Uridine Diphosphate Glucose Pyrophosphorylase from Sorghum vulgare

PURIFICATION AND KINETIC PROPERTIES*

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SUMMARY

The enzyme uridine diphosphate glucose pyrophosphorylase was purified to a final specific activity of approximately 1200 units per mg from etiolated Sorghum vulgare seedlings. The purified enzyme was highly specific for both UTP and UDP-glucose. The divalent cation requirement was most readily satisfied by magnesium, but manganese and cobalt were also effective.

The enzyme was characterized kinetically with regard to the mechanism of catalysis. Product inhibition studies indicate an ordered Bi Bi reaction mechanism in which the nucleotide substrate adds first and is released last as product. Evidence was obtained supporting mechanisms in which either free magnesium ion activates catalysis of UDP-glucose synthesis by forming a complex with enzyme in addition to its role in formation of MgUTP²⁻, or UTP⁻ inhibits the reaction. In contrast, magnesium ion had no effect on the rate of pyrophosphorylization beyond its role in formation of MgPP₁⁻. Michaelis constants for each substrate and dissociation (inhibition) constants for nucleotides combining with enzyme were determined in the presence of excess or limiting concentrations of free magnesium ion.

The influence of these mechanisms on the regulation of carbohydrate flow in higher plants is discussed.

EXPERIMENTAL PROCEDURE

Materials and Methods

Reagents—Crystalline phosphoglucomutase (rabbit muscle), crystalline glucose-6-P dehydrogenase (yeast), and nucleotide substrates were purchased from Calbiochem. UDP-glucose dehydrogenase (bovine liver), having a specific activity of approximately 0.008 unit per mg, was purchased from Sigma Chemical Co. Uniformly labeled α-D-[¹⁴C]glucose 1-phosphate was ob-
Kinetics of UDP-glucose Pyrophosphorylase

Vol. 247, No. 5

stopped from New England Nuclear. All other chemicals used were reagent grade.

Spectrophotometry—A Beckman DB-G spectrophotometer, equipped with a Sargent SRL recorder, was used for all spectrophotometric measurements unless otherwise stated.

Fluorometry—A Farrand automatic scanning and recording spectrophotometer connected to a Honeywell Electronik 15 recorder providing a full scale response in 1 sec was used for most fluorometric determinations. Reduced pyridine nucleotides were excited with monochromatic light at 350-nm wavelength and fluorescence was analyzed at 450-nm wavelength. In a few experiments a Turner fluorometer (model III) was used instead of the spectrophotometer.

Electrophoresis—Discontinuous acrylamide gel electrophoresis was performed with an EC 470 Vertical Gel Electrophoresis Cell (EC Apparatus Corp.). A modification of the Ornstein and Davis procedure was employed. The system described by these authors was modified in that a 7% stacking gel was used, and also in that all the buffers employed were prepared at half-strength. For analytical studies the stacking gel was slotted so that several samples could be electrophoretically treated separately on the same gel. Electrophoresis was performed at 300 volts for 4 to 5 hours. Following electrophoresis, the gels were stained with Amido black in 7% acetic acid solution followed by destaining in 7% acetic acid.

Protein Assays—Protein was estimated in fractions collected during column chromatography purity steps by measuring absorbance at 280 nm. Aside from this, the protein concentration in preparations obtained at the various stages of purification was determined on the residue precipitated with trichloroacetic acid and resolubilized in alkali with the Lowry modification of the Folin assay (13). In the latter stages of purification, when most of the phenolic substances which interfere in the Folin assay had been removed from enzyme preparations, dialysis was used for eliminating 2-mercaptoethanol before protein was assayed had been removed from enzyme preparations, dialysis was used for eliminating 2-mercaptoethanol before protein was determined by the Folin procedure. Bovine serum albumin was used as the standard with the Folin assay.

Assays of Enzymic Activity

Assay A—The routine assay used for quantitating enzymic activity during purification studies was analogous to that described by Munch-Petersen (1). Reaction mixtures contained, in a total volume of 1.0 ml, 50 μmoles of Tris-HCl (pH 8.5), 2.0 μmoles of MgSO4, 1.0 μmole of PPI, 0.5 μmole of UDP-glucose, 0.4 μmole of NADP+, 10⁻⁴ μmole of α-d-glucose 1,6-diphosphate, 0.1 unit of phosphoglucomutase, 0.2 unit of glucose 6-phosphate dehydrogenase, and sufficient UDP-glucose pyrophosphorylase to produce an absorbance change at 340 nm of 0.02 to 0.1 per min. Reactions were initiated by addition of UDP-glucose pyrophosphorylase and the change in absorbance at 340 nm was followed continuously. The temperature of the assay mixtures was maintained at 37° by circulating water, at this temperature, through the jacket of the cuvette holder.

Both PPI and UDP-glucose were required for producing a change in absorbance with crude extracts as well as purified preparations of the enzyme. The substrate concentrations defined above were approximately saturating, and hence the rates of pyrophosphorylase measured were approximately maximum. The rate of NADPH formation was quantitated with a value of 0.2 x 10⁴ as the molar extinction coefficient for NADPH at 340 nm. One unit of enzyme was defined as that amount of enzyme catalyzing the synthesis of 1 μmole of NADPH (or 1 μmole of glucose-1-P) per min under the assay conditions employed.

Assay B—A fluorometric coupled enzyme assay, analogous to the spectrophotometric assay described above, was used also for measuring initial rates of pyrophosphorylation. The assay mixtures contained, in a total volume of 0.2 ml, 10 μmoles of Tris-HCl (pH 8.5), 0.1 μmole of NADP+, 10⁻⁴ μmole of α-d-glucose 1,6-diphosphate, 0.01 unit of phosphoglucomutase, 0.02 unit of glucose 6-phosphate dehydrogenase, specified quantities of UDP-glucose, PPI, MgSO₄, and sufficient purified UDP-glucose pyrophosphorylase to catalyze the synthesis of 0.03 to 0.6 μmole of NADPH per min. The rate of formation of NADPH was followed continuously. The assay temperature was 21°.

Assay C—The initial rates of pyrophosphorylation were also measured with a two-step assay. Reaction mixtures containing, in a total volume of 0.5 ml, 50 μmoles of Tris-HCl (pH 8.5) and specified quantities of UDP-glucose, PPI, and MgSO₄ were equilibrated in a 37° constant temperature bath. Following equilibration, pyrophosphorylation was initiated by addition of UDP-glucose pyrophosphorylase, and the reaction tubes were incubated for 5- or 10-min time periods. Reactions were terminated by placing the reaction tubes in a boiling water bath for 1 min. The tubes were then cooled in an ice bath, and 0.5 ml of 0.1 M Tris-HCl (pH 8.5) containing 50 μmoles of NADP+, 0.02 unit of glucose 6-phosphate dehydrogenase, and MgSO₄ were added to each tube. The NADPH produced was then added to each tube. The NADPH was determined fluorometrically. The time interval of initial incubation was adjusted such that less than 5% of the limiting substrate was converted to product.

Assay D—A fluorometric coupled enzyme assay was also used for determining initial rates of UDP-glucose synthesis. Reaction mixtures containing, in a total volume of 0.2 ml, 10 μmoles of Tris-HCl (pH 8.5), approximately 0.003 unit of UDP-glucose dehydrogenase, 0.1 μmole of NAD⁺, specified quantities of UTP, glucose-1-P, and MgSO₄ were prepared. The reactions were initiated by addition of UDP-glucose pyrophosphorylase and the rate of NADH formation was followed continuously with the spectrophotometer. The assay temperature was 20°. In this assay 1 μmole of NAD⁺ was reduced for each 0.5 μmole of UDP-glucose oxidized.

Assay E—A two-step assay analogous to Assay C was also used for determining initial rates of UDP-glucose synthesis. Reaction mixtures containing, in a total volume of 0.2 ml, 20 μmoles of Tris-HCl (pH 8.5), and specified quantities of UTP, glucose-1-P, and MgSO₄ were equilibrated in a 37° constant temperature water bath. Reactions were initiated by addition of UDP-glucose pyrophosphorylase. After 5 or 10 min of incubation, reactions were terminated by placing the reaction tubes in a boiling water bath for 1 min. After the tubes had cooled, 0.5 ml of 0.1 M Tris-HCl (pH 8.5) containing 0.1 μmole of NAD⁺ and approximately 5 x 10⁻⁵ unit of UDP-glucose dehydrogenase was added to each reaction tube. The NADH was quantitated fluorometrically and related to the UDP-glucose produced as described under Assay D.

Assay F—An isotope assay of UDP-glucose synthesis, analo-
... gous to that described by Ghosh and Preiss (14) for the measurement of ADP-glucose synthesis, was employed in specificity studies where nucleotides other than UTP were tested as substrates. Reaction mixtures, containing in a total of 0.2 ml, 15 μmoles of Tris-HCl (pH 8.5), approximately 0.1 μCi of [3H]glucose-1-P (specific activity 141 μCi per μmole), 0.02 μmole of glucose-1-P, 0.2 μmole of MgSO4, 0.1 μmole of nucleoside triphosphate were initiated with addition of specified amounts of UDP-glucose pyrophosphorylase and incubated for specified time intervals. Reactions were terminated by placing the reaction tubes in a boiling water bath for 10 min. Following the heat treatment, 0.023 unit of E. coli alkaline phosphatase was added to each tube and the tubes were incubated for 40 min. The unreacted [3H]glucose-1-P was hydrolyzed to [3H]glucose by the phosphatase and the sugar nucleotide remained intact. Fifty-microliter aliquots of the reaction mixtures were then spotted on DEAE-cellulose paper discs (2.5 cm in diameter) which were pinned to a screen disc 5 inches in diameter. The [3H]glucose was then eluted from the discs by swining the containing discs in five separate 2-ml aliquots of distilled water. The discs were then dried in an oven at 80°C. The dried discs were counted by the liquid scintillation technique described by Shen and Preiss (16).

Purification of Sorghum UDP-glucose Pyrophosphorylase: Homogenization and Protamine Sulfate Treatment—Seeds of S. vulgare variety Rox Orange were a gift of Northrup King Seed Co. The seeds were planted in moist vermiculite contained in large glass trays (0 x 30 x 45 cm). The trays were placed at 27°C in the dark for 2 days followed by 3 days in the dark at 20°C. Unless stated otherwise, all procedures in the enzyme fractionation were performed at 4°C and centrifugations were performed at 16,000 x g for 30 min. In a typical purification, enzyme was extracted from 1,000 g of shoots harvested from etiolated seedlings. Shoots, 200 g, were placed in a Waring blender with 300 ml of crushed ice and 300 ml of 0.02 mM PPi-HCl buffer (pH 6.6) containing 28 μM 2-mercaptoethanol. The blender was run at top speed for 1 min. This procedure was repeated four additional times to homogenize the remaining 800 g of shoots. The resulting homogenate was filtered through four layers of cheesecloth. Solid protamine sulfate was added to the homogenate to a concentration of 2 mg per ml. After stirring for 1 hour, this mixture was centrifuged. The resulting supernatant fluid was made 5 mM in EDTA, and an additional 1 ml of 2-mercaptoethanol per liter of extract was added.

Ammonium Sulfate Fractionation—Solid ammonium sulfate was added slowly with stirring to bring the supernatant fluid to 80% saturation with ammonium sulfate. The mixture was stirred for an additional 1 hour after all of the ammonium sulfate had dissolved. The mixture was then centrifuged, the supernatant fluid decanted and discarded. The pelleted precipitate was collected and suspended in 1 liter of 15 mM PPi-HCl (pH 6.6) buffer, 60% saturated with ammonium sulfate and containing 14 mM 2-mercaptoethanol and 5 mM EDTA. This mixture was stirred for 3 hours and then centrifuged. Solid ammonium sulfate was added to the supernatant fluid to a concentration of 80% saturation. After the ammonium sulfate had been added, the mixture was stirred for an additional 1 hour and then centrifuged. The supernatant fluid was discarded. The pelleted precipitate was collected and dissolved in approximately 50 ml of 5 mM PPi-HCl (pH 6.6) containing 14 mM 2-mercaptoethanol. This solution was then dialyzed overnight against 2 liters of the same buffer.

First DEAE-cellulose Chromatography—Whatman DE-23 diethylenetriamine cellulose was cycled with HCl and NaOH and was charged with pyrophosphate by washing with 5-volume portions of 50 mM Na2P2O7. The DEAE-cellulose was then packed in a column and washed with 5-column volumes of 50 mM Na2P2O7, followed by washing with 50 mM Na4P2O6 (pH 6.6) to reduce the pH of the effluent to pH 6.6. The column was then equilibrated with 5 mM PPi-HCl buffer containing 14 mM 2-mercaptoethanol. The dialyzed enzyme solution was chromatographed on a DEAE-cellulose column, 2.5 x 33 cm. The fractionation of the enzyme with the first DEAE-cellulose chromatography is shown in Fig. 1. As indicated by the bar at the top of the figure, Fractions 31 to 45 were combined. This solution of combined fractions was concentrated to approximately 3 ml with an Amicon ultrafiltration cell equipped with a UM-10 series membrane.

Gel Filtration—The concentrated enzyme solution was fractionated further by exclusion chromatography on a column of Sephadex G-75 previously equilibrated with 0.015 mM PPi-HCl (pH 6.6). The column was washed with 0.015 mM PPi-HCl (pH 6.6) to elute the enzyme in 4.5-ml fractions. The elution profile is shown in Fig. 2. Fractions 35 to 40, representing the peak of enzyme activity, were combined.

Second DEAE-cellulose Chromatography—The enzyme solution, representing the pooled fractions from the previous step, was diluted 3-fold with deionized distilled water containing 14 mM 2-mercaptoethanol. This solution was added to a DEAE-cellulose column, 1 x 20 cm, previously equilibrated with 5 mM PPi-HCl (pH 6.6) containing 14 mM 2-mercaptoethanol. The column was irrigated with 10 mM PPi-HCl (pH 6.6) containing 14...
Fig. 2. Gel filtration of UDP-glucose pyrophosphorylase. Concentrated enzyme solution obtained from the first DEAE-cellulose step was applied to a column (3 × 60 cm) of G-75 Sephadex previously equilibrated with elution medium (15 mM PPi·HCl at pH 6.6 containing 14 mM 2-mercaptoethanol). The flow rate was maintained at 1 ml per min, and 4.5-ml fractions were collected: Δ — Δ, protein; O — O, enzyme activity as determined by assay A. V₀ indicates the void volume of column. The fractions indicated by the brackets were combined, and further purification was carried out on the combined fractions.

Table I

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity</th>
<th>Total protein</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protamine sulfate-treated homogenate</td>
<td>18,000 units</td>
<td>2070 mg</td>
<td>8.7 units/mg</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ fractionation</td>
<td>12,600 units</td>
<td>292 mg</td>
<td>50 units/mg</td>
</tr>
<tr>
<td>First DEAE-cellulose chromatography</td>
<td>8,050 units</td>
<td>17.5 mg</td>
<td>450 units/mg</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>5,400 units</td>
<td>5.55 mg</td>
<td>980 units/mg</td>
</tr>
<tr>
<td>Second DEAE-cellulose chromatography</td>
<td>3,260 units</td>
<td>2.72 mg</td>
<td>1200 units/mg</td>
</tr>
</tbody>
</table>

* An enzyme unit is that amount forming 1 μmole of product per min under the conditions of Assay A described under "Experimental Procedure."

a Protein was determined with the Lowry modification of the Folin assay.

mm 2-mercaptoethanol. The flow rate was maintained at approximately 0.5 ml per min, and 4.5-ml fractions were collected. Fractions 15 to 35 were combined and will hereafter be referred to as the purified enzyme.

RESULTS

Enzyme Purification

Table I summarizes the stepwise purification of the sorghum UDP-glucose pyrophosphorylase. Addition of 2-mercaptoethanol to the buffers used during the early stages of purification was essential for maintaining active enzyme. Furthermore, less than 5% loss in enzymic activity was observed upon storage of the purified enzyme for up to 1 month at 4°C in the presence of either 10 mM PPi·HCl (pH 6.6) or in 0.1 mM Tris·HCl (pH 8.5) buffer, each containing 14 mM 2-mercaptoethanol with protein concentrations of 0.05 to 0.5 mg per ml.

The period of time utilized for the ammonium sulfate fractionation appeared to influence the protein banding observed on acrylamide gel after electrophoresis of the purified enzyme. In preliminary purification studies the ammonium sulfate fractionation was performed over approximately 16 hours. Using this procedure, purified enzyme preparations were obtained which yielded two protein bands as illustrated in Lane I of the gel photograph in Fig. 3. In subsequent purification studies the time utilized for the ammonium sulfate fractionation was reduced to approximately 4 hours. Using this latter procedure, purified enzyme preparations exhibiting only one major protein band with electrophoresis were obtained. A typical preparation exhibiting only one major band is illustrated in Lane II of the gel photograph in Fig. 3. Whereas this modification did influence the protein banding of the purified enzyme, it did not alter the specific activity of the purified enzyme. Enzyme preparations exhibiting either one or two bands with electrophoresis had specific activities in the range of 1160 to 1250 units per mg.

Sedimentation velocity ultracentrifugation studies performed with purified enzyme preparations indicated a Svedberg constant of approximately 2 S, suggesting a molecular weight of less than 50,000. Chromatography of the enzyme on Sephadex G-75 also supported a molecular weight of less than 50,000, possibly as low as 20,000 (see Fig. 2).
**General Catalytic Properties**

**Stoichiometry**—With the fluorimetric coupled enzyme assays of UDP-glucose synthesis and pyrophosphorolysis it was possible to pull the reaction essentially to completion in either direction. Utilizing excess quantities of NADP⁺ with Assay B, it was found that the total amount of NADPH produced was equal to the quantity of limiting substrate (UDP-glucose or PPi) in the reaction mixture. Similarly, utilizing excess amounts of NAD⁺ with Assay D, the total quantity of NADH produced was equal to twice the quantity of UTP or glucose 1-P, whichever was limiting.

**Optimum pH**—Broad pH maxima were observed for catalysis in both directions of the reaction. In the direction of UDP-glucose synthesis, maximum activity was observed at pH 8 to 9, and the enzyme was about 85% as active at pH 7 as at the higher pH values. In the direction of pyrophosphorolysis, no significant change in activity was observed when the pH was varied in the range of 7 to 9. Tris-HCl buffer was used in all assays.

**Specificity**—Table II shows that none of the four common nucleoside triphosphates could replace UTP, and of the four corresponding nucleotide sugars, only TDP-glucose yielded detectable activity.

**Tests for Possible Effectors**—Ghosh and Preiss (14) and Sanwal et al. (16) have demonstrated that the ADP-glucose pyrophosphorylases isolated from a variety of plant leaf tissues are activated by 3-phosphoglycerate, phosphoenolpyruvate, fructose-6-P, and fructose 1,6-diphosphate. None of these substances had any effect on the rate of synthesis or pyrophosphorolysis of UDP-glucose catalyzed by the sorghum UDP-glucose pyrophosphorylase. In addition, p-hydroxymandelonitrile-β-D-glucopyranoside, a phenolic glucoside found at about 50 mM in young etiolated sorghum seedlings (17), had no effect on either the rate of UDP-glucose synthesis or its rate of pyrophosphorolysis.

**Effect of 2-Mercaptoethanol**—Although 2-mercaptoethanol was not essential for maintaining enzymic activity during storage of the purified enzyme, storage in its absence resulted in a modification of the enzyme reflected by increases in the Michaelis constants determined for the substrates. This modification could be readily reversed either by making the assay mixtures 140 mM in 2-mercaptoethanol or by incubating the modified enzyme overnight in the presence of 14 mM 2-mercaptoethanol. Fig. 4 illustrates the effect of adding 2-mercaptoethanol. This figure shows that 2-mercaptoethanol activated pyrophosphorolysis of UDP-glucose, especially at low MgPPi⁺ concentrations. A similar activation of UDP-glucose synthesis by 2-mercaptoethanol was observed when low concentrations of UTP and glucose-1-P were employed. In both directions of catalysis the activation was observed almost immediately with the coupled enzyme assays. Increased rates of reaction were established within 1 min after addition of 2-mercaptoethanol. Addition of 2-mercaptoethanol to 140 mM to assay mixtures containing enzyme preparations previously stored in the presence of 14 mM 2-mercaptoethanol, had no effect on rates of reaction in either direction of catalysis at high or low substrate concentrations.

**Equilibrium Constant**—Effect of MgSO₄ on the apparent equilibrium was measured by allowing reactions to approach equilibrium in the direction of pyrophosphorolysis. Table III shows that the apparent equilibrium was shifted toward UDP-glucose synthesis with increasing concentrations of MgSO₄.

**Cation Requirement**—The purified sorghum UDP-glucose pyrophosphorylase showed an absolute requirement for a divalent cation for catalysis of both synthesis and pyrophosphorolysis of UDP-glucose. Figs. 5 and 6 show that magnesium ion was more effective than either manganese or cobalt ions in activating catalysis of the reversible reaction. Fig. 5 shows hyperbolic saturation curves obtained with low concentrations of all three metal ions in the direction of pyrophosphorolysis, whereas in the direction of UDP-glucose synthesis (Fig. 6), the saturation curves were sigmoidal. The direction of pyrophosphorolysis: maximum activity was obtained when the cation was present at a concentration equal to PPi, and manganese or cobalt in excess of PPi was inhibitory. In the direction of UDP-glucose

### Table II

**Specificity of sorghum UDP-glucose pyrophosphorylase**

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Relative reaction rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>UTP</td>
<td>100</td>
</tr>
<tr>
<td>TTP</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>CTP</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ATP</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>GTP</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>UDP-glucose</td>
<td>100</td>
</tr>
<tr>
<td>TDP-glucose</td>
<td>0.125</td>
</tr>
<tr>
<td>CDP-glucose</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ADP-glucose</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>GDP-glucose</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

![Fig. 4. Effect of 2-mercaptoethanol on initial rates of pyrophosphorolysis with purified enzyme stored in the absence of 2-mercaptoethanol. Assay B was employed. Assays performed in the presence of added 2-mercaptoethanol in the presence of 0.05 and 0.2 mM UDP-glucose are represented by O---O and •—•, respectively. In contrast, △---△ and ▲—▲ represent 0.05 and 0.2 mM UDP-glucose, respectively, in assays performed in the presence of 140 mM 2-mercaptoethanol added to reaction mixtures immediately before initial rates of reaction were determined. Velocity units (V) are nmoles of glucose-1-P per min per mg.](https://example.com/fig4.png)
TABLE III
Influence of MgSO₄ on apparent equilibrium of UDP-glucose
pyrophosphorylase-catalyzed reaction

Reaction mixtures containing in a total volume of 0.5 ml, approximately 0.1 pmole of PPi, 0.1 pmole of UDP-glucose, 20 pmoles of Tris-HCl (pH 8.5), specified quantities of MgSO₄, and 0.123 unit of purified pyrophosphorylase were incubated for 20, 40, or 60 min at 37°. Reactions were terminated by heating the reaction tubes in a boiling water bath. The concentrations of glucose-1-P were estimated spectrophotometrically by enzymic conversion of the glucose-1-P to 6-phosphogluconate. All reactions reached equilibrium within the 20-min time interval, and the reactant concentrations represent averages of the three time intervals. The concentrations of UTP, UDP-glucose, and PPi in each reaction tube were calculated according to the stoichiometry of the reaction.

<table>
<thead>
<tr>
<th>MgSO₄ concentration (mM)</th>
<th>Concentration of reactants at equilibrium (mM)</th>
<th>Apparent equilibrium ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UDPG</td>
<td>PPi</td>
</tr>
<tr>
<td>0.2</td>
<td>0.048</td>
<td>0.055</td>
</tr>
<tr>
<td>0.4</td>
<td>0.063</td>
<td>0.060</td>
</tr>
<tr>
<td>0.6</td>
<td>0.067</td>
<td>0.064</td>
</tr>
<tr>
<td>1.0</td>
<td>0.063</td>
<td>0.070</td>
</tr>
<tr>
<td>4.0</td>
<td>0.068</td>
<td>0.075</td>
</tr>
<tr>
<td>10.0</td>
<td>0.087</td>
<td>0.092</td>
</tr>
</tbody>
</table>

a UDPG, UDP-glucose.

Fig. 5. Effect of divalent cations on initial rates of pyrophosphorylase synthesis. Assay C was employed. The concentrations of UDP-glucose and PPi were 0.4 mM in all assays. Velocity units (V) are nmoles of UDP-glucose per min per ml.

synthesis, an excess of cation in relation to UTP was required for obtaining maximum activity.

In evaluating the fraction of magnesium ion in the catalysis of the reversible reaction it is necessary to consider the ability of the substrates to bind the cation. Of the four reactants, PPi and UTP would be expected to form a complex much more readily with magnesium ion than either UDP-glucose or glucose-1-P. Lambert and Watters (18) have calculated a value of 3.9 x 10⁻⁶ M for the dissociation constant of MgPPi₂⁻ complex. Since the K_d for HPo₄²⁻ is equal to 1.12 x 10⁻⁴ M (19), the calculated apparent dissociation constant at pH 8.5 is 1.5 x 10⁻⁵ M. Ray and Roscelli (20) have determined values for the dissociation constant of magnesium glucose-1-P in the range of 10⁻⁹ M. Although a value for the dissociation constant of MgUDP-glucose has not been determined, it is likely that it is of the same magnitude as that for magnesium glucose-1-P assuming that magnesium ion binds only to the phosphorous oxyanions in the formation of these complexes.

The dissociation constant for MgUTP⁻ was determined by the method of Burton (21) in which the spectral changes of 8-hydroxyquinoline were used to determine the amount of free magnesium ion present in solutions containing UTP and cation. With Burton’s procedure [Mg]₀ values (the concentration of free magnesium when MgUTP⁻ equals half the total UTP) were determined at several concentrations of UTP. Extrapolating [Mg]₀ to zero concentration UTP as shown in Fig. 7, a dissociation constant of 1.1 x 10⁻⁴ M was determined. That is, the dissociation constant is given by [Mg]₀ as UTP → 0.

Fig. 8 illustrates plots of initial rates of pyrophosphorylase as a function of magnesium ion concentration with different fixed concentrations of UDP-glucose and PPi. Hyperbolic curves were obtained with all substrate concentrations. Maximum rates of pyrophosphorylase were obtained when magnesium was added at approximately the same concentration as PPi.
and magnesium ion in moderate excess of PPi did not inhibit catalysis. The direct relationship between the concentration of magnesium ion required and the concentration of PPi employed in the reactions suggests that MgPPi\(^2^+\) is the substrate. However, utilizing the value of $1.5 \times 10^{-5}$ M for the dissociation constant of MgPPi\(^2^+\), it was calculated that at maximum velocity only 60% of the pyrophosphate formed a complex with magnesium ion (Curves 2 and 3, Fig. 8).

Whereas in the direction of pyrophosphorylisis the magnesium requirement was directly related to the concentration of PPi, and independent of the UDP-glucose concentration, in the direction of UDP-glucose synthesis the requirement for magnesium was dependent on the concentrations of both UTP and glucose-1-P. With concentrations of UTP of 0.2 mM, plots of velocity of UDP-glucose synthesis as a function of MgSO\(_4\) concentration yielded sigmoid curves as shown with Curves 1 and 3 in Fig. 9. As indicated in Fig. 9, sigmoid curves were also observed with manganese and cobalt ions as well as with magnesium ion when 0.4 mM UTP was employed. In contrast, at 0.025 mM UTP, velocity was a hyperbolic function of MgSO\(_4\) concentration (Curve 2, Fig. 9).

Utilizing a value of $1.1 \times 10^{-5}$ M for the dissociation constant for MgUTP\(^2^+\), the percentage of the UTP forming a complex with magnesium at half-maximum velocity was calculated for each curve in Fig. 9. In Curve 1 72% of the UTP formed a complex whereas in Curves 2 and 3 95% formed a complex at half-maximum velocity. That more magnesium was required for obtaining half-maximum velocity than was necessary to form a complex of 50% of the UTP indicated that either (a) free UTP inhibits catalysis or (b) free magnesium ion activates catalysis.

Fig. 10 illustrates an experiment in which the rate of decrease in velocity of UDP-glucose synthesis in the presence of either 0.1 or 0.2 mM glucose-1-P was compared with (a) the calculated rate of decrease in free magnesium ion concentration after addition of the indicated quantities of UTP, and (b) the calculated increase in UTP\(^\text{Mg}^+\) concentration. As indicated, the calculated concentration of free magnesium decreased slightly more rapidly than did the velocities of UDP-glucose synthesis. At the point where free magnesium ion was depleted the velocities plateaued.

The velocities at the plateau levels were about 10% of the maximum velocities obtainable with excess magnesium ion and the same substrate concentrations. It is not possible to clearly distinguish between the two alternatives mentioned above regarding the role of magnesium ion in the catalysis from this figure, however the fact that the velocities do plateau, rather than approach zero as free UTP increases linearly, indicates that if free UTP does inhibit, it is not competitive with MgUTP\(^2^+\) since no UDP-glucose is formed in the absence of any magnesium (see Fig. 9).
Product Inhibition and Initial Velocity Studies

Product Inhibition Studies—As described by Cleland (22, 23) product inhibition studies can be used to obtain information regarding the sequence of addition of substrates and release of products from an enzyme during catalysis. Following the general procedure outlined by Cleland, initial velocities of pyrophosphorylation and synthesis of UDP-glucose were measured as a function of the concentration of one of the substrates while maintaining the other substrate at a fixed concentration at each of several concentrations of one of the products. The reciprocal of the initial velocity was plotted versus the reciprocal of the variable substrate concentration at each fixed concentration of added product. The patterns of inhibition observed with double reciprocal plots were then compared with those predicted for a variety of mechanisms.

Figs. 11 to 14 illustrate product inhibition studies performed in which concentrations of magnesium ion at least 1.0 mM in excess of UTP, or PPi, or both, were employed. This excess quantity of magnesium added to all assays was sufficient to give maximum activation of catalysis. Assuming dissociation constants of $1.1 \times 10^{-4}$ and $1.5 \times 10^{-5}$ M for the dissociation constants for MgUTP$^-$ and MgPPi$^-$, respectively, it was calculated that in all assays performed for the data shown in

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**Fig. 11.** Product inhibition of pyrophosphorylation by MgUTP$^-$ with UDP-glucose as the variable substrate, and MgSO$_4$ added at a concentration 1.0 mM in excess of UTP and PP$_i$. Assay B was used. The fixed concentration of MgPPi$^-$ was 0.1 mM. The reciprocal of initial velocity of glucose-1-P formation, in nmoles per min per ml, is plotted as a function of the reciprocal of the UDP-glucose (UDPG) concentration in the primary plot. The slopes of the primary plot are replotted as a function of the concentration of MgUTP$^-$ in the secondary plot.

**Fig. 12.** Product inhibition of UDP-glucose synthesis by MgPPi$^-$ with MgUTP$^-$ as the variable substrate, and glucose-1-P added at a fixed, nonsaturating concentration. Assay D was used. The fixed concentration of glucose-1-P was 0.1 mM. Magnesium sulfate was added to reaction mixtures at a concentration 1.0 mM in excess of UTP and PP$_i$ concentration. In the primary plot the reciprocal of initial velocity of UDP-glucose synthesis in nmoles per min per ml is plotted as a function of the reciprocal of MgPPi$^-$. The slopes and intercepts of the primary plot are replotted as a function of the concentration of MgUTP$^-$ in the secondary plot.

**Fig. 13.** Product inhibition of UDP-glucose synthesis by MgPPi$^-$ with glucose-1-P as the variable substrate. Assay D was used. Magnesium sulfate was added to all reaction mixtures at a concentration 1.0 mM in excess of UTP and PP$_i$. The fixed concentration of MgUTP$^-$ was 0.2 mM. In the primary plot the reciprocal of the initial velocity of UDP-glucose synthesis in nmoles per min per ml is plotted as a function of the reciprocal of the concentration of glucose-1-P (GIP). In the secondary plots the slopes and intercepts of the primary plot are replotted as a function of the concentration of MgPPi$^-$.}

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**Fig. 14.** Product inhibition of UDP-glucose synthesis by MgPPi$^-$ with MgUTP$^-$ as the variable substrate, and glucose-1-P added at a fixed, saturating concentration. Assay D was used. The fixed concentration of glucose-1-P was 10 mM. Magnesium sulfate was added to all reaction mixtures at a concentration 2.0 mM in excess of UTP and PP$_i$. In the primary plot the reciprocal of the initial velocity of UDP-glucose synthesis in nmoles per min per ml is plotted as a function of the reciprocal of the concentration of MgUTP$^-$ in the secondary plot.
Figs. 11 to 14, greater than 98.5% of the PPi and UTP formed a complex with magnesium ion.

Fig. 11 shows the results obtained when MgPPi− was the product added, and initial velocities of pyrophosphorolysis were measured as a function of UDP-glucose concentration. Inhibition was competitive indicating that UDP-glucose and MgUTP− combined with the same form of the enzyme. The linearity of the replots of the slopes as a function of the MgUTP− concentration indicates that MgUTP− combined with only one form of the enzyme. As shown in Fig. 12, MgPPi− was a noncompetitive inhibitor of the UDP-glucose when MgUTP− was the variable substrate, and glucose-1-P was added at a fixed nonsaturating concentration of 0.01 mM. The replots of the slopes and intercepts as a function of MgPPi− were linear indicating that MgPPi−, like MgUTP−, combined with only one form of the enzyme. MgPPi− was also a noncompetitive inhibitor of UDP-glucose synthesis when glucose-1-P was the variable substrate as shown in Fig. 13. Fig. 14 represents data from a product inhibition study analogous to that which was shown in Fig. 12 except that glucose-1-P was added at a saturating concentration of 10 mM. With saturating concentration of glucose-1-P, parallel lines were obtained indicating noncompetitive inhibition between MgPPi− and MgUTP−.

In contrast to the product inhibition studies illustrated in Figs. 11 to 14, the product inhibition study presented in Fig. 15 was performed utilizing a limiting concentration of magnesium ion. This study was analogous to that presented in Fig. 11 except that magnesium was added at a concentration equal to the sum of UTP and PPi concentration rather than in excess of these reactants. Linear, competitive inhibition was observed in the primary plot of Fig. 15 as was observed for the analogous study shown in Fig. 11. When the slopes of the primary plot were replotted as a function of total UTP and the calculated concentration of MgUTP−, nonlinear curves were obtained. Since the same amount of enzyme per assay was used in this study as for that illustrated in Fig. 11, the replot from Fig. 11 was superimposed on the replot of Fig. 15 as a dashed line to allow for a direct comparison of the inhibition observed in the two experiments. As can be seen in comparing these replots in Fig. 15, with concentrations of UTP above 0.05 mM, significantly more inhibition was observed per unit of UTP added when magnesium ion was limiting.

Initial Velocity Studies—In further characterizing the sorghum UDP-glucose pyrophosphorylase reaction initial velocities were measured as a function of substrate concentrations in the absence of added products. Fig. 16 illustrates a reciprocal plot of

![Graph](http://www.jbc.org/)

FIG. 16. Initial velocity of UDP-glucose synthesis as a function of the concentrations of MgUTP− and glucose-1-P with MgSO₄ added at a concentration 1.0 mM in excess of UTP. Assay D was used. In the primary plot the reciprocal of the initial velocities of UDP-glucose synthesis in nmoles per min per ml were plotted as functions of the reciprocal of the concentration of MgUTP− at each designated concentration of glucose-1-P (GIP). In the secondary plot the slopes and intercepts of the primary plots are replotted as functions of the reciprocal of the concentration of glucose-1-P (GIP).

![Graph](http://www.jbc.org/)

FIG. 17. Initial velocity of pyrophosphorolysis as a function of the concentration of UDP-glucose and MgPPi− with MgSO₄ added at a concentration 1.0 mM in excess of PPi. Assay D was used. In the primary plot the reciprocal of the initial velocity of glucose-1-P synthesis, in nmoles per min per ml, is plotted as a function of the reciprocal of the concentration of UDP-glucose (UDPG). In the secondary plot the slopes and intercepts of the primary plots are replotted as functions of the reciprocal of the concentration of MgPPi−.

![Graph](http://www.jbc.org/)
Tovey and Roberts (25) have shown that UDP-glucose pyrophosphorylase from wheat has a UTP to magnesium ratio of 1:2 for maximum activity, and UDP-glucuronic acid pyrophosphorylase from barley seedlings also shows a similar dependence on magnesium ion (26).

Fig. 15 indicates that UTP (either free or complexed) is a competitive inhibitor with respect to UDP-glucose. Fig. 11 on the other hand indicates that completely complexed UTP is also competitive with UDP-glucose. It thus follows that free UTP, if catalytically active, should be a competitive inhibitor with respect to MgUTP++, an hypothesis which is untenable with the results of Figs. 9 and 10 as previously indicated. Hence on the two alternative explanations for the requirement of magnesium in the direction of UDP-glucose synthesis, that involving activation by free magnesium seems the most likely.

The simplest mechanism which qualitatively satisfies the data obtained with 1.0 mM excess magnesium ion added to assays shows the sequential binding of substrates and release of products.

\[ E \rightleftharpoons E \cdot MgUTP \rightleftharpoons E \cdot MgUTP \cdot GIP \rightleftharpoons E \cdot UDPG \rightleftharpoons E \cdot ADP \rightleftharpoons E \cdot STARCH \]

where GIP = glucose-1-P and UDPG = UDP-glucose. The ordered addition and release of reactants indicated for the catalysis is consistent with the product inhibition patterns observed. The noncompetitive inhibition between MgPP++ and glucose-1-P, and the shift from noncompetitive to uncompetitive inhibition between MgPP++ and MgUTP++ when glucose-1-P is increased from a nonsaturating to a saturating concentration eliminate Theorell-Chance, "ping-pong," and random-type mechanisms as possibilities (22, 23).

Although the sequential binding and release of substrates and products from S. vulgare UDP-glucose pyrophosphorylase is the same as that proposed for the enzyme isolated from human erythrocytes (3), the kinetic properties of the two enzymes differ considerably. Tsuibo et al. (10) in comparing the kinetic constants for the mammalian UDP-glucose pyrophosphorylases isolated from cardiac muscle and human erythrocytes with that obtained for the mung bean enzyme, observed that the K_{UDPG} value obtained for mammalian enzymes was 10-fold lower than that obtained for the mung bean enzyme. The K_{UDPG} value obtained for the sorghum enzyme is approximately 3-fold lower than that de-
termed by the latter authors for the mung bean enzyme. In comparing the Michaelis and inhibition constants observed for the sorghum UDP-glucose pyrophosphorylase, the most significant differences observed are in the values for \( K_{\text{UTP}} \) and \( K_{\text{UTP}} \) and the effect of magnesium ion concentration on these values.

The physiological significance of the differential effect of magnesium ion in activating the catalysis of synthesis and pyrophosphorylation of UDP-glucose cannot be assessed at this time since data regarding the intracellular concentration of free magnesium is not available. However, providing that sufficient quantities of substrate were available, it is obvious that the sorghum plant might regulate the direction of catalysis by the pyrophosphorylase if it could regulate the intracellular or intracompartment concentration of free magnesium ion. Whereas, it is generally assumed that enzyme reactions releasing PP\(_i\) are essentially irreversible in vivo due to rapid hydrolysis of PP\(_i\) by pyrophosphatases (27), deFekete and Cardini (28) have proposed a coupled sequence of reactions for the conversion of sucrose to starch in plant tissues involving the pyrophosphorylation of UDP-glucose. The reaction pathways proposed by these authors is represented in Fig. 18. Their proposal can be extended to include other nucleoside diphosphate glucopyranosides; for instance, GTP in place of ATP would provide the necessary GDP-glucose for the conversion of the glucosyl moiety of sucrose to cellulose in plants. The coupled reactions allow the synthesis of the necessary nucleotide sugars presumably involved in starch (29) and cellulose (30) synthesis directly from the glucosyl group of sucrose without losing the energy of the glucosyl anomeric linkage. Furthermore, carbohydrate funneled from the transpired sucrose could be either shunted directly into a polysaccharide, glycoprotein or glycolipid for those polymers in which the UDP-sugar was the glycosyl donor, or through the UDP-glucose pyrophosphorylase-catalyzed reaction be converted to a nucleotide sugar such as GDP-glucose, ADP-glucose, or CDP-glucose without further energetic requirements and then be incorporated into polymers. It is suggested that MgUTP\(_i\) and magnesium ion are instrumental in regulating this process through the UDP-glucose pyrophosphorylase catalyzed reaction. Potent inhibition of pyrophosphorylation by UTP decreases the rate of UTP accumulation under limiting concentration of magnesium ion. The concentration of MgUTP\(_i\) and its turnover rate would also depend on the extent to which it serves as a transphosphorylating agent and the rate at which the various nucleoside diphosphate sugars are incorporated into polymers. In contrast to the sorghum UDP-glucose pyrophosphorylase, the mammalian enzyme appears to function primarily in the synthesis of UDP-glucose and inhibition by UDP-glucose is one major factor in controlling UDP-glucose synthesis. Thus, UDP-glucose pyrophosphorylase may have different functions in plants than in animals.

Reay and Conn (31) demonstrated that UDP-glucose is the glycosyl donor in \( p\)-hydroxymandelonitrile-\( \beta\)-D-glucopyranoside biosynthesis in sorghum seedlings. This substance is present at a concentration of about 50 mm in the youngest of sorghum seedlings and it is rapidly turned over in these plants (17). Evidence from Conn's laboratory (32) suggests that the glucoside is actively transported in young seedlings before they have leafed out and photosynthesis has become an active source of sucrose synthesis. It is tempting to consider that the glucoside at least in part substitutes for sucrose as a source of glucosyl donor in a coupled sequence of reactions, similar to that outlined in Fig. 18, prior to the onset of photosynthesis. Studies to test this speculation are being carried out in this laboratory.

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