ENZYMOLOGY:

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Uridine Diphosphate Glucose Pyrophosphorylase

IV. CRYSTALLIZATION AND PROPERTIES OF THE ENZYME FROM HUMAN LIVER*

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SUMMARY

A purification and crystallization procedure has been developed for uridine diphosphate glucose pyrophosphorylase from human liver. The 500-fold purified enzyme was found to be almost homogeneous by polyacrylamide gel electrophoresis and sedimentation velocity.

The crystalline enzyme has an absolute requirement for divalent cations. Magnesium at 3.0 mM and manganese at 1.0 mM gave about equal and maximum activation while cobalt at 1.0 mM resulted in 40% activation of the pyrophosphorylase. The pH optimum is broad, ranging between 7.6 and 9.2. In the direction of uridine diphosphate glucose synthesis, the equilibrium constant is 0.15.

The apparent Michaelis constants from Lineweaver-Burk plots for uridine diphosphate glucose, glucose 1-phosphate, and uridine triphosphate are 5.0×10^{-5}, 9.5×10^{-5}, and 4.8×10^{-5} M, respectively. With the pyrophosphorylase, sigmoidal kinetics was observed when the reaction was initiated with inorganic pyrophosphate. The slope of the Hill plot decreased from 2.5 to 1.5 when the enzyme was previously incubated in 2 mM inorganic pyrophosphate for 5 min. The apparent K_m for inorganic pyrophosphate was estimated to be 2.1 to 2.6 $\times 10^{-4}$ M. Uridine diphosphate is a competitive inhibitor of uridine diphosphate glucose.

The enzyme was not specific for either nucleoside or the hexose component of the nucleoside diphosphate hexose. At nonlimiting substrate concentrations, the activity ratio of the pyrophosphorylase with uridine diphosphate glucose and uridine diphosphate galactose as substrates remained constant throughout the purification and crystallization procedures. Thus, human liver does not appear to contain a separate uridine diphosphate galactose pyrophosphorylase.

In human tissues the biosynthesis of uridine diphosphate glucose from uridine triphosphate and glucose 1-phosphate is catalyzed by a pyrophosphorylase which appears to be ubiquitous in nature.

Crystallization of the pyrophosphorylase was first achieved from calf liver in which it occurs abundantly (1). For the calf liver enzyme, some of the unique structural features and a proposed catalytic mechanism have been previously described (2). A pathway of galactose metabolism could function in humans which involves a UDP-glucose and a UDP-galactose pyrophosphorylase (3, 4). A key role in carbohydrate metabolism has been shown for the biosynthetic products, UDP-glucose and UDP-galactose; therefore, it is important to characterize these enzymes in human tissue. A fractionation procedure for human liver leading to crystalline UDP-glucose pyrophosphorylase has now been developed. The isolation procedure and some of the properties of the crystalline enzyme are the subject of this report.

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EXPERIMENTAL PROCEDURE

Materials and Methods

Crystalline UDP-glucose pyrophosphorylase was prepared from calf liver according to Gillett, Levine, and Hansen.[1] All other chemicals, enzymes, and supplies were purchased commercially as indicated in previous papers (1, 2). The methods and procedures which were used have also been described and hence only the exceptions are noted.

Fractionation of Liver

Unless otherwise stated, all of the fractionation steps were carried out at 4°, and all centrifugations were performed at 23,000 $\times$ *g* for 30 min.

Step 1: Extraction—Liver specimens were obtained from accident victims and held at −20°. The 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 for 2 min. The resulting homogenate was stirred for 10 min; 12 g of protamine sulfate per kg of liver, suspended in a small amount of deionized distilled water, were added slowly with stirring. The pH was then adjusted to 6.8 with glacial acetic acid. The resulting mixture was allowed to stir for 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; 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. The pH was maintained 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

[1] T. A. Gillett, S. Levine, and R. G. Hansen, manuscript submitted for publication.

20 min, it was then centrifuged and 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 (0.2 volume of Fraction I) in 0.01 M Tricine-chloride ((*N*-methyl)tris-hydroxymethylglycine) buffer (pH 8.5), 0.001 M in EDTA and 0.005 M in mercaptoethanol and dialyzed overnight against 20 liters of the same buffer (Fraction II).

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

Step 4: DEAE-cellulose Column Chromatography I—The DEAE-cellulose adsorption column was prepared as previously described.[1] Fraction III was rinsed onto the column and eluted with 1.5 liters of 0.01 M Tricine buffer, pH 8.5, 0.02 M in NaCl and 0.02 M in mercaptoethanol. Twenty-milliliter fractions were collected and tubes containing 10 units or more per ml were combined (Fraction IV).

Step 5: Second Ammonium Sulfate Fractionation—Fraction IV was brought to 58% saturation with ammonium sulfate while maintaining the pH at 8.5, stirred for 30 min, and centrifuged. The resulting precipitate was dissolved in about 0.2 volume of Fraction IV in 0.01 M Tricine buffer, pH 8.0, 0.001 M in EDTA and 0.02 M in mercaptoethanol (Buffer A), and dialyzed overnight against 20 liters of the same buffer (Fraction V).

Step 6: DEAE-cellulose Column Chromatography II—Fraction V was applied to 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 0.01 M Tricine buffer, pH 8.0, 0.02 N in NaCl and 0.02 M mercaptoethanol. A linear gradient (0.02 to 0.20 N NaCl in 0.02 M mercaptoethanol-0.01 M Tricine buffer, pH 8.0) was used to elute the activity from the column. Tubes with specific activity above 5 were combined (Fraction VI).

Step 7: Crystallization—Fraction VI was brought to 60% saturation with ammonium sulfate at pH 8.5, stirred for 20 min, and centrifuged. The precipitate was dissolved in 8 to 10 ml of Buffer A by warming in a 37° water bath for 5 to 6 min. Any undissolved material was removed by centrifugation at room temperature.[2] The supernatant was dialyzed against 250 ml of 32% saturated ammonium sulfate in Buffer A for 24 hours. Crystals were evident after 8 hours.

Step 8: Recrystallization—Crystals were removed from the supernatant by a low speed centrifugation (2000 × *g* for 10 min). The crystals were dissolved in 2 to 3 ml of Buffer A by warming in a 37° water bath for 2 to 3 min; any undissolved material was removed by centrifugation at room temperature.[2] The solution was then dialyzed against the 32% saturated ammonium sulfate solution described above for 24 hours. Crystals usually appeared within 1 to 2 hours. The specific activity became constant after the second recrystallization. The crystals formed during the first crystallization appeared as long needles which were clearly visible with a phase contrast microscope equipped with an oil immersion lens. Upon recrystallization the needles were generally smaller (Fig. 1). A summary of a typical purification procedure is given in Table I.

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

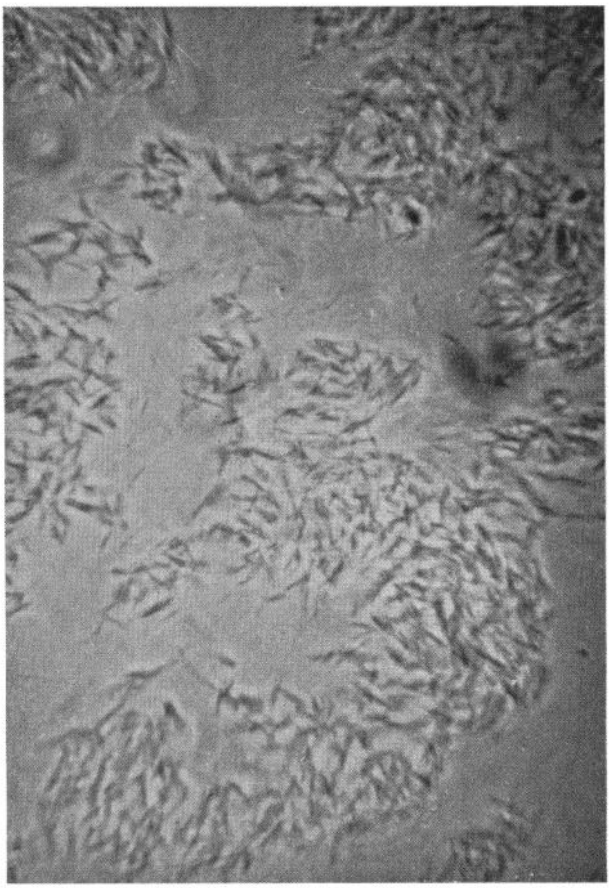

FIG. 1. Photomicrograph of crystals of UDP-glucose pyrophosphorylase. Magnification under phase contrast is × 100.

TABLE I

Purification and crystallization of UDP-glucose pyrophosphorylase from human liver

Step	Volume	Protein	Total activity	Specific activity	Yield
	ml	*mg*	*units*	*units/mg*	%
1. Extraction	3,200	163,000	62,000	0.38	100
2. $(NH_4)_2SO_4$, 35 to 58%	570	79,000	55,000	0.69	89
3. $Ca_3(PO_4)_2$ gel	1,860	24,000	23,000	0.85	37
4. DEAE-cellulose I	470	6,400	17,000	2.6	27
5. $(NH_4)_2SO_4$ 60%	92	4,100	15,000	3.7	27
6. DEAE-cellulose II	350	840	9,300	11.0	15
7. Crystallization	2.4	60	5,100	85.0	8.3
8. Recrystallization I	2.4	28	4,800	181.0	7.7
9. Recrystallization II	1.7	20	4,100	200.0	6.6

RESULTS

Protection against Oxidizing Conditions and Pyrophosphate Dependence

To prevent loss of enzymatic activity during the purification and crystallization procedures, it was necessary to add mercaptoethanol to all of the preparations. Either dithiothreitol or

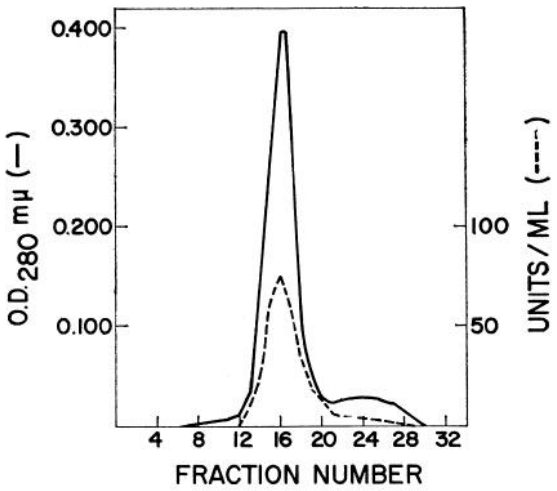

FIG. 2. Density gradient sedimentation of UDP-glucose pyrophosphorylase in sucrose. Gradients were developed in a SW 50 rotor at 35,000 rpm for 12 hours at 4°. After puncturing the bottom of the tubes, the contents were forced out and through a flow cell and 200-μl fractions were collected. ——, optical density at 280 mμ; – – –, activity in units per ml.

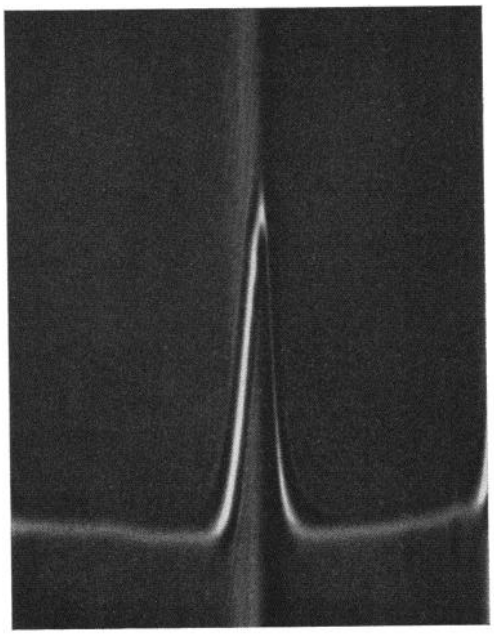

FIG. 3. Sedimentation velocity pattern of UDP-glucose pyrophosphorylase from human liver. The enzyme was 3.3 mg per ml in 0.01 M Tricine buffer (pH 8.0), 0.02 M in mercaptoethanol, and 0.10 M in NaCl. The picture was taken 28 min after attaining 56,108 rpm. The diaphragm angle was 65° and the temperature was 4.4°. Sedimentation is from *left* to *right*.

mercaptoethanol was added to all dilution media at an optimum concentration of 2 mM. The crystalline enzyme maintained good activity when stored at 4° in 0.02 M dithiothreitol.

With the standard procedure to determine enzyme activity, the reaction velocity was found to be proportional to the enzyme concentration. For the formation of UTP and glucose-1-P from UDP-glucose, the crystalline enzyme exhibited complete dependence upon the presence of pyrophosphate but displayed a pronounced lag period after initiation of the reaction with pyrophosphate. If the reaction were initiated with UDP-glucose, the observed lag period was shortened but not entirely eliminated when the enzyme was previously incubated with the other components of the reaction.

TABLE II

Equilibrium constant for UDP-glucose pyrophosphorylase from human liver

Micromolar quantities of substrates were incubated in the presence of enzyme, 1.0 μmole of magnesium acetate, 2.0 μmoles of mercaptoethanol, 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 unit of enzyme; after incubating for 1 hour at 30°, the reactions were stopped and the products were determined. The direction of the reaction is indicated:

$$\text{Glucose-1-P} + \text{UTP} \underset{\text{II}}{\overset{\text{I}}{\rightleftharpoons}} \text{UDP-glucose} + \text{PP}_i$$

K_{eq} is defined as: [UDP-glucose] [PP_i]/[Glucose-1-P] [UTP].

Experiment	Direction	Glucose-1-P	UTP	UDP-Glucose	PP_i	K_{eq}
1	I	0.77	0.79	0.31	0.29	0.15
2	I	0.77	0.79	0.31	0.30	0.16
3	I	0.77	0.76	0.31	0.29	0.15
4	II	0.66	0.66	0.26	0.26	0.16
5	II	0.64	0.68	0.24	0.26	0.15
6	II	0.68	0.71	0.29	0.29	0.16

Homogeneity and Sedimentation—Electrophoresis of twice recrystallized UDP-glucose pyrophosphorylase on polyacrylamide gel generally resulted in only one protein band. As the preparation aged another slower moving minor component appeared during electrophoresis. This slower moving component also exhibited UDP-glucose pyrophosphorylase activity and probably is a dimer as was previously found to occur in preparations of the calf liver enzyme (2). Even when gel columns were heavily loaded with enzyme (0.5 mg of protein per gel), only one major and the slower moving faint band were visible after staining.

On sucrose density gradient centrifugation a major protein peak and also a minor slower moving, rather than a faster moving, peak were found (Fig. 2).

In schlieren patterns (Fig. 3) produced in the analytical ultracentrifuge, one major protein peak was visible with a second minor slight discontinuity in the base line suggesting the possibility of a slower moving component. Further investigation is needed to ascertain whether there is some relation between these fractions which could be interconvertible forms of the same protein (2). The twice recrystallized enzyme was, therefore, nearly homogeneous. From the sedimentation velocity patterns for the major component, a Svedberg coefficient $s^0_{20,w}$ corrected for standard conditions, was calculated to be 12.8.

Optimum pH—The crystalline enzyme exhibited maximal activity over a broad pH range from 7.6 to 9.2. As has been reported for UDP-glucose pyrophosphorylase from other sources (1, 5, 6), the activity of this enzyme dropped off sharply with pH changes outside these broad limits. At pH 7.0 or 9.9, approximately 65% of the maximal activity was attained.

Cation Requirements—For catalytic activity, the crystalline enzyme has a requirement for divalent cations. Magnesium at an optimum concentration of 3 mM and manganese at an optimum concentration of 1 mM gave maximum activity. Cobalt at a concentration of 1 mM was 40% as effective as magnesium. In

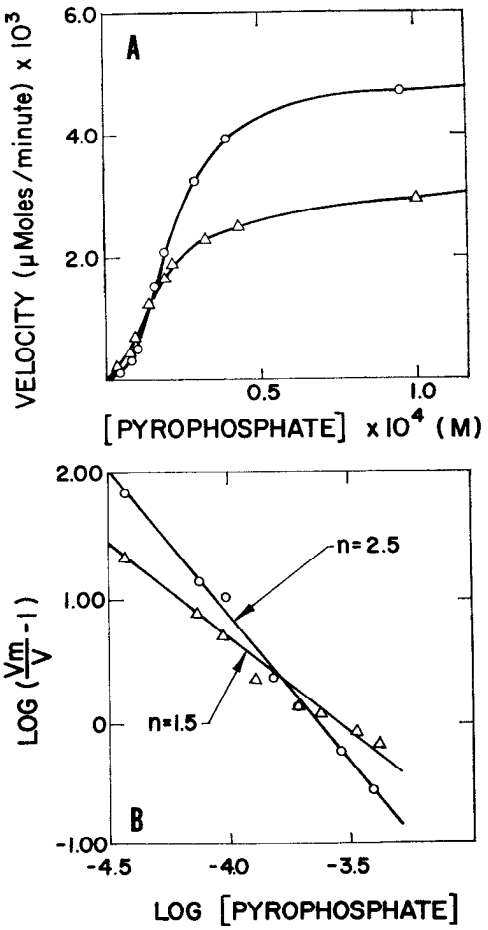

FIG. 4. The effect of pyrophosphate concentration on the reaction velocity. In one case (△——△) the enzyme was previously incubated in 2 mM pyrophosphate for 5 min before addition to the reaction mixture.

the presence of 3 mM magnesium, all divalent metals tested were inhibitory.

Equilibrium Constant—The equilibrium constant for the UDP-glucose pyrophosphorylase reaction was determined by initiating the reaction in either direction (Table II). In the direction of UDP-glucose biosynthesis an equilibrium constant of 0.15 was obtained which is similar to that for the calf liver enzyme (1).

Substrate Affinity—The affinity of the crystalline enzyme for UDP-glucose, UTP, and glucose 1-P was determined form Lineweaver-Burk plots. For UDP-glucose, UTP, and glucose-1-P the apparent Michaelis constants are 5.0×10^{-5} M, 4.8×10^{-5} M, and 9.5×10^{-5} M, respectively. A plot of velocity as a function of concentration of pyrophosphate was sigmoidal (Fig. 4*A*), and the resulting Lineweaver-Burk plots curved upward. In this case, the maximum velocity was estimated from the Lineweaver-Burk plots; the Michaelis constant of 2.1×10^{-4} M was obtained when the log $((V_{max}/V)^{-1})$ was plotted against the log of pyrophosphate concentration (Hill plot) (Fig. 4*B*). From these data, the Hill coefficient (n) of 2.5 was obtained, indicating that more than 1 molecule of PP_i was binding to the enzyme. Previously incubating the enzyme for 5 min in 2 mM PP_i did not eliminate the sigmoidal slope, but a figure nearer unity (1.5) was obtained, suggesting a decrease in the number of PP_i molecules binding to the enzyme. The apparent Michaelis constant calculated from these data changed only slightly to 2.6×10^{-4} M, which is within experimental error. Because there was still a lag period in the reaction even when the enzyme was previously incubated with PP_i, the initial velocities measured for the three lowest concentration points probably were not real. If the three points of lowest concentration were not included on the graph, the line drawn through the remaining points yields a value for n approaching 1.0, indicating that only 1 molecule of PP_i was binding to the enzyme after preliminary incubation in PP_i. If the three points of lowest concentration were excluded when the enzyme was not previously incubated with PP_i, the slope of the Hill plot would decrease to 2.2. Further experimentation is required before conclusions can be drawn on the effect of pyrophosphate on the enzyme or the number of PP_i molecules that bind to it. With a Dixon plot of $1/v$ against inhibitor concentration, it was determined that UDP was a competitive inhibitor of UDP-glucose, with a K_i of about 1.0×10^{-4} M.

TABLE III

Specificity of human liver UDP-glucose pyrophosphorylase

All substrates at a final concentration of 0.40 μM.

Substrate	Initial reaction velocity
UDP-glucose	100
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

TABLE IV

Substrate specificity during purification and crystallization of UDP-glucose pyrophosphorylase

Step	Activity[a]: UDP-Glucose: UDP-galactose
Extraction	10.8
$Ca_3(PO_4)$ gel	11.3
Recrystallization I	14.0
Recrystallization II	13.8

[a] UDP-glucose and UDP-galactose were at a final concentration of 0.40 and 4.0 μM.

Specificity—The most active substrate for the enzyme is UDP-glucose; however, the enzyme also catalyzes the pyrophosphorolysis of several other compounds but less rapidly. At a substrate concentration of 0.4 mM, UDP-xylose, UDP-galactose, and TDP-glucose yielded from 1.5 to 2.2% of the rate of reaction with UDP-glucose as a substrate (Table III). Less than 1% of the rate of activity of UDP glucose was measured when CDP-glucose, GDP-glucose, or UDP-mannose was used as a substrate. Activity with IDP-glucose was just perceptible; no activity could be detected with ADP-glucose at 1000 times the enzyme concentration used for UDP-glucose measurements.

UDP-galactose is required in high concentration (4.0 mM) to saturate the human liver UDP-glucose pyrophosphorylase. With a concentration of 0.4 mM for UDP-glucose and 4.0 mM for UDP-galactose, the ratio of activity for these two substrates was

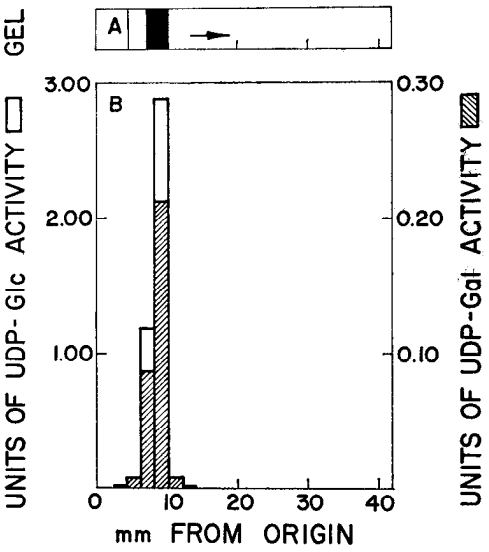

FIG. 5. Activity of UDP-glucose pyrophosphorylase eluted from polyacrylamide gel in the presence of UDP-glucose or UDP-galactose (*UDP-gal*). The direction of electrophoresis is indicated by the *arrow;* conditions for electrophoresis and the elution of activity from the gel are described in Fig. 2. One gel was stained for protein with Coomassie blue, and another gel was sliced into 2-mm segments for activity determinations. *A*, schematic reproduction of gel stained with Coomassie blue; *B*, activity of UDP-glucose pyrophosphorylase in the presence of UDP-glucose and UDP-galactose.

about 12.0 and did not vary significantly during the purification or crystallization procedures (Table IV).

Since upon electrophoresis of the crystalline enzyme a slower moving, minor component had been observed, the possibility of this fraction being UDP-galactose pyrophosphorylase was investigated. Also, to test the possibility that a UDP-galactose pyrophosphorylase was eluted from the polyacrylamide gels before the electrophoresis period was completed, activity with both substrates was determined on all protein fractions eluted from the gels (Fig. 5). The activity ratio remained constant throughout at about 12.0. This ratio was the same for the recrystallized enzyme, indicating that the UDP-glucose and UDP-galactose activities were inseparable by the technique used.

DISCUSSION

In purifying and crystallizing UDP-glucose pyrophosphorylase from human liver, several factors must be considered. Among these is the addition of reducing agents to the enzyme preparation to prevent loss of activity. Without reducing agents present, nearly two-thirds of the activity was lost on the first DEAE-cellulose column, and, after elution, the remaining activity decreased rapidly. Appreciable quantities of enzyme were found in livers from all subjects and also from livers that had been stored at −20° for several months. The largest loss of activity during purification occurred with the calcium phosphate gel treatment; however, proportionately larger amounts of the contaminating proteins were also lost with this treatment.

The crystalline enzyme was found to be nearly homogeneous by polyacrylamide gel electrophoresis and velocity sedimentation. The coefficient of 12.8 S calculated from the sedimentation velocity experiments was comparable to that for the calf liver enzyme.

The broad pH optimum, the equilibrium constant, the substrate specificity, the apparent Michaelis coefficients for UDP-glucose, UTP-glucose-1-P, and PP_i, and the inhibitor constant for UDP of the human liver UDP-glucose pyrophosphorylase were similar to reported values for the enzymes from calf liver and human brain and erythrocytes (1, 7, 8).

A UDP-glucose pyrophosphorylase has been purified from human erythrocytes and some of its properties have been defined (8). The enzymes from human erythrocytes and liver appear to be similar, if not identical. At this time the specificity is of major interest since the enzyme from human erythrocytes, even though less pure, was reported to be less active toward galactose-containing substrates than that found in the present studies for the enzyme from human liver. This apparent difference in specificity is somewhat puzzling, and is presently being investigated.

The sigmoidal kinetics displayed by PP_i, although not reported for the calf liver enzyme, has been found for the enzyme from rabbit muscle (6), *Escherichia coli* K-12 (9), guinea pig brain, and rat liver (10). Villar-Palasi and Larner (6) reported that the degree of curvature of their Lineweaver-Burk plots was dependent upon the Mg^{+2}:PP_i ratio and suggested that the actual substrate for the enzyme was a magnesium-pyrophosphate complex.

The formation of UDP-glucose is believed to be the major physiological function of the pyrophosphorylase (1). However, at a slower rate, the enzyme also catalyzed the pyrophosphorolysis of the following sugar nucleotides: UDP-galactose, UDP-mannose, UDP-xylose, TDP-glucose, CDP-glucose, GDP-glucose, and IDP-glucose.

The saturating concentration for UDP-galactose is 10 times that of UDP-glucose. Under normal physiological conditions, this may not be significant, but, in a galactosemic patient, 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-galactose from galactose-1-P and UTP (3). Gitzelmann, on the other hand, has suggested that a pyrophosphorylase may be responsible for the elevated galactose-1-P levels found in the blood of some galactosemics on supposedly galactose-free diets (11). Thus, a pyrophosphorylase has been suggested to catalyze both the biosynthesis and the pyrophosphorolysis of UDP-galactose in galactosemics.

Abraham and Howell (12) extracted UDP-galactose pyrophosphorylase activity from human liver and, without purification, the catalytic properties were elaborated upon. The specificity of their extract for substrate was not reported and, in view of the present findings, it was undoubtedly highly active toward UDP-glucose. Abraham and Howell did not differentiate between two alternative possibilities: (*a*) a specific pyrophosphorylase for UDP-glucose and for UDP-galactose, and (*b*) a nonspecific pyrophosphorylase with UDP-glucose as principal substrate and with some activity for UDP-galactose. In the present study the ratio of activity for UDP-glucose and UDP-galactose remained constant throughout the fractionation procedures and also during sedimentation and electrophoresis; therefore, Alternative *b* is indicated. Thus, a nucleoside diphosphate hexose pyrophosphorylase is present in human liver in abundance. Uridine diphosphate glucose is the most active but not the only substrate for this enzyme.

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