PURIFICATION, CRYSTALLIZATION, AND PROPERTIES OF UDP-GLUCOSE PYROPHOSPHORYLASE FROM HUMAN LIVER

Thesis for the Degree of M. S.
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Janice K. Knop
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ABSTRACT

PURIFICATION, CRYSTALLIZATION, AND PROPERTIES
OF UDP-GLUCOSE PYROPHOSPHORYLASE
FROM HUMAN LIVER

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The biosynthesis of uridine diphosphate glucose, an important intermediate in carbohydrate metabolism, from uridine triphosphate and glucose-1-phosphate is catalyzed by uridine diphosphate glucose pyrophosphorylase. In this research, the enzyme was purified 500-fold from human liver and crystallized.

The crystalline enzyme was found to be nearly homogeneous by several analytical techniques. The equilibrium constant, specificity, pH optimum, metal requirement, and substrate affinity of the crystalline enzyme were determined. The activity ratio UDP-glucose/UDP-galactose was measured at several stages of purification and on the crystalline enzyme and was found to remain constant, indicating that there is not a separate uridine diphosphate galactose pyrophosphorylase in human liver.

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INTRODUCTION

Uridine diphosphate glucose pyrophosphorylase (UTP: α-D-glucose-1-phosphate uridylyltransferase, E. C. 2.7.7.9) catalyzed the synthesis of the important intermediate in carbohydrate metabolism, UDP-Glc,[1] from UTP and Glc-1-P.

$$\text{UTP} + \text{Glc-1-P} \xrightleftharpoons{Mg^{+2}} \text{UDP-Glc} + PP_i \qquad (1)$$

This enzyme has been found to be widely distributed in nature (1-15) and has been reported in several human tissues (12, 16-19). With respect to human tissue, the enzyme has been only partially purified and characterized from human brain (12). Recently, the enzyme has been obtained in crystalline

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[1]The following abbreviations are used: UMP, UDP, and UTP, uridine mono-, di-, and tri-phosphate; UDP-Glc, uridine diphosphate glucose; UDP-Gal, uridine diphosphate galactose; UDP-Man, uridine diphosphate mannose; UDP-Xyl, uridine diphosphate xylose; AMP, ADP, and ATP, adenosine mono-, di-, and tri-phosphates; ADP-Glc, adenosine diphosphate glucose; CTP, cytidine tri-phosphate; CDP-Glc, cytidine diphosphate glucose; GTP, guanosine tri-phosphate; GDP-Glc, guanosine diphosphate glucose; ITP, inosine tri-phosphate; IDP-Glc, inosine diphosphate glucose; TDP-Glc, thymidine diphosphate glucose; Glc-1-P, glucose-1-phosphate; Gal-1-P, galactose-1-P; PP_i, inorganic pyrophosphate; P_i, inorganic orthophosphate; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; MSH, 2-mercaptoethanol; EDTA, ethylenediaminetetraacetic acid; K_m, Michaelis constant; K_i, inhibitor constant; K_{eq}, equilibrium constant.

form from calf liver (15) and constituted more than 0.3% of the extractable protein.

This thesis describes: (1) the purification and crystallization procedure for UDP-Glc pyrophosphorylase from human liver; (2) the homogeneity, specificity, pH optimum, cation requirement, and some of the kinetic properties of the crystalline enzyme. The ratio of activities of UDP-Glc to UDP-Gal was found to remain relatively constant throughout the purification procedure, indicating that there is not a separate UDP-Gal pyrophosphorylase in human liver.

LITERATURE REVIEW

Uridine diphosphate glucose plays an important role in the interconversion of hexoses, resulting in the formation of other hexoses, pentoses and uronic acids, and in the biosynthesis of glycogen and other polysaccharides from hexose phosphate (20-23). The enzyme catalyzing the biosynthesis of UDP-Glc from UTP and Glc-1-P is UDP-Glc pyrophosphorylase.

$$\text{UTP} + \text{Glc-1-P} \xrightleftharpoons{Mg^{+2}} \text{UDP-Glc} + PP_i \qquad (1)$$

The reaction is reversible <u>in vitro</u> (3,24), however, physiologically the biosynthesis of UDP-Glc is favored because of its rapid removal by other biosynthetic reactions or interconversions (15).

UDP-Glc pyrophosphorylase has been reported to be present in several human tissues (12,16-19). A 30-fold purification of this enzyme from human brain has been reported by Basu and Bachhawat (12). This pyrophosphorylase had an absolute and specific requirement for Mg^{+2} and no activity in the presence of Mn^{+2}, Zn^{+2}, Ni^{+2}, Cc^{+2}, Cu^{+2}, or Ca^{+2}. The activity of the enzyme was not affected by Ca^{+2} in the presence of Mg^{+2}. Neither cysteine nor glutathione had any affect on the activity; however, at 10^{-4} M p-chloromercuribenzoate, only

50% of the activity remained. The enzyme exhibited a sharp pH optimum at 8.0. The Michaelis constants for UDP-Glc and PP_i were 7.5×10^{-5} M and 2.0×10^{-4} M, respectively. A slight Gal-1-P exchange into UDP-Gal was found in the presence of UTP, Mg^{+2}, and pyrophosphate, but Gal-1-P did not inhibit the exchange with Glc-1-P. Glc-1-P contamination of their Gal-1-P preparation was postulated to account for the exchange, however, this was not tested.

Recently, UDP-Glc pyrophosphorylase was crystallized from calf liver (15). The crystalline calf liver enzyme had Michaelis constants for UDP-Glc, PP_i, UTP, and Glc-1-P of 6.0×10^{-5} M, 8.4×10^{-5} M, 2.0×10^{-4} M, and 5.5×10^{-5} M, respectively. On a rate basis, the enzyme was not highly specific for either nucleoside or hexose component of the nucleoside diphosphate hexose (Table I) and was inhibited by P_i and UDP with K_i's of 3.7×10^{-3} M and 1.5×10^{-4} M, respectively. The activity of the calf liver UDP-Glc pyrophosphorylase was not stimulated by mercaptoethanol, glutathione, or cysteine. The equilibrium constant at 30^{o} C (pH 7.8) was 0.20, using micromolar quantities of substrates (24).

The divalent cation requirement of the calf liver enzyme was best met by magnesium at an optimum concentration of 2×10^{-3} M. At the same concentration, Co^{+2} and Mn^{+2} had 25% of the catalytic activity of magnesium, and Ca^{+2} had 10%. The enzyme had no activity in the presence of Fe^{+2}, Ni^{+2},

TABLE I

Specificity of Calf Liver UDP-Glc Pyrophosphorylase*(15)

Substrate	Reaction Rate
UDP-Glucose	100.0%
TDP-Glucose	2.7
CDP-Glucose	0.8
GDP-Glucose	0.1
IDP-Glucose	very low
ADP-Glucose	very low
UDP-Galactose	3.5
UDP-Xylose	3.9
UDP-Mannose	0.3
UDP-Glucuronic Acid	very low
UDP-N-acetylglucosamine	very low

*All substrates were added at a level of 0.4 μmole per ml since UDP-glucose saturates the enzyme at this concentration.

Cu^{+2}, or Zn^{+2}, and in the presence of magnesium, all the divalent ions tested were somewhat inhibitory.

The enzyme exhibited a broad pH optimum which ranged from 7.0 to 9.5 with maximal activity at 8.5 and 85% of this activity at pH's 7.0 and 9.5.

Electrophoresis of the crystalline calf liver UDP-Glc tyrophosphorylase on polyacrylamide gels resulted in four distinct protein bands when the gels were stained with Coomassie blue (25). When unstained gels were sliced into 2 mm segments and the activity eluted and measured, all four protein bands were found to be active. These same four forms have also been found in 5 to 20% sucrose density gradients (25). The four forms of the enzyme correspond to monomer, dimer, trimer, and tetramer forms with the monomer being by far the predominant form. However, during sedimentation velocity experiments in the Model E analytical ultracentrifuge, usually only two forms, monomer and dimer, were seen.

The sedimentation coefficient of the monomer (0.4% protein, determined by extinction coefficient) in 0.10 M MSH, 0.10 N NaCl, 0.01 M tricine buffer, pH 8.5, has ranged from 12.8 to 13.2 (26). From sedimentation and diffusion experiments, the molecular weight of the monomer has been calculated to be about 472,000 (25).

The pyrophosphorylase has also been crystallized from lamb, goat, and rabbit livers and has been obtained in an amorphous but homogeneous form from rabbit muscle (34).

The enzyme from calf, goat, and lamb livers crystallized as regular bipyramids; however, the rabbit liver enzyme crystallized as plump rods (larger in the middle than at the ends). Unlike the pyrophosphorylases from calf, lamb, and goat livers, the rabbit liver and muscle enzymes required mercaptoethanol to maintain activity. Only the calf liver pyrophosphorylase has been extensively characterized.

The biosynthesis of UDP-Gal from Gal-1-P and UTP, is also catalyzed by a pyrophosphorylase.

$$\text{Gal-1-P} + \text{UTP} \xrightleftharpoons{Mg^{+2}} \text{UDP-Gal} + PP_i \qquad (2)$$

The synthesis of UDP-Gal by this mechanism has been reported in yeast (27), mung beans (7), and mammalian liver (15,28-31), but it was believed absent in human erythrocytes (31). The presence of a UDP-Gal pyrophosphorylase has been proposed by some of these investigators (7,28,29). Isselbacher (28,29) found this activity in rat, beef, pigeon, and human livers. He reported that the UDP-Gal pyrophosphorylase activity from beef liver was partially separable from UDP-Glc pyrophosphorylase activity by polyvinyl geon electrophoresis, and concluded the former activity was distinct from UDP-Glc pyrophosphorylase. Further, he suggested that the UDP-Gal pyrophosphorylase activity may be responsible for the increased ability to metabolize galactose with age in some galactosemics, according to the following accessary pathway:

$$\text{Galactose} + \text{ATP} \xrightarrow{\text{kinase}} \text{Gal-1-P} + \text{ADP} \qquad (3)$$

$$\text{Gal-1-P} + \text{UTP} \underset{}{\overset{\text{pyrophosphorylase}}{\rightleftharpoons}} \text{UDP-Gal} + PP_i \qquad (2)$$

$$\text{UDP-Gal} \underset{}{\overset{\text{epimerase}}{\rightleftharpoons}} \text{UDP-Glc} \qquad (4)$$

$$\text{UDP-Glc} + PP_i \underset{}{\overset{\text{pyrophosphorylase}}{\rightleftharpoons}} \text{Glc-1-P} + \text{UTP} \qquad (1)$$

Sum of Equations (1, 2, and 4): $\text{Gal-1-P} \rightleftharpoons \text{Glc-1-P}$

Recently, Gitzelmann found that human erythrocytes, both normal and galactosemic, can form Gal-1-P from UDP-Gal, and that this reaction was dependent upon the presence of PP_i (32). The synthesis of Gal-1-P required Mg^{+2} and was inhibited by UDP, P_i, and UDP-Glc. In the presence of UTP, Gal-1-P, and Mg^{+2}, the hemolysates formed a UDP-hexose, however, not in sufficient quantities to characterize. Gitzelmann concluded that his findings were best explained by the presence of a UDP-Gal pyrophosphorylase in human erythrocytes. However, because of the crude system used as an enzyme source, he could not determine whether the pyrophosphorolysis of UDP-Gal was catalyzed by a distinct UDP-Gal pyrophosphorylase or a non-specific UDP-hexose pyrophosphorylase.

The specificity of the crystalline calf liver UDP-Glc pyrophosphorylase was examined with respect to UDP-Gal by Ting and Hansen (30). At a concentration of 0.4 mM, the activity for UDP-Gal was only 1.5% of the UDP-Glc activity. Throughout the purification and crystallization procedures, the activity ratio $\frac{\text{UDP-Glc}}{\text{UDP-Gal}}$ remained relatively constant

(61 to 77), and the two activities were not separated by several electrophoretic techniques, including polyvinyl geon electrophoresis. They suggested that the significance of their findings would depend upon the relative concentrations of Glc-1-P, UTP, Gal-1-P, etc., in the liver tissue and upon the results of similar studies on human liver. Therefore, they suggested exercising caution in attributing significance to a UDP-Gal pyrophosphorylase pathway as a major auxilliary pathway for galactose metabolism.

EXPERIMENTAL PROCEDURE

Materials and Methods

Reagents

All nucleotides and sugar-1-phosphates were purchased from commercial sources with the exception of IDP-Glc which was synthesized by the method of Michelson (33). Ultra pure sucrose and tris(hydroxymethyl)aminomethane were purchased from Mann Research Laboratories. Crystalline UDP-Glc pyrophosphorylase from calf liver was prepared according to Albrecht *et al.* (15), as modified by Levine *et al.* (25). Crystalline rabbit liver and muscle UDP-Glc pyrophosphorylases were prepared according to Gillette *et al.* (34). All other chemicals, enzymes, and supplies were purchased commercially.

Spectrophotometry

Either a Beckman DU Spectrophotometer equipped with a Gilford automatic sample changer and recorder (35) or a Gilford Model 240 Spectrophotometer equipped with a Gilford automatic sample changer and a Sargent SRL recorder was used for spectrophotometric measurements.

Definition of Unit and Specific Activity

One unit of enzyme activity is defined according to Albrecht et al. (15) as that amount which forms 1 μmole of product per min at 25° C. The method of Warburg and Christian (36) was used to determine protein. Specific activity (S.A.) is defined as units of enzyme per mg of protein.

Electrophoresis

Polyacrylamide gel (5% acrylamide) electrophoresis was conducted according to Davis (37) in a pH 8.7 buffer system. In addition, the upper buffer was made 0.001 M in thioglycolate. In each 4 mm I.D. x 70 mm tube, 0.8 ml of running gel and 0.2 ml of spacer gel were used. Dialyzed protein samples (0.02 to 0.20 mg) were layered on top of the spacer gel in 5 to 10% sucrose, followed by careful layering of the upper buffer on the protein sample. The gels were run at constant amperage (5 ma/tube) for 2½ hours at 4° C. The gel columns were either stained for protein with Coomassie blue according to the method of Chrambach et al. (38) or sliced into 2 mm segments for activity determinations. The activity was eluted from each 2 mm segment with 0.4 ml of 0.02 M MSH, 0.01 M tricine buffer, pH 8.0, by gently mashing the gel and allowing the mixture to extract for 10-15 min with occasional stirring. Aliquots of the extract were then taken for activity determinations.

Sedimentation

A Spinco Model E analytical ultracentrifuge equipped with phase plate schlieren optics was used for sedimentation velocity experiments. A Bausch and Lomb Microcomparator was used to read the Schlieren patterns.

A Spinco Model L preparative ultracentrifuge was used for sucrose density gradient experiments. Dialyzed protein samples (50 µl containing 0.1 to 0.5 mg protein) were layered on 5.2 ml of a 5 to 20% sucrose density gradient prepared in 0.10 M Tris-acetate buffer, pH 8.0. The gradients were spun in a SW 50 rotor at 35,000 rpm for 12 hours (4° C). The tubes were punctured and the contents were forced from the bottom and through a flow-cell with 2% sucrose, 0.10 M Tris-acetate buffer, pH 8.0, containing o-bromophenol blue. The optical density was monitored at 280 mµ, and 200 µl fractions were collected each min. The activity of the fractions was determined immediately.

Measurement of Pyrophosphorylase Activity

Pyrophosphorylase activity was quantitatively determined by one of the four assays described below. In the direction of pyrophosphorolysis, either Glc-1-P (Assay 1 or 3) or nucleoside triphosphate (Assay 2 or 3) was measured.

Assay 1: Glc-1-P was quantitatively converted to Glc-6-P and then to P-6-Gluconate with the concomitant formation of NADPH essentially according to the procedure of Munch-Petersen (3). The NADPH formation was measured at 340 mµ.

A typical reaction mixture contained, in a final volume of 0.5 ml, 45 μmoles of Tris-acetate buffer (pH 7.8), 0.2 μmole of NADP, 1.5 μmoles of magnesium acetate, 1.0 μmole of MSH, 1.0 μmole of PP_i, 0.2 μmole of nucleoside diphosphate glucose, excess phosphoglucomutase and glucose-6-P dehydrogenase, and sufficient pyrophosphorylase to produce an absorbance change of 0.06 to 0.4 in 15 min at 25° C.

Assay 2: The assay procedure of Verachtert et al. (39) was used when the formation of the nucleotide triphosphate, ATP, GTP, ITP, or UTP, was followed. 3-P-glycerate was phosphorylated by the nucleotide triphosphate resulting in the ultimate formation of glyceraldehyde-3-P with the concomitant oxidation of NADH. The oxidation of NADH was followed at 340 mμ. The assay mixture contained, in a final volume of 0.5 ml, 20 μmoles of triethanolamine buffer (pH 7.8), 0.5 μmole of hydrazine sulfate, 0.6 μmole of 3-P-glycerate, 0.12 μmole of NADH, 0.2 μmole of nucleoside diphosphate hexose, 1.0 μmole of magnesium acetate, 1.0 μmole of MSH, 1.0 μmole of PP_i, excess 3-P-glycerate kinase and glyceraldehyde-3-dehydrogenase, and sufficient pyrophosphorylase to produce the same absorbance change specified in Assay 1. 3-P-glycerate kinase and glyceraldehyde-3-P dehydrogenase were dissolved in 0.05 M triethanolamine buffer, pH 7.8; in addition, the solution containing the dehydrogenase was made 0.01 M in dithiothreitol.

Assay 3: This assay was a two-step assay. Since the concentrations of the reagents, the length of incubation, and

the temperature varied depending upon the experiment, the exact conditions of the incubation mixture, etc., will be given with the results. However, in all cases, the reaction was stopped by heating for 2 min at 100° C, followed by cooling in an ice bath. Aliquots of the incubation mixtures were then taken; and the concentrations of the products and/or reactants were quantitatively determined in end-point assays as follows: Glc-1-P, by using excess phosphoglucomutase and glucose-6-P dehydrogenase (3); UTP, by using Assay 2 and omitting PP_i, UDP-Glc, MSH, and pyrophosphorylase (39); PP_i, by using Assay 1, excess crystalline calf liver UDP-Glc pyrophosphorylase, and omitting PP_i and MSH (40); and UDP-Glc, by using Assay 1, excess calf liver UDP-Glc pyrophosphorylase, and omitting UDP-Glc and MSH (24).

In the direction of synthesis of UDP-Glc and in the determinations of the Michaelis constants for UTP and Glc-1-P, the UDP-Glc formed was quantitatively determined with UDP-Glc dehydrogenase (41) by following NAD reduction at 340 mμ (Assay 4). For each micromole of UDP-Glc converted to UDP-Gluruconic acid, 2 micromoles of NAD are reduced.

Assay 4: In a final volume of 0.5 ml, the reaction mixture contained 45 μmoles of Tris-acetate buffer (pH 7.8), 0.10 μmole of NAD, 1.0 μmole of MSH, 1.5 μmoles of magnesium acetate, 1.0 μmole of Glc-1-P, 0.5 μmole of UTP, excess UDP-Glc dehydrogenase and sufficient pyrophosphorylase to produce 0.06 to 0.4 absorbance change in 15 min at 25° C. In Figure 1, example optical density tracings of Assays 1 and 4 are shown.

Figure 1. Spectrophotometric measurement of pyrophosphorylase activity.

These lines are optical density tracings of two of the assays used to quantitatively determine pyrophosphorylase activity. Line A: Assay 4, recording of the reaction in the direction of UDP-Glc synthesis. Line B: Assay 1, recording of the reaction in the direction of pyrophosphorolysis of nucleotide diphosphate glucose (in this case, UDP-Glc). The conditions for the assays are described in Experimental Procedure. The same amount of protein, approximately 4×10^{-5} mg, was added to each cuvette.

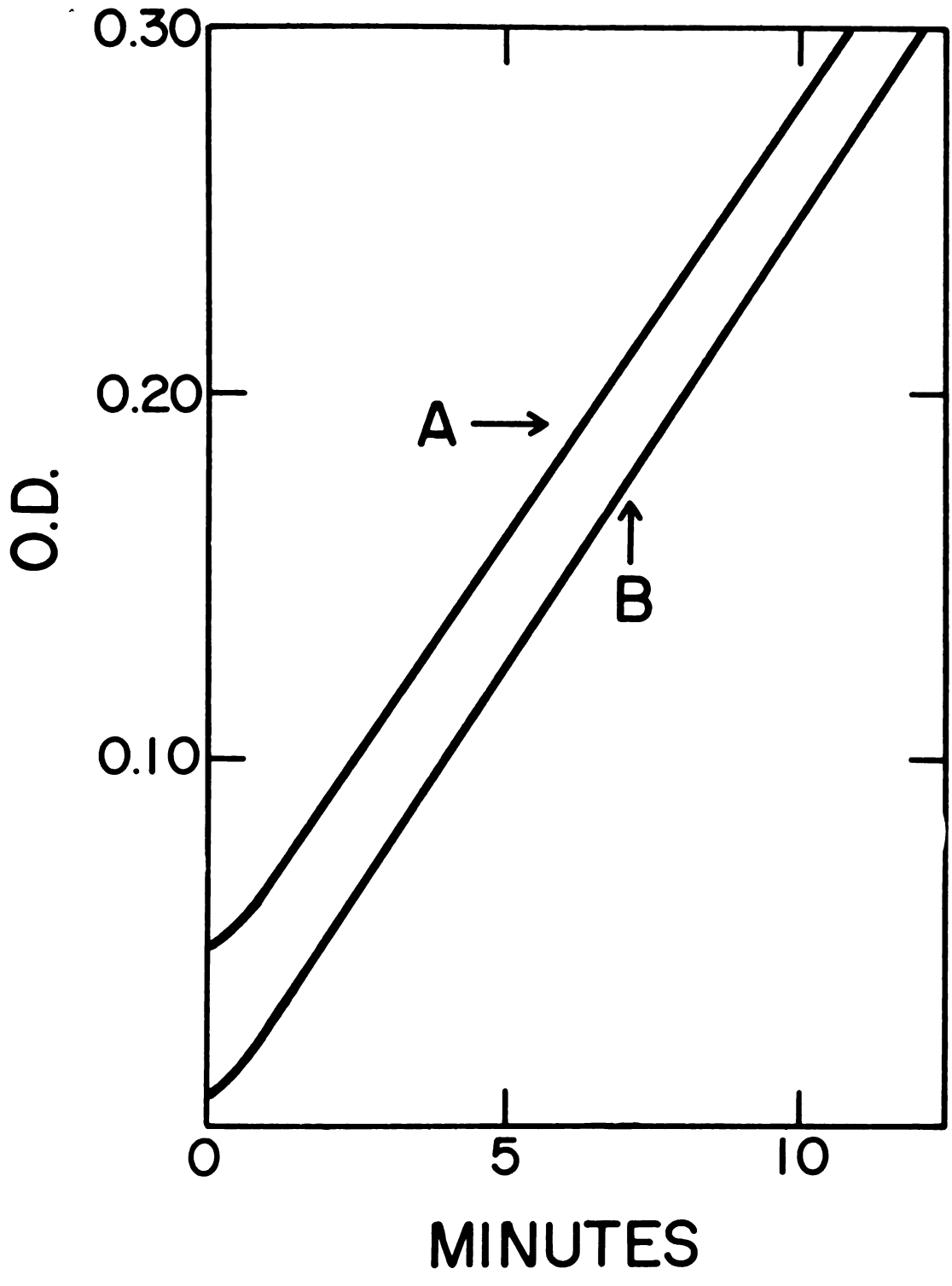

Figure 1

Fractionation of Human Liver

All of the fractionation steps were conducted at 4° C and the centrifugation steps at 23,000 x g for 30 min, unless otherwise noted.

Step 1: Extraction--Frozen liver, 1.7 kg, was cut into 1 cm cubes and homogenized in 1.5 volumes of 0.03 M KOH-0.005 M EDTA-0.005 M mercaptoethanol (MSH) for 2 min. The resulting homogenate was stirred for 10 min and protamine sulfate (12 gm/kg of liver), dissolved in a small amount of deionized distilled water, was added slowly with stirring. The pH was then adjusted to 6.8 with glacial acetic acid. The resulting mixture was allowed to stir an additional 10 to 15 min and centrifuged. After the supernatant was filtered through glass wool, the pH was adjusted to 8.5 with concentrated NH_4OH, and the resulting solution constituted Fraction I.

Step 2: Ammonium Sulfate Fractionation--Fraction I was brought to 35% saturation with the addition of solid ammonium sulfate while stirring and maintaining the pH at 8.5 with the addition of small amounts of concentrated NH_4OH (the addition was completed within 10 min). After stirring the solution for 20 min, and then centrifuging, the supernatant was filtered through glass wool and brought to 58% saturation with ammonium sulfate as described above. This solution was then stirred for 30 min and centrifuged. The precipitate was dissolved in 0.001 M EDTA-0.005 M MSH-0.01 M tricine buffer, pH 8.5 (about 0.2 volumes of Fraction I),

and dialyzed overnight against 20 liters of the same buffer (Fraction II).

Step 3: Calcium Phosphate Gel Treatment--Calcium phosphate gel (0.6 mg dry weight per mg protein) was added to Fraction II with constant stirring, and after 10 min was immediately centrifuged. The supernatant was retained (Fraction III).[2]

Step 4: Diethylaminoethyl Cellulose Column Chromatography I--The diethylaminoethyl cellulose (DEAE-cellulose) was prepared as previously described (15), except that it was washed four times with deionized distilled water (2 volumes) after treatment with 0.5 M Na_3PO_4. The DEAE-cellulose was then suspended in 0.01 M tricine buffer, pH 8.5 as a thick slurry, added to a column (4.0 x 40 cm), and packed with 2 liters of 0.01 M tricine buffer, pH 8.5. Fraction III was put on the column and washed with 1.5 liters of 0.02 N NaCl-0.02 M MSH-0.01 M tricine buffer, pH 8.5. Twenty milliliter samples were collected, and tubes containing 10 or more units per ml were combined (Fraction IV). A typical elution pattern is shown in Figure 2.

---

[2]Occasionally, nearly all of the activity was bound to the gel. Activity can be eluted from the gel by making a slurry of the gel in 0.10 M sodium pyrophosphate-0.02 M MSH-0.01 M tricine buffer, pH 8.5, stirring for 30 min, and centrifuging. The supernatant is dialyzed against 20 liters of 0.001 M EDTA-0.005 M MSH-0.01 M tricine buffer, pH 8.5, overnight, and then put on the first DEAE-cellulose column as described above. Crystals obtained from such a preparation required several more recrystallizations (at least two) to remove the colored proteins.

Figure 2. Chromatography of UDP-Glc pyrophosphorylase, Fraction IV, on DEAE-cellulose.

The column dimensions were 4.0 x 40 cm; the flow rate was 1 ml per min; and 20 ml fractions were collected. The activity was eluted by washing the column with 1.5 liters of 0.02 N NaCl, 0.02 M MSH, 0.01 M tricine buffer, pH 8.5. (-----), milligrams of protein per ml; (———), units per ml.

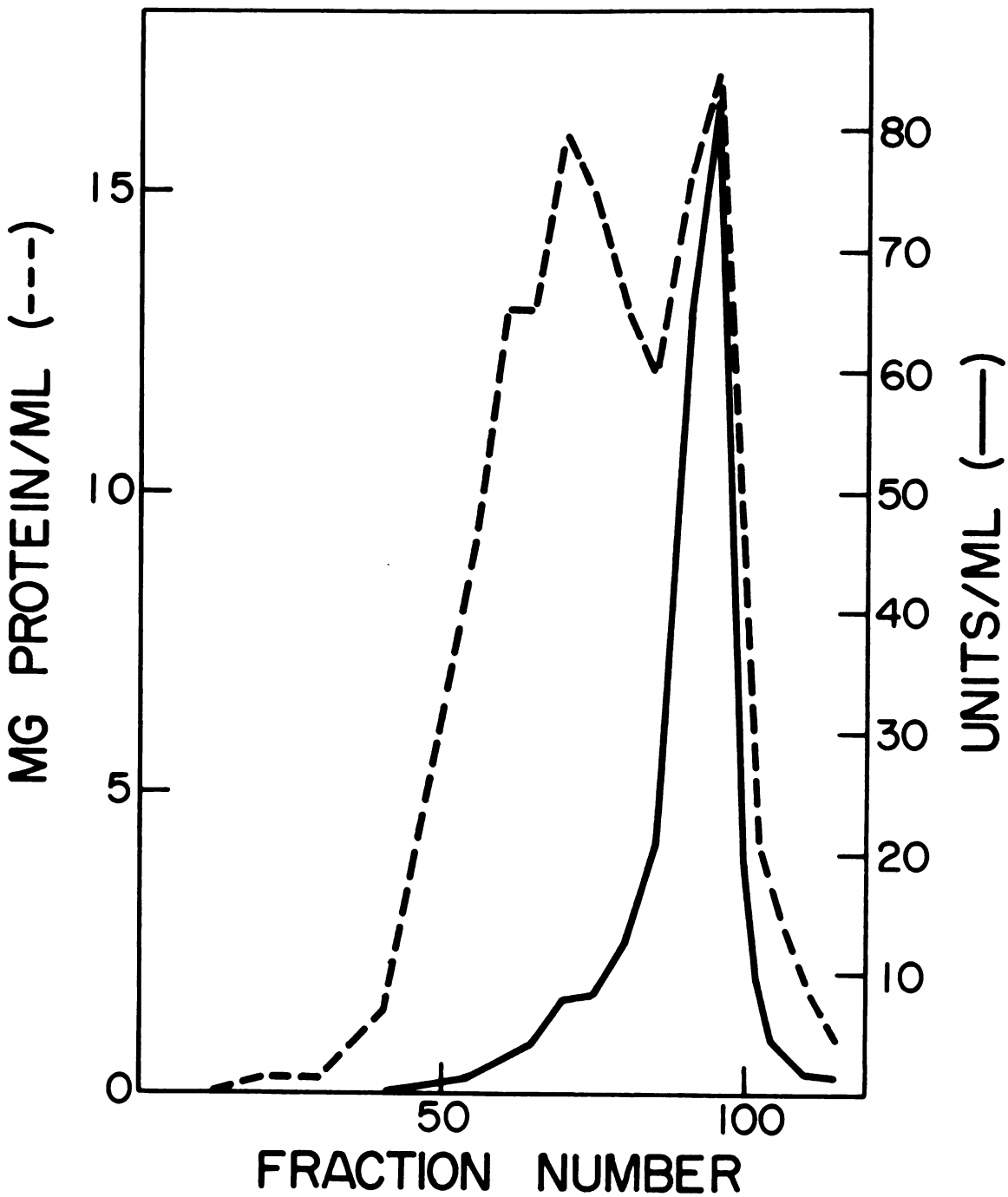
MG PROTEIN/ML (---)
UNITS/ML (—)
FRACTION NUMBER
0
5
10
15
50
100
10
20
30
40
50
60
70
80

Figure 2

Step 5: Second Ammonium Sulfate Fractionation--Fraction IV was made 58% saturated with ammonium sulfate while maintaining the pH at 8.5, stirred for 30 min and centrifuged. The resulting precipitate was dissolved in 0.001 M EDTA-0.02 M MSH-0.01 M tricine buffer, pH 8.0 (about 0.2 volumes of Fraction IV), and dialyzed overnight against 20 liters of the same buffer (Fraction V).

Step 6: DEAE-Cellulose Column Chromatography II--Fraction V was placed on a DEAE-cellulose column similar to the one described in Step 4 except that the DEAE-cellulose had been suspended and packed in 0.01 M tricine buffer, pH 8.0. The column was washed with 3 liters of 0.02 N NaCl-0.02 M MSH-0.01 M tricine buffer, pH 8.0. A linear gradient was used to elute the activity from the column (0.02 to 0.20 N NaCl in 0.02 M MSH-0.01 M tricine buffer, pH 8.0). Tubes with specific activity above 5 were combined (Fraction VI). See Figure 3 for an example elution pattern.

Step 7: Crystallization--Fraction IV was brought to 60% saturation with ammonium sulfate at pH 8.5, stirred for 20 min, and centrifuged. The precipitate was dissolved in 8-10 ml of 0.02 M MSH-0.001 M EDTA-0.01 M tricine buffer, pH 8.0, by warming in a 37° C water bath for 5-6 min. Any undissolved material was removed by centrifugation at room temperature.[3] The supernatant was dialyzed against 250 ml of 32%

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[3]The amount of buffer added varied somewhat with each preparation; therefore, the precipitate obtained after the centrifugation is always checked for activity.

Figure 3. Chromatography of UDP-Glc pyrophosphorylase, Fraction VI, on DEAE-cellulose.

The column dimensions were 4.0 x 40 cm; the flow rate was about 1.5 ml per min; 15 to 17 ml fractions were collected. The column was first washed with 3 liters of 0.02 N NaCl, 0.02 M MSH, 0.01 M tricine buffer, pH 8.0. The activity was then eluted with a linear NaCl gradient, 0.02 N NaCl to 0.20 N NaCl in 0.02 M MSH, 0.01 M tricine buffer, pH 8.0. (-----), milligrams of protein per ml; (———), units per ml.

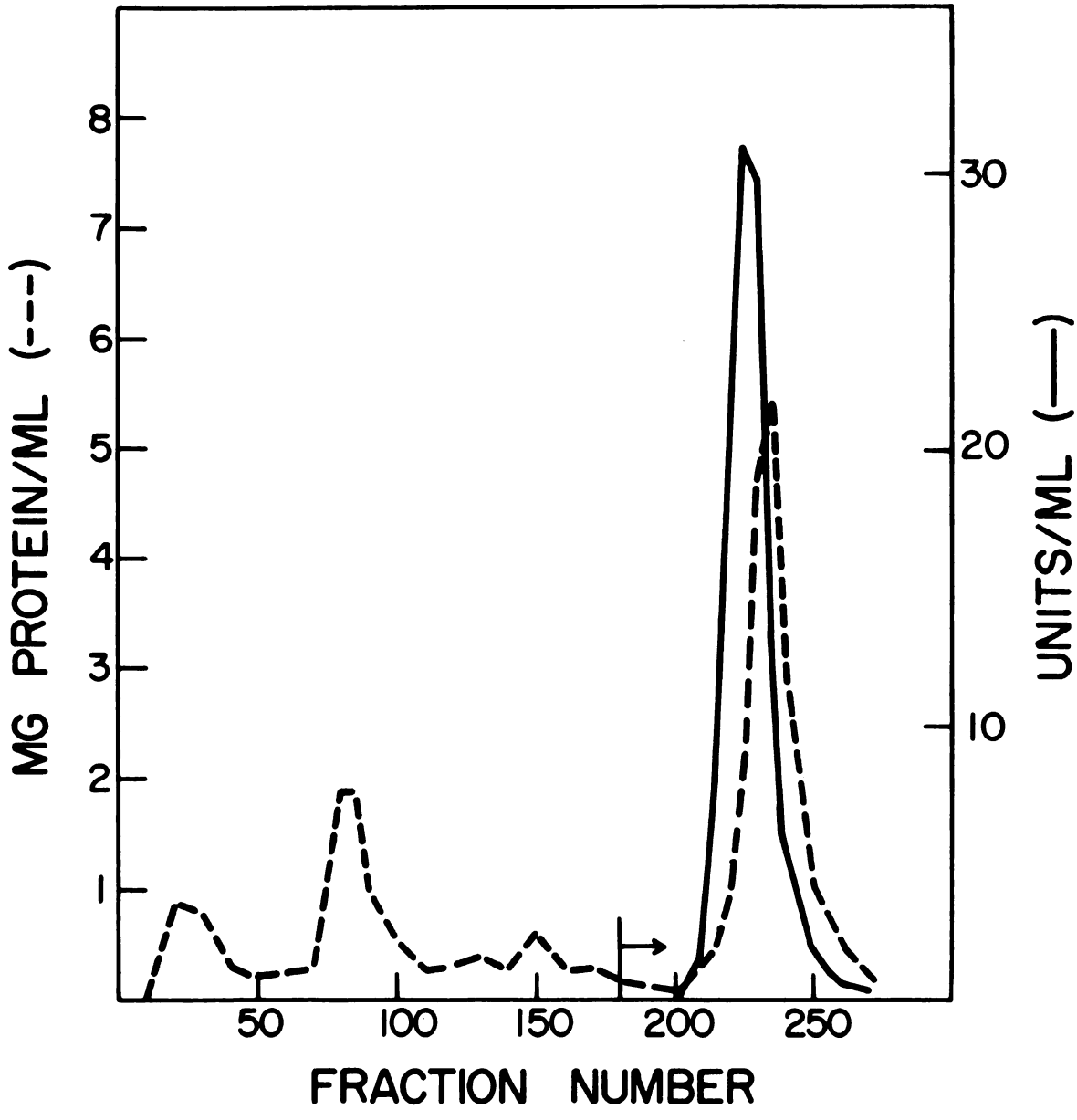
MG PROTEIN/ML (---)
UNITS/ML (—)
FRACTION NUMBER
1
2
3
4
5
6
7
8
10
20
30
50
100
150
200
250

Figure 3

saturated ammonium sulfate in 0.001 M EDTA-0.02 M MSH-0.01 M tricine buffer, pH 8.0 for 24 hr at 4° C. Crystals began to appear 6 to 8 hrs later.

Step 8: Recrystallization--Crystals were removed from the supernatant by a low speed centrifugation (2000 x g for 10 min). The crystals were dissolved in 0.02 M MSH-0.001 M EDTA-0.01 M tricine buffer, pH 8.0 (2 to 3 ml) by warming in a 37° C water bath for 2 to 3 min, and any undissolved material removed by centrifugation at room temperature.[4] The solution was then dialyzed against the 32% saturated ammonium sulfate solution described above for 24 hr. Crystals usually appeared within 1 to 2 hr. The specific activity became constant after the second recrystallization. The crystals formed during the first crystallization were long needles which were clearly visible using a microscope equipped with an oil immersion lens and phase contrast. Upon recrystallization the needles became very small (Figure 4). A summary of a typical purification procedure is given in Table II.

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[4] Ibid.

Figure 4. Photomicrograph of crystals of UDP-Glc pyrophosphorylase.

A microscope equipped with both an oil immersion lens and phase contrast was needed to view these needles from twice crystallized enzyme.

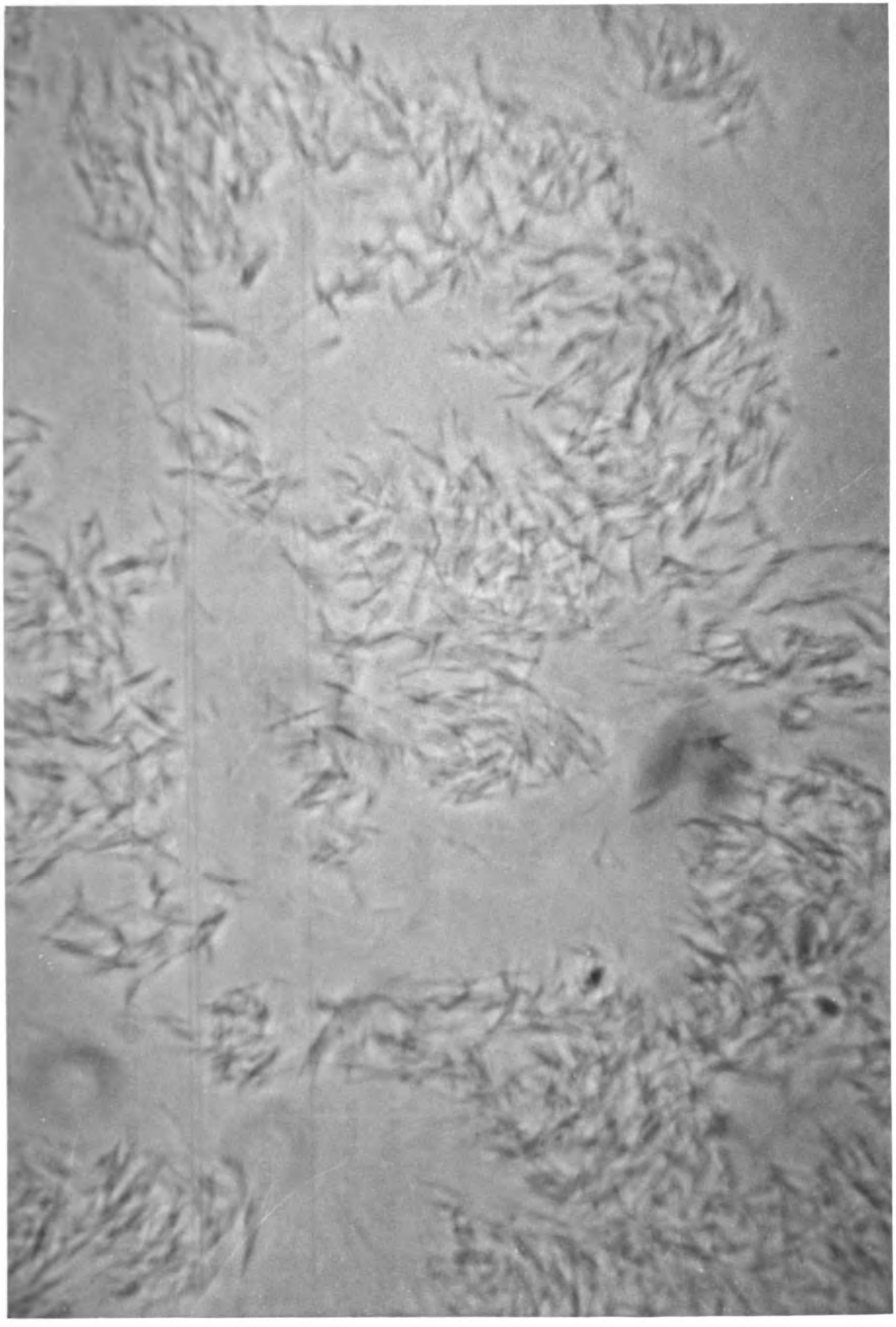

Figure 4

TABLE II

Purification and Crystallization of UDP-Glucose Pyrophosphorylase from Human Liver

Step	Volume (ml)	Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Yield (%)
1. Extraction	3200	163,200	62,080	0.38	100
2. $(NH_4)_2SO_4$, 35 to 58%	570	79,230	55,176	0.69	89
3. $Ca_3(PO_4)_2$ gel	1860	24,180	22,878	0.85	37
4. DEAE-cellulose I	470	6,439	16,685	2.59	27
5. 60% $(NH_4)_2SO_4$	92	4,140	15,134	3.66	27
6. DEAE-cellulose II	350	840	9,310	11.1	15
7. Crystallization	2.4	60	5,110	85.2	8.3
8. Recrystallization I	2.4	27.6	4,760	181.	7.7
9. Recrystallization II	1.7	20.4	4,080	200.	6.6

RESULTS

Assay Linearity, Pyrophosphate Dependence, and Protection Against Oxidizing Conditions

Using the assay conditions described in the Experimental Procedure to determine enzyme activity, the reaction velocity was found to be proportional to the enzyme concentration. In Figure 5, the linearity of Assay 1 is depicted. This assay was routinely used for the majority of the activity determinations.

The crystalline enzyme exhibited complete dependence upon the presence of pyrophosphate for the formation of UTP and Glc-1-P from UDP-Glc (Figure 6). As has been previously reported (3,15), this enzyme also displayed a definite lag period after the reaction had been initiated (Figure 6). The lag period could be reduced but not eliminated when the enzyme and all assay reagents except UDP-Glc were mixed together in the cuvette, and the reaction initiated with UDP-Glc. A slight lag period also existed in the reverse direction (Figure 1), the synthesis of UDP-Glc from UTP and Glc-1-P (Assay 4); but the lag period was not eliminated or reduced by preincubating the enzyme with either Glc-1-P or UTP.

Figure 5. Effect of enzyme concentration on reaction velocity.

The formation of Glc-1-P was followed using Assay 1. In a final volume of 0.5 ml, the reaction mixture contained 45 μmoles of Tris-acetate buffer (pH 7.8), 0.2 μmole of NADP, 1.5 μmoles of magnesium acetate, 1.0 μmole of MSH, 1.0 μmole of PP_i, 0.2 μmole of UDP-Glc, excess phosphoglucomutase and glucose-6-P dehydrogenase, and the indicated amounts of UDP-Glc pyrophosphorylase (1.2 to 30 x 10^{-6} mg of protein). The reaction was initiated with the addition of UDP-Glc pyrophosphorylase and was measured at 25^{o} C.

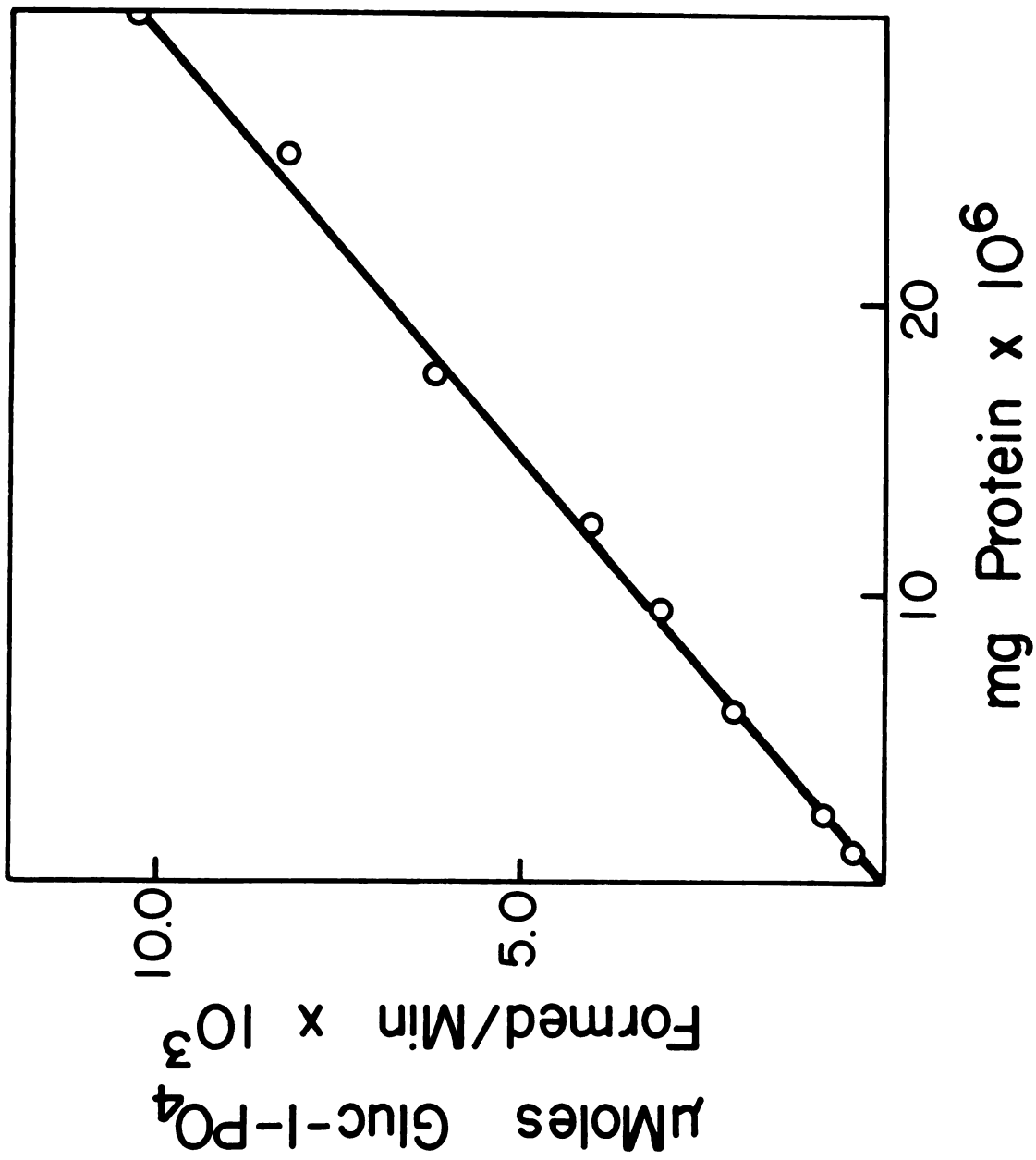

Figure 5

Figure 6. Dependence of UDP-Glc pyrophosphorylase on PP_i.

These lines are optical density tracings from a Gilford converter and recorder of the enzyme reaction. Line A, complete system; Line B, minus PP_i until 12 min. In a final volume of 0.5 ml, the complete system contained 45 μmoles of Tris-acetate buffer (pH 7.8), 0.2 μmole of NADP, 1.5 μmoles of magnesium acetate, 1.0 μmole of MSH, 1.0 μmole of PP_i, 0.2 μmole of UDP-Glc, excess phosphoglucomutase and glucose-6-P dehydrogenase, and pyrophosphorylase. The reaction was initiated by the addition of pyrophosphorylase.

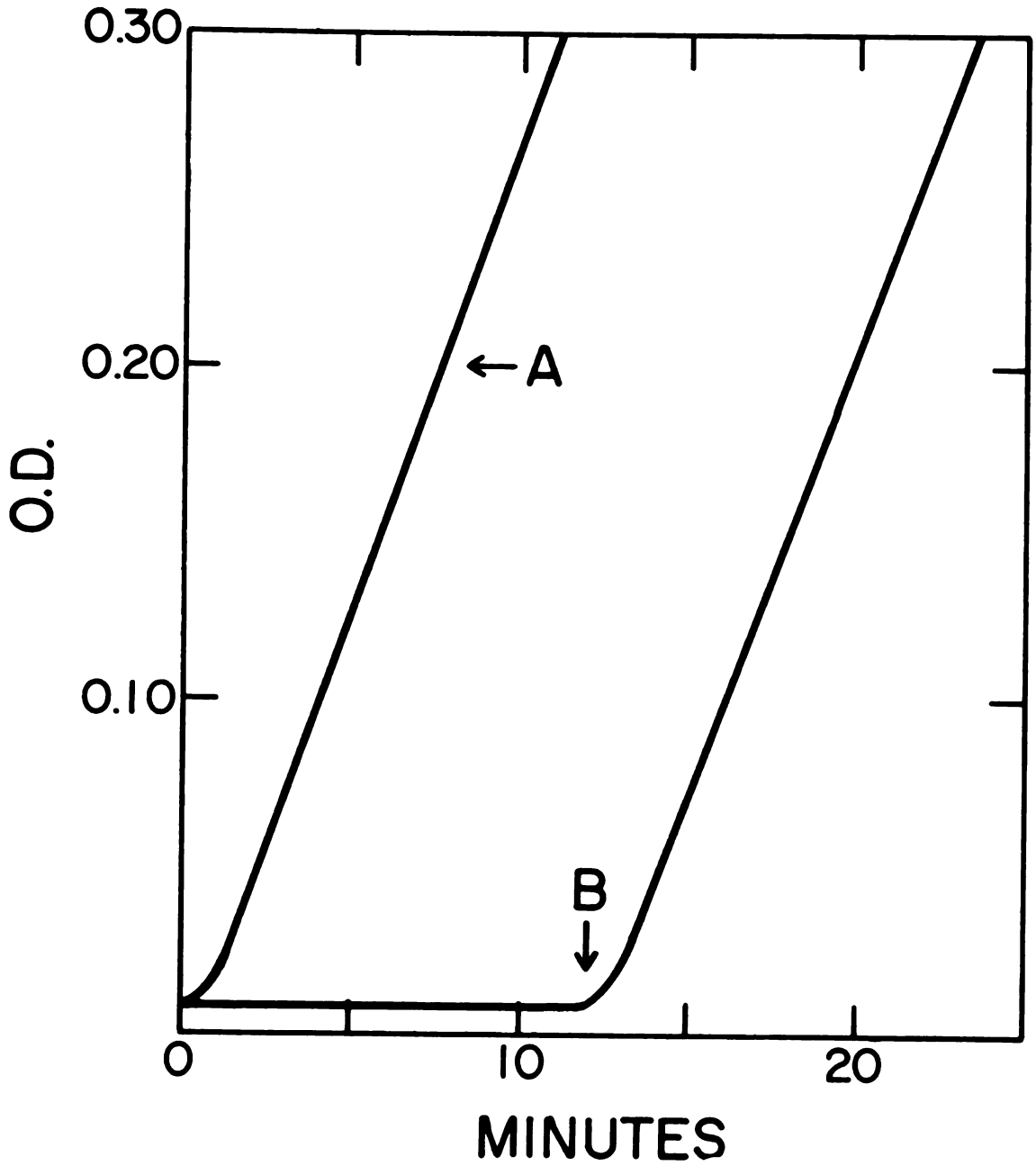

Figure 6

To prevent loss of enzymatic activity during the purification and crystallization procedures, mercaptoethanol was routinely added to the preparations. Either dithiothreitol or mercaptoethanol was added to the dilution media and assay mixtures at an optimum concentration of 2 mM. However, the crystalline enzyme maintained maximum specific activity longer if it were stored at 4° C in 0.02 M dithiothreitol rather than 0.02 M mercaptoethanol-buffer system.

Homogeneity and Sedimentation

Electrophoresis of twice recrystallized UDP-Glc pyrophosphorylase on polyacrylamide gel resulted in only one protein band when the gels were stained with Coomassie blue (Figures 7 and 8). As the human liver enzyme preparation aged, another slower moving minor component appeared during electrophoresis. The slower moving component corresponded in mobility to the calf liver dimer band and was also active. Even when gel columns were overloaded (0.5 mg protein/gel), only two protein bands were visible after staining. When compared to UDP-Glc pyrophosphorylases from other sources (Figure 8), the human liver enzyme (Gel B) moved slower during electrophoresis than the rabbit muscle enzyme (Gel D) and the monomer of the calf liver enzyme (Gel A, fastest moving protein band). The human liver enzyme moved somewhat faster than the major band of the rabbit liver enzyme (Gel C).

Figure 7. Electrophoresis of UDP-Glc pyrophosphorylase on polyacrylamide gel.

A pH 8.7 buffer system was used for electrophoresis (37); and the upper buffer was made 0.001 M in thioglycolate. Protein samples were dialyzed overnight in 0.02 M MSH, 0.01 M Tricine buffer, pH 8.0. Approximately 0.05 mg of protein was layered on each gel. Constant amperage, 5 mg/gel, was used for 2½ hrs at 4° C. Gels were either stained for protein with Coomassie blue (38), or sliced into 2 mm segments and eluted for activity determinations. 0.4 ml of 0.02 M MSH, 0.01 M Tricine buffer, pH 8.0, was used to elute each 2 mm segment. The origin and direction of electrophoresis are indicated by arrows. A, a tracing of an optical density recording, produced by a Gilford gel scanner and converter, of a Coomassie blue stained gel; B, schematic reproduction of the same gel (this gel is also shown as Gel B in Figure 8); C, activity eluted from an unstained gel which had been electrophoresed at the same time.

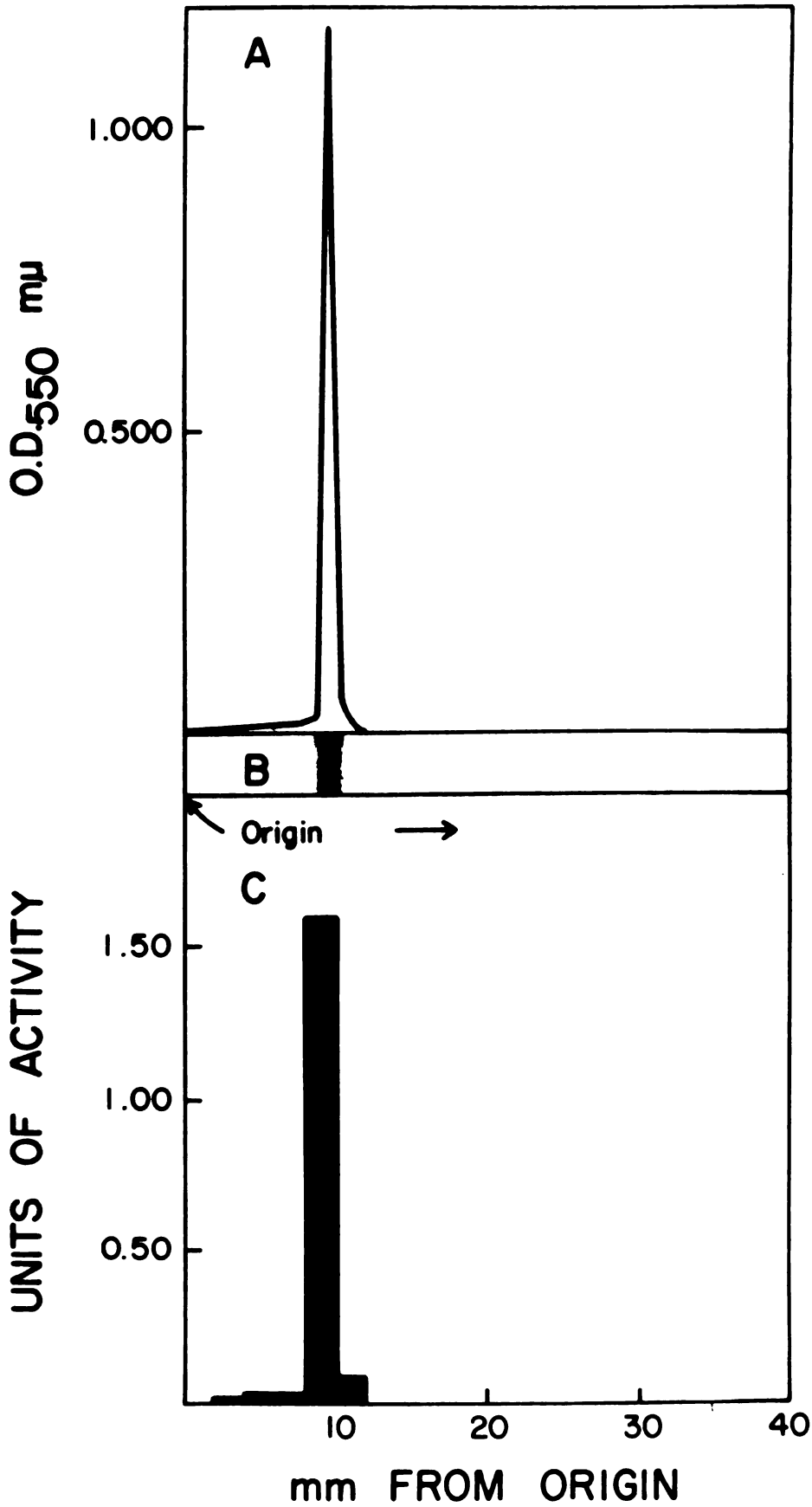
A
1.000
0.500
O.D550 mμ
B
Origin
C
1.50
1.00
0.50
UNITS OF ACTIVITY
10
20
30
40
mm FROM ORIGIN

Figure 7

Figure 8. Polyacrylamide gel electrophoresis of UDP-Glc pyrophosphorylase from four different sources.

The samples were dialyzed as described in Figure 7, and a solution containing about 0.05 mg of protein was layered on each gel as described in Experimental Procedure. The direction of electrophoresis was from the top to the bottom of the gel. The gels were then stained with Coomassie blue (38). Gel A, twice recrystallized calf liver UDP-Glc pyrophosphorylase (15); Gel B, twice recrystallized human liver enzyme; Gel C, six times recrystallized rabbit liver enzyme (34); Gel D, rabbit muscle enzyme (34). Only the upper, more darkly stained band of Gel C is active.

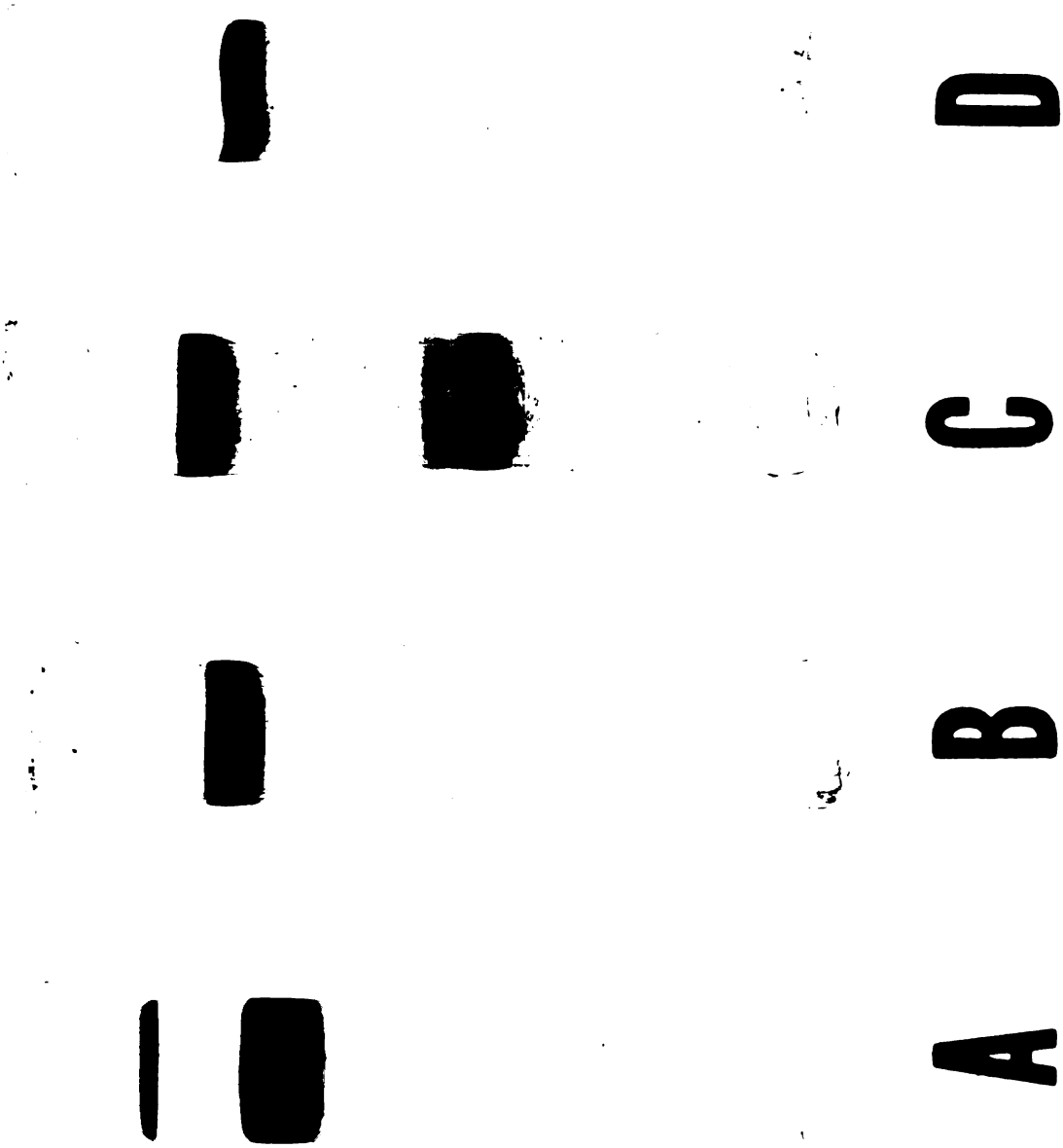

Figure 8

From sucrose density gradient centrifugation absorbance patterns (Figure 9), a major protein peak and also a minor, slower moving peak near the top of the tube were found. When this minor band was concentrated, dialyzed, and electrophoresed on polyacrylamide gel, only a faint protein band appeared after staining. The faint band corresponded in mobility to the usual major band found during gel electrophoresis. Bovine serum albumin, which was used as a protein of smaller molecular weight for comparison, was about 2 mm from the bottom of the gel in 2 hrs.

In Schlieren patterns (Figure 10) produced in the Model E Analytical Ultracentrifuge, a sharp major protein peak was visible. A slight discontinuity was also visible in the base line indicating again the possibility of a slower moving component. It is of interest that the protein concentration was nearly seven times greater for this experiment than it was in the sucrose density gradient experiment; yet, the slower moving component was just barely visible in the Schlieren patterns. The twice recrystallized enzyme was therefore nearly homogeneous. Further investigation is needed to determine the origin of this contaminating material and if it can be eliminated by further recrystallization or changes in the purification procedure.

A sedimentation coefficient (0.4% protein), was calculated to be 12.8 from the sedimentation velocity patterns.

Figure 9. Density gradient sedimentation of UDP-Glc pyrophosphorylase in sucrose.

Gradients from 5 to 20% in sucrose were spun in a SW 50 rotor at 35,000 rpm for 12 hrs at 4° C. After puncturing the bottom of the tubes, the contents were forced out and through a flow-cell as described in Experimental Procedure. 200 µl fractions were collected. (———), optical density at 280 mµ; (-----), activity in units/ml.

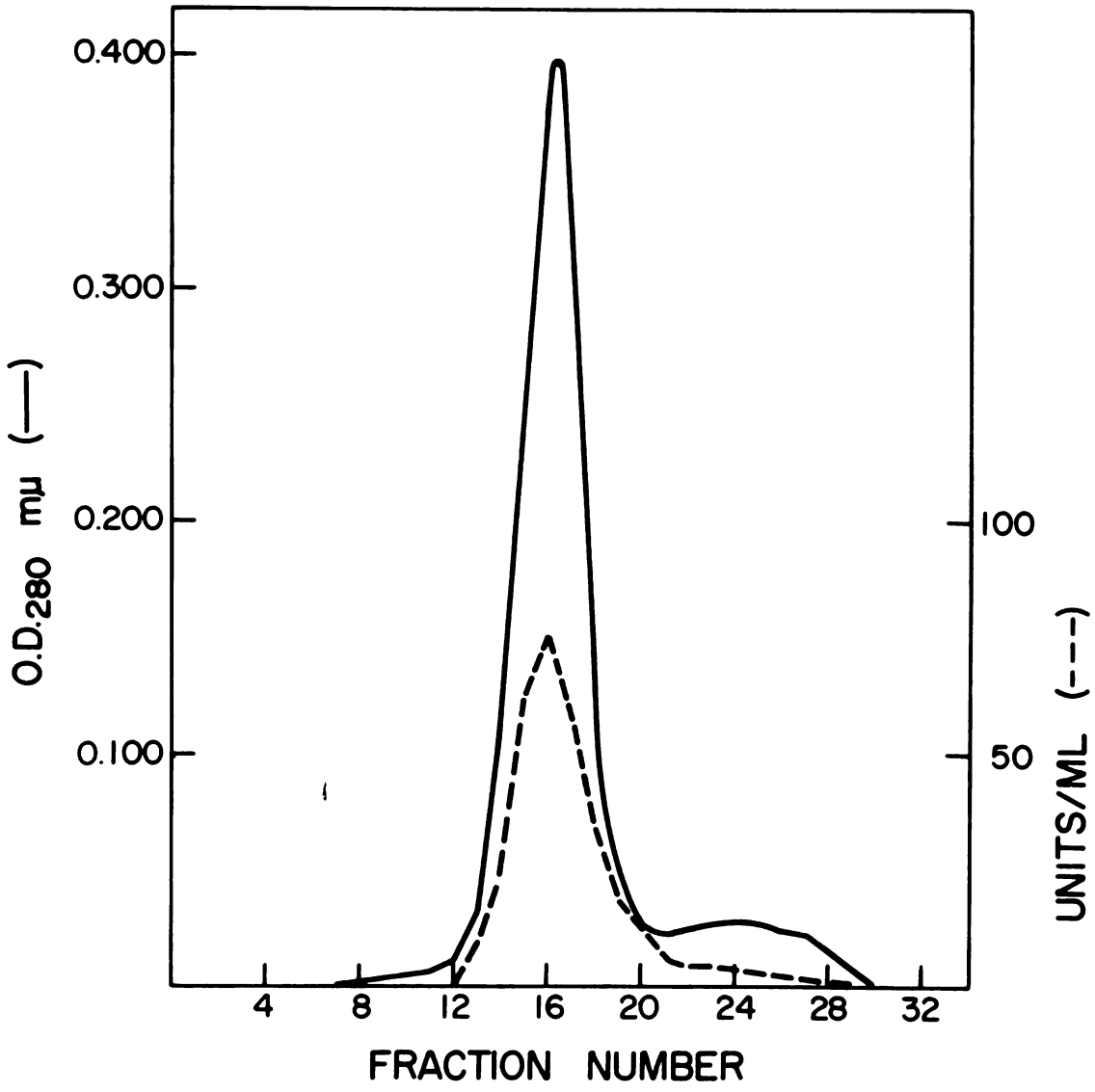
0.400
0.300
0.200
0.100
O.D.280 mμ (——)
100
50
UNITS/ML (---)
4
8
12
16
20
24
28
32
FRACTION NUMBER

Figure 9

Figure 10. Sedimentation pattern of UDP-Glc pyrophosphorylase from human liver.

The experimental conditions were as follows: buffer system, 0.02 M MSH, 0.10 N NaCl, 0.01 M tricine buffer, pH 8.0; protein concentration, 3.3 mg per ml; temperature, 4.4° C; speed, 56,108 rpm; diaphragm angle, 65°; time, 28 min after speed was attained. Sedimentation was from left to right.

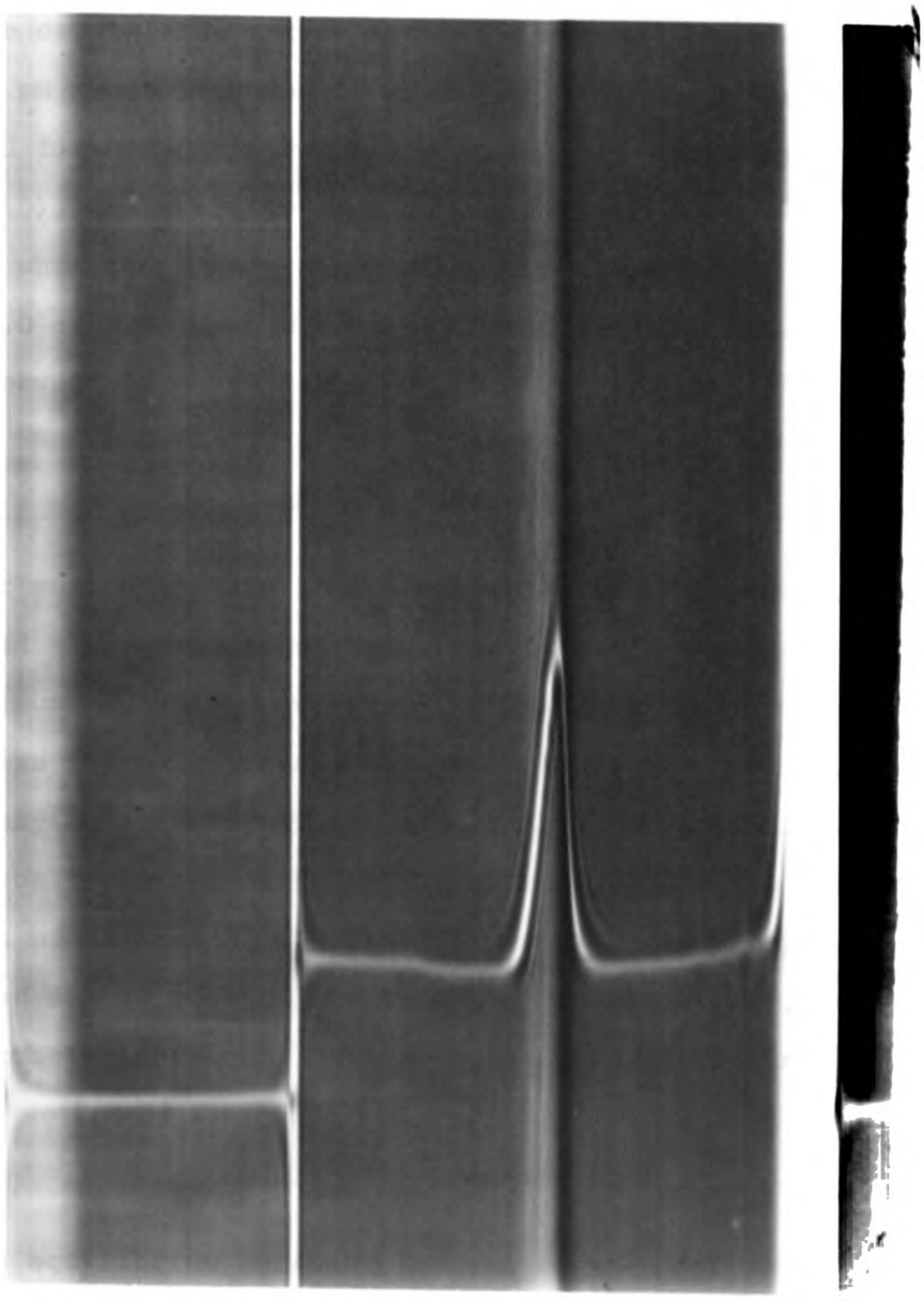

Figure 10

Optimum pH

The crystalline enzyme exhibited maximal activity over a broad pH range (Figure 11) from 7.6 to 9.2. As has been reported for UDP-Glc pyrophosphorylase from other sources (10,11,15), the activity of this enzyme also dropped off sharply when the pH was changed outside these broad limits. At pH 7.0 or 9.9, approximately 65% of the maximal activity was observed.

Cation Requirement

For maximum catalytic activity, the crystalline enzyme exhibited a specific requirement for divalent magnesium. The optimum magnesium concentration was 3 mM (Figure 12). Cobalt was the only other divalent metal tested that had any catalytic activity (Table III). At a concentration of 3 mM, cobalt was only 14% as effective as magnesium. In the presence of magnesium, all divalent metals except calcium inhibited 65% or more of the catalytic activity. NH_4^+ was only slightly inhibitory in the presence of magnesium.

Equilibrium Constant

The equilibrium constant for the UDP-Glc pyrophosphorylase reaction was determined in both directions (Table IV). A K_{eq} value of 0.15 (as defined by the equation in Table IV) was obtained when equilibrium was approached from

Figure 11. Effect of the pH on the reaction rate.

The incubation mixtures contained in a final volume of 0.5 ml, 1.0 μmole of UDP-Glc, 1.0 μmole of PP_i, 1.5 μmoles of magnesium acetate, 1.0 μmole of MSH, and 45 μmoles of buffer. The reaction was initiated by the addition of pyrophosphorylase. Reaction mixtures were incubated for 2 min at 25° C, heated 2 min at 100° C, and cooled in an ice bath; and the pH remeasured. The Glc-1-P formed was measured in aliquots using NADP, phosphoglucomutase, and glucose-6-P dehydrogenase (3). (—□——□—), citrate buffer; (—○——○—), Tris-acetate buffer; (—△——△—), glycyl-glycine buffer.

Figure 12. Effect of the magnesium concentration on the reaction rate.

Assay 1, described in Experimental Procedure, was used except that the magnesium concentration was varied from 10^{-4} to 10^{-2} M as indicated.

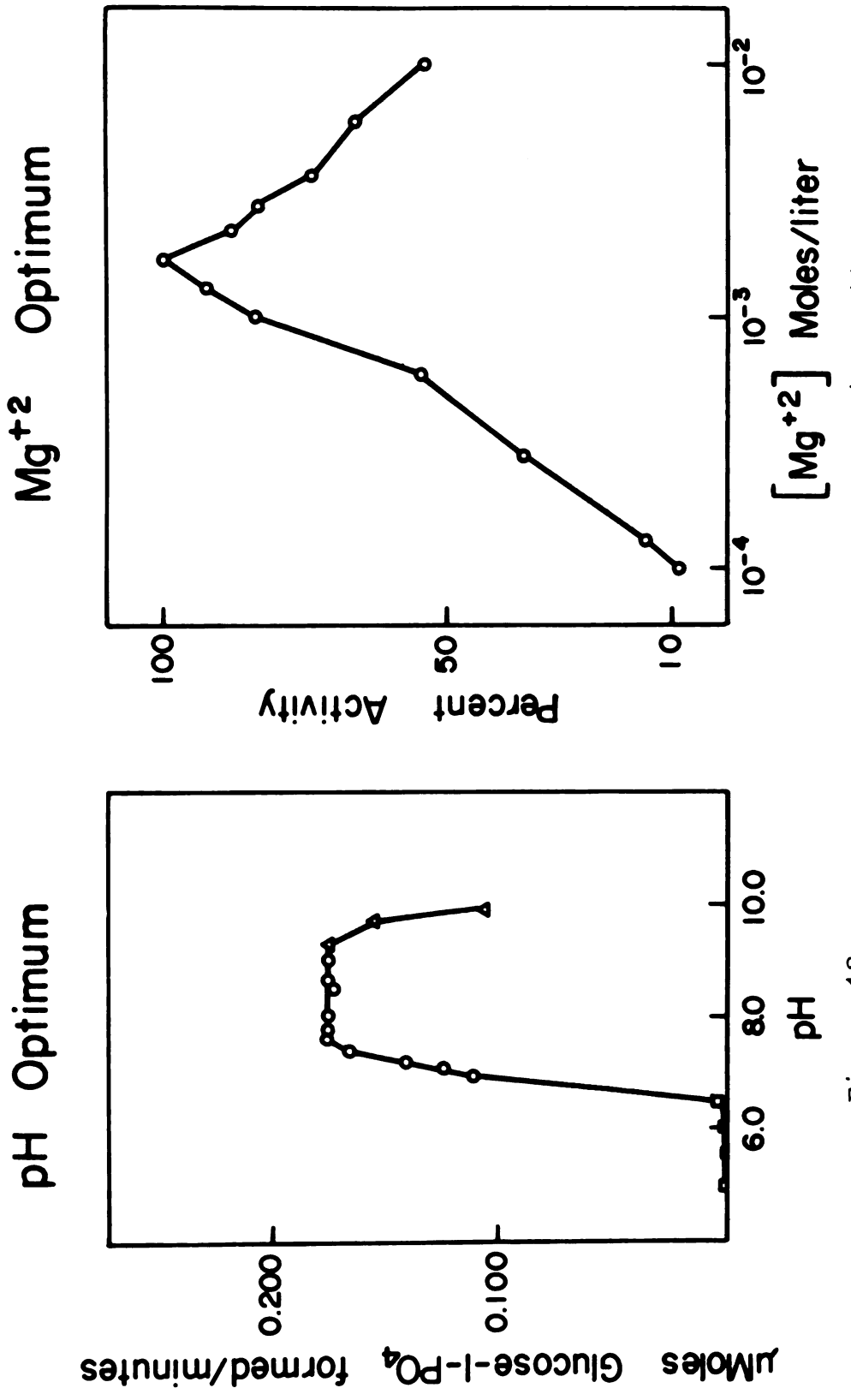

Figure 11

Figure 12

TABLE III

Cation Requirement of UDP-Glc Pyrophosphorylase from Human Liver

Cation	Percent Activity*	Percent Activity in the Presence of Mg^{++}
None	0	100.0
Mg^{++}	100.0	-----
Mn^{++}	0	1.8
Co^{++}	13.8	20.9
Ca^{++}	0	52.8
Fe^{++}	0	7.2
Ni^{++}	0	29.5
Cu^{++}	0	34.2
Zn^{++}	0	1.5
NH_4^+	0	94.2

*Based on Mg^{++} as 100%

Magnesium, manganese, copper, calcium, and ammonium were the acetate salts; all other cations were the chloride salts. All metals were 3.0×10^{-3} M. The cations were incubated with 1.0 μmole of UDP-Glc, 1.0 μmole of PP_i, 90.0 μmoles of Tris-acetate buffer, pH 7.8, and enzyme (final volume, 1.0 ml) for 5 min. at 25° C. The reactions were stopped and the Glc-1-P present determined according to Assay 4.

TABLE IV

The Determination of the Equilibrium Constant for UDP-Glc Pyrophosphorylase from Human Liver

Experiment	Direction	Glc-1-P	UTP	UDP-Glc	PP_i	K_{eq}
1	I	0.774	0.790	0.306	0.290	0.14
2	I	0.774	0.790	0.306	0.304	0.15
3	I	0.774	0.757	0.306	0.290	0.15
4	II	0.660	0.660	0.255	0.258	0.15
5	II	0.636	0.676	0.242	0.258	0.15
6	II	0.676	0.708	0.290	0.290	0.15

Micromolar quantities of substrates were incubated in the presence of enzyme, 1.0 μmole of magnesium acetate, 2.0 μmoles of MSH, and 90.0 μmoles of 0.10 M Tris-acetate buffer, pH 7.8, in a final volume of 1.0 ml. The reactions were started by the addition of 0.24 units of enzyme; and after incubating for one hour at 30° C, the reactions were stopped as previously described (Assay 4). The products were determined as outlined in Assay 4. The direction of the reaction is indicated:

$$\text{Glc-1-P} + \text{UTP} \underset{\text{II}}{\overset{\text{I}}{\rightleftharpoons}} \text{UDP-Glc} + PP_i .$$

K_{eq} is defined as: $\frac{[\text{UDP-Glc}]\ [PP_i]}{[\text{Glc-1-P}]\ [\text{UTP}]}$. All concentrations are micromolar.

either direction which is near the value of 0.20 obtained for the calf liver enzyme (24). Assuming equimolar quantities of the substrates UDP-Glc and PP_i initially, 70% of the UDP-Glc was converted to UTP and Glc-1-P.

Apparent Michaelis Constants

The affinity of the crystalline enzyme for UDP-Glc, UTP, Glc-1-P, and PP_i was determined. Lineweaver-Burk (42) plots were used to estimate Michaelis constants for three of the substrates (Figures 13, 14, and 15). The apparent K_m's for UDP-Glc, UTP, and Glc-1-P were 5.0×10^{-5} M, 4.8×10^{-5} M, and 9.5×10^{-5} M, respectively. The velocity-concentration plots with pyrophosphate, however, were sigmoidal and the resulting Lineweaver-Burk plots curved upward (Figure 16). In this case, the maximum velocity was estimated from the Lineweaver-Burk plot; and the log $(\frac{V_m}{V} - 1)$ was plotted against the log (PP_i) (Figure 17). The Michaelis constant for PP_i was estimated from this Hill plot and found to be 2.1×10^{-4} M. A n value (slope of the graph) of 2.5 was obtained from this data, indicating cooperative binding of PP_i to the enzyme.

In preliminary attempts to eliminate the sigmoidal plots, the enzyme was preincubated for 5 min in 2 mM PP_i. The sigmoidal kinetics were not eliminated by this procedure; however, a n value closer to unity, 1.5, was obtained, indicating a decrease in the cooperative interaction of PP_i.

Figure 13. Effect of UDP-Glc concentration on the velocity of the UDP-Glc pyrophosphorylase reaction.

The assay conditions were the same as those described in Assay 1 of the Experimental Procedure except the UDP-Glc concentration was varied as indicated, and the reaction was initiated by the addition of UDP-Glc to the cuvette. The inset is a Lineweaver-Burk plot of the same data.

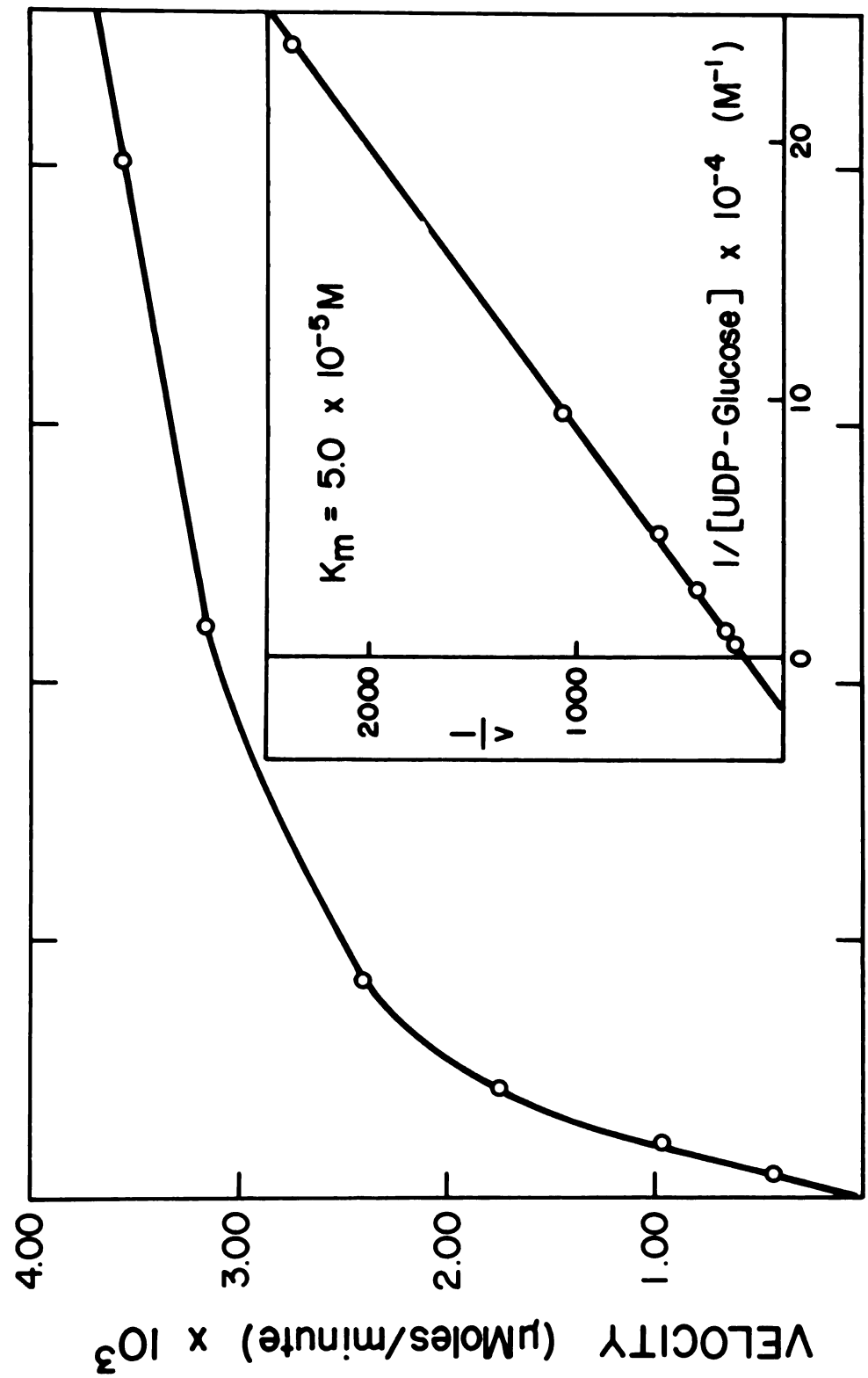
VELOCITY (μMoles/minute) x 10³
4.00
3.00
2.00
1.00
0
5
10
15
20
[UDP-Glucose] x 10⁵ (M)
1/v
2000
1000
0
10
20
1/[UDP-Glucose] x 10⁻⁴ (M⁻¹)
Kₘ = 5.0 x 10⁻⁵M

Figure 13

Figure 14. Effect of UTP concentration of the velocity of the UDP-Glc pyrophosphorylase reaction.

The assay conditions are described in the Experimental Procedure as Assay 4 except the UTP concentration was varied. The inset is a Lineweaver-Burk plot of the same data.

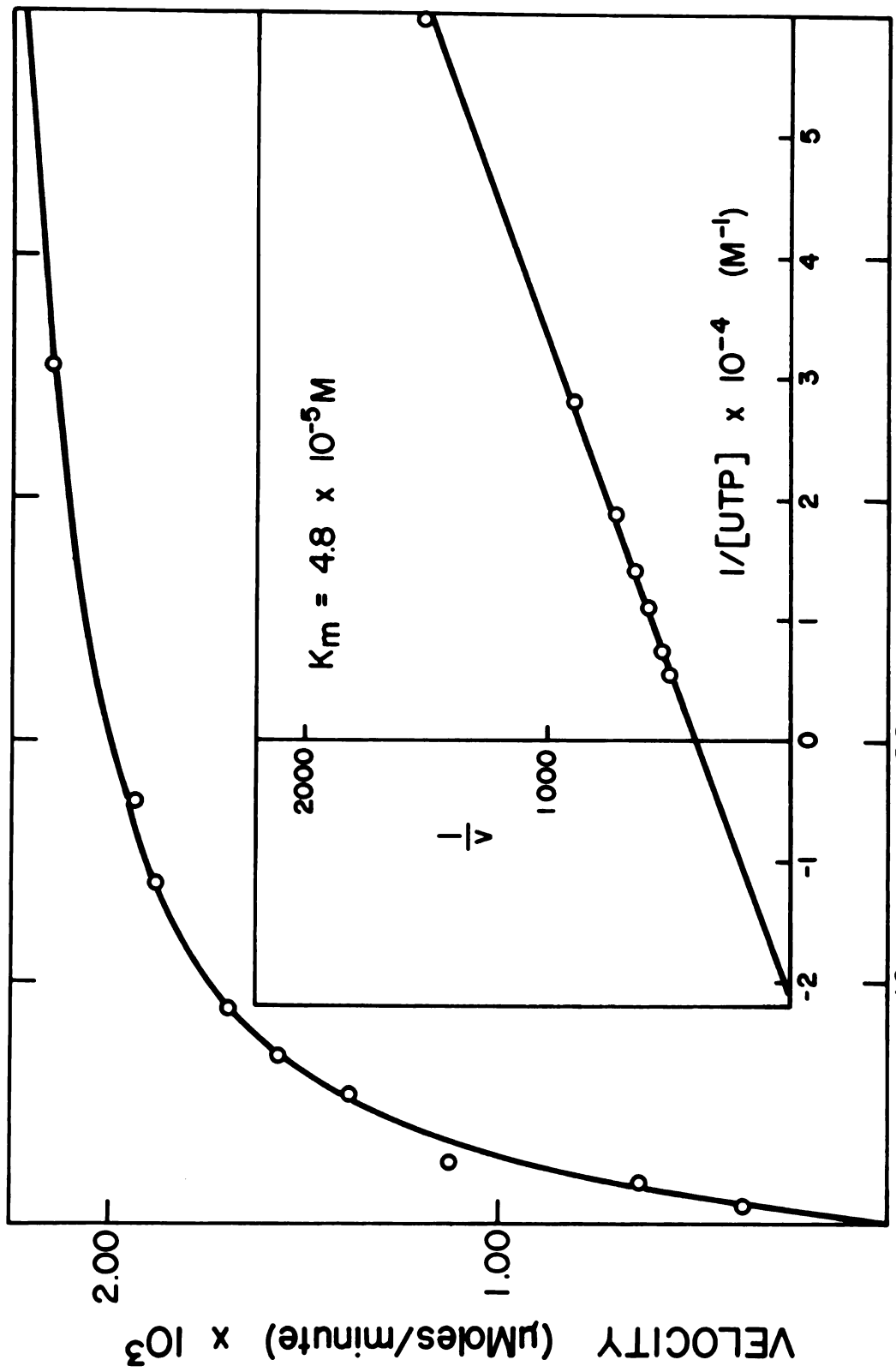
VELOCITY (μMoles/minute) x 10^3
2.00
1.00
10
20
30
40
[UTP] x 10^5 (M)
K_m = 4.8 x 10^{-5}M
$\frac{1}{v}$
2000
1000
0
-1
-2
1
2
3
4
5
1/[UTP] x 10^{-4} (M^{-1})

Figure 14

Figure 15. Effect of Glc-1-P concentration on the velocity of the UDP-Glc pyrophosphorylase reaction.

The assay conditions are described in the Experimental Procedure as Assay 4 except the Glc-1-P concentration was varied. The inset is a Lineweaver-Burk plot of the same data.

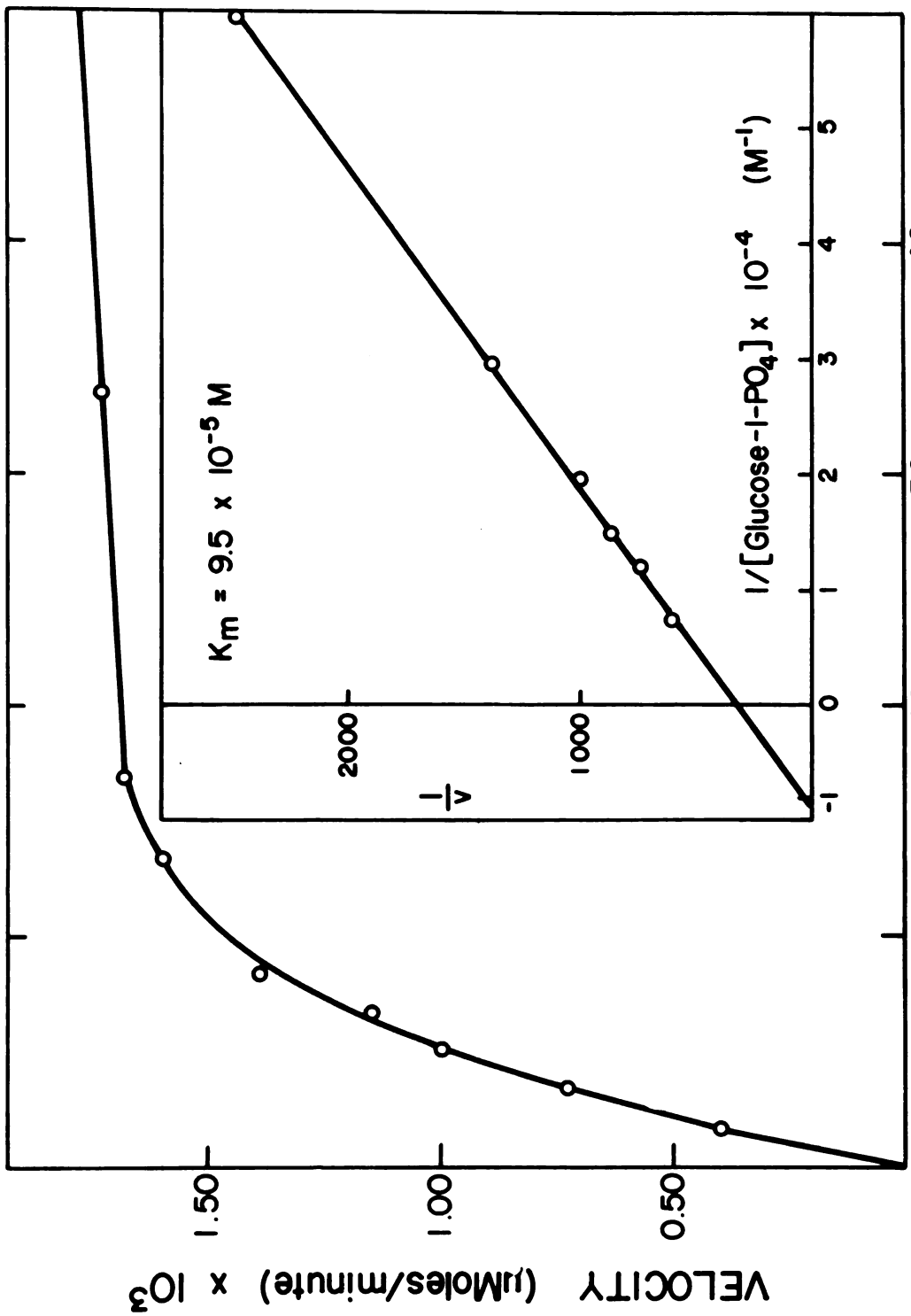
VELOCITY (μMoles/minute) x 10³
1.50
1.00
0.50
10
20
30
40
[Glucose-1-PO4] x 10⁵ (M)
Km = 9.5 x 10⁻⁵ M
1/v
2000
1000
-1
0
1
2
3
4
5
1/[Glucose-1-PO4] x 10⁻⁴ (M⁻¹)

Figure 15

Figure 16. Effect of pyrophosphate concentration on the velocity of the UDP-Glc pyrophosphorylase reaction.

Assay conditions were as described in Assay 1 of the Experimental Procedure except the pyrophosphate concentration was varied. In one case, (—△——△—), the enzyme was preincubated in 2 mM pyrophosphate for 5 min before addition to the reaction mixture. Upper graph, velocity versus pyrophosphate concentration plot; Lower graph, Lineweaver-Burk plot of the same data. (—○——○—), no enzyme preincubation; (—△——△—), enzyme preincubated in 2 mM pyrophosphate for 5 min before addition to the reaction mixture.

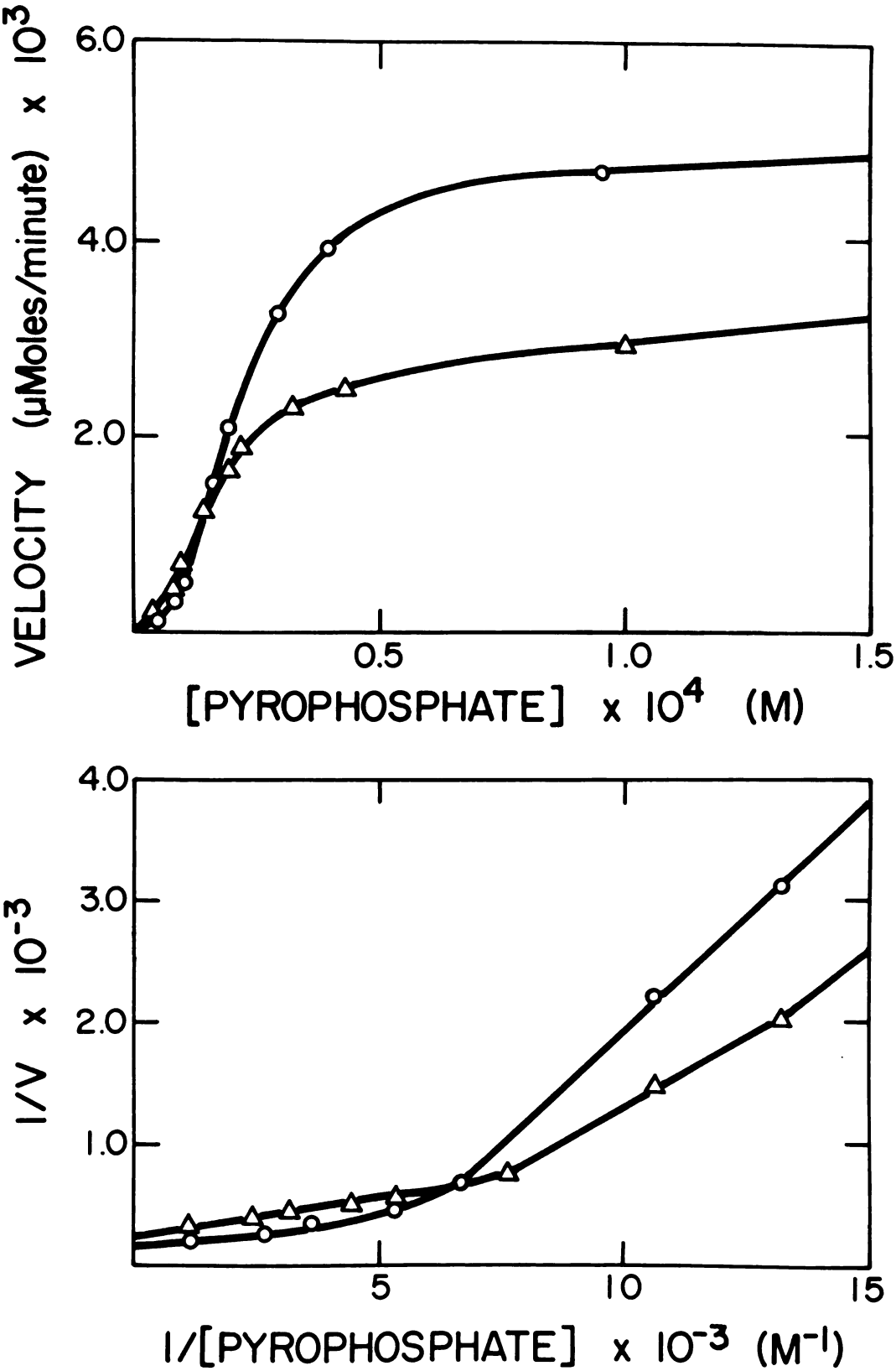
VELOCITY (μMoles/minute) x 10^3
6.0
4.0
2.0
0.5
1.0
1.5
[PYROPHOSPHATE] x 10^4 (M)
1/V x 10^{-3}
4.0
3.0
2.0
1.0
5
10
15
1/[PYROPHOSPHATE] x 10^{-3} (M^{-1})

Figure 16

Figure 17. Hill plot of the effect of pyrophosphate concentration on the reaction velocity.

Data was taken from Figure 16. V_m was estimated from the Lineweaver-Burk plots in Figure 16. (—o——o—), enzyme not preincubated; (—Δ——Δ—), enzyme preincubated in 2 mM pyrophosphate for 5 min before addition to the reaction mixture.

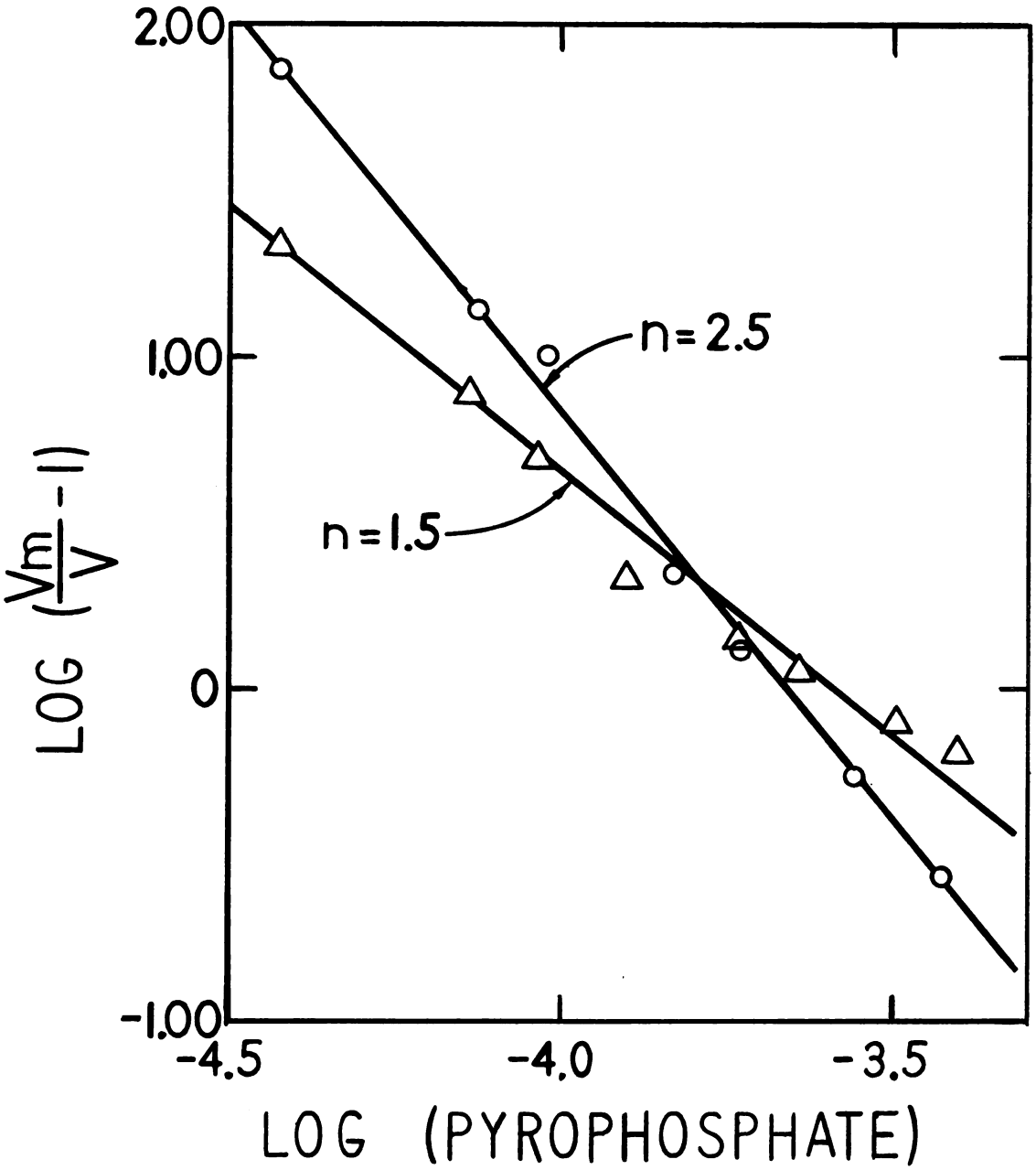

Figure 17

The K_m changed only slightly to 2.6×10^{-4} M which is within experimental error. Because there was still a slight lag period in the reaction, even when the enzyme was preincubated with PP_i, the initial velocities measured at the three lowest concentrations probably were not the true initial velocities. If the three points of lowest concentration on the graph were not included, the line drawn through the remaining points would give a n value of 1.0, indicating no cooperative interaction of PP_i after preincubation. If the three points of lowest concentration were excluded on the first set of PP_i data (enzyme not preincubated), the Hill plot slope would decrease to 2.2.

Preliminary results indicated that UDP was a competitive inhibitor with UDP-Glc, with a K_i of about 1.9×10^{-4} M. The K_i was determined using a Dixon plot of 1/v versus inhibitor concentration (43).

Specificity

The activity of the enzyme was measured in the presence of several sugar nucleotides (Table V). The most active sugar nucleotide was UDP-Glc; however, the enzyme also catalyzed the pyrophosphorolysis of several other sugar nucleotides to a lesser extent. At a substrate concentration of 0.4 mM, TDP-Glc, UDP-Gal, and UDP-Xyl produced 2.2, 2.0, and 1.5%, respectively, of the activity which was measured using UDP-Glc as a substrate. Less than 1% of the UDP-Glc

TABLE V

Specificity of Human Liver UDP-Glc Pyrophosphorylase*

Substrate	Reaction Rate
UDP-Glucose	100.0%
TDP-Glucose	2.2
CDP-Glucose	0.5
GDP-Glucose	0.1
IDP-Glucose	very low
ADP-Glucose	undetectable
UDP-Galactose	2.0
UDP-Xylose	1.5
UDP-Mannose	0.4

*All substrates were added at a level of 0.4 μmole per ml. Enzyme activity in the presence of glucose containing sugar nucleotides was determined as described in Assay 1. Assay 2 was used to determine UDP-Gal, UDP-Xyl, and UDP-Man activities. UDP-Glc activity was determined with both Assays 1 and 2.

activity was measured when CDP-Glc, GDP-Glc, or UDP-Man was used as a substrate. IDP-Glc activity was very low, and no activity could be detected in the presence of ADP-Glc, even at 1000 times the enzyme concentration used for UDP-Glc assays.

In preliminary attempts to determine the UDP-Gal activity present at each step in the purification procedure, inconsistent results were obtained. Upon further investigation, it was discovered that UDP-Gal was not saturating the human liver enzyme at a concentration of 0.4 mM. UDP-Gal saturated the human liver UDP-Glc pyrophosphorylase at a concentration of 4.0 mM. When the UDP-Glc/UDP-Gal activity ratio was determined using 4.0 mM UDP-Gal, the activity ratio did not vary significantly during the purification or crystallization procedures (Table VI). The ratio ranged from 10.8 to 14.0.

With this discovery, the activity ratio was remeasured on the crystalline calf liver enzyme. The UDP-Glc/UDP-Gal ratio thus obtained was about 7.6, nearly one-tenth the reported ratio (30) for which a UDP-Gal concentration of 0.4 mM was used. A value of 4.6 was obtained for the ratio on the calf liver extraction step (Table VI).

Since a slower moving, minor component had been observed during sedimentation experiments, the possibility of this component being a UDP-Gal pyrophosphorylase was investigated. Since the component could run through the polyacrylamide gels

TABLE VI

Activity Ratio $\frac{\text{UDP-Glc}}{\text{UDP-Gal}}$ During Purification and Crystallization of UDP-Glc Pyrophosphorylase

Step	UDP-Glc/UDP-Gal*	
	Human Liver	Calf Liver**
Extraction	10.8	4.6
$Ca_3(PO_4)_2$ gel	11.3	
Recrystallization I	14.0	
Recrystallization II	13.8	7.6**

*Both UDP-Glc and UDP-Gal activities were determined with Assay 2 except that in the latter case the UDP-Gal concentration was 4 mM.

**Calf liver enzyme was prepared according to the procedure of Albrecht et al. (15). The step designated recrystallization II for the calf liver enzyme corresponds to their step designated 3rd crystallization.

before the electrophoresis period is completed, the UDP-Glc/ UDP-Gal activity ratio was determined on the protein eluted from the gels (Figure 18). The ratio remained constant, at about 14.0, throughout both of the protein bands eluted from the gel, and this was the same ratio as that of the recrystallized enzyme, indicating that the two activities were inseparable by this technique.

Figure 18. Activity of UDP-Glc pyrophosphorylase eluted from polyacrylamide gel in the presence of UDP-Glc or UDP-Gal.

The direction of electrophoresis is indicated by the arrow; conditions for electrophoresis and the elution of activity from the gel are described in the Experimental Procedure and Figure 7. One gel was stained for protein with Coomassie blue, and another gel was sliced into 2 mm segments for activity determinations. Both Assays 1 and 2 were used to determine UDP-Glc activity; UDP-Gal activity was determined as described in Assay 2, except 2 μmoles of UDP-Gal were added to the reaction mixture. A, activity ratio, $\frac{\text{UDP-Glc}}{\text{UDP-Gal}}$; B, schematic reproduction of gel stained with Coomassie blue; C, activity of UDP-Glc pyrophosphorylase in the presence of UDP-Glc and UDP-Gal.

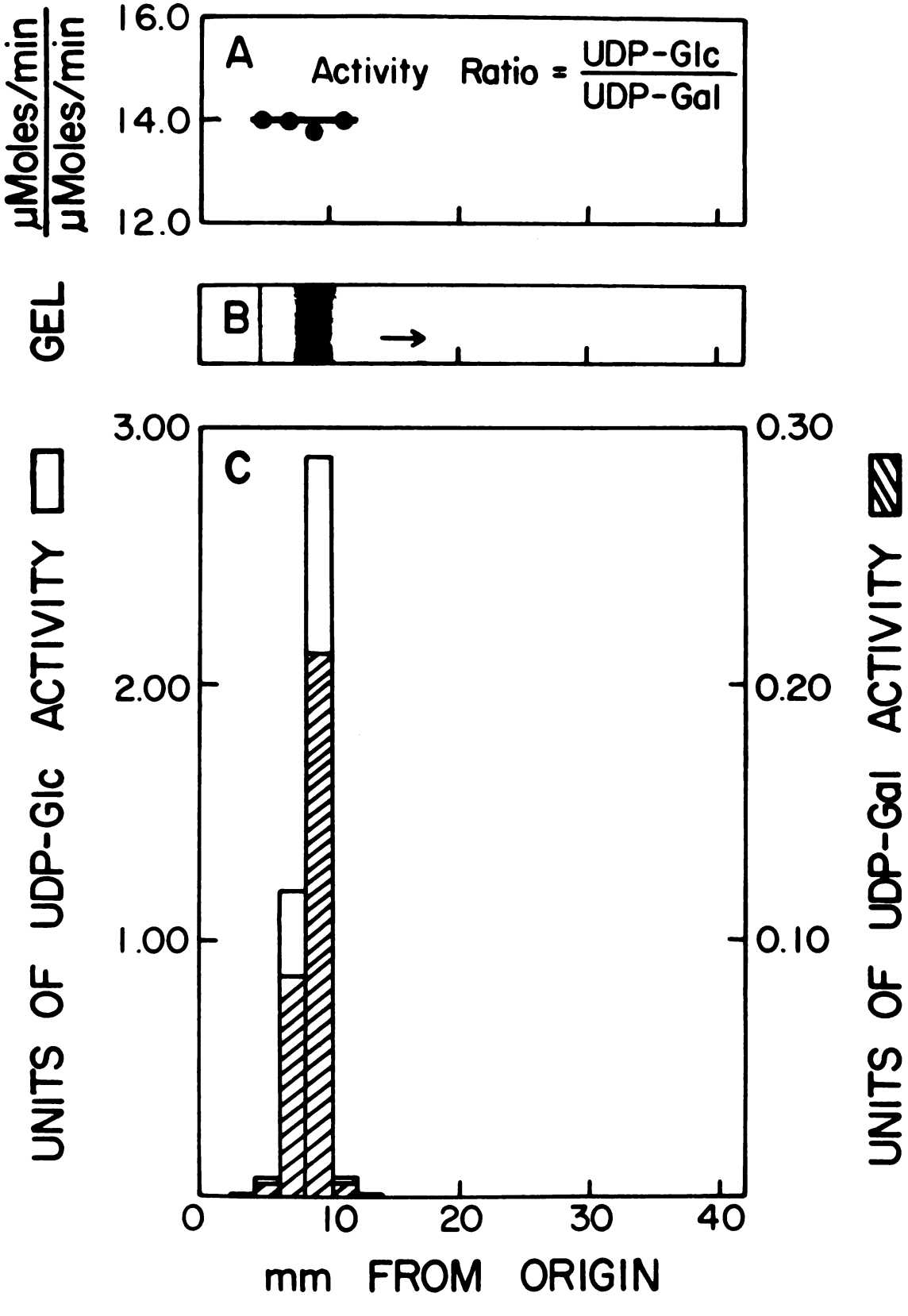
µMoles/min
µMoles/min
16.0
14.0
12.0
A
Activity Ratio = UDP-Glc / UDP-Gal
GEL
B
3.00
2.00
1.00
0
C
0.30
0.20
0.10
10
20
30
40
UNITS OF UDP-Glc ACTIVITY
UNITS OF UDP-Gal ACTIVITY
mm FROM ORIGIN

Figure 18

DISCUSSION

In purifying and crystallizing UDP-Glc pyrophosphorylase from human liver, several important factors must be considered. Among these factors is the addition of mercaptoethanol to the enzyme preparations to prevent large losses of activity. Without mercaptoethanol present, nearly two-thirds of the activity was lost on the first DEAE-cellulose column; and after elution from the column, the remaining activity decreased rapidly.

The age and physical condition of the liver appeared to have an effect on the amount of contaminating proteins present in the liver extracts. The major contaminating proteins eluted from the DEAE-cellulose columns as a greenish-brown band, halfway through the activity peak, and appeared to be more abundant in older livers. The general physical condition of these livers was purely an individual matter; however, it was noted that the more darkly colored livers contained more of these greenish-brown proteins. Neither varying the pH of the gradient nor using a more gradual gradient altered the elution pattern of these proteins significantly. The activity per gram of tissue was not significantly affected by either the age of the liver or the storage of the liver at -20° C for several months.

The pH of the DEAE-cellulose columns was also important. If the pH of the first column were above 8.7, significant amounts of activity was lost on the column. Further, if the pH of the second DEAE-cellulose column was above 8.2, the activity washed off the column. An additional 500 mls of buffer should be used to wash the columns if the designated amounts of buffer in the Experimental Procedure have not attained the desired pH.

As is apparent from Table II, the largest loss of activity during purification occurred by treatment with calcium phosphate gel; however, proportionately larger amounts of the contaminating proteins were lost with this treatment. If nearly all of the activity bound to the gel, it was eluted as described in the text. The binding of the enzyme to the gel may have been caused by insufficient washing of the gel before use. Such an enzyme preparation, if it crystallized, required at least two extra recrystallizations to remove the colored contaminants.

The crystalline enzyme was found to be nearly homogeneous by polyacrylamide gel electrophoresis, sucrose density gradient sedimentation, and sedimentation velocity experiments. The removal of the minor component may be obtained by several more recrystallizations or changes in the purification procedure. However, the possibility also exists that this component may be in slow equilibrium with the enzyme and may be either a subunit of the enzyme or a small molecular weight protein essential for enzymatic activity.

Fresh preparations produced only one protein band during gel electrophoresis; however, as the preparation aged, a second, slower moving protein band appeared, corresponding in mobility to the calf liver dimer form. In fresh calf liver preparations, all four multimer forms of the enzyme were found to be present (26). A comparison of the conditions which result in multimer formation for the human liver and the calf liver enzymes should be interesting.

The sedimentation coefficient of 12.8, calculated from the sedimentation velocity experiments, was the same as the lowest value reported for the calf liver enzyme. However, an extinction coefficient should be determined on the human liver enzyme before further comparison of sedimentation coefficients are made.

The broad pH optimum, the equilibrium constant, the sugar nucleotide specificity, the apparent Michaelis constants for UDP-Glc and Glc-1-P, and the K_i for UDP of the human liver UDP-Glc pyrophosphorylase were similar to the reported values for the calf liver enzyme. The pyrophosphorylase from human brain had K_m's for UDP-Glc and PP_i of 7.5×10^{-5} M and 2.0×10^{-4} M, respectively, which are in agreement with the values determined for the human liver enzyme.

The sigmoidal kinetics displayed by PP_i, although not reported for the calf liver enzyme, have been found for the enzyme from rabbit muscle (11), E. coli. K-12 (14), guinea pig brain and rat liver (13). Villar-Palasi and Larner (11)

reported that the degree of curvature of their Lineweaver-Burk plots was dependent upon the Mg^{+2}/PP_i ratio and suggested the actual substrate for their enzyme was a magnesium-pyrophosphate complex. A similar complex may also exist during catalysis by the pyrophosphorylase from human liver. Further investigation is required before any conclusions can be drawn on the effect of pyrophosphate on the enzyme, i.e., preincubation of the enzyme in PP_i versus no preincubation.

The human liver enzyme required divalent magnesium for maximal catalytic activity; at the same concentration, divalent cobalt was only 14% as effective as magnesium in producing catalytic activity. The calf liver enzyme displayed 25% of its maximal activity in the presence of either cobalt or manganese and 10% in the presence of calcium (15). In comparison, the human liver enzyme was not active in the presence of either manganese or calcium and was almost completely inhibited when magnesium and manganese were present in combination.

The formation of UDP-Glc is believed to be the major catalytic function of the pyrophosphorylase physiologically (15). However, the enzyme also catalyzed the pyrophosphorolysis of the following sugar nucleotides in vitro: UDP-Galactose, UDP-Mannose, UDP-Xylose, TDP-Glucose, CDP-Glucose, GDP-Glucose, and IDP-Glucose. This lack of specificity cannot be fully evaluated until the activity of the pyrophosphorylase toward the different substrates in competition

with UDP-Glc as well as the intracellular concentrations of the various substrates can be determined.

The human liver UDP-Glc pyrophosphorylase differed from the calf liver enzyme in crystalline form and electrophoretic mobility of the monomer. The human liver pyrophosphorylase required the presence of a reducing reagent to maintain activity whereas the calf liver enzyme did not. However, the two enzymes had similar sedimentation coefficients, equilibrium constants, specificity, and kinetic properties. The differences between the two enzymes probably are the result of differences in amino acid composition and sequence. The changes in composition and sequence probably do not occur at the active site because of the similarities between the two enzymes in catalytic and kinetic properties. However, appropriate amino acid studies are required to test this hypothesis.

When UDP-Galactose was used as a substrate for the human liver enzyme, it was discovered that a UDP-Gal concentration 10 times that of the saturating concentration of UDP-Glc was necessary to attain a non-limiting UDP-Gal concentration. Under normal physiological conditions, this data may not have any significance; but in a galactosemic, in which the normal galactose metabolism is impaired, the pyrophosphorylase may participate in an abnormal role. Isselbacher has suggested that a pyrophosphorylase is responsible for the increased ability of some galactosemics to

metabolize galactose by synthesizing UDP-Gal from Gal-1-P and UTP (28,29). As a result of recent research, Gitzelmann has suggested that a pyrophosphorylase may be responsible for the elevated Gal-1-P levels found in the blood of some galactosemics on supposedly galactose-free diets (30). Thus, a pyrophosphorylase has been implicated in catalyzing both the biosynthesis and the pyrophosphorolysis of UDP-Gal in galactosemics. Further speculation about the possible auxiliary function of UDP-Glc pyrophosphorylase in galactosemics should be avoided until results are available on competition studies between the various substrates (UDP-Glc, UDP-Gal, Glc-1-P, and Gal-1-P) involved.

Since the activity ratio UDP-Glc/UDP-Gal remained constant throughout the purification and crystallization procedures and also during polyacrylamide gel electrophoresis, there is probably not a separate UDP-Gal pyrophosphorylase in human liver, but rather a nonspecific UDP-Glc pyrophosphorylase. The enzyme is present in sufficient quantities in both human liver and erythrocytes (32) to account for the physiological observations made by both Gitzelmann and Isselbacher, as well as other researchers.

SUMMARY

A purification and crystallization procedure for uridine diphosphate glucose pyrophosphorylase from human liver was developed. The 500-fold purified enzyme was found to be nearly homogeneous by polyacrylamide gel electrophoresis, sucrose density gradient sedimentation, and velocity sedimentation experiments.

The pH optimum, cation requirements, and equilibrium constant were determined on the crystalline enzyme. The enzyme was not specific for either the nucleoside or the hexose component of the nucleoside diphosphate hexose.

The Michaelis constants were determined for UDP-Glc, Glc-1-P, and UTP from Lineweaver-Burk plots. The K_m for PP_i had to be estimated from a Hill plot since PP_i displayed sigmoidal kinetics with this enzyme. The Hill plot slope decreased from 2.5 to 1.5 when the enzyme was preincubated in 2 mM PP_i for 5 min before addition to the cuvette. UDP was found to be a competitive inhibitor of UDP-Glc.

At non-limiting substrate concentrations, the activity ratio UDP-Glc/UDP-Gal was found to remain constant throughout the purification and crystallization procedures. As a result of this data, it is believed that human liver does not contain a separate UDP-Gal pyrophosphorylase.

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