Crystallization and Properties of Uridine Diphosphate Glucose Pyrophosphorylase from Liver

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SUMMARY

The purification and crystallization of uridine diphosphate glucose pyrophosphorylase from calf liver has been described. As much as 0.3% of the extractable protein is the pyrophosphorylase, which catalyzes the synthesis of uridine diphosphate glucose. After recrystallizing the enzyme twice, constant specific activity is attained; it appears to be almost homogeneous on polyacrylamide gel electrophoresis and on ultracentrifugation.

The recrystallized enzyme is not highly specific for either the nucleoside or the sugar component of the nucleoside diphosphate sugar. When thymidine, cytidine, or guanosine are substituted for uridine, or when galactose, mannose, or xylose are substituted for glucose, the resulting compounds have from 0.1 to 4% of the activity of uridine diphosphate glucose.

The molecular weight of the recrystallized enzyme is about 400,000; the specific activity, 240; and the turnover number, 83,000. The following $K_m$ values were obtained for each substrate: glucose 1-phosphate, $5.5 \times 10^{-5}$ M; uridine triphosphate, $2 \times 10^{-5}$ M; pyrophosphate, $8.4 \times 10^{-5}$ M; and uridine diphosphate glucose, $6 \times 10^{-4}$ M. Inorganic phosphate inhibits the enzyme competitively with pyrophosphate at a $K_i$ of $4 \times 10^{-4}$ M and uridine diphosphate competitively with uridine diphosphate glucose at a $K_i$ of $1.5 \times 10^{-4}$ M.

A number of novel nucleoside diphosphate sugars have been biosynthetically prepared from nucleoside triphosphates and sugar 1-phosphates with enzymes extracted from liver or mammary tissue (1-3). Nucleoside diphosphate sugars have been formed when the nucleoside is adenosine, guanosine, inosine, uridine, thymidine, or cytidine in combination with one of the following sugars: glucose, galactose, mannose, or xylose. Since a physiological function is not necessarily implied for all of the nucleosides which have been formed, it became of interest to fractionate the extracts and examine the specificity and other properties of the catalysts. Consequently, a 500-fold purified guanosine diphosphate hexose pyrophosphorylase (4) and a 300-fold purified and crystalline uridine diphosphate glucose pyrophosphorylase have been obtained. This latter enzyme, constituting 0.2 to 0.3% of the extractable protein of calf liver, is the UDP-glucose pyrophosphorylase (UTP:α-1-glucose 1-phosphate uridylyltransferase, EC 2.7.7.9) which catalyzes the biosynthesis of UDP-glucose from UTP and glucose-1-P (4-12). The fractionation and crystallization procedures, together with some of the properties of the UDP-glucose pyrophosphorylase, are the subject of this report.

EXPERIMENTAL PROCEDURE

Materials and Methods

Reagents—The enzymes, chemicals, and other supplies were purchased from commercial sources with the exception of TDP glucose, mannose-1-P, and galactose-1-P which were synthesized according to procedures which have been described (13, 14).

Chromatography—For qualitative identification of the various substrates and reaction products, chromatographic separation was achieved with a polyvinylideneimine-impregnated paper (15). The chromatograms were developed with LiCl; the ultraviolet-absorbing compounds were detected with a Mineralight ultraviolet lamp, and phosphorus with a molybdate spray (16). Spot prints of ultraviolet-absorbing spots were made by the procedure of Markham and Smith (17).

Spectrophotometry—A Beckman model DU spectrophotometer equipped with a Gilford automatic sample changer and recorder (18) was used for spectrophotometric measurements.

Definition of Unit and Specific Activity—One unit of enzyme is defined as that amount which liberates 1 μmole of product per min at 25°. Specific activity is defined as units of enzyme per mg of protein present. Protein was determined by the procedure of Lowry et al. (19) or that of Warburg and Christian (20).

Electrophoresis and Sedimentation—Electrophoresis on polyacrylamide gel gave a qualitative index of purity of the fractions. The columns were observed visually after staining with naphthol blue black (21). The sedimentation characteristics were determined by the sucrose density gradient procedure of Martin and Ames (22) and in the Spinco model E analytical ultracentrifuge.

Measurement of Pyrophosphorylase Activity—In the direction of pyrophosphorolysis of the nucleoside diphosphate sugars, the enzyme reaction was determined quantitatively by two assays. Assay 1: When the sugar was glucose, the formation of glucose-1-P was estimated quantitatively by converting it to glucose-6-P and subsequently to 6-P-gluconate (10). The concomitant formation of NADPH was measured at 340 mμ. The assay mixture contained, in a final volume of 0.5 ml, 45 μmoles of
Fig. 1. Chromatography of UDP-glucose pyrophosphorylase, Fraction III, on DEAE-cellulose. Protein was not eluted until the washing was begun at tube 92 with 0.02 M triethanolamine (pH 8.5) containing 0.02 M NaCl. At tube 296 the NaCl concentration was progressively increased to 0.2 M. The column dimensions were 2.5 × 50 cm; flow rate was about 1 ml per min, and 15-ml fractions were collected. ---, milligrams of protein per ml; --, units per ml.

Tris-acetate buffer (pH 7.8), 1 μmole of magnesium acetate, 0.2 μmole of NADP, 1 μmole of PPi, 0.2 μmole of nucleoside diphosphate glucose, excess phosphoglucomutase and glucose-6-P dehydrogenase, and enough pyrophosphorylase to produce an absorbance change of 0.06 to 0.3 in 15 min at 25°C. Assay 2: When ATP, GTP, ITP, or UTP were formed in the reaction, they were substrates for the phosphorylation of 3-P-glycerate and reduction by NADH to glyceraldehyde-3-P (23-25). The oxidation of NADH was the quantitative index in a reaction mixture which contained, in a final volume of 0.5 ml, 20 pmoles of triethanolamine buffer (pH 7.8), 1 μmole of magnesium acetate, 1 μmole of PPi, 0.5 μmole of hydrazine sulfate, 0.6 μmole of 3-P-glycerate, 0.12 μmole of NADH, 0.2 μmole of nucleoside diphosphate sugar, excess 3-P-glycerate kinase and glyceraldehyde-3-P dehydrogenase, and enough pyrophosphorylase to produce an absorbance change of 0.06 to 0.3 in 15 min at 25°C.

In the direction of synthesis of nucleoside diphosphate sugar, the products were quantitatively determined by hydrolysis with venom pyrophosphatase followed by determination of glucose-1-P (26). The quantity of UDP-glucose formed was also specifically measured with the calf liver UDP-glucose dehydrogenase (27).

Fractionation of Liver

Step 1: Extraction—All steps were carried out at 4°C, and all centrifugation was carried out at 23,000 × g for 20 min, unless otherwise stated. A portion, 1 kg, of calf liver was cut into pieces about 1 cm thick, homogenized in 2 liters of 0.03 M KOH-0.005 M EDTA for 2 min, and allowed to stand for 15 min. The mixture was centrifuged, the sediment was discarded, and the supernatant fluid was filtered through glass wool. After adjusting the supernatant fluid to pH 6.9 with glacial acetic acid, the pH of the extract was adjusted to 6.9 with glacial acetic acid and 400 ml of 2% protamine sulfate solution were added directly to this extract. After stirring the mixture for 10 to 15 min, it was centrifuged, and the supernatant fluid filtered through glass wool.
0.2 volume of a 2% protamine sulfate solution was added with stirring; the mixture was then stirred for 10 to 15 min, and centrifuged. The resulting supernatant fluid was adjusted to pH 8.5 with concentrated NH₄OH and is Fraction I. Throughout the balance of the purification procedure, the pH was maintained at 8.5, and triethanolamine buffer used subsequently was also at pH 8.5.

Step 2: Ammonium Sulfate Fractionation—The ammonium sulfate concentration was brought to 40% saturation (28) in Fraction I by adding the solid salt slowly while stirring (small quantities of concentrated NH₄OH were necessary to maintain the pH at 8.5). After 30 min of stirring, the precipitate was centrifuged and discarded; ammonium sulfate was added to the supernatant to bring the concentration to 58% saturation. The mixture was again stirred for 30 min and centrifuged. The precipitate was dissolved in 0.02 M triethanolamine buffer (about 0.1 of the volume of Fraction I) and dialyzed overnight against the same buffer (Fraction II).

Step 3: Calcium Phosphate Gel Treatment—Calcium phosphate gel was added to Fraction II (29) (1 mg, dry weight, per mg of protein); after stirring 10 min, it was centrifuged. The precipitate was discarded, and the supernatant (Fraction III) was retained.

Step 4: Diethylaminoethyl Cellulose Column Chromatography—The DEAE-cellulose was prepared by successive treatments of the commercial product with the following: 1 N NaOH, water until the pH reached about 9.0, 0.5 M Na₂PO₄, and finally deionized distilled water until the pH of the eluate was 7.0. The DEAE-cellulose was suspended in 0.02 M triethanolamine buffer and added to a column (2.5 x 50 cm) which was rinsed overnight with the same buffer to complete packing and equilibration. Fraction III was placed on the column and washed with 2 liters of 0.02 M triethanolamine buffer containing 0.02 M NaCl; 15-ml samples were collected with a flow rate of about 1 ml per min. The elution gradient consisted of 1 liter of 0.02 M triethanolamine buffer plus 0.02 M NaCl in a mixing flask, and 2 liters of 0.02 M triethanolamine buffer containing 0.2 M NaCl in the overhead container. Only the tubes with the highest specific activity (preferably over 10) were combined into Fraction IV. The pattern of elution of the enzyme from DEAE-cellulose and its separation from other proteins is shown in Fig. 1.

Step 5: Second Ammonium Sulfate Fractionation and Crystallization—Fraction IV was brought to 60% saturation with solid ammonium sulfate, and the mixture was stirred for 30 min. After centrifugation, the precipitate was redissolved at 30° by addition of the smallest possible amount of 0.02 M triethanolamine buffer (about 5 ml), and then cooled in an ice bath. After a few

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**TABLE I**

Purification and crystallization of UDP-glucose pyrophosphorylase from calf liver

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Extract</td>
<td>2,260</td>
<td>109,600</td>
<td>84,900</td>
<td>0.77</td>
<td>100</td>
</tr>
<tr>
<td>2. (NH₄)₂SO₄, 40 to 58%</td>
<td>335</td>
<td>30,100</td>
<td>53,900</td>
<td>1.78</td>
<td>63.4</td>
</tr>
<tr>
<td>3. Ca₃(PO₄)₂; gel, 1:1</td>
<td>1,210</td>
<td>9,700</td>
<td>25,300</td>
<td>2.60</td>
<td>28.6</td>
</tr>
<tr>
<td>4. DEAE-cellulose</td>
<td>520</td>
<td>1,560</td>
<td>23,400</td>
<td>15.0</td>
<td>26.4</td>
</tr>
<tr>
<td>5. 1st crystallization</td>
<td>2.0</td>
<td>102</td>
<td>17,000</td>
<td>167</td>
<td>19.0</td>
</tr>
<tr>
<td>2nd crystallization</td>
<td>3.1</td>
<td>40</td>
<td>9,600</td>
<td>240</td>
<td>11.5</td>
</tr>
<tr>
<td>3rd crystallization</td>
<td>3.0</td>
<td>18</td>
<td>4,240</td>
<td>236</td>
<td>5.0</td>
</tr>
</tbody>
</table>

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**Fig. 3.** Velocity sedimentation pattern of crystalline UDP-glucose pyrophosphorylase during ultracentrifugation. Experimental conditions are as follows: protein concentration, 3.5 mg per ml; medium, 0.02 M triethanolamine (pH 7.8) + 0.1 M NaCl; temperature, 4.5°; speed, 42,040 rpm; diaphragm angle, 65°; time, 48 min after speed was attained. Sedimentation is from right to left.

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**Fig. 4.** Dependence of UDP-glucose pyrophosphorylase upon PPi. This is a tracing from the recording of the reaction by the Gilford converter (18). A, complete system; B, minus PPi, until 9 min. The complete system contained, in a volume of 0.5 ml, 45 pmoles of Tris-acetate buffer (pH 7.8), 1 µ mole of magnesium acetate, 0.2 µ mole of NADP, 0.2 µ mole of nucleoside diphosphate glucose, excess phosphoglucomutase and glucose-6-P dehydrogenase, and enough pyrophosphorylase to produce an absorbance change of 0.06 to 0.3 in 15 min at 25°.
FIG. 5. Chromatographic evidence of the reaction products of UDP-glucose pyrophosphorylase. This is a contact print of a chromatogram exposed with ultraviolet light (17). The reaction mixtures contained 0.5 μmole of each substrate, 1 μmole of magnesium, and 83 μg of enzyme in 0.2 ml of 0.1 M Tris-acetate buffer (pH 7.8). After incubation for 1 hour at 30°, the mixture was heated to 100° for 2 min, cooled, centrifuged, and spotted on polyethyleneimine-impregnated filter paper (15). Lanes 1 to 4 were developed with 0.3 M LiCl and Lanes 5 to 8 with 0.5 M LiCl. Unincubated standard solutions were spotted in Lanes 1 and 8.

The synthesis of UDP-glucose is shown in Lanes 2 to 4. The composition of the incubation mixtures was: in Lane 2, UTP and UDP-glucose without enzyme; in Lane 3, UTP and glucose-1-P with enzyme; and in Lane 4, UTP and enzyme without glucose-1-P. The enzyme-dependent pyrophosphorolysis of UDP-glucose is illustrated in Lanes 6 to 8. The composition of incubation mixtures was: in Lane 6, UTP and UDP-glucose without enzyme; in Lane 7, UDP-glucose, PPi, and enzyme; and in Lane 8, UDP-glucose and enzyme without PPi. Trace quantities of UMP in the UDP-glucose and UDP in the UTP were initially present.

minutes, fine crystals appeared; the solution usually became heavily opaque if crystallization was proceeding. If no crystals formed after cooling, crystallization was readily induced by gradually increasing the concentration of ammonium sulfate by rapidly stirring in a few drops of saturated solution. The enzyme crystallized in the form of regular bipyramids which were separated from the supernatant at low speed centrifugation (about 2000 x g). The supernatant solution contained approximately 25% of the total activity; it could be used for further crystallization if the protein were first reprecipitated at 60% ammonium sulfate saturation, dissolved, and treated as described above.

Step 6: Recrystallization—The crystalline protein was dissolved in the smallest possible volume of 0.02 M triethanolamine buffer (pH 8.5), about 2 to 3 ml, by warming the sample to 30°. The denatured protein was then removed by centrifugation, and the supernatant solution was dialyzed against 0.02 M triethanolamine buffer overnight. The sample was dialyzed against increasing
of enzyme per ml, aliquots were removed at various time intervals in 0.1 M Tris-acetate buffer (pH 7.8). After addition of 0.35 µg of enzyme per ml, aliquots were removed at various time intervals and heated to boiling to stop the reaction. Equilibrium was attained in about 10 min at 30°. The concentrations of the substrates and reaction products were determined as follows: glucose-1-P by phosphoglucomutase and glucose-6-P dehydrogenase (10), UDP-glucose after hydrolysis with venom pyrophosphatase and then measuring glucose-1-P as above, and PPi by difference. All concentrations are millimolar.

The concentrations of ammonium sulfate in 0.02 M Tris-acetate buffer at pH 7.8, beginning with 10% and proceeding at increments of 2.5% to be affected by the magnesium concentration unlike the plant enzyme (8).

centrations of ammonium sulfate in 0.02 M triethanolamine buffer, beginning with 10% and proceeding at increments of 2.5% until crystallization occurred. Somewhat dependent on the concentration of protein inside the tubing, crystallization began when the ammonium sulfate concentration reached approximately 20% saturation. The crystals formed under these conditions were more uniform and larger in size, but had the same characteristic shape (Fig. 2). The specific activity became constant after the second recrystallization. A summary of the procedure is given in Table 1.

The enzyme, dissolved in a minimal volume of either 0.02 M triethanolamine (pH 8.5) or 0.1 M pyrophosphate (pH 8.5), may be stored at 4° for several weeks. The enzyme has been repeatedly prepared by this procedure over a period of 6 months with essentially the same results. Crystalline enzyme has been obtained from calf liver stored frozen at −20° as well as from fresh liver.

**RESULTS**

**Homogeneity and Molecular Weight of UDP-glucose Pyrophosphorylase**—The twice recrystallized enzyme is almost homogeneous on polyacrylamide gel electrophoresis, but a slower moving trace component is sometimes visible. The sedimentation pattern in the ultracentrifuge (Fig. 3) indicates the possibility of a more rapidly sedimenting component. There is some preliminary evidence that the molecule tends to form aggregates, and the electrophoresis and sedimentation patterns may be indicative of this property.

The molecular weight has been estimated from sucrose density gradient centrifugation (22) to be 350,000 ± 50,000. From a Svedberg constant of 14.8 and assuming a spherical molecule with a partial specific volume of 0.725, a molecular weight of 424,000 has been calculated from velocity sedimentation. The molecular weight of approximately 400,000 and the specific activity of 238, were used to calculate a turnover number of 83,000 moles of substrate per mole of enzyme per min at 25°.

**Stoichiometry and Pyrophosphate Dependence**—The crystalline enzyme has a complete dependence upon pyrophosphate for the formation of glucose-1-P and UTP from UDP-glucose (Fig. 4). It may be noted from this figure that, as reported by Munch-Petersen (10), a slight lag period occurs as activity is initiated. The glucose-1-P and UTP produced in the reaction were measured by the spectrophotometric procedures described as Assay 1 and 2 which are specific for glucose-1-P and nucleoside triphosphate. In addition, the reaction products starting with either glucose-1-P and UTP or with UDP-glucose and PPi have been chromatographed for qualitative identification of the UDP and UDP-glucose (Fig. 5). The UDP-glucose has been further identified with the specific UDP-glucose dehydrogenase. The rate of pyrophosphorylization of UDP-glucose is proportional to enzyme concentration, and the products UTP and glucose-1-P are produced in equimolar amounts (23).

**Equilibrium Constant**—The equilibrium constant in the direction of synthesis of UDP-glucose has been determined to be between 0.28 and 0.34 (Table II). The equilibrium appears not to be affected by the magnesium concentration unlike the plant enzyme (8).

**TABLE II**

**Equilibrium of UDP-glucose pyrophosphorylase reaction**

The substrates were incubated with magnesium acetate, 2 mM, in 0.1 M Tris-acetate buffer (pH 7.8). After addition of 0.35 µg of enzyme per ml, aliquots were removed at various time intervals and heated to boiling to stop the reaction. Equilibrium was attained in about 10 min at 30°. The concentrations of the substrates and reaction products were determined as follows: glucose-1-P by phosphoglucomutase and glucose-6-P dehydrogenase (10), UDP-glucose after hydrolysis with venom pyrophosphatase and then measuring glucose-1-P as above, and PPi by difference. All concentrations are millimolar.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Initial</th>
<th>Final</th>
<th>Initial</th>
<th>Final</th>
<th>Initial</th>
<th>Final</th>
<th>K_eq</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.56</td>
<td>0.28</td>
<td>0.48</td>
<td>0.26</td>
<td>0.10</td>
<td>0.10</td>
<td>0.31</td>
</tr>
<tr>
<td>2</td>
<td>2.82</td>
<td>2.50</td>
<td>0.48</td>
<td>0.14</td>
<td>0.34</td>
<td>0.34</td>
<td>0.33</td>
</tr>
<tr>
<td>3</td>
<td>0.35</td>
<td>0.24</td>
<td>0.29</td>
<td>0.15</td>
<td>0.11</td>
<td>0.11</td>
<td>0.28</td>
</tr>
</tbody>
</table>

**TABLE III**

**Inhibition of UDP-glucose pyrophosphorylase**

Assay 1 was used to determine the constants which were calculated according to Dixon and Webb (31).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Competitive substrate</th>
<th>K_i</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDP, 2 × 10^{-3} M</td>
<td>UDP-glucose, 8 × 10^{-6} M</td>
<td>1.5 × 10^{-4}</td>
</tr>
<tr>
<td>Pr, 3.2 × 10^{-3} M</td>
<td>PPi, 4 × 10^{-6}-3 × 10^{-5} M</td>
<td>3.7 × 10^{-3}</td>
</tr>
</tbody>
</table>

**TABLE IV**

**Specificity of UDP-glucose pyrophosphorylase**

All substrates were added at a level of 0.4 µmole per ml since UDP-glucose saturates the enzyme at this concentration. See "Materials and Methods" for assay procedures.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Assay</th>
<th>Reaction rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDP-glucose</td>
<td>1 and 2</td>
<td>100</td>
</tr>
<tr>
<td>TDP-glucose</td>
<td>1</td>
<td>2.7</td>
</tr>
<tr>
<td>GDP-glucose</td>
<td>1</td>
<td>0.8</td>
</tr>
<tr>
<td>UDP-glucose</td>
<td>1 and 2</td>
<td>0.1</td>
</tr>
<tr>
<td>ADP-glucose</td>
<td>1 and 2</td>
<td>Very low</td>
</tr>
<tr>
<td>UDP-galactose</td>
<td>2</td>
<td>Very low</td>
</tr>
<tr>
<td>UDP-xyllose</td>
<td>2</td>
<td>3.9</td>
</tr>
<tr>
<td>UDP-mannose</td>
<td>2</td>
<td>0.3</td>
</tr>
<tr>
<td>UDP-GlcUA</td>
<td>2</td>
<td>Very low</td>
</tr>
<tr>
<td>UDP-GlcNAl</td>
<td>2</td>
<td>Very low</td>
</tr>
</tbody>
</table>

**TABLE V**

**Homogeneity and Molecular Weight of UDP-glucose Pyrophosphorylase**

The twice recrystallized enzyme is almost homogeneous on polyacrylamide gel electrophoresis, but a slower moving trace component is sometimes visible. The sedimentation pattern in the ultracentrifuge (Fig. 3) indicates the possibility of a more rapidly sedimenting component. There is some preliminary evidence that the molecule tends to form aggregates, and the electrophoresis and sedimentation patterns may be indicative of this property.

The molecular weight has been estimated from sucrose density gradient centrifugation (22) to be 350,000 ± 50,000. From a Svedberg constant of 14.8 and assuming a spherical molecule with a partial specific volume of 0.725, a molecular weight of 424,000 has been calculated from velocity sedimentation. The molecular weight of approximately 400,000 and the specific activity of 238, were used to calculate a turnover number of 83,000 moles of substrate per mole of enzyme per min at 25°.

**Stoichiometry and Pyrophosphate Dependence**—The crystalline enzyme has a complete dependence upon pyrophosphate for the formation of glucose-1-P and UTP from UDP-glucose (Fig. 4). It may be noted from this figure that, as reported by Munch-Petersen (10), a slight lag period occurs as activity is initiated. The glucose-1-P and UTP produced in the reaction were measured by the spectrophotometric procedures described as Assay 1 and 2 which are specific for glucose-1-P and nucleoside triphosphate. In addition, the reaction products starting with either glucose-1-P and UTP or with UDP-glucose and PPi have been chromatographed for qualitative identification of the UDP and UDP-glucose (Fig. 5). The UDP-glucose has been further identified with the specific UDP-glucose dehydrogenase. The rate of pyrophosphorylization of UDP-glucose is proportional to enzyme concentration, and the products UTP and glucose-1-P are produced in equimolar amounts (23).

**Equilibrium Constant**—The equilibrium constant in the direction of synthesis of UDP-glucose has been determined to be between 0.28 and 0.34 (Table II). The equilibrium appears not to be affected by the magnesium concentration unlike the plant enzyme (8).
Optimum pH—The pyrophosphorylase is optimally active over a broad pH range. At pH 8.5, the enzyme has maximum activity; and at pH 7.0 or 9.5, approximately 85% of the maximum activity is measured. Outside of these broad limits, the activity falls off sharply with a change in pH. These data are in agreement with the properties of the enzyme cited in some other sources (0, 8).

Cation Requirement—For catalytic activity, the crystalline enzyme has a requirement for a divalent metal which is most readily met by magnesium at 2 \times 10^{-3} M. Cobalt and manganese in the same concentration are only about 25% as effective as magnesium. Of the other divalent and monovalent cations tested, i.e., calcium, iron, nickel, copper, and zinc, only calcium has slight activity. When added in combination with magnesium, all divalent cations are somewhat inhibitory, while sodium ions are without effect.

Substrate Affinity—The affinity of the enzyme for the substrates and some compounds of similar structure has been measured. Michaelis constants have been estimated by extrapolation of the linear portion of Lineweaver-Burke plots. For the substrates, the \( K_m \) values are as follows: glucose-1-P, 5.5 \times 10^{-3} M; UDP-glucose, 6.0 \times 10^{-4} M; PP_i, 8.4 \times 10^{-4} M; UTP, 2 \times 10^{-4} M; and for TDP-glucose, 3.5 \times 10^{-4} M. The crystalline UDP-glucose pyrophosphorylase is inhibited by P_i and UDP (Table III). Inhibition with P_i is competitive with PP; at a \( K_i \) of 3.7 \times 10^{-3} M; and with UDP, inhibition is competitive with UDP-glucose at a \( K_i \) of 1.5 \times 10^{-4} M.

Specificity—UDP-glucose is the most active substrate for the crystalline pyrophosphorylase, but the enzyme is not highly specific for either the nucleoside or hexose component of the nucleoside diphosphate hexose. On substitution of thymidine, cytosine, or guanosine for the uridine of UDP-glucose, the reaction rate was about 0.1 to 3% (Table IV). On substitution of inosine or adenosine for uridine, only a trace of activity remained. When the glucose component of UDP-glucose was replaced with xylose, galactose, or mannose, from 0.3 to 4% of the activity remained; while replacement of glucose with N-acetylglucosamine or glucuronic acid resulted in only a trace of activity. As the low rate of activity with these nucleoside diphosphate sugars might be presumed to be due to contamination with UDP-glucose, a large excess of the recrystallized enzyme (100 to 10,000 times that used for assays with UDP-glucose) was added to each experiment in order to determine activity on the other substrates. Contaminating UDP-glucose would have been evidenced by higher initial activity, or by a change in slope as the UDP-glucose was removed; instead, a slight lag period was observed followed by a linear reaction rate. Furthermore, the formation of products has also been qualitatively followed by polyethyleneimine chromatography under conditions where rates of migration for each compound were characteristic (Fig. 6). Thus, the reactions are due to the compounds indicated.

DISCUSSION

An important consideration in the purification of this enzyme was the maintenance of an alkaline pH whenever possible during the fractionation. The enzyme could be held in dilute solution at 30° at pH 8.5 to 9.6 for several hours without appreciable loss in activity. In the example given in Table I, the largest loss in activity occurred during the calcium phosphate gel treatment. With some preparations, a slightly lower gel to protein ratio has also been used with good yields. The DEAE-cellulose step has consistently given an appreciable purification with minimal loss in activity.

Increasing the concentration of ammonium sulfate in liver extracts by increments of 2%, between 30 and 60% saturation, resulted in two distinct peaks of activity, with 40% salt saturation precipitating one, and leaving the other in solution. Therefore, in the procedure described here, about 25% of the pyrophosphorylase activity for UDP-glucose was discarded with the precipitate which formed on addition of ammonium sulfate to 40% saturation (32). It has not been determined whether this represents a coprecipitation of UDP-glucose pyrophosphorylase with other inactive protein, or whether a separate enzyme is involved. The molecule tends to form aggregates which may be seen as a component less mobile than the enzyme on polyacrylamide gel and which sediment more rapidly in the ultracentrifuge. Thus, two interconvertible forms of the same enzyme could account for the seemingly anomalous behavior in ammonium sulfate.

The intracellular function of the pyrophosphorylase is to catalyze the biosynthesis of UDP-glucose. Since this enzyme constitutes 0.2 to 0.5% of the liver extracts, the low \( K_m \) for UTP and glucose-1-P, the high turnover number, and the favorable equilibrium, all suggest that this enzyme is a major catalyst for UDP-glucose formation. The significance of the activity toward other substrates is not apparent. From this point it would be of interest to determine the activity of the pyrophosphorylase toward the various substrates in competition.

The biosynthesis of the following nucleoside diphosphate sugars has been shown with the recrystallized enzyme: UDP-glucose, UDP-galactose, UDP-mannose, UDP-xylose, TDP-glucose, CDP-glucose, and GDP-glucose. Whether the activity for all of these compounds is due to one nonspecific pyrophosphorylase or a contaminating protein is unclear. In support of one relatively nonspecific enzyme is a constant ratio of activity toward UDP-glucose and UDP-galactose throughout purification. Some preparations of the enzyme have been obtained which are homogeneous on polyacrylamide gel and which manifest the same lack of specificity. The question must remain open until the aggregation characteristics of the purified enzyme are understood, controlled, and until further purification of the enzyme is achieved.

A GDP-hexose pyrophosphorylase has also been purified about 500-fold from liver extracts (3). This enzyme is precipitated with ammonium sulfate at 40% saturation, and catalyzes the biosynthesis of GDP-glucose and GDP-mannose equally well. Thus, there are at least two enzymes which give rise to GDP-glucose formation. Since GDP-hexose pyrophosphorylase is also nonspecific, it and the UDP-glucose pyrophosphorylase may account for the biosynthesis of most of the nucleoside diphosphate hexoses which we have found. In the course of purification, activity for the compounds UDP-glucose and GDP-glucose were principally used as substrates. Thus, other similar enzymes could have been discarded during fractionation.

It has been reported that inorganic phosphate noncompetitively inhibits UDP-glucose pyrophosphorylase from rat liver (11). With a concentration of 2.0 \times 10^{-3} M, about 40% inhibition was observed. The crystalline calf liver enzyme appears to be more sensitive to P_i, since this compound inhibits competitively with PP; at a \( K_i \) of 4 \times 10^{-3}, a concentration which is in a

range to imply a physiological function. Thus, in the cell, when glucose and ATP are in excess and Pi is low, the pyrophosphorylase could function in the provision of UDP-glucose for glycogen storage; but when the Pi is high and the cell is in need of energy, the formation of UDP-glucose is limited by the Pi. It also may be of physiological significance that UDP, a product of glycogen formation, inhibits the enzyme competitively with UDP-glucose at a $K_i$ of $1.5 \times 10^{-4}$ M. Thus, for glycogen formation to proceed, the UDP must be removed by resynthesis to UTP at a sufficient rate to prevent accumulation of UDP and inhibition of the formation of the glucosyl donor, UDP-glucose.

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