Yeast Glycogen Synthetase in the Glucose 6-Phosphate-dependent Form

I. PURIFICATION AND PROPERTIES

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SUMMARY

The glucose-6-P-dependent form of glycogen synthetase has been purified about 5,000-fold from yeast extracts. The enzyme was separated from the glucose-6-P-independent form by DEAE-cellulose chromatography and from other proteins by a two-step agarose chromatography, using complex formation with glycogen to change the elution properties of the synthetase. The purified material shows a single band on disc gel electrophoresis and possesses a molecular weight of 300,000, as measured by sedimentation equilibrium. Acrylamide electrophoresis in the presence of sodium dodecyl sulfate yields a major band, molecular weight about 77,000, and a minor band of variable intensity, with molecular weight about 71,000. The faster band is believed to be a proteolysis degradation product of the slow band. Thus, the native enzyme would consist of four subunits of equal molecular weight. The enzyme was labeled with radioactive phosphate by conversion into the glucose-6-P-independent form followed by incubation with \[^{32}P\]ATP. The amount of radioactivity incorporated corresponds to 1.4 phosphate groups per subunit. Incubation with a muscle protein phosphatase led to a liberation of radioactivity and a parallel increase in glucose-6-P-independent synthetase activity.

Lineweaver-Burk plots with the purified enzyme, using either UDP-glucose or glycogen as the variable substrate, showed a biphasic curve, as previously observed with crude enzymes. Increasing concentrations of glucose-6-P led to a gradual elimination of the line corresponding to a higher \(K_m\) value.

Previous work from this laboratory has uncovered a correlation between the accumulation of glycogen which takes place in yeast at the end of the logarithmic growth phase and an increase in that activity of glycogen synthetase which can be measured in the absence of glucose-6-P (1). These results were interpreted as indicating a transformation from the glucose-6-P-dependent (D) form into the glucose-6-P-independent (I) form of the enzyme. Such transformations were also obtained in vitro (1, 2). The event or events that trigger the in vivo conversion of D to I are unknown. Therefore, it seemed desirable to obtain more information about the pertinent enzymes, i.e. the two forms of glycogen synthetase and the enzymes which interconvert them. A study on the sensitivity of the I form to cold and a method for the separation of the two forms from a mixture have been recently published (3, 4). The present article describes the purification of the D form to an essentially homogeneous state and a study of its properties.

EXPERIMENTAL PROCEDURE

Materials

UDP-glucose, glucose-6-P, shellfish glycogen, rabbit liver glycogen, bovine serum albumin, and Tris were obtained from Sigma. UDP-\[^{32}P\]glucose (uniformly labeled in the glucose moiety, 22 Ci per mole), \[^{32}P\]ATP (14 Ci per mmole), and Aquasol were from New England Nuclear Corp. Chromatographic sheet (ITLG type, 20 x 20 cm) was the product of Gelman Instrument Company. DEAE-cellulose (DE52) was purchased from Whatman and Bio-Gel A-5m (200 to 400 mesh) from Bio-Rad Laboratories. Ovalbumin, pepsin, phosphorylase, and \(\alpha\)-amylase were obtained from Worthington and pyruvate kinase from Boehringer Mannheim Corp. Glycogen synthetase phosphatase from muscle was kindly provided by Dr. F. Zieve of the National Institute of Child Health and Human Development.

Methods

Yeast Growth—Saccharomyces cerevisiae S28C (American Type Culture Collection 26108) was grown as previously described by guest on November 11, 2017 http://www.jbc.org/ Downloaded from

#1 The abbreviations used are: D, glucose 6-phosphate-dependent form of glycogen synthetase; I, glucose 6-phosphate-independent form of glycogen synthetase; RI, ratio of independence (for definition see text).

#2 In a mixture of I and D form, the ratio of independence is proportional, although not necessarily identical, to the percentage

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out glucose-6-P divided by the activity with 8 mm glucose-6-P, the result being multiplied by 100 (1).

**Purification of High Molecular Weight Glycogen—Rabbit liver glycogen type III from Sigma (20 g) was dissolved in 4 liters of 0.05 M Tris-chlorate at pH 7.5, containing 1 mM mercaptoethanol.** The solution was passed through a DEAE-cellulose column (4 X 20 cm) previously equilibrated with the same buffer. Ethanol was added to the effluent to a concentration of 60% (v/v) and the precipitated glycogen was collected in a Sorvall continuous flow rotor. The precipitate was washed with absolute ethanol and ether and dried in a desiccator under reduced pressure. A high molecular weight fraction was isolated by chromatography on agarose. A 2-g portion of glycogen purified on DEAE-cellulose was dissolved in 5 ml of water and the mixture was applied to a DEAE-cellulose column (5 X 90 cm) previously equilibrated with 45 mM Tris-chlorate at pH 7.5, containing 1 mM mercaptoethanol and 10% glycerol (Buffer A). Elution was carried out with the same buffer and the material excluded from the column was collected. From this fraction, glycogen was precipitated with ethanol and washed as described above.

**Treatment of α-Amylase with Phenylmethylsulfonyl Fluoride—**A solution containing 3 mg per ml of α-amylase and 0.01 M phenylmethylsulfonyl fluoride was incubated at 30° for 40 min and then dialyzed in the cold against Buffer A. A precipitate which appeared after dialysis was centrifuged off at 20,000 X g for 10 min. The supernatant fluid, which was free of protease activity as measured by an Azocoll assay (5), was used for treatment of yeast extracts.

**Polyacrylamide Gel Electrophoresis—**Disc gel electrophoresis of native glycogen synthetase was carried out as described by Ornstein and Davis (6, 7) with 7%, gels. For determination of subunit size, sodium dodecyl sulfate treatment and subsequent gel electrophoresis were carried out according to Weber and Osborn (8). A linear calibration curve was obtained with proteins ranging in subunit size from 35,000 to 135,000 daltons, which corresponded to a relative migration range of 80 to 29%. All gels were stained with Coomassie brilliant blue overnight and destained in a mixture of 40% ethanol and 7% acetic acid.

**Ultracentrifugation—**Meniscus depletion sedimentation equilibrium experiments were performed at 3 to 5° in a Spinco model E ultracentrifuge equipped with a temperature control unit and an electronic speed control. Double sector interference cells and a Rayleigh optical system were utilized. Rotor speed varied from 6,000 to 12,000 rpm. The lack of significant pattern changes in the last 4 to 8 hours was accepted as an indication that equilibrium had been achieved. In all experiments overspeeding demonstrated that the meniscus had in fact been depleted of protein.

**Results**

**Enzyme Purification**

The yeast used for preparation of the enzyme was harvested about 30 min after the onset of the stationary phase of growth. At this point the percentage of D form is lower than during the logarithmic phase (1), but this disadvantage is more than compensated for by the large increase in total glycogen synthetase activity which occurs during the early stationary phase. Furthermore, under the conditions used for extraction the I form appeared to be lower than previously found and could be easily eliminated by DEAE-cellulose chromatography as described earlier (4).

**Table I**

<table>
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<tr>
<th>Fraction</th>
<th>Total volume</th>
<th>Total activity</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Recovery</th>
<th>Relative purification</th>
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<td>units</td>
<td>mg</td>
<td>umol/mg protein</td>
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<td>0.766</td>
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<td>140</td>
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<td>300</td>
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<td>Second gel filtration</td>
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<td>42</td>
<td>37</td>
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</table>

All purification steps were carried out between 0 and 4° unless otherwise stated.

1. **Extraction—**Yeast was harvested by centrifugation in a continuous Sharplies centrifuge and washed twice with cold distilled water. In order to obtain good breakage in the subsequent step the cells should be processed immediately. A slurry was made with 240 g of yeast (wet weight) and 20 to 30 ml of 50 mM Tris-chlorate at pH 7.5, containing 1 mM EDTA, 1 mM mercaptoethanol, and 0.05 mM phenylmethylsulfonyl fluoride (Buffer B); 50-ml portions were extruded through a French pressure cell at 8000 p.s.i. The broken cell suspension was diluted with 120 ml of Buffer B and centrifuged for 10 min at 20,000 X g. The supernatant fluid (S1) was retained. The pellet consisted of two easily distinguishable layers: the top one contained cell walls and cell debris and the bottom one, intact cells. The top layer was carefully removed with a spatula and saved; the bottom layer was again submitted to treatment in the French press followed by centrifugation. The supernatant fluid (S2) was saved. The top layer of the new pellet was mixed with that obtained previously and the combined material was washed once with 100 ml of Buffer B. The supernatant liquid from this washing was combined with S1 and S2 (crude extract, see Table 1).

2. **DEAE-cellulose Chromatography—**The crude extract (415 ml) was incubated for 30 min at 30° with 6 mg of phenylmethylsulfonyl fluoride-treated α-amylase. After incubation, 300 ml of 45 mM Tris-chlorate at pH 7.5, containing 1 mM mercaptoethanol, 0.05 mM phenylmethylsulfonyl fluoride, and 10% glycerol (Buffer C) were added and the mixture was applied to a DEAE-cellulose column (4 X 17 cm) previously equilibrated with the same buffer. The column was eluted successively with 190 ml of Buffer C and 570 ml of the same buffer containing 0.5 M KC1 in the reservoir. Fractions containing glycogen synthetase emerged at about 0.25 M KC1 and were pooled.

3. **Ammonium Sulfate Fractionation—**Solid ammonium sulfate, 277 g per liter (45% of saturation), was added to the pooled fractions from the previous step. After stirring for 1 hour the precipitate was collected by centrifugation at 37,000 X g during 10 min and dissolved in a minimal amount of Buffer C containing 0.02% sodium azide.

4. **First Chromatography on Bio-Gel A-5m—**The fraction from
the ammonium sulfate precipitation, 7 ml, was applied to a Bio-Gel A-5m column (2.5 × 90 cm) previously equilibrated with Buffer C containing 0.02% sodium azide. Elution was carried out with the same buffer. Fractions of 3.5 ml were collected and those containing glycogen synthetase activity (Fractions 79 to 98) were pooled (see Fig. 1). The solution was concentrated to a volume of 3 to 4 ml by ultrafiltration with an Amicon filtration apparatus and a UM-10 membrane.

6. Second Chromatography on Bio-Gel A-5m—High molecular weight glycogen (see "Methods") was added to the concentrated fraction to yield a final concentration of 180 mg per ml. The viscous solution was applied to the same Bio-Gel A-5m column used previously. Buffer C was again used, both for equilibration and washing from the column. Glycogen synthetase now emerged with the excluded glycogen peak, whereas most of the protein was eluted at the position which corresponded to the synthetase in the chromatography of Step 4 (see Fig. 1). Fractions 40 to 68 were pooled.

6. Second Chromatography on DEAE-cellulose—The glycogen synthetase fraction obtained from the second agarose column was diluted with enough Buffer A to yield a final glycogen concentration of 5 mg per ml and was applied to a DEAE-cellulose column (1.5 × 12 cm) previously equilibrated with Buffer A. Elution was started with 20 ml of the same buffer, followed by 40 ml of 0.2 M KCl, also dissolved in Buffer A. Thereafter a linear gradient was applied with 110 ml of 0.2 M KCl in Buffer A in the mixing chamber and an identical volume of 0.38 M KCl in the same buffer in the reservoir. Glycogen emerged in the void volume and could be recovered for further use by precipitation with 66% ethanol. Glycogen synthetase was eluted by the KCl gradient. The effluent fractions with high glycogen synthetase activity were pooled and concentrated to 2 to 3 ml in an Amicon ultrafiltration cell fitted with a UM-10 membrane. A summary of the results appears in Table I. A purification of 5500-fold was attained; both the over-all purification and the final specific activity were reproducible in different batches.

**Properties of Purified Enzyme**

**Stability**—The purified enzyme was stable for several months when stored at −20° in the presence of Buffer A. Incubation with 10 mM oxidized glutathione in Buffer A at 30° for 30 min (9) did not affect the activity.

**Disc Gel Electrophoresis**—When submitted to disc gel electrophoresis, purified preparations showed a single protein band with a slightly diffuse trailing edge (Fig. 2). After slicing and extracting the gel, glycogen synthetase activity was found to coincide with the protein band.

**Ultracentrifugation**—A plot of the logarithms of protein concentration in the cell versus the square of the distance from the center of rotation yielded a straight line (see Fig. 3). From these data and the assumption of \( \bar{
u} = 0.725 \) a molecular weight of 300,000 was calculated.

**Subunit Structure**—Acrylamide electrophoresis in sodium dodecyl sulfate resulted in a major protein band, with variable...
amounts of a faster band, which in some preparations rivaled the slow moving one in intensity (see Fig. 4). The apparent molecular weights of the two components are 77,000 and 71,000, as determined with a series of standard proteins (Fig. 5). As is discussed in the accompanying paper (10), it appears probable that the slow component is a subunit of the native enzyme and the faster one is a product of proteolysis. The amount of the fast component was smaller when strict precautions against proteolysis were taken; these included addition of phenylmethylsulfonyl fluoride during the first few steps of purification and of sodium azide to the agarose column to prevent bacterial contamination.

On the basis of the molecular weights assigned to the subunits and to the native enzyme, it would appear that the latter is a tetramer.

Kinetics—The RI of the purified enzyme was high compared to that of earlier preparations (1, 2). Even in a preparation which appeared to have undergone very little proteolysis (see Fig. 4A) the RI was 26. Another difference was found in the activation curve yielded by glucose-6-P in the presence of ATP (see Fig. 6). The shape of the curve was intermediate between those previously obtained with the D and I forms (1, 2).

With partially purified preparations a biphasic curve was observed in reciprocal plots of reaction rate versus UDP-glucose.

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**Fig. 4.** Acrylamide gel electrophoresis of D form glycogen synthetase in the presence of sodium dodecyl sulfate. Four different preparations of the enzyme are shown. The wires inserted at the bottom of the gels correspond to the position of the tracking dye. The arrow points to a faint band present in all the gels. Its position would correspond to that of a dimer.

**Fig. 5.** Determination of the apparent molecular weight of the subunits of glycogen synthetase from the electrophoretic mobility of standard proteins in the presence of sodium dodecyl sulfate.

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**Fig. 6 (left).** Effect of glucose-6-P on enzyme activity in the presence of ATP, ADP, and AMP, each at 4 mM. The upper curve shows the activity without added nucleotide. The assays were carried out under standard conditions, except that UDP-glucose was 0.4 mM and the buffer was 50 mM cacodylate at pH 6.5. The activity at 8 mM glucose-6-P in the absence of inhibitors was taken as 100.

**Fig. 7 (center).** Effect of UDP-glucose concentration on reaction rate at different levels of glucose-6-P. The assay was carried out under standard conditions, except for the concentrations of UDP-glucose (UDPG) and glucose-6-P (G-6-P) as indicated.

**Fig. 8 (right).** Effect of UDP-glucose concentration on reaction rate at different levels of glycogen. The assay was carried out under standard conditions except for the concentrations of UDP-glucose (UDPG) and glycogen, as indicated. The concentration of glucose-6-P was 8 mM.
concentration (2); the same behavior was manifested by the homogeneous enzyme (Fig. 7). In the presence of high concentrations of glucose-6-P, a single straight line was obtained (Fig. 7), although in some cases the component which corresponds to the higher $K_m$ could still be detected (see Fig. 8, lowest curve). The average values of the two Michaelis constants were 0.4 and 1.1 mM, respectively, whereas those obtained previously were 0.42 and 1.4 mM (2). It should be mentioned that the older measurements were made at pH 6.5 and the present ones at pH 7.5.

Previous preparations of the synthetase contained large amounts of glycogen, whereas those obtained in this study were devoid of the polysaccharide. Therefore it was possible to examine the effect of glycogen concentration on the kinetics. As shown in Fig. 8, the break in the reciprocal plot persisted at different levels of the polysaccharide, although it was more marked at low concentrations. The $K_m$ values did not seem to be affected by glycogen. When UDP-glucose was kept constant and glycogen was changed, a biphasic curve was again observed, and the lower of the two apparent $K_m$ values varied within a narrow range (Fig. 9). The effect of glucose-6-P on the reciprocal plot of rate versus glycogen concentration was also examined (Fig. 10); in this case it is difficult to assess if the phosphoric ester modifies the biphasic character of the curve. Glucose-6-P did cause a substantial change in the smaller $K_m$, in contrast with the results obtained when UDP-glucose rather than glycogen was the variable substrate.

Effects of $-SH$ Reagents—Inubcation of the enzyme for 30 min at 30°C with 10 μM $p$-chloromercuribenzoate led to a 90% decrease in the activity. Further incubation with 10 mM di-thioerythritol partially reversed the inhibition. In the presence of 5 mM UDP-glucose the mercurial inhibited only 60%. When the incubation time was reduced to 10 min, the effect of UDP-glucose was more marked, i.e., the inhibition was 85% without and 10% with UDP-glucose. Glycogen (24 mg per ml) or glucose-6-P (10 mM) were without effect on the inhibition.

Other $-SH$ reagents were less effective. After 30-min incubation at 30°C with each of the following substances, the inhibitions observed were: 3 mM $N$-ethylmaleimide, 83%; 1 mM 5,5'-dithiobis(2-nitrobenzoic acid), 77%; 1 mM iodo benzole, 22%; and 10 mM iodo acetic acid, 10%.

Preparation of $^{32}P$-Labeled Enzyme

Previous evidence that the yeast D-form synthetase is phosphorylated was based on the ATP requirement for the I to D transformation (2). In order to investigate the presence of phosphate and to eventually determine the phosphate to protein ratio, the I form of the enzyme was converted into D form in the presence of $[^{32}P]ATP$ and the product was resolated.

A crude extract (450 ml) was obtained from 390 g of yeast as described above for the purification of the D form synthetase and dialyzed overnight against two 4-liter changes of 0.05 M Tris-chloride, pH 7.5, containing 1 mM mercaptoethanol and 0.05 mM phenylmethylsulfonyl fluoride. During dialysis the D form of glycogen synthetase was converted into the I form (4). The dialyzed extract was incubated with 7 mg of phenylmethylsulfonyl fluoride treated a-amylase for 30 min at 30°C and then applied to a DEAE-cellulose column (4 x 17 cm) previously equilibrated with Buffer C. The column was eluted successively with 190 ml of Buffer C and with 570 ml of 0.2 M KCl in Buffer C. The latter solution eluted a peak of glycogen synthetase in the I form, which also contained protein kinase activity (4). Solid ammonium sulfate (35.1 g/100 ml) was added to the pooled peak fractions. After stirring for 1 hour, the precipitate was collected by centrifugation at 37,000 × g for 10 min. The pellet was taken up with Buffer C and dialyzed overnight against the same buffer. The dialysate was incubated for 15 min at 30°C in the presence of 10 mM glycglycine at pH 7.5 and 5 mM magnesium acetate in order to reactivate any cold-inactivated enzyme (3). Thereafter, $[^{32}P]ATP$ (specific activity, $5 \times 10^{5}$ cpm per μmole) was added to a final concentration of 5 mM and the reaction mixture (total volume, 75 ml) was further incubated at 30°C for 30 min, to convert the I into the D form. Solid ammonium sulfate (0.28 g per ml) was added and the suspension was centrifuged at 37,000 × g for 10 min. The precipitate was dissolved in Buffer C containing 0.02% sodium azide and applied to a Bio-Gel A-5m column (2.5 × 90 cm). The remainder of the purification procedure, including the second agarose column and the second chromatography on DEAE-cellulose, was as described above for the purification of the D form. Only a small proportion of the $^{32}P$ incorporated into protein accompanied the synthetase through the agarose column and the final DEAE-cellulose column. In the latter, the enzymatic activity was eluted in a peak coincident with those of $^{32}P$ and protein (see Fig. 11).

The radioactive phosphate incorporated into the purified enzyme was 19 μmoles per mg of protein which corresponds to about 1.4 moles/77,000 g. Incubation of the labeled enzyme with a muscle phosphoprotein phosphatase resulted in release of radioactivity and a parallel increase in glucose-6-P independent activity, as shown in Fig. 12.
three subunits, each containing 12 alkali-labile phosphate groups. The rat liver glycogen synthetase, D form, would also consist of about 7 moles per subunit. According to Lin and Segal (15), the latter authors found a high content of phosphorus in the protein, and of the subunits it would appear that the enzyme is a multimer. However, it was not possible to obtain enzymes showing the biphasic character of the Lineweaver and Burk plot, obtained with the muscle enzyme (10), it was determined that the RI of Enzyme A in Fig. 4 was 20 and that of Enzyme D, 52. Furthermore, precautions against proteolysis resulted in a decrease of the fast component in the final preparation. However, it was not possible to obtain enzymes showing a single electrophoretic band.

From the molecular weight determinations of the intact protein and of the subunits it would appear that the enzyme is a tetramer. A similar finding was reported by Soderling et al. (13) for the muscle enzyme, whereas the data of Smith et al. (14) are more in agreement with a trimeric structure. The latter authors found a high content of phosphorus in the protein, about 7 moles per subunit. According to Lin and Segal (15) the rat liver glycogen synthetase, D form, would also consist of three subunits, each containing 12 alkali-labile phosphate groups.

**FIG. 11 (left).** DEAE-cellulose chromatography of 32P-labeled enzyme. For details see text. Protein was measured from the difference in absorbance at 210 and 220 nm (11). Protein phosphatase and the appearance of glucose-6-P independent activity. The reaction mixture contained 40 μg of 32P-labeled glycogen synthetase, 0.3 units of muscle glycogen synthetase phosphatase (12), 0.01 M imidazole-chloride (pH 7.4), 1.5 mM MβCl₂, 0.01% bovine serum albumin, and 0.1 mM dithiothreitol in a total volume of 240 μl. The incubation was carried out at 30°. At timed intervals 50 μl aliquots, containing about 1100 cpm, were withdrawn and the reaction was terminated by adding 0.35 ml of 10% trichloroacetic acid and 0.1 ml of 1% bovine serum albumin. After centrifugation, the precipitate was redissolved in 0.5 ml of 0.1 N NaOH and the protein was reprecipitated by the addition of 0.5 ml of 10% trichloroacetic acid. The final precipitate was dissolved in 0.5 ml of 95% formic acid and counted in a scintillation counter after adding 10 ml of Aquasol. The amount of radioactive phosphate liberated was calculated by difference. In other aliquots glycogen synthetase was measured. The plotted values represent the difference between the activity at any given time and the zero time activity when the assay was performed in the absence of glucose-6-P. mU, milliunits.

**FIG. 12 (right).** Correlation between 32P release by a phosphoprotein phosphatase and the appearance of glucose-6-P independent activity. The reaction mixture contained 40 μg of 32P-labeled glycogen synthetase, 0.3 units of muscle glycogen synthetase phosphatase (12), 0.01 M imidazole-chloride (pH 7.4), 1.5 mM MβCl₂, 0.01% bovine serum albumin. After centrifugation, the precipitate was redissolved in 0.5 ml of 0.1 N NaOH and the protein was reprecipitated by the addition of 0.5 ml of 10% trichloroacetic acid. The final precipitate was dissolved in 0.5 ml of 95% formic acid and counted in a scintillation counter after adding 10 ml of Aquasol. The amount of radioactive phosphate liberated was calculated by difference. In other aliquots glycogen synthetase was measured. The plotted values represent the difference between the activity at any given time and the zero time activity when the assay was performed in the absence of glucose-6-P. mU, milliunits.

**DISCUSSION**

The purification of yeast glycogen synthetase, D form, depends on two critical steps: the chromatography on DEAE-cellulose whereby any I form present is separated out and the double chromatography on agarose. In the latter procedure, glycogen synthetase is first isolated along with those proteins which have a similar Stokes radius. After adding high molecular weight glycogen to the eluate and repeating the chromatography, most of the proteins emerge in the same fractions, except for those which bind to glycogen and will therefore be eluted in the void volume together with the polysaccharide. Because of the element of specificity involved, this method results in a large purification and probably could be adapted to analogous situations.

The purified enzyme appears to be homogeneous by the criteria of DEAE-cellulose chromatography, ultracentrifugation, and disc gel electrophoresis. Nevertheless, aerylamide electrophoresis in the presence of sodium dodecyl sulfate gave rise to two bands of variable relative intensity. As will be discussed to a greater extent in the accompanying paper (10) it seems probable that the fast band is the product of proteolytic action. In agreement with the finding that proteolysis causes an increase both in electrophoretic mobility and in RI (11), it was determined that the RI of Enzyme A in Fig. 4 was 20 and that of Enzyme D, 52. Furthermore, precautions against proteolysis resulted in a decrease of the fast component in the final preparation. However, it was not possible to obtain enzymes showing a single electrophoretic band.

In contrast, our results with the 32P-labeled enzyme correspond to a content of 1.4 moles of phosphate per subunit. If either the conversion of D to I form, previous to incubation with [γ-32P]-ATP, or the I to D transformation obtained in the presence of the radioactive nucleotide had been incomplete, less than the theoretical amount of 32P would have been incorporated. In addition, some of the acceptor sites for phosphate might have been missing because of previous proteolysis (10). Conversely, it is possible that some phosphate was introduced at unspecific locations in the protein. Thus, some uncertainty remains about the phosphate content of the native, completely phosphorylated D form, but it seems probable that the correct value should be either one or two groups per subunit. The phosphate content of the rabbit muscle and rat liver enzyme appears to be excessive and it is possible that some of it was bound at unspecific sites.

The labeling of the synthetase with 32P is the first direct evidence that the yeast D form is phosphorylated. The correlation between the loss of label and the increase in I activity during incubation with a phosphatase is in agreement with this interpretation.

The preparations of purified D form synthetase obtained in this work were less dependent on glucose-6-P than the crude enzymes of previous studies (1, 2). The lowest RI values determined in recent preparations were in the 20 to 30 range. Furthermore, the curve of activity versus glucose-6-P concentration, in the presence of ATP, was intermediate between those previously obtained with the D and I forms (1, 2). There is no explanation for these discrepancies. One difference between older and more recent preparations is that the former came from lyophilized yeast in the logarithmic phase of growth, whereas the latter were obtained by breaking fresh cells from the stationary phase. The purification of synthetase from logarithmic phase yeast has not yet been carried out with the procedure of this study because of the low yield of cells and enzyme in the early stage of growth.

The biphasic character of the Lineweaver and Burk plot, ob-
served previously with a relatively crude preparation from yeast (2) and more recently with glycogen synthetases from other sources (16, 17) was also found with the purified D form. Since the latter was free of glycogen, the effect of varying concentrations of the polysaccharide on the kinetics could be studied. No change in the two $K_m$ values for UDP-glucose was detected at different concentrations of glycogen. When UDP-glucose was maintained constant and glycogen was varied, the break in the reciprocal plot was again observed. A simplistic explanation of these results would be that the enzyme consists of two populations of molecules, which differ in their affinity for substrate and thus generate the two $K_m$ values. The finding that the enzyme in sodium dodecyl sulfate gave rise to a variable mixture of two subunits, presumably arising from partial proteolysis, suggested that the two types of molecules could represent the intact and the partially degraded enzyme. Nevertheless, as shown in the accompanying paper (10), there seems to be no correlation between extent of proteolysis and kinetic results.

In one experiment the crude enzyme was converted to the $I$ form by dialysis, then submitted to DEAE-cellulose chromatography and finally reconverted into the $D$ form by incubation with ATP and Mg$^{2+}$; this is the same procedure used in the preparation of the $3^P$-labeled enzyme. The regenerated $D$ form also showed the biphasic Lineweaver-Burk plot. Since this property survived through the two conversions, it appears to be an intrinsic attribute of the enzyme rather than an experimental artifact.

Another explanation of the abnormal inverse plot could be negative cooperativity (18). However, since the effect is shown both by UDP-glucose and glycogen, this interpretation would imply that more than 1 molecule of polysaccharide would bind to the enzyme. This is certainly possible, but sterically difficult to visualize, since glycogen is much larger than the synthetase.

The two $K_m$ values for UDP-glucose did not vary with glycogen concentration nor did those for glycogen change with the UDP-glucose concentration. These results differ from those obtained with the rabbit muscle $D$ form, where the double-reciprocal plot for each of the two substrates yielded a series of parallel lines when the other substrate was varied (19). The lack of effect of glucose-6-P concentration on the smaller $K_m$ for UDP-glucose was also reported for the enzyme from trout liver (17), whereas the synthetases from rat and tadpole liver showed a different behavior (20, 21). Thus, glycogen synthetases from different sources exhibit quite different kinetics, both with respect to the character (normal or bimodal) of double-reciprocal plots and to the variations of $K_m$ values. For this reason and because of the complex kinetics observed, attempts to determine the mechanism of the reaction by an analytical treatment of the results appear premature. The situation is further complicated by the nature of one of the substrates, i.e. glycogen; this is a large molecule to which the enzyme may bind in more than one way (22); furthermore, in each round of reaction the growing point of the chain is regenerated; chain growth may take place by processive or random mechanism (23), and individual chains differ in distance to a branching point and therefore probably in steric properties. Changes in some of these parameters rather than in the basic reaction mechanism may account for some of the differences observed between synthetases from various sources.

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