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Uridine Diphosphate Glucose Pyrophosphorylase

CRYSTALLIZATION AND PROPERTIES OF THE ENZYME FROM RABBIT LIVER AND SPECIES COMPARISONS*

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SUMMARY

Rabbit liver UDP-glucose pyrophosphorylase has been crystallized and further purified to a specific activity of 200 by using preparative centrifugation with sucrose density gradients.

The pyrophosphorylase requires a divalent cation. Magnesium is preferred, although manganese, cobalt, and calcium can serve as less effective alternate cation activators. Maximum activity requires a reducing agent, and the enzyme has a broad pH range from 7.0 to 10.5.

The molecular weight of the enzyme is approximately 400,000, with eight identical subunits. While the enzyme shows no tendency to form multimolecular aggregates as do other liver pyrophosphorylases, the eight subunits are arranged in a stacked tetrameric configuration. Amino acid analysis, peptide mapping, gel electrophoresis, and sedimentation showed the enzyme to be homogeneous with a single NH_2-terminal amino acid being serine. The subunits of the rabbit liver enzyme are chemically identical as confirmed by the foregoing analyses.

Although UDP-glucose is the most active substrate, the enzyme will catalyze the pyrophosphorylation of a variety of nucleoside diphosphate hexoses. Rates as high as 14% of that for UDP-glucose were obtained using UDP-galactose as a substrate. The ratio of glucose to galactose activity remained constant throughout purification and no evidence was found for a separate enzyme for UDP-galactose.

The equilibrium constant in the direction of UDP-glucose formation was 0.16. The apparent Michaelis constants for UDP-glucose, UTP, and glucose 1-phosphate were 6.6×10^{-5}, 3.8×10^{-5}, and 4.6×10^{-5}, respectively, and that for UDP-galactose was 4.2×10^{-4}. UDP-galactose and galactose 1-phosphate are competitive inhibitors of UDP-glucose and glucose 1-phosphate with apparent constants of 2.2×10^{-4} and 7.3×10^{-3}, respectively. Either 8 moles of UDP-glucose or UDP-galactose were bound per mole of enzyme, and the bound UDP-galactose could be replaced stoichiometrically by equivalent quantities of UDP-glucose.

The measured chemical and physical properties of the enzyme from calf, rabbit, and human livers are compared.

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The enzyme uridine diphosphate glucose pyrophosphorylase (UTP-D-glucose 1-phosphate uridyltransferase, EC 2.7.7.9) catalyzes the formation of UDP-glucose from UTP and glucose 1-phosphate and is therefore critical to mono- and polysaccharide synthesis. The crystallization of this pyrophosphorylase from calf and human liver has already been described (1, 2). A catalytic mechanism has been proposed for the enzyme from calf liver (3), and a somewhat unusual polymeric and subunit structure has been described (4).

Pyrophosphorylation of UDP-galactose is an important reaction with special implications for human galactosemics[1] (5). While the reactions with UDP-glucose and UDP-galactose have been considered to be catalyzed by separate enzymes (6, 7), evidence in this paper and elsewhere indicates that a single catalyst may be functional in liver.

In this report we describe the crystallization and many of the properties of the pyrophosphorylase from rabbit liver and compare the properties with those of the human and calf liver enzymes.

EXPERIMENTAL PROCEDURE

Assay and Supplies—The measurement of pyrophosphorylase activity (Assays 1 and 2) have been described (1). The final mixture in Assay 1, used to determine reaction rates of UDP-glucose and TDP-glucose, contained in a total volume of 0.5 ml, 1 μmole of magnesium acetate, 0.2 μmole of NADP, 1 μmole of PP_i, 45 μmoles of Tris-acetate buffer (pH 7.8), 0.2 μmole of the nucleoside diphosphate glucose, and excess phosphoglucomutase and glucose-6-P dehydrogenase. Assay 2, used to measure activity with UDP-galactose and UDP-xylose, contained in 0.5 ml, 45 μmoles of Tris-acetate buffer (pH 7.8), 1 μmole of magnesium acetate, 1 μmole of PP_i, 0.6 μmole of 3-P-glycerate, 0.12 μmole of NADH, 0.2 μmole of the nucleoside diphosphate sugar, and excess 3-P-glycerate kinase and glyceraldehyde-3-P dehydrogenase. When analysis concerned the enzymes from rabbit and human livers, the reaction mixtures contained 0.02 M mercaptoethanol.

Rabbit livers were purchased from Pel-Freeze Biologicals and all chemicals and reagents were purchased from commercial sources.

[1] R. Gitzelmann, and R. G. Hansen, submitted for publication.

In developing a procedure to crystallize the enzyme from the liver of rabbits, we started with that which was successful for human liver (2). Better results were obtained with enzymes from both sources, however, with the following modification. Immediately after the DEAE-cellulose column (Column I), the fractions containing over 10 units per ml of enzyme were combined and brought to 60% saturation by adding crystalline ammonium sulfate (pH 8.5). The mixture was stirred for 30 min, then centrifuged and decanted. The precipitate was dissolved in a minimal amount of buffer (0.01 M Tricine[2] containing 0.02 M mercaptoethanol at pH 8.0, and hereinafter referred to as a Tricine buffer) and dialyzed overnight in the same buffer. Two milliliters of the dialyzed solution were then layered on each sucrose gradient column (5 to 20% linear gradient) and centrifuged in a Beckman SW 41 rotor for 17 hours at 200,000 × *g*. The cellulose nitrate tubes containing the gradients were then punctured and 0.5-ml fractions collected. Fractions with a specific activity of 10 or greater were combined, precipitated with ammonium sulfate, and crystallized as reported previously. Ten- to twelve-fold purifications have been obtained without loss of activity using the sucrose density gradient step as a modification. Following recrystallization, specific activities of 200 were obtained.

Polyacrylamide Gel Electrophoresis—Polyacrylamide gel electrophoresis was performed according to the procedure of Davis (8). Where enzymatic activity was monitored, the columns were divided lengthwise. One-half of the column was stained for protein (9) and the remaining half sliced into cross-sections 1-mm thick and, for measurement of activity, homogenized in 100 μl of Tricine buffer.

Sucrose Density Gradient—Linear sucrose density gradients (5 to 20%) in Tricine buffer were made according to the procedure of Martin and Ames (10). Twelve-milliliter cellulose nitrate centrifuge tubes were filled to 11.3 ml with the sucrose gradients. Two milligrams each of reference solutions of proteins and the various crystalline enzymes were layered on the top of individual gradients. The tubes were then placed in the Beckman SW 41 rotor and centrifuged at 210,000 × *g* (mean) for 22 hours (4°). To measure protein the tubes were punctured, and the contents emptied through a flow-through cell by applying water at the top of the gradient with a syringe pump. Optical density was monitored spectrophotometically at 280 nm. Fractions containing 200 μl were collected and assayed for enzymatic activity. Molecular weight was plotted on a semilog scale.

Electron Microscopy—Electron micrographs were produced using a droplet technique for negative staining (11). Small drops of Tricine containing 0.05 to 0.1 mg per ml of pyrophosphorylase were placed on carbon-coated, mesh copper grids. After 2 to 5 min, the excess enzyme solution was removed by blotting with filter paper. The samples were stained with 2% potassium phosphotungstate for 1 to 5 min and again blotted with filter paper. Grids were then examined on a Jelco 100 B3 electron microscope.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis—Subunit molecular weight was determined by sodium dodecyl sulfate gel electrophoresis (12). The standard proteins and the enzyme solutions containing approximately 0.7 mg per ml of buffer (0.01 M phosphate containing 0.02 M mercaptoethanol) were denatured in 1% sodium dodecyl sulfate at 37° for 2 hours. Trypsin, ovalbumin, bovine serum albumin, catalase, and glucose oxidase were used as reference standards. Fifty microliters of the denatured standards and enzyme were layered on the top of the columns (5% acrylamide) and electric current applied for 2.3 hours at a constant voltage of 8 volts per cm. Following staining for protein (9), migration distance was measured and subunit molecular weight was estimated from projections on semilogarithmic paper.

Equilibrium Dialysis—To estimate substrate binding by the enzyme quantitatively, dialysis cells were constructed using silicone rubber sheeting (13). Two milligrams of enzyme were added to one cell. Both cells were then filled to 0.5-ml volume with 8×10^{-4} M UDP-galactose, 2 mM magnesium acetate, and 2 μCi of radioactive [^{14}C]UDP-galactose (254.5 μCi per μM) in Tricine buffer. The equilibrium was monitored by placing 5-μl aliquots in 15 ml of Aquasol and measuring the radioactivity in a liquid scintillation counter. After 46 hours, UDP-glucose (2×10^{-4} M) was added to the equilibrium mixture to displace bound UDP-galactose. The displacement of [^{14}C]UDP-galactose from the enzyme was then followed as described.

Sephadex Chromatography—Sephadex chromatography on G-25-80 columns has been described (3).

Amino Acid Analysis—Amino acid analysis was performed according to the procedure of Zumwalt *et al.* (14) by the Analytical Biochemistry Laboratory at Columbia, Missouri.

Trypsin Digestion and Peptide Mapping—Lyophilized enzyme (about 25 mg) was digested with 0.518 mg of L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin in 0.001 N HCl for 16 hours. An aliquot of the protein digest dissolved in dilute ammonia containing approximately 3 mg of the digest was then chromatographed two-dimensionally using the procedures of Ingram (15) and Katz *et al.* (16). Peptide and amino acid spots were visualized with multiple dipping techniques (17) using ninhydrin, Ehrlich's stain, and Sakaguchi reagent.

Cyanogen Bromide Cleavage—Cleavage with cyanogen bromide was performed by the procedure of Grass *et al.* (18) as modified by Steers *et al.* (19). Three to four milligrams of the reduced and *S*-carboxymethylated protein (prepared according to the procedure of Crestfield *et al.* (20)) were dissolved in 2.0 ml of 70% formic acid. Cyanogen bromide was next added in approximately 50-fold excess over the methionine amount. The reaction was allowed to proceed at room temperature for 16 to 20 hours before the solution was lyophilized. The dry digest was dissolved in 10 M urea, and then placed on 7.5% polyacrylamide gel columns containing 5 M urea and developed in the pH 8.7 system described by Davis (8) at 4° and 5 ma per tube.

End Group Analysis—The end group analysis was performed according to the procedure of Edman as modified by Schroeder (21) and of Sanger as modified by Fraenkel-Conrat *et al.* (22).

Metal Cofactors—Metal cofactors were determined using the standard assay procedure varying the concentration of the metals and comparing their activity to maximum magnesium activity taken as 100%.

Equilibrium Constants—Equilibrium constants were determined spectrophotometrically as described by Albrecht *et al.* (1) except incubation mixtures with rabbit and human enzyme contained 0.02 M mercaptoethanol.

RESULTS AND DISCUSSION

The uridine diphosphate glucose pyrophosphorylase from rabbit liver crystallizes as a tapered rod. Fig. 1 (*upper frame*) illustrates the shapes of the crystals from rabbit (*tapered rod*), human (*rectangle* or *needle*), and calf liver (*diamond*) pyrophosphorylases. The molecular weight of the undenatured pyrophosphorylases (octamers) may be seen in the semilog scale in Fig. 2.

Molecular aggregation of the enzyme from calf liver has been shown by polyacrylamide electrophoresis and sucrose gradient centrifugation (4). The enzyme from rabbit liver, however, migrates as one active protein band on acrylamide gels (Fig. 3*A*). The other, faster moving bands which are present in the crystalline preparation appear to be contaminants. They have been successfully removed (Fig. 3*B*) by sucrose density gradient centrifugation, or reverse gradient purification which is the stepwise reduction of ammonium sulfate concentration to dissolve contaminants while leaving the enzyme precipitated. Evidence of molecular aggregation of the enzyme from human liver is limited and, although molecular aggregates of the human enzyme do not always appear on polyacrylamide gel, the presence of an apparent dimer peak has been noted on centrifugation in sucrose gradient.

Fig. 2 also illustrates a semilogarithmic plot of subunit molecular weight determined by electrophoresis on acrylamide containing sodium dodecyl sulfate gels. The molecular weight of the subunits of the pyrophosphorylases was approximately 50,000 from the rabbit, 56,000 from the human, and 60,000 from the calf. The presence of a single band on the polyacrylamide

[2] The abbreviation used is: Tricine, *N*-tris(hydroxymethyl)-methylglycine.

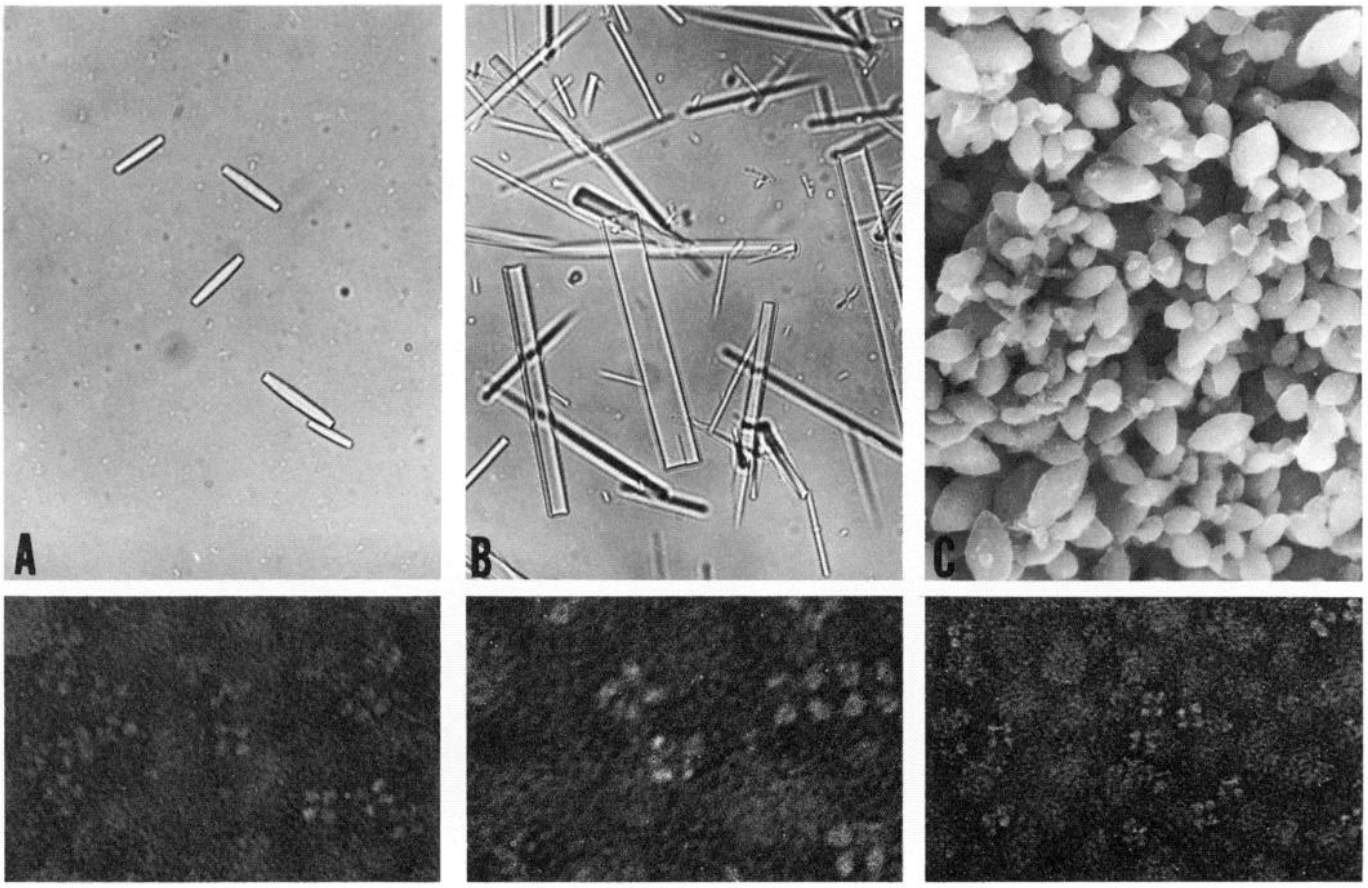

FIG. 1. *Upper frame*, photomicrographs of rabbit liver (*A*) and human liver (*B*) UDP-glucose pyrophosphorylase magnified 780 and 400 times, respectively. Also a scanning electron micrograph of calf liver enzyme (*C*) magnified 1400 times. *Lower frame*, negatively stained electron micrographs of rabbit, human, and calf liver enzymes magnified 510,000, 810,000, and 350,000 times, respectively.

gel containing sodium dodecyl sulfate indicated that within each species each pyrophosphorylase may have identical subunits.

Measurements of the diameter of the subunits on electron micrographs of the three pyrophosphorylases (Fig. 1, *lower frame*) show that calf and human liver enzyme subunits are about 50 A, while the rabbit liver enzyme has a slightly smaller diameter. Molecular weights calculated from these diameters agree approximately with the molecular weights calculated from migration on acrylamide gels containing sodium dodecyl sulfate. Although the electron micrographs all reveal arrangements that apparently contain four subunits, the situation is believed to involve two superimposed tetrads or an octamer. The molecular weight of the native pyrophosphorylases and their subunits are substantiated with the presence of eight subunits of equal weight.

Equilibrium dialysis has shown that the enzyme from rabbit liver will bind 8 moles of UDP-galactose per mole (Fig. 4) or 1 molecule of substrate per subunit assuming an octameric enzyme. Furthermore, the UDP-galactose can be displaced by UDP-glucose, a substrate having greater affinity for the enzyme. In the binding of UDP-glucose to the rabbit enzyme at a mole ratio of approximately 8:1, the enzyme-bound substrate was clearly separated from unbound substrate by the Sephadex G-25-80 column (Fig. 5).

Amino acid analysis of the pyrophosphorylases from the three species were similar with the exception of the very low methionine content of the rabbit enzyme as determined by the procedure of Zumwalt *et al.* (14). Earlier results using ion exchange procedures (23) indicated higher methionine levels. With calf liver enzyme, 10 bands usually result from cyanogen bromide cleavage confirming the higher estimate of methionine. Cyanogen bromide cleavage has not been performed on the human and rabbit pyrophosphorylase.

The enzymes from rabbit and human livers (unlike that from calf liver) both require a reducing agent (mercaptoethanol) to achieve maximum activity and stability. Some of the chemical and physical properties of the pyrophosphorylases from the three species are summarized in Table I. Amino acid analysis indicated that the number of lysine and arginine residues total 375 for the rabbit enzyme. Hence the number of peptides expected from trypsin treatment is 376, assuming no repeating sequences in the primary structure. Assuming identical subunits and an octameric molecule, the predicted number of peptide spots would be reduced 8-fold plus 1, or from 376 to 48. Actual peptide mapping of the trypsin digest produced a total of 47 to 50 peptide spots. This good agreement between the predicted and actual number of trypsin peptides strongly supports the existence of a molecule containing eight identical subunits. Based on the octamer model, the rabbit liver pyrophosphorylase would be expected to have approximately 14 arginine spots. Peptide mapping after staining indicated 13. The pyrophosphorylase from calf and human enzymes also proved to have values very near to those predicted. The calf enzyme showed 58 to 63 total spots after peptide mapping, compared to 59 predicted from amino acid analysis, while the human enzyme showed 51 to 55 compared to 53 predicted. The 21 arginine spots predicted from the amino acid analysis of the calf pyrophosphorylase were also in good agreement with the 19 arginine spots detected upon peptide mapping. Similarly, agreement is noted with

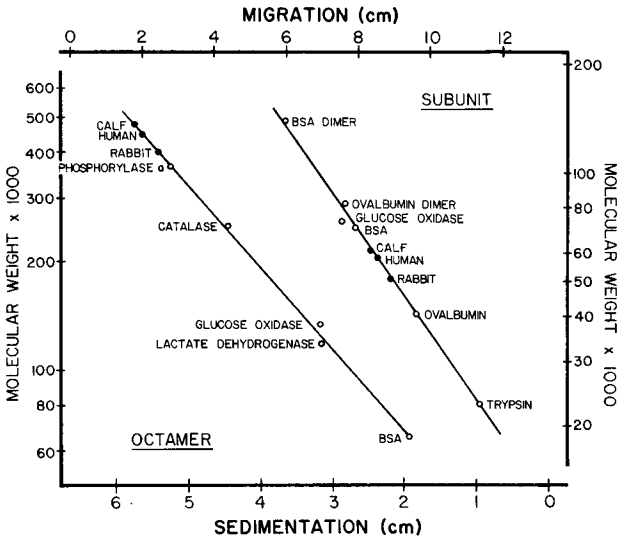

FIG. 2. *Left curve*, semilogarithmic graph of molecular weight of undenatured UDP-glucose pyrophosphorylase (*octamer*) from calf, human, and rabbit liver based on sedimentation rates in sucrose density gradients. *Right curve*, graph of subunit molecular weight of denatured calf, human, and rabbit UDP-glucose pyrophosphorylase based on migration rates upon electrophoresis in polyacrylamide gel containing sodium dodecyl sulfate. *BSA*, bovine serum albumin.

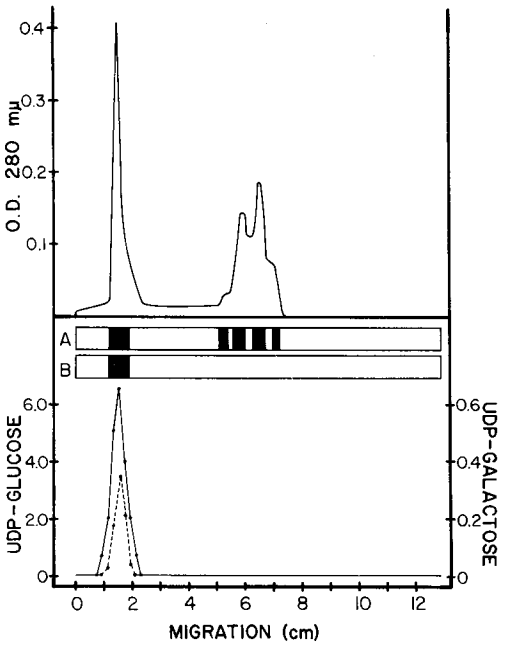

FIG. 3. *A*, electrophoretic profile of crystalline rabbit UDP-glucose pyrophosphorylase on polyacrylamide gel and associated 280-nm absorbance (*above*). *B*, electrophoretic profile of UDP-glucose pyrophosphorylase following purification on sucrose density gradients or by reverse gradient recrystallization. Lower peaks illustrate a graph of enzyme activity for UDP-glucose (——) and UDP-galactose (– – –).

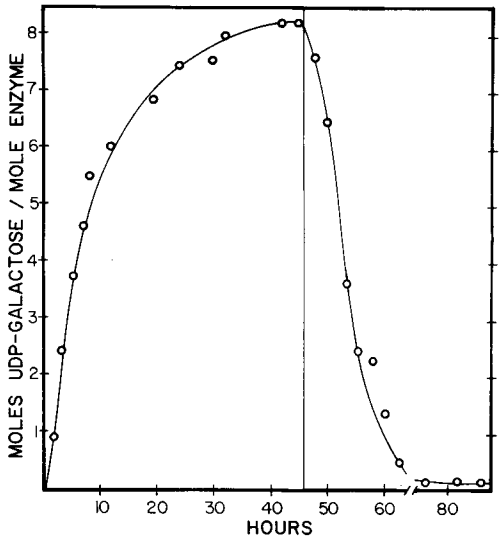

FIG. 4. Substrate binding ratio for rabbit UDP-glucose pyrophosphorylase based on equilibrium dialysis with [^{14}C]UDP-galactose followed by progressive displacement of UDP-galactose with addition of UDP-glucose. At equilibrium, 8.2 moles of UDP-galactose were bound per mole of enzyme. Approximately 98% of the UDP-galactose was displaced by UDP-glucose.

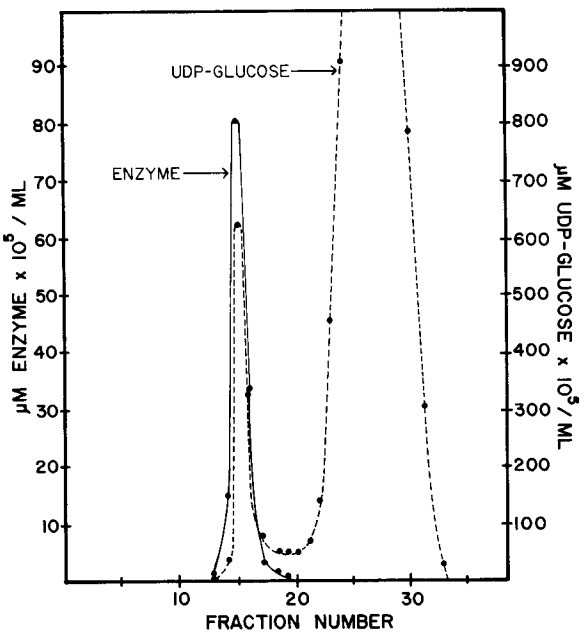

FIG. 5. Elution pattern of UDP-[^{3}H]glucose from Sephadex G-25-80 column following incubation for 15 min at 32° with rabbit UDP-glucose pyrophosphorylase. Incubation mixture contained 0.2 μM of magnesium, 0.272 μM of UDP-[^{3}H]glucose (specific activity 4850 μCi per μM), and 1.8×10^{-3} μM of enzyme dissolved in 0.65 ml of 0.01 M Tricine buffer at pH 8.0. Fractions (1 ml) were collected and assayed for enzyme activity using Assay 1 and counted for radioactivity.

human enzyme (17 predicted *versus* 16 actual). Thus, the model proposed (with eight identical subunits) appears similar for all species. Based on end group analysis, with no attempt made to quantify the results, serine is the only detectable NH_2-terminal amino acid for all three species.

Magnesium appears to be the principal metal cofactor for all three enzymes, although Mn^{2+}, Co^{2+}, and Ca^{2+} do, however, activate the enzyme. The per cent of activity showed by each of these cations, when compared to an optimum concentration of Mg^{2+} taken as 100% is given in Table I. Manganese (1 mM) was approximately as effective as Mg^{2+} for all three liver enzymes. Cobalt, at its optimum concentration, was about 75% as active in calf enzyme and 49% as active in rabbit enzyme as was magnesium. The optimum Co^{2+} concentration for the human enzyme was 1 mM and was 47% as effective as 3 mM magnesium. Calcium at its optimum concentration was less

TABLE I

Comparison of chemical and physical properties of UDP-glucose pyrophosphorylase from calf, rabbit, and human liver

Property	Calf	Rabbit	Human
1. Crystalline shape	Diamond	Tapered rod	Rectangular plate or needle
2. Molecular weight (sucrose gradient)	480,000	400,000	450,000
3. Molecular aggregation	Monomer Dimer Trimer Tetramer	Monomer	Monomer Dimer
4. Subunit mol wt, by sodium dodecyl sulfate acrylamide gels	60,000	50,000	56,000
5. Electron micrograph			
Subunit diameter	50 A	45–50 A	50 A
Subunit arrangement	Two superimpossible tetrads (octameric)		
6. Binding ratio, substrate to enzyme	8 moles	8 moles	8 moles
7. Amino acid analysis			
Lysine residues per mole	289	257	280
Arginine residues per mole	171	118	137
8. Peptide maps, trypsin digest			
Arginine spots	19	13	14–16
Total spots	58–63	47–50	51–55
9. Methonine by cyanogen bromide cleavage	10–11 bands		
10. NH_2-Terminal amino acid	Serine	Serine	Serine
11. Reducing agent	N.R.[a]	Required	Required
12. Metal cofactors Mg^{2+} optimum	1 mM	2 mM	3 mM
Mn^{2+} opt. conc.[b]	1 mM-100%	1 mM-100%	1 mM-96%
Co^{2+} opt. conc.[b]	2 mM-75%	1 mM-49%	1 mM-47%
Ca^{2+} opt. conc.[b]	1 mM-28%	1 mM-50%	2 mM-16%
13. pH optimum	7.0–9.5	7–10.5	7.6–9.2 (2)

[a] N.R., not required.

[b] Per cent of activity compared to optimum concentration of Mg^{2+}, taken as 100%.

TABLE II

Michaelis constants and substrate specificity of uridine diphosphate glucose pyrophosphorylase from liver

	Calf	Rabbit	Human
Michaelis constants ($K_m \times 10^5$)			
UDP-glucose	6.0	6.6	5.0
UTP	20.0	3.8	4.8
Glucose-1-P	5.5	4.6	9.5
PP_i	8.4		21.0
UDP-galactose		42.0	
Equilibrium constants	0.20	0.16	0.15
Specificity (%)			
UDP-glucose	100	100	100
UDP-galactose	3.5	5.9–14.3[a]	2.0–12[a]
UDP-xylose	3.9	4.6	1.5
TDP-glucose	2.7	0.44	2.2

[a] Where concentration of nucleoside base was 10 times the level of UDP-glucose (3.0 mM).

active than Mn^{2+} and Co^{2+}, being 28, 50, and 16% as effective as magnesium with calf, rabbit, and human pyrophosphorylases, respectively. The pyrophosphorylases from the three species had wide pH optimums, with the optimum for the rabbit enzyme ranging from pH 7.0 to 10.5.

Michaelis constants for the pyrophosphorylases are shown in Table II. Of particular interest is the rather low K_m for UDP-galactose. This indicates the affinity of the phosphorylases for UDP-galactose and suggests that they could be easily mistaken as UDP-galactose pyrophosphorylases. UDP-galactose and galactose 1-phosphate are competitive inhibitors of UDP-glucose and glucose 1-phosphate with K_i values of 2.2×10^{-4} and 7.3×10^{-3} (rabbit). The equilibrium constants for the reaction involving UDP-glucose synthesis are also shown in Table II. As expected, good agreement between the species may be noted.

The pyrophosphorylases showed a broad specificity for sugars and nucleoside bases (Table II). Glucose, galactose, and xylose are suitable sugars. UDP-galactose was an adequate substrate at 10 times the concentration of UDP-glucose, showing 14.3% of the activity of UDP-glucose with calf enzyme and 12.0% with human enzyme. This has practical significance in galactosemic individuals where the galactose levels are elevated even when galactose intake is avoided.[1]

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