/mg)* | *Total units* | *Recovery (%)* | *Purification* |
|---|---|---|---|---|---|
| Homogenate | 0.49 | 0.018 | 2156 | 100 | -- |
| Extract | 0.67 | 0.037 | 2010 | 93 | 2 |
| 1st Ethanol (30%) | 3.57 | 0.092 | 1285 | 60 | 5 |
| 78 000 × *g* pellet | 0.62 | 0.110 | 732 | 34 | 6 |
| DEAE fraction | 1.07 | 2.70 | 535 | 25 | 149 |
| 2nd Ethanol (30%) | 64.18 | 5.19 | 674 | 31 | 285 |
| 3rd Ethanol (15%) | 23.28 | 9.36 | 350 | 16 | 514 |
| 4th Ethanol (15%) | 35.66 | 12.01 | 357 | 17 | 660 |

*Salivary α-amylase treatment of transferase*

One of the aims of these experiments was to determine the molecular weight of rabbit muscle glycogen synthase D. Since gel filtration experiments with Sepharose 4-B demonstrated that the enzyme could not be separated from glycogen by gel filtration, it was found necessary to remove the glycogen by α-amylase digestion. Human salivary α-amylase (100 μg) was added to 25–30 mg of protein and the mixture dialyzed against 1 l of Tris–EDTA–mercaptoethanol buffer (pH 6.8) for 16–20 h at 3°. The progress of the digestion was followed by removing aliquots of the dialysate and testing for carbohydrate by the phenol–sulfuric acid method. When no more phenol–sulfuric acid reacting material appeared in the dialysate, the protein was removed from the dialysis sac and the pH adjusted to 7.8 with 2 M unneutralized Tris. The mixture was then incubated at 30° for 30 min to resolubilize any protein which had precipitated. It was later found that equivalent α-amylase digestion could be carried out at room temperature using much shorter times (2–5 h) with more frequent buffer changes. The enzyme prepared at room temperature was found to be somewhat less stable than that prepared at 3° and the recovery of activity was somewhat more variable (50–85%) (Table II). The most striking effect of the α-amylase digestion at room temperature was on the apparent molecular size (see following section).

TABLE II

EFFECT OF α-AMYLASE DIGESTION AND SEPHAROSE 4-β GEL FILTRATION ON RABBIT MUSCLE GLYCOGEN SYNTHASE D

Highest specific activity attained in any preparation was 20 units/mg.

| *Synthase* | *Total activity (μmoles/ml per min)* | *Total units* | *Specific activity (μmoles/mg per min)* | *Recovery* | *Purification* |
|---|---|---|---|---|---|
| 30% Ethanol ppt. | 59.3 | 178 | 5.62 | 1000 | — |
| α-Amylase treated | 46.1 | 138 | 7.62 | 77.5 | 1.35 |
| 34 000 × *g* supt. | 40.2 | 121 | 11.50 | 68.0 | 2.10 |
| Sepharose 4-B peak* | 11.9 | 112 | 15.10 | 63.0 | 2.70 |

* Carbohydrate concentration as measured by the phenol–sulfuric method: 11.2 μg carbohydrate per mg of protein.

*Gel filtration experiments*

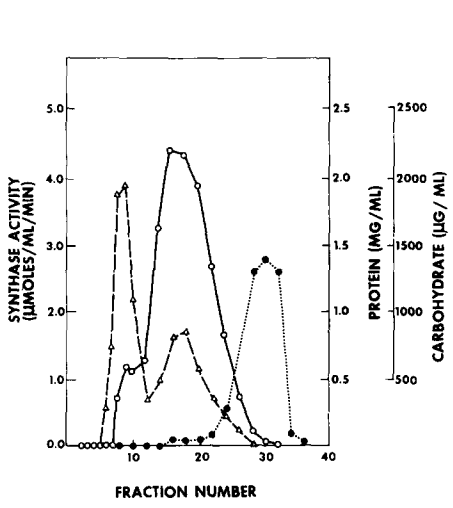
*At 3°*

*Sepharose 4-B gel filtration.* The α-amylase treated enzyme (3°) (20–25 mg in approx. 20 ml Tris–EDTA–mercaptoethanol buffer, pH 7.8) was applied to a 200-ml Sepharose 4-B column (2 cm × 62 cm) equilibrated with the same buffer at 3°. The enzyme was eluted from the column with the same buffer at a flow rate of 0.25 ml/min. The column fractions were read at 3 wavelengths: 280, 260 and 250 nm. The protein concentration was corrected for nucleotide present by the method of WARBURG AND CHRISTIAN[13]. The column fractions were assayed for synthase activity and for carbohydrate content (Fig. 1). The Sepharose 4-B column separated the preparation into 2 protein peaks (both having synthase activity), a carbohydrate peak separated from the protein and a nucleotide peak containing adenine (not shown). The nucleotide was probably ATP or ADP bound to the enzyme during the I to D conversion. The fractions containing the 2 protein peaks were pooled separately, placed in dialysis sacs and concentrated by placing the sacs in dry Ficoll at 3°. After the volume was reduced to 1/3 of the original volume, the sacs were then dialyzed for 3–4 h against Tris–EDTA–mercaptoethanol buffer, pH 7.8 at 3°. The protein content of the concentrated peaks was determined with the method of LOWRY *et al.*[12]. The protein peak excluded from the Sepharose 4-B was called the "heavy" fraction and comprised about 10–20% of the protein. The included peak which contained the bulk of the enzyme activity and the protein was termed the "medium" fraction. The 2 peaks were completely separated from each other by rechromatography over Sepharose 4-B under the same conditions.

*Sephadex G-200 gel filtration.* When the "medium" fraction was applied to a Sephadex G-200 column with a 200-ml bed volume (2 cm × 62 cm) under the same conditions used for the Sepharose 4-B gel filtration, a single excluded protein peak was found which contained all the synthase activity (Fig. 2).

*At room temperature*

*Sepharose 4-B gel filtration.* If the α-amylase treatment was carried out at room temperature, rather than at 3°, and the Sepharose 4-B gel filtration run at this same temperature, all other conditions remaining the same, the "heavy" fraction was



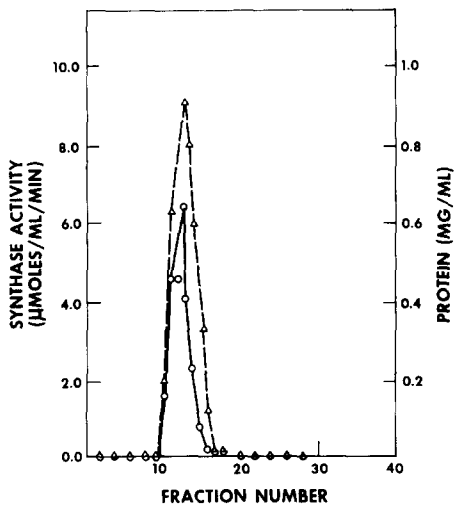
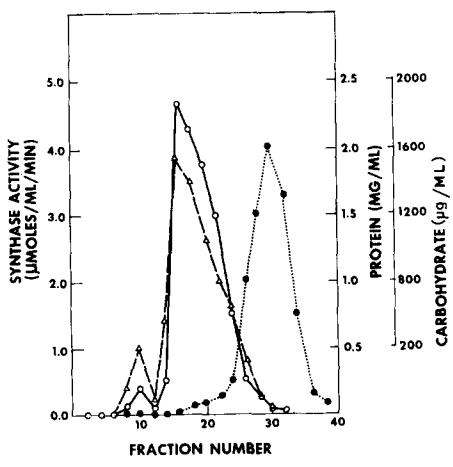




Fig. 1. Sepharose 4-B gel filtration of α-amylase (3°) treated enzyme. The conditions were the same as those specified in Fig. 3. ○—○, enzyme activity; △—△, protein; ● . . . ●, carbohydrate.

Fig. 2. Sephadex G-200 gel filtration of the α-amylase (3°) treated Sepharose 4-B included fraction. The temperature (from Fig. 1) of the column was 3°. ○—○, enzyme activity; △—△, protein.



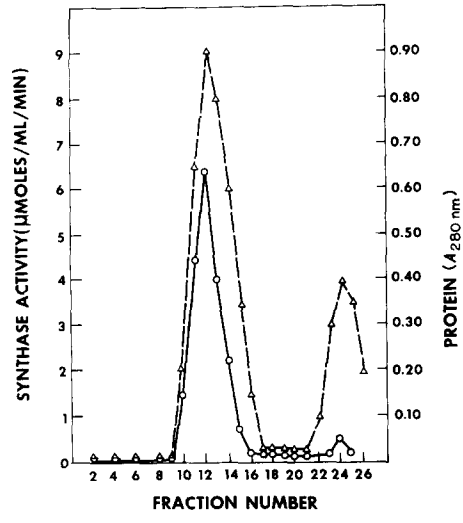




Fig. 3. Sepharose 4-B gel filtration of α-amylase (25°) treated enzyme. The column was operated at room temperature. The buffer used was 50 mM Tris–HCl (pH 7.8)–5 mM EDTA–5 mM mercaptoethanol. Protein was determined by the method of LOWRY *et al.*[12]. Fractions were stored at room temperature rather than at 3°. ○—○, enzyme activity; △--△, protein; ● . . . ●, carbohydrate.

Fig. 4. Sephadex G-200 gel filtration of the α-amylase (25°) treated Sepharose 4-B included fraction. Conditions for this experiment were the same as described in Fig. 3. ○—○, enzyme activity; △--△, protein.

markedly reduced (protein content) to about 5% or less (Fig. 3). The increased "medium" fraction was concentrated with Ficoll and dialyzed against Tris–EDTA–mercaptoethanol buffer, pH 7.8 at room temperature.

*Sephadex G-200 gel filtration.* When the room temperature treated "medium" fraction from the Sepharose 4-B column was applied to a Sephadex G-200 column at room temperature, 2 protein peaks were now found. The largest peak was excluded and represented 80–90% of the activity and protein applied. There was also an included peak which contained varying amounts of activity, but only about 10–20% of the protein (Fig. 4). These peaks were pooled and concentrated with Ficoll and dialyzed against Tris–EDTA–mercaptoethanol buffer, pH 7.8 at room temperature.

*Determination of sedimentation coefficients, diffusion coefficients and molecular weight of glycogen Synthase D*

Sedimentation coefficients and diffusion coefficients were determined in the Spinco Model E ultracentrifuge, which was equipped with a Schlieren optical system. Photographs were taken at appropriate times and were developed. The peaks from the photographic plates were enlarged, using a photographic enlarger and traced onto graph paper. In the determination of the diffusion coefficients, the areas under the peaks were determined with a KE planimeter. Standard procedures for calculating sedimentation and diffusion constants and molecular weight by sedimentation velocity were followed[17].

TABLE III

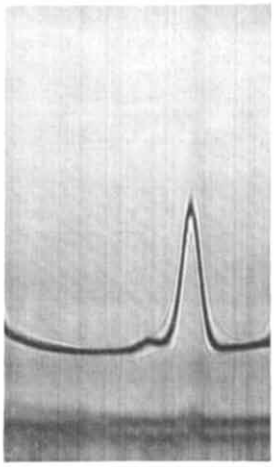
SEDIMENTATION AND DIFFUSION COEFFICIENT OF RABBIT MUSCLE GLYCOGEN SYNTHASE D

The α-amylase treated enzyme which had been treated on Sepharose 4-B and Sephadex G-200 was used in these experiments. The preparation was kept at room temperature in the presence of fresh mercaptoethanol for the α-amylase treatment and the subsequent gel filtration. The buffer was 50 mM Tris–HCl (pH 7.8)–5 mM EDTA-50 mM mercaptoethanol. The determination of the sedimentation and diffusion coefficients were done at 25° in the Spinco Model E. The protein concentration ranged from 2.5–5 mg/ml and the fraction used was the "medium" fraction.

| $S_{20}$, $H_2O$ | $D_{20}$, $H_2O$ |
|---|---|
| 13.2 | — |
| 10.0 | $4.8 \cdot 10^{-7}$ |
| 11.2 | — |
| 11.7 | $4.4 \cdot 10^{-7}$ |

*Enzyme in the presence of glycogen.* When sedimentation velocity experiments were run on the second 15% ethanol fraction at 3°, two distinct peaks were obtained: a 19-S peak which comprised 60–70% of the protein; a 15.6-S peak which made up 20–30% of the protein. Neither temperature (2–25°), protein concentration (0.1 to 1%) nor pH (6–9) was found to have any appreciable effect on the sedimentation velocity pattern of the preparation. Increasing KCl (1–3 M) concentration was found to decrease the sedimentation velocity pattern of this preparation.

*Amylase treated enzyme.* The "heavy" fraction which was produced when the α-amylase treatment was done at 3° settled out of solution on standing. No attempt was made to determine its sedimentation characteristics. Sedimentation coefficients and diffusion coefficients were determined on the "medium" and "light" fractions



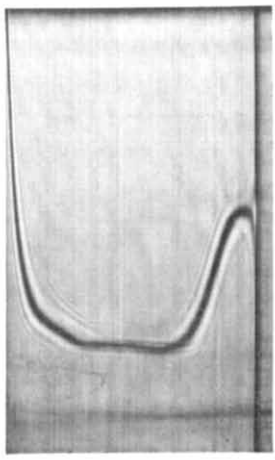


Fig. 5. Sedimentation from right to left. "Medium" fraction as prepared by methods described in the text at room temperature. The buffer was 50 mM Tris–HCl (pH 7.8)–5 mM EDTA–50 mM mercaptoethanol and the temperature was 22°.

Fig. 6. Sedimentation from right to left. "Light" fraction as prepared by methods described in the text at room temperature. The buffer was 50 mM Tris–HCl (pH 7.8)–5 mM EDTA–50 mM mercaptoethanol and the temperature was 22°. The Schlieren optical system was used with a phase plate angle of 30° and a protein concentration of 6 mg/ml.

produced when the amylase, gel filtration and centrifugation were all carried out at room temperature. The average of several experiments were used to obtain an average $s_{20}$, $H_2O$ value of $11.7 \cdot 10^{-13}$ and a $D_{20}$, $H_2O$ of $4.6 \cdot 10^{-7}$ to yield a molecular weight of 250 000 for the "medium" fraction (Table III, Fig. 5).

The "light" fraction had a sedimentation coefficient of about 0.9 S and a diffusion coefficient of $7.3 \cdot 10^{-7}$. These data yield a molecular weight of 11 800 (Table IV, Fig. 6)*.

*Kinetic experiments on purified glycogen synthase D*

ROSELL-PEREZ AND LARNER[7] in 1964 reported that the $K_m$ for UDP-glucose

TABLE IV

SEDIMENTATION AND DIFFUSION COEFFICIENTS OF RABBIT MUSCLE GLYCOGEN SYNTHASE D "LIGHT" FRACTION

The "light" fraction was obtained as described in th etext and was dissolved in 50 mM Tris–HCl (pH 7.8)–5 mM EDTA–50 mM mercaptoethanol. The Spinco Model E was operated at 25°. The protein concentrations ranged from 1–3 mg/ml.

| $s_{20}$, $H_2O$ | $D_{20}$, $H_2O$ |
|---|---|
| 0.92 | $7.9 \cdot 10^{-7}$ |
| 0.76 | $6.7 \cdot 10^{-7}$ |
| 1.04 | — |
| 0.97 | — |

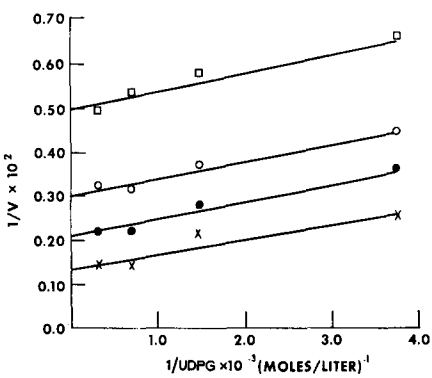
* The $s$ and $D$ values yield the stated molecular weight, but they are both smaller than those of many native globular proteins of the same mass. One explanation of this may be that the molecule became partially denatured and unfolded during the long isolation procedure at room temperature.

of rabbit muscle glycogen synthase D in the presence of glucose-6-*P* was $2.6 \cdot 10^{-4}$ M. These experiments were carried out using preparations which contained glycogen and therefore the effect of glycogen on the $K_m$ for UDP-glucose could not be determined. KORNFELD AND BROWN[18] demonstrated that the reaction was slightly reversible and tried to determine whether a glucosyl–enzyme intermediate might be formed during the reaction. With no added glycogen, they found no exchange of radioactivity between [$^{14}$C]UDP and UDPG and concluded that no glucosyl–enzyme intermediate was formed. These early preparations of the enzyme undoubtedly contained glycogen.

The goal of the following experiments was to determine the two substrate kinetic behavior of glycogen synthase D to study the reaction mechanism. For these experiments [$^{14}$C]UDPG was made up to a final concentration of 10 mM with a specific activity of 50 000 counts/min per $\mu$mole. This [$^{14}$C]UDPG was diluted appropriately with buffer, a standard constant amount of glucose-6-*P* and varying amounts of glycogen were added in that order. If no glycogen was added, no reaction occurred under standard assay conditions. The reaction was therefore begun by adding aliquots of the appropriately diluted preincubated enzyme. After incubating for 2 min at 30°, aliquots were spotted on filter papers and processed. Duplicates were run for each point.

All data were plotted as the reciprocal of the initial velocity *versus* the reciprocal of the concentration of the variable substrate. Secondary plots were made by plotting the slopes *versus* the reciprocal concentration of the changing fixed substrate and plots of intercepts *versus* the reciprocal concentration of the changing fixed substrate. The interpretation of the data was that outlined by CLELAND[19,20].

The primary reciprocal plots showed that a family of lines was obtained which were parallel for plots with either substrate (Figs. 7, 8). Secondary slope plots were



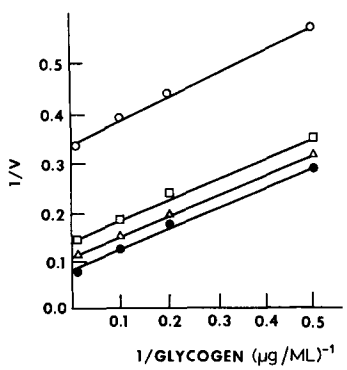




Fig. 7. Kinetic studies on transferase D at constant glycogen and varying UDPG. The specific activity of the UDPG was 50 000 counts/min per $\mu$mole. The buffer used was 50 mM Tris–HCl (pH 7.8)–20 mM EDTA–25 mM KF–50 mM mercaptoethanol. The glucose-6-*P* concentration was $4 \cdot 10^{-3}$ M. The assay was incubated for 2 min at 30°. The rest of the procedure was described in the standard procedure. Glycogen concentrations: □—□, 2 $\mu$g/ml; ○—○, 5 $\mu$g/ml; ●—●, 10 $\mu$g/ml; ×—×, 107 $\mu$g/ml.

Fig. 8. Kinetic studies on transferase D at constant UDPG concentration and varying glycogen concentration. The conditions are the same as in Fig. 7. UDPG concentrations: ○—○, $6.67 \cdot 10^{-5}$ M; □—□, $2.67 \cdot 10^{-4}$ M; △—△, $6.67 \cdot 10^{-4}$ M; ●—●, $3.33 \cdot 10^{-3}$ M.

TABLE V

VARIATION OF THE $K_M$ FOR GLYCOGEN WITH CHANGING UDPG CONCENTRATION

| *UDPG (moles/l)* | *$K_m$ (Glycogen) (μg/ml)* | *$K_m/v_{max}$* |
|---|---|---|
| $6.67 \cdot 10^{-5}$ | 1.32 | 0.46 |
| $2.67 \cdot 10^{-4}$ | 3.06 | 0.55 |
| $6.67 \cdot 10^{-4}$ | 3.92 | 0.41 |
| $3.33 \cdot 10^{-3}$ | 5.72 | 0.44 |

also linear, but essentially horizontal. The ratios of $K_m/v_{max}$ for both variable substrates were constant. As the concentration of the fixed (non-varied) substrate increased, the apparent $K_m$ increased to a limiting value (Tables V, VI).

According to CLELAND[19,20], these data indicate a ping-pong mechanism; that is, a mechanism in which one or more products are released before all substrates have been added to the enzyme. The enzyme thus exists in two or more stable forms between which it oscillates during the reaction. This type of mechanism must be further tested by inhibitor studies with UDP. The order of binding of the substrates must also be determined. An exchange between [$^{14}$C]UDP and UDPG should also be retested in the absence of glycogen.

TABLE VI

VARIATION OF THE $K_m$ FOR UDPG WITH CHANGING GLYCOGEN CONCENTRATION

| *Glycogen (μg/ml)* | *$K_m$ (UDPG) (moles/l)* | *$K_m/v_{max}$ ($\times 10^7$)* |
|---|---|---|
| 2 | $7.52 \cdot 10^{-5}$ | 3.71 |
| 5 | $1.07 \cdot 10^{-4}$ | 3.24 |
| 10 | $1.46 \cdot 10^{-4}$ | 3.18 |
| 107 | $2.14 \cdot 10^{-4}$ | 3.10 |

## DISCUSSION

This paper details methods for the preparation and purification of the D form of glycogen synthase from rabbit muscle which is essentially free of glycogen (about 10 μg carbohydrate per mg protein). The conversion of the I to the D form was done at 3° for 3 days because the synthase I kinase which was present in the preparation and was used for the conversion was not stable enough at 30° to give a product which was essentially totally D. 3′,5′-Cyclic adenylate was probably necessary because the kinase prepared under these conditions was highly dependent on the cyclic nucleotide for activity.

The glycogen could not easily be separated from the enzyme by gel filtration, but α-amylase digestion removed the glycogen. After α-amylase treatment, only traces of glycogen remained and when glycogen was omitted in the assay, no enzyme activity was present. After amylase digestion, the enzyme aggregated variably depending on the temperature. At 3° there was a higher yield of the excluded "heavy"

fractions. At room temperature there was an increased yield of the "medium" included fraction and, in addition, a "light" fraction was also obtained. The medium fraction contained the bulk of the activity and protein. This behavior suggests that in the absence of glycogen (stabilizer), the protein is capable of aggregating into a less active form at lower temperature and dissociates when the temperature is raised.

Sedimentation velocity and diffusion studies indicate that the enzymatically active medium fraction has a molecular weight of 250 000. This value was calculated assuming the partial specific volume to be 0.74. Since the sedimentation was determined at a single protein concentration, this is only an approximate value. The molecular weight of the light fraction is more uncertain because of the small $s$ value (0.9). The molecular weight is in the range of 11 000–13 000*.

After this study was completed, SODERLING *et al.*[21] described another procedure for purification of the enzyme. While there are some similarities between it and the technique described in this report, several important differences are apparent. In the published report, phosphorylation was conducted at 30° rather than in the cold and conversion to total glucose 6-phosphate dependency was not accomplished under these conditions. In the present study the enzyme was purified as a glycogen–protein complex and amylase digestion was postponed to a near final step. Amylase digestion can thus be controlled and all but traces of measurable carbohydrate eliminated. This allows a longer retention of stabilization by glycogen and makes possible purification by ethanol fractionation. Perhaps, as a result, specific activities 2–3-fold greater, are therefore obtained. In the published study, the sedimentation coefficient and molecular weight were significantly larger than those reported here. Other than to suggest that the preparation described may still retain bound carbohydrate, we cannot account for this discrepancy at present.

The kinetic data obtained are compatible with a stable enzyme–substrate intermediate being formed during the reaction. All the data are consistent with a ping-pong mechanism in which one or more products are released before all the substrates are added, the enzyme thus existing in more than one stable form. The problem with kinetic analysis of this enzyme is that it is not possible to molecularly define the glycogen acceptor. Glycogen may also act to stabilize the enzyme and bind at a site other than the active site.

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* Considering the analytical data reported in the succeeding paper, it has not escaped our attention that this molecule may be a small subunit of the enzyme.

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