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PURIFICATION, PROPERTIES AND KINETIC ANALYSIS OF UDP-GLUCOSE PYROPHOSPHORYLASE FROM BOVINE MAMMARY TISSUE*

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SUMMARY

UDP-glucose pyrophosphorylase was purified from bovine mammary acetone powders. Evidence is presented that multiple forms of the enzyme exist. Kinetic studies are consistent with a steady state ordered mechanism and values of the true Michaelis and inhibition constants for $MgUTP^{2-}$ and Glc-1-*P* are presented.

INTRODUCTION

UDP-glucose pyrophosphorylase (UTP:α-D-glucose-1-*P* uridyltransferase, Ec 2.7.7.9) catalyzes the following reaction:

$$UTP + \text{Glc-1-}P \overset{Mg^{2+}}{\rightleftarrows} \text{UDP-glucose} + P_2O_7^{4-}$$

The enzyme is common to all tissues studied including the mammary gland[1,2]. The enzyme has been crystallized from bovine[3] and human liver[4] and may represent 0.2–0.3% of the extractable protein. The bovine liver[5] and human liver enzymes[4] exist as polymeric species. Initial velocity and product inhibition studies with the human erythrocyte enzyme[6] indicate a steady-state ordered mechanism whereby $MgUTP^{2-}$ is the first substrate to add and UDP-glucose the last product released. Previous studies with the enzyme in crude mammary extracts and other tissues showed that there was a time dependent increase in activity of the enzyme[7]. The purpose of this investigation was to purify the enzyme from bovine mammary tissue and to study some of the physical and kinetic properties in order to gain insight into the mode of action and control aspects of this enzyme.

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MATERIALS

Tris, UTP, Glc-1-*P*, NAD^+, UDP-glucose, $NADP^+$, piperazine, glycylglycine, bovine serum albumin, lactate dehydrogenase (Type III from beef heart), phosphoglucomutase (rabbit muscle), glucose 6-phosphate dehydrogenase (Type IV from yeast) and protamine sulfate were obtained from Sigma, St. Louis; $MgCl_2$, quinine sulfate (N.F. grade), triethanolamine (certified), ethylenediaminetetraacetic acid from Fisher; UTP from P-L Biochemicals; Whatman DE-32 from Reeve Angel; Bio-Gel P from Bio Rad; and Sephadex from Pharmacia. All other chemicals were reagent grade.

UDP-glucose dehydrogenase was purified from bovine liver through Step V by the procedure of STROMINGER *et al.*[8] Hydroxylapatite was prepared following the procedure of SIEGELMAN *et al.*[9] Lactating bovine udders were obtained immediately after slaughter from the Wilson Packing Plant, Oklahoma City, Okla.

METHODS

Purification procedures

UDP-glucose pyrophosphorylase was assayed in the direction of UDP-glucose synthesis by determining UDP-glucose with UDP-glucose dehydrogenase as previously described[10]. Assays were done in a 1.0-ml final volume and contained 0.2 M Tris–HCl, (pH 8.0), 2 mM $MgCl_2$, 5 mM Glc-1-*P*, 1 mM NAD^+, 4–6 units UDP-glucose dehydrogenase, enzyme, and 1 mM UTP. The reaction was initiated by the addition of 0.1 ml of 10 mM UTP. Assays with a change in absorbance at 340 nm of 0.04 per min or less were proportional to enzyme concentration. 1 unit is defined as that amount of enzyme which forms 1 μmole of UDP-glucose per min. Assays were performed at 23° on a Cary-14 Spectrophotometer.

Ion exchange resins and molecular sieve gels were prepared and equilibrated as recommended. Sucrose density gradient centrifugation in 5–20% sucrose was performed as described by MARTIN AND AMES[11] in a Beckman L2-65 centrifuge. Disc gel electrophoresis was performed as suggested in the Canalco Model 6 System with the standard 7% separating gel but no sample gel was used. The best method for concentrating the enzyme was by precipitation with $(NH_4)_2SO_4$. Protein was estimated by the absorbance at 280 nm assuming that an absorbance of 1.0 was equal to 1 mg of protein. A small crystal of 1,1,1-trichloro-2-methyl-2-propanol was added to buffers and enzymatic solutions to control microbial contamination.

Kinetic studies

For kinetic studies in the direction of UDP-glucose synthesis, UDP-glucose pyrophosphorylase from bovine mammary tissue was purified through Step VII, chromatographed on DEAE-cellulose at pH 10.0, precipitated by $(NH_4)_2SO_4$, dissolved in a minimal volume of 0.2 M potassium phosphate, and desalted on a Bio-Gel P-10 column (1.15 cm × 22.5 cm) equilibrated with 20 mM triethanolamine (pH 8.0), to remove $(NH_4)_2SO_4$ since it is a potent inhibitor of UDP-glucose dehydrogenase (5.5% in the assay inhibits the reaction by 45%)

For kinetic studies in the direction of Glc-1-*P* formation, UDP-glucose pyrophosphorylase from bovine mammary tissue was purified through Step VII, chromatographed on DEAE-cellulose at pH 9.7, precipitated by $(NH_4)_2SO_4$, dissolved in a

minimal volume of 0.2 M glycine (pH 9.7) chromatographed on a Sephadex G-200 column (3 cm × 100 cm), precipitated by ammonium sulfate and dissolved in a minimal volume of 20 mM triethanolamine, pH 8.5. Since dilutions of 1:10 000 or greater were usually used in these kinetic analyses, the enzyme preparation was not desalted.

Assays for UDP-glucose synthesis were similar to the assay described above except that 100 mM triethanolamine pH 8.0, was used in place of Tris–HCl and the temperature was 30°. The reagents without enzyme were preincubated at 30° and were stable. $MgCl_2$, UTP, and Glc-1-*P* were varied according to the experiment.

Fixed-time assay for UDP-glucose pyrophosphorylase

Reaction mixtures contained, in a total volume of 1.0 ml: 100 mM triethanolamine at pH 8.0, UTP and $Na_4P_2O_7$ at the desired concentration, and sufficient $MgCl_2$ to maintain the concentration of Mg^{2+} at 1 mM (see RESULTS). Before the addition of enzyme the tubes were incubated for 5 min at 30°. The enzyme was added with a Hamilton microliter syringe with a stop. All assays were run for at least four different time intervals to insure that initial velocities were measured. The reaction was stopped by adding 0.10 ml of 1.0 M HCl. After 2.5 min 0.05 ml of 2.0 M KOH was added to return the pH to 8.0. Either Eppendorf or Oxford micropipets were used for the addition of acid and base. Blank values were obtained by adding acid, immediately followed by enzyme, and neutralizing 2.5 min later with base.

Glc-1-*P* was estimated by using the reaction of phosphoglucomutase and Glc-6-*P* dehydrogenase. To each of the reaction mixtures were added 0.1 ml of the coupling system containing 0.1 mM EDTA, 10 mM triethanolamine, pH 8.0, 6 mM $NADP^+$, 0.002 ml of phosphoglucomutase (2 units), and 0.001 ml of Glc-6-*P* dehydrogenase (2 units). NADPH was estimated by measuring the absorbance at 340 nm or by measuring the natural fluorescence of NADPH (excitation at 350 nm, emission at 460 nm)[12].

Absorbance measurements were made on a Beckman DU spectrophotometer and fluorescence measurements were done on an Aminco-Bowman Spectrophotofluorimeter equipped with a water-jacketed sample chamber with thermoregulation at 25°.

Analysis of data

The type of curve obtained was determined by graphical analysis of the data which were then analyzed by an appropriate computer program[13,14]. Linear intersecting initial velocity plots were fitted to Eqn. 1; linear noncompetitive inhibition plots were fitted to Eqn. 2.

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \quad (1)$$

$$v = \frac{VA}{K\left(1 + \frac{I}{K_{is}}\right) + A\left(1 + \frac{I}{K_{ii}}\right)} \quad (2)$$

RESULTS

Purification of UDP-glucose pyrophosphorylase

Acetone powder was prepared from bovine mammary glands[10] and yielded 20 g powder per 100 g of tissue. The powder was stable for two years when stored desiccated at −20°. All purification steps unless otherwise indicated were at 4° and centrifugations were at 10 000 × *g* for 30 min.

Step I. 50 g acetone powder were extracted with 750 ml 0.1 M Tris–HCl, pH 8.0 for 1–1.5 h. After centrifugation, the supernatant solution was filtered through glass wool.

Step II. 1 ml 0.2% protamine sulfate solution (2 g dissolved in 3.2 ml 0.1 M KOH and diluted to 100 ml) was added slowly to each 12 ml of supernatant solution from Step 1. The solution was stirred 15 min after the last addition and centrifuged.

Step III. 313 g $(NH_4)_2SO_4$ were added slowly to each liter of solution from Step II, stirred for 15 min and centrifuged. The precipitate was dissolved in 250 ml 0.2 M potassium phosphate pH 8.0.

Step IV. The dissolved precipitate from Step III was heated to 50° and held at this temperature for 30 min. The solution was stirred constantly during the heating process and it was critical that the temperature did not exceed 52°. After heating, the mixture was cooled rapidly to 0–4°.

Step IV. 100 g $(NH_4)_2SO_4$ were added to each liter of solution from Step IV, stirred for 15 min and centrifuged. The precipitate was discarded.

Step VI. 114 g $(NH_4)_2SO_4$ were added to each liter of solution from Step V, stirred for 15 min, centrifuged and the precipitate was dissolved in a minimum volume of 0.2 M potassium phosphate pH 8.0.

Step VII. Material from Step VI was placed on a 6.4 × 12 cm DEAE-cellulose column (10 mg of protein per 2 ml bed volume of column) equilibrated with 20 mM potassium phosphate, pH 8.0. The column was eluted with 2 column volumes each of 20, 50 and 500 mM potassium phosphate, pH 8.0. 20-ml fractions were collected and the activity eluted with the 500 mM potassium phosphate, pH 8.0. The enzymatic activity was precipitated with 313 g $(NH_4)_2SO_4$ per l and the precipitate was dissolved in a minimum volume 0.2 M potassium phosphate, pH 8.0.

Step VIII. The enzyme was purified further on DEAE-cellulose between pH 8.8 and 10.0 and on Sephadex G-200 columns. For example, 60 mg of protein containing 45 I.U. of enzyme were chromatographed on a 3 cm × 28 cm column of DEAE-cellulose equilibrated with 20 mM glycine, pH 9.7. The column was washed with 600 ml 20 mM glycine, pH 9.7 and then eluted with a linear gradient formed from 1000 ml each of 20 mM and 1.5 M glycine, pH 9.7. 3.2-ml fractions were collected and 8 protein peaks were observed of which two were enzymatically active. Varying the pH between 9.0 and 10.0 gave similar results. In general the total enzymatic units recovered from the DEAE cellulose columns were 10–30 times that put on the columns and several protein peaks containing enzymatic activity were observed. The major enzymatic activity peak was pooled, precipitated with $(NH_4)_2SO_4$ and purified by chromatography on a 2 cm × 100 cm Sephadex G-200 column equilibrated and eluted with 20 mM triethanolamine and 250 mM KCl, pH 8.5. The enzymatic activity eluted near the void volume in a symmetrical peak and the specific activity was from 16–18. A summary of the purification is shown in Table I.

TABLE I

SUMMARY OF THE PURIFICATION OF UDP-GLUCOSE PYROPHOSPHORYLASE FROM 50 g OF BOVINE MAMMARY ACETONE POWDER

Step	*Vol. (ml)*	*Activity (I.U.)*	*Protein (mg)*	*Specific activity*	*Purification (-fold)*
I. Extraction	640	681	22 100	0.03	1
II. Protamine sulfate	680	429	13 000	0.03	1
III. $(NH_4)_2SO_4$	250	668	4280	0.16	5.3
IV. Heat	250	688	5180	0.13	4.3
V. $(NH_4)_2SO_4$	242	472	2400	0.20	6.7
VI. $(NH_4)_2SO_4$	17.8	433	1175	0.37	12.3
VII. DEAE-cellulose	14.5	440	280	1.57	52.3
VIII. DEAE-cellulose, pH 9.7, Sephadex G-200	—	—	—	16.0	533

Properties of bovine mammary UDP-glucose pyrophosphorylase

Temperature stability. The enzyme from Step III is stable when incubated for 30 min at 50° between pH 7.5 and 9.0 in the presence of 250 mM potassium phosphate. About 70% of the enzymatic activity is lost when the enzyme is held at 52° for 30 min and no activity remained when held for 5 min at 60°. The observed heat stability may be explained by the observation that phosphate is a competitive inhibitor of the bovine liver enzyme[3].

pH stability. The effect of pH on the stability of the enzyme in a borate–citrate–phosphate buffer at 4° is presented in Fig. 1. At pH 3.0, 80% of the activity was lost immediately but 86 days were required for the remainder to be lost. At pH 4.0, the enzyme lost 20% of its activity at zero time, about 50% after two days and still retained detectable activity at 86 days. Above pH 4.0 there seemed to be an increase

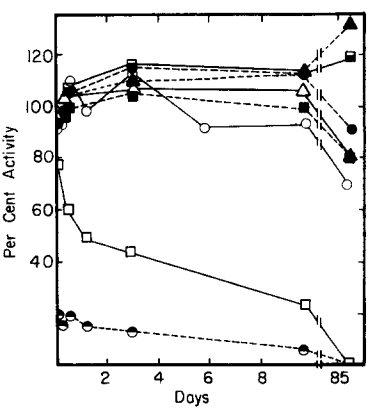

Fig. 1. The effect of pH on the stability of UDP-glucose pyrophosphorylase in borate–citrate–phosphate buffer, pH 3.0–7.5. Enzyme from Step VII was diluted 10-fold in the buffer containing 57.3 mM borate, 33.3 mM citrate and 72.5 mM phosphate and stored at 4°. The control was diluted 10-fold in 0.2 M Tris, pH 8.0 which is the assay buffer. Since the buffer was slightly inhibitory, 0.005 ml of each buffer was added to each assay. Aliquots of enzyme were removed at various times and assayed in the standard assay at pH 8.0. The variation in the assay (5 separate assays in 1 h) was ±3%. 100% activity equals 0.51 I.U./ml and the results are expressed as percentages of the control in the Tris buffer. ◓---◓, pH 3.0; □—□, pH 4.0; ○—○, pH 5.0; ■---■, control; △—△, pH 6.0; ●---●, pH 6.5; ◒—◒, pH 7.0; ▲---▲, pH 7.5.

in activity for about 70 h and at 86 days the highest activity was similar to the increase in activity of the enzyme observed in crude mammary gland homogenates[7]. The pH stability was measured over a shorter time period in 0.1 M piperazine and 0.1 M glycylglycine buffer[15] between pH 4.4 and 7.5. This buffer did not inhibit the reaction and the results obtained showed that there were oscillations in enzymatic activity ±30% which occurred on a weekly basis. The oscillations were more pronounced at the higher pH values. Another experiment, using piperazine–glycylglycine buffer from pH 8.0 to 10.8, for 20 days showed similar oscillations in enzymatic activity which could not be explained on the variation in the enzymatic assays (Fig. 1).

Multiple forms. Chromatography on various hydroxylapatite columns between pH 8.0 and 9.6 were investigated as a possible purification step. Multiple protein peaks having varying specific activities were observed. Step-wise elution of the protein with phosphate buffer usually resulted in the elution of one protein peak with each concentration of phosphate. A typical pattern is shown in Fig. 2. This procedure was not satisfactory as a purification step, but the results showed that the enzyme was eluted in a differential manner suggesting the presence of different forms of the enzyme or that there was inhomogeneity of binding sites on hydroxylapatite.

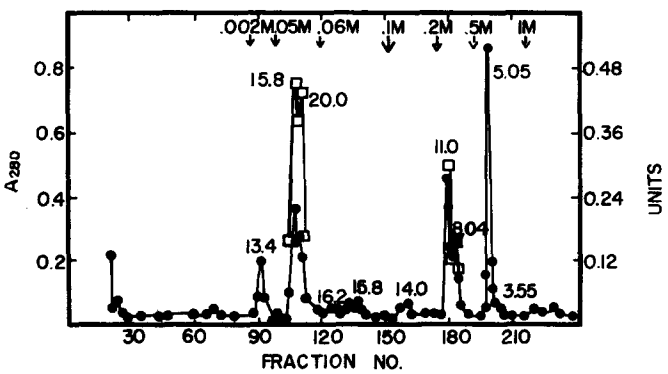

Fig. 2. Elution profile of a hydroxylapatite column at pH 8.5. Enzyme from Step VII, containing 125 mg protein, specific activity 9.0 was placed on a 2 cm × 12 cm hydroxylapatite column equilibrated with water after the enzyme was desalted by passing through a Bio-Gel P-10 column equilibrated and eluted with water. The column was eluted stepwise with potassium phosphate buffer pH 8.5 and 3.0-ml fractions were collected. The arrows on top of the figure indicate the concentration of the buffer change. The number above the peaks are the specific activity of the peak tube in units/ml. ●, $A_{280\ nm}$; □, activity in units/ml.

Chromatography on DEAE-cellulose columns between pH 8.0 and 10.0 also gave rise to multiple protein peaks and multiple enzymatic peaks. Usually two to three protein peaks had enzymatic activity. Usually, there was a 10–30-fold increase in the total number of units of enzyme after chromatography on the DEAE-cellulose column. Multiple activity peaks were occasionally observed when the purified enzyme was chromatographed on a Bio Gel P-300 column. The results from chromatography on hydroxylapatite and DEAE-cellulose showed the presence of multiple peaks of enzymatic activity, suggesting that there may be different forms of the enzyme.

Molecular weight. An estimate of the molecular weight was performed on 5–20% sucrose gradients according to MARTIN AND AMES[11]. Catalase was used as a marker

and the best estimate of the molecular weight by this technique was 500 000 at 20°. The enzymatic activity peak was symmetrical and no evidence for other active species was present. Attempts to determine the molecular weight on Sephadex G-200[16] and Bio Gel P-300 gave values greater than 400 000 since the enzyme was in the non-linear region of the elution pattern.

Inhibitors. EDTA did not affect the enzymatic activity until it sufficiently complexed Mg^{2+}. Sulfhydryl reagents, 1 mM PCMB and 10 mM iodoacetate, 10 mM mercaptoethanol or 1 mM dithiothreitol did not affect the activity or storage of the enzyme. A 2% sucrose solution inhibited the enzyme 54% but did not inhibit the UDP-glucose dehydrogenase.

Kinetic Studies

Effect of Mg^{2+}. The stability constants of $MgP_2O_7^{2-}$, $MgUTP^{2-}$ and MgGlc-1-*P* at pH 8.0 were considered to be 250 000[17], 70 000[18,19] and 20[20,21], respectively. The stability constant of UDP-glucose was considered to be similar to that of UDP which is low[18,19]. Since the stability constants for UTP and $P_2O_7^{4-}$ are so large, they will essentially exist as their Mg^{2+} complexes in solution. Glc-1-*P* and UDP-glucose were assumed to react in the non-complexed form and binding of Mg^{2+} to the other components of the assay were negligible including the buffer, triethanolamine[18]. During the analysis free Mg^{2+} was held constant at 1 mM so that any effect of free Mg^{2+} on the enzyme was constant.

Kinetic studies in the direction of UDPG formation. Initial velocity studies with varying $MgUTP^{2-}$ and fixed Glc-1-P (Fig. 3) and varying Glc-1-*P* and fixed

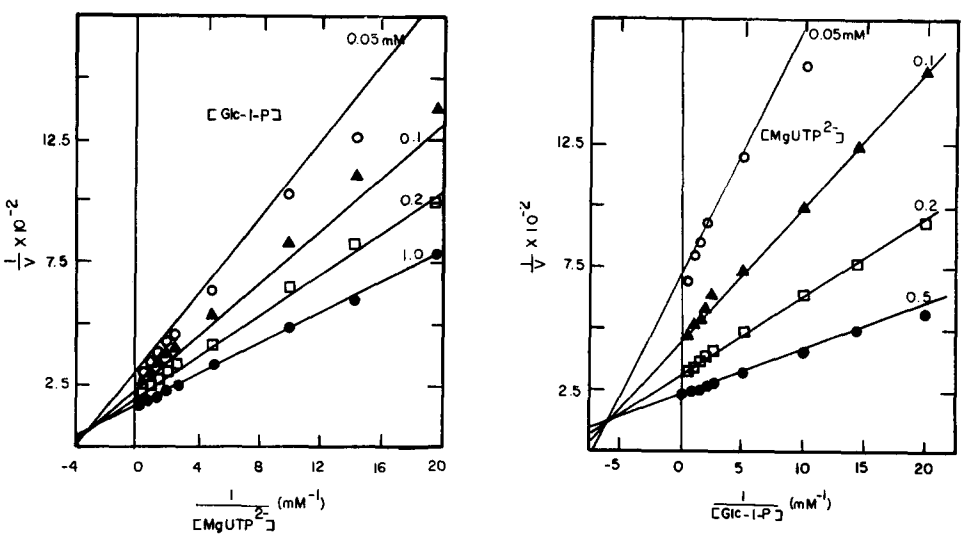

Fig. 3. Double reciprocal plot with $MgUTP^{2-}$ as the variable substrate at fixed concentrations of Glc-1-*P*. Reaction mixtures contained 2.2 μg enzyme, $MgUTP^{2-}$ and Glc-1-*P* as indicated, free Mg^{2+} at 1 mM, and 100 mM triethanolamine at pH 8.0. *v*, initial rate of UDP-glucose formation in μmoles/ml per min.

Fig. 4. Double reciprocal plot with Glc-1-*P* as the variable substrate at fixed concentrations of $MgUTP^{2+}$. Reaction mixtures contained 2.2 μg enzyme, $MgUTP^{2-}$ and Glc-1-*P* as indicated, free Mg^{2-} at 1 mM, and 100 mM triethanolamine at pH 8.0. *v*, initial rate of UDP-glucose formation in μmoles/ml per min.

TABLE II

KINETIC CONSTANTS FOR UDP-GLUCOSE SYNTHESIS FROM INITIAL VELOCITY STUDIES

*Kinetic constant**	*Exp. I*	*Exp. II*	*Weighted mean***
v	69.9 ± 1.71	73.0 ± 2.63	70.8 ± 1.43
$K_{MgUTP^{2-}}$	0.153 ± 0.010	0.167 ± 0.022	0.156 ± 0.009
$K_{iMgUTP^{2-}}$	0.489 ± 0.116	0.342 ± 0.12	0.417 ± 0.084
$K_{Glc\text{-}1\text{-}P}$	0.053 ± 0.009	0.042 ± 0.007	0.046 ± 0.006
$K_{iGlc\text{-}1\text{-}P}$	0.168 ± 0.021	0.085 ± 0.026	0.135 ± 0.016

* μmoles/min per 2.2 μg enzyme for v; the kinetic and inhibition constants are in mM.

** Weighted mean and the standard error (S.E.) of the mean for three experiments were calculated using the following formulas[23]:

$$\text{Mean} = \frac{\Sigma x_i w_i}{\Sigma w_i}; \quad \text{S.E.} = \frac{1}{\sqrt{\Sigma w_i}}; \quad \text{where } w_i = 1/(\text{S.E. for each experiment})^2.$$

$MgUTP^{2-}$ (Fig. 4) gave intersecting patterns to the left of the ordinate. The experimental points are plotted and the lines are drawn using a statistical fit to Eqn. 1 with a weighting factor of v^4. The intersecting lines indicate that the reaction is sequential[22] and the intersection point above the abscissa indicates that $K_{ia} > K_a$. This differs from the erythrocyte enzyme[6] where the lines intersect below the abscissa indicating that $K_{ia} < K_a$. The secondary plots of slope and intercept are linear. The true kinetic constants obtained from the initial velocity studied are presented in Table II. No substrate inhibition was observed up to 2 mM UTP or 2.5 mM Glc-1-*P*.

Product inhibition by $MgP_2O_7^{2-}$ is shown in Fig. 5 when $MgUTP^{2-}$ is varied and in Fig. 6 when Glc-1-*P* is varied. The points are the experimental data and the

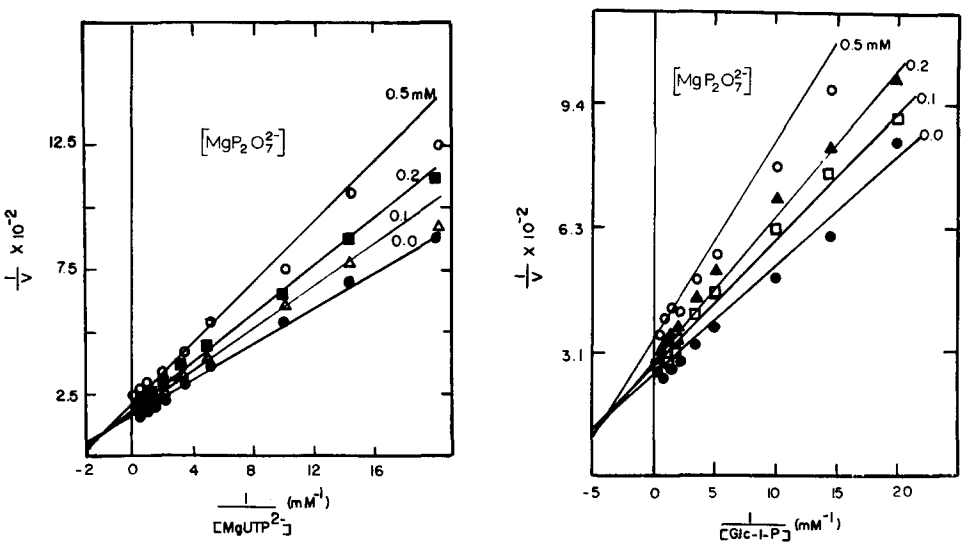

Fig. 5. Inhibition of UDP-glucose synthesis by $MgP_2O_7^{2-}$ with $MgUTP^{2-}$ as the variable substrate. Reaction mixtures contained 2.2 μg enzyme, $MgP_2O_7^{2-}$ and $MgUTP^{2-}$ as indicated, free Mg^{2+} and Glc-1-*P* at 1 mM, and 100 mM triethanolamine at pH 8.0. v, initial rate of UDP-glucose formation in μmoles/ml per min.

Fig. 6. Inhibition of UDP-glucose synthesis by $MgP_2O_7^{2-}$ with Glc-1-*P* as the variable substrate. Reaction mixtures contained 2.2 μg enzyme, $MgP_2O_7^{2-}$ and Glc-1-*P* as indicated, free Mg^{2+} and $MgUTP^{2-}$ at 1 mM, and 100 mM triethanolamine at pH 8.0. v, initial rate of UDP-glucose formation in μmoles/ml per min.

lines represent the best fit to Eqn. 2. The secondary plots of slopes and intercepts are linear. The noncompetitive inhibition of Glc-1-*P* and $MgUTP^{2-}$ by $MgP_2O_7^{2-}$ is consistent with an ordered mechanism where $MgP_2O_7^{2-}$ is the first product released However, the data cannot distinguish the ordered addition of $MgUTP^{2-}$ and Glc-1-*P*. The kinetic constants obtained from the product inhibition analysis are presented with their standard errors in Table III.

TABLE III

APPARENT KINETIC CONSTANTS FOR PRODUCT INHIBITION OF UDP-GLUCOSE PYROPHOSPHORYLASE BY $MgP_2O_7^{2-}$

*Apparent kinetic constant**	*Variable substrate*	
	Glc-1-P	*$MgUTP^{2-}$*
K	0.108 ± 0.008	0.253 ± 0.017
K_{ts}	0.633 ± 0.172	0.766 ± 0.193
K_{ti}	1.46 ± 0.24	1.44 ± 0.27

* Values for the kinetic constants (mM) with the standard errors.

Kinetic studies in the direction of UTP formation. Initial velocity studies at substrate concentrations adequate for reading NADH by absorbance were unsatisfactory in obtaining reliable constants, since they were apparently very small. Fluorometric measurement of the reduced pyridine nucleotide concentration was in a satisfactory range and a linear correspondence existed between relative fluorescence and 0 — 15 nmoles of Glc-1-*P* and the velocity showed a linear dependence upon enzyme concentration.

However, when one substrate was held constant and the velocity determined at several concentrations of the second, the lines on a plot of relative fluorescence versus

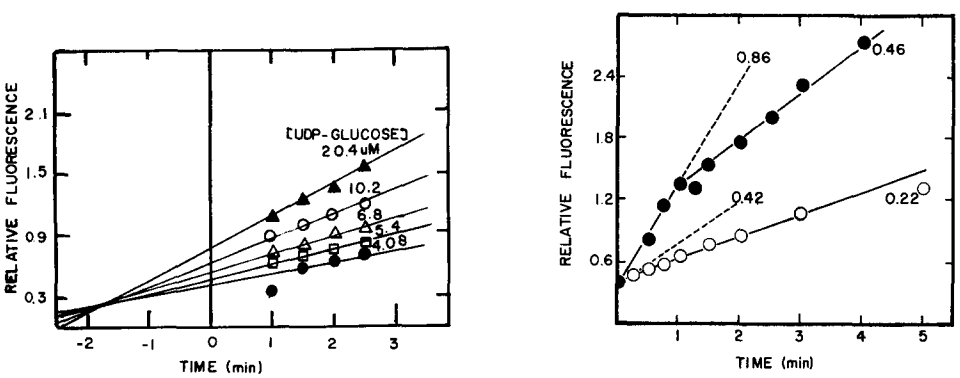

Fig. 7. Assays of pyrophosphorololysis at a fixed $MgP_2O_7^{2-}$ concentration and variable UDP-glucose concentration. Reaction mixtures contained 2 μl of 1:10 dilution of enzyme, 0.36 mM $MgP_2O_7^{2-}$, Mg^{2+} at 1 mM, 100 mM triethanolamine, pH 8.0, and UDP-glucose at 4.08 (●), 5.1 (□), 6.8 (△), 10.2 (○), or 20 μM (▲).

Fig. 8. Biphasic time course of the fluorescence assay at two enzyme concentrations. Reaction mixtures contained 0.36 mM $MgP_2O_7^{2-}$, 20.4 μM UDP-glucose, Mg^{2+} at 1 mM triethanolamine at pH 8.0, and enzyme. ○, 0.001 ml of 1:10 dilution of enzyme; ●, 0.002 ml of 1:10 dilution of enzyme. Rates are indicated on the figure and are expressed as the change in fluorescence per min.

time did not intersect at a common point on the ordinate, but rather intersected at a common point at negative time (Fig. 7). An explanation of such a phenomena is a two step process in which the velocity of each step is very different. Fig. 8 indicates a 2-step process when shorter time intervals are examined. The velocity for each step is proportional to enzyme concentration. An initial velocity study in which the rate of the slower process was used and $MgP_2O_7^{2-}$ was varied from 0.22 to 1.08 mM and UDP-glucose was varied from 4.08 to 20.4 μM gave an intersecting pattern to the left of the ordinate. However, there is considerable scatter in the data as reflected in the large standard errors of the kinetic constants (Table IV).

TABLE IV

KINETIC CONSTANTS FOR PYROPHOSPHOROLYSIS FROM INITIAL VELOCITY STUDIES

Data are values for the kinetic constants with the standard errors.

Assay	$K_{UDP\text{-}glucose}$ (μM)	$K_{iUDP\text{-}glucose}$ (μM)	$K_{MgP_2O_7^{2-}}$ (μM)	$K_{iMgP_2O_7^{2-}}$ (μM)
Bovine mammary				
Absorbance	81.9 ± 37.7	−8 ± 16	893 ± 220	−93 ± 154
UDP-glucose (0.1 to 0.5 mM)				
$MgP_2O_7^{2-}$ (0.2 to 1.0 mM)				
Fluorescence	21.3 ± 5.6	6.7 ± 2.6	608 ± 185	191 ± 64.7
UDP-glucose (4.08 to 20.4 μM)				
$MgP_2O_7^{2-}$ (0.216 to 1.08 mM)				

DISCUSSION

UDP-glucose pyrophosphorylase purified from bovine mammary acetone powder had a specific activity of about 16.0 I.U. compared to a specific activity of 80 for the crystalline bovine liver enzyme[3]. The molecular weight is between 400 000 and 500 000 as determined by sucrose density grandient and gel filtration experiments compared to 480 000 for the bovine liver enzyme[3]. The enzyme is stable to 50° for at least 1 h at pH 8.0 and the purified enzyme is stable between pH 5.0 and 10.8.

There is evidence to suggest that more than one form of enzyme exists. Multiple enzymatic peaks were observed readily upon chromatography on DEAE cellulose, hydroxylapatite, and less readily on Bio Gel P-300 though the number of peaks varied with the preparation. A single peak of activity was obtained on the sucrose density gradients which is a less descriminating technique than the previous ones. The pH instability at pH 3.0 may be interpreted as evidence for two enzymatic forms one of which is very sensitive to pH since 80% of the activity was lost in 2 min but 86 days were required to loose the remainder. Lowering the pH to 2.0 for 2.5 min did not sufficiently inactivate the enzyme for the fixed-time assays. Kinetic experiments have also indicated (Fig. 8) two forms of enzyme. The present evidence would suggest that multiple forms of the enzyme exist and is consistant with previous observations on a time-dependent increase of the enzyme activity in crude extracts which was due to a change in V of the enzyme suggesting a conversion from a less active to a more

active enzyme form[7]. The observed increase in enzymatic activity on DEAE cellulose chromatography may also reflect this process.

The kinetic studies are consistent with a steady state ordered mechanism similar to the erythrocyte enzyme where an ordered addition of $MgUTP^{2-}$ and Glc-1-*P* was followed by an ordered release of $MgP_2O_7^{2-}$ and UDP-glucose[6]. In this study the order of release of $MgP_2O_7^{2-}$ and UDP-glucose was established by the product inhibition studies but the order of addition of $MgUTP^{2-}$ and Glc-1-*P* was not determined. Since the secondary plots of slopes and intercepts were linear, no dead-end complexes or sigmoidal kinetics were indicated. Quantitatively the bovine mammary and erythrocyte enzymes differ in that K_{ia} is greater than K_a for the bovine mammary enzyme and is less than K_a for the human liver enzyme.

The true Michaelis constants for the human erythrocyte and bovine mammary enzyme indicate that $K_{Glc\text{-}1\text{-}P} < K_{MgUTP}$ in both cases and kinetic constants for the bovine mammary gland and bovine liver[3] enzymes are similar. The bovine mammary and liver enzymes are unaffected by dithiothreitol or mercaptoethanol whereas one of these is required for the human erythrocyte or human liver enzymes.

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REFERENCES

1 J. B. SHATTON, M. GRUENSTEIN, H. SHAY AND S. WEINHOUSE, *J. Biol. Chem.*, 240 (1965) 22.
2 R. S. EMERY AND R. L. BALDWIN, *Biochim. Biophys. Acta*, 136 (1967) 223.
3 G. J. ALBRECHT, S. T. BASS, L. L. SEIFERT AND R. G. HANSEN, *J. Biol. Chem.*, 241 (1966) 2968.
4 J. K. KNOP AND R. G. HANSEN, *J. Biol. Chem.*, 245 (1970) 2499.
5 S. LEVINE, T. A. GILLET, E. HAGEMAN AND R. G. HANSEN, *J. Biol. Chem.*, 244 (1969) 5729.
6 K. K. TSUBOI, K. FUKUNAGA AND J. C. PETRICCIANI, *J. Biol. Chem.*, 244 (1968) 1008.
7 D. K. FITZGERALD, S. CHEN AND K. E. EBNER, *Biochim. Biophys. Acta*, 178 (1969) 491.
8 J. L. STROMINGER, E. S. MAXWELL, F. AXELROD AND H. M. KALCKAR, *J. Biol. Chem.*, 244 (1957) 79.
9 H. W. SIEGELMAN, G. A. WIECZOEREK AND B. C. TURNER, *Anal. Biochem.*, 13 (1965) 402.
10 V. S. STEELMAN AND K. E. EBNER, *Biochim. Biophys. Acta*, 128 (1966) 92.
11 R. G. MARTIN AND B. H. AMES, *J. Biol. Chem.*, 236 (1961) 1372.
12 O. H. LOWRY, J. V. PASSONNEAU, F. X. HASSELBERGER AND D. W. SCHULTZ, *J. Biol. Chem.*, 239 (1964) 18.
13 W. W. CLELAND, *Nature*, 198 (1963) 463.
14 W. W. CLELAND, *Adv. Enzymol.*, 29 (1967) 1.
15 M. E. SMITH AND L. B. SMITH, *Biol. Bull.*, 96 (1949) 233.
16 P. ANDREWS, *Biochem. J.*, 96 (1965) 595.
17 R. E. GOSSELIN AND E. R. COGHLON, *Arch. Biochem. Biophys.*, 45 (1953) 301.
18 W. J. O'SULLIVAN AND D. D. PERIN, *Biochemistry*, 3 (1964) 18.
19 E. WALAAS, *Acta Chem. Scand.*, 12 (1958) 528.
20 H. B. CLARKE, D. C. CUSWORTH AND S. P. DATTA, *Biochem. J.*, 58 (1954) 146.
21 W. E. TREVELYAN, P. F. E. MANN AND J. S. HARRISON, *Arch. Biochem. Biophys.*, 39 (1952) 440.
22 W. W. CLELAND, *Biochim. Biophys. Acta*, 67 (1963) 104.
23 J. F. MORRISON AND E. JAMES, *Biochem. J.*, 97 (1965) 39.