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URIDINE DIPHOSPHOGLUCOSE PYROPHOSPHORYLASE AND URIDINE DIPHOSPHOGALACTOSE PYROPHOSPHORYLASE IN HUMAN SKIN FIBROBLASTS DERIVED FROM NORMAL AND GALACTOSEMIC INDIVIDUALS

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SUMMARY

UDP-Gal pyrophosphorylase (UTP:α-D-galactose-1-phosphate uridyltransferase, EC 2.7.7.10) and UDP-Glc pyrophosphorylase (UTP:α-D-glucose-1-phosphate uridyltransferase, EC 2.7.7.9) activities have been detected in cultivated human skin fibroblasts and their properties compared. UDP-Glc pyrophosphorylase is more stable at 50 and 56° than UDP-Gal pyrophosphorylase while both enzymes have similar K_m values, pH optima and electrophoretic mobility. On the basis of the difference in thermal stability, it has been suggested that UDP-Gal pyrophosphorylase is an enzyme distinct from UDP-Glc pyrophosphorylase. A possible alternate pathway for the metabolism of galactose in the galactosemic individuals is discussed.

INTRODUCTION

The conversion of galactose to glucose in biological systems has been shown by LELOIR[1] and KALCKAR[2] to be intimately linked with the metabolism of uridine nucleotides. In order to enter the glucose metabolic pathway, α-D-galactose is first converted by galactokinase to α-D-galactose 1-phosphate (Gal-1-*P*). The subsequent conversion of Gal-1-*P* to glucose 1-phosphate (Glc-1-*P*) involves the following reactions.

$$\text{Gal-1-}P + \text{UDP-Glc} \rightleftharpoons \text{Glc-1-}P + \text{UDP-Gal}$$
$$\text{UDP-Gal} \rightleftharpoons \text{UDP-Glc}$$
$$\text{UDP-Glc} + PP_i \rightleftharpoons \text{Glc-1-}P + \text{UTP}$$

The enzymes catalysing these reactions are respectively, Gal-1-*P* uridyltransferase (transferase), UDP-Gal 4-epimerase (epimerase), and UDP-Glc pyrophosphorylase.

Transferase has been shown to be absent in the tissues of patients with galac-

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tosemia[3]. The deficiency of transferase results in an impaired utilization of ingested galactose resulting in the accumulation of Gal-1-*P* in tissues[4]. Small amounts of galactose are metabolized in galactosemics despite the reported absence of transferase. In fact, as these patients get older they appear to develop increased ability to utilize ingested galactose[5]. UDP-Gal pyrophosphorylase discovered by KALCKAR *et al.*[6] in yeast and by ISSELBACKER[7] in mammalian liver may participate in the metabolism of galactose and may be responsible for its increased metabolism. The level of UDP-Gal pyrophosphorylase has been shown to be higher in adult organs as compared to fetal or neonatal livers[8].

Previous studies have shown that skin fibroblasts derived from control individuals, heterozygotes for galactosemia, and galactosemics clearly demonstrate their genotype[9]. In order to investigate the possibility of alternate pathways of galactose metabolism, a comparative study of UDP-Glc pyrophosphorylase and UDP-Gal pyrophosphorylase was carried out on skin fibroblasts derived from normal controls and galactosemic patients. UDP-Glc pyrophosphorylase and UDP-Gal pyrophosphorylase were studied with respect to specific activity, Michaelis–Menten constants, thermal stability, electrophoretic mobility and pH optima. Our studies have shown that these are two distinct enzymes in fibroblasts and that the specific activity of UDP-Gal pyrophosphorylase is about one third that of UDP-Glc pyrophosphorylase.

MATERIALS AND METHODS

Skin biopsies were obtained from three patients with galactosemia and from age-matched controls by techniques previously described[10]. Bits of tissue approx. 2 mm^2 were immobilized under coverslips in 35-mm Petri dishes, containing 2.0 ml of growth medium, nutrient mixture F-10 (ref. 11) containing 15% fetal calf serum, 50 units/ml of penicillin, 50 μg/ml of streptomycin and 25 units/ml fungizone (BBL Cockeysville, Md.) and incubated at 37° in a 5% CO_2 atmosphere. After 2–3 weeks, the cell monolayer was trypsinized and transferred into 75-cm^2 falcon flasks. The medium was changed every 2nd day unless otherwise specified and the fibroblasts were harvested on the 4th or 5th day for enzyme studies. The fibroblasts were washed 2 times with isotonic saline and treated with 3.0 ml of a mixture of 1 part of 0.25% trypsin to 3 parts of 1:5000 versene (Grand Island). The fibroblasts which detach from the surface of the flasks in about 15 min were collected, washed with isotonic saline 3 times, suspended in 1.0 mM triethanolamine buffer (pH 7.8) and ruptured by rapid freezing and thawing. The cell homogenate was spun at 60 000 $\times$ *g* for 30 min and the supernatant used in the studies reported. The enzyme preparation was dialyzed against 1.0 mM triethanolamine buffer (pH 7.8) for kinetic studies.

Growth experiment

Two sets of 500 000 cells per flask were seeded and supplemented with nutrient mixture F-10 containing 15% fetal calf serum. In one set the media was changed daily and in the other set the media was not changed for 168 h. Cells were harvested at 24, 48, 72, 96 and 168 h after seeding, processed and enzyme activity measured as described in Tables I and II.

TABLE I

BIOCHEMICAL PROPERTIES OF UDP-Glc PYROPHOSPHORYLASE OBTAINED FROM NORMAL AND GALACTOSEMIC FIBROBLASTS

Specific activity determinations were carried out at 37° in cuvettes with 1-cm light path. A final volume of 1.0 ml reaction mixture contained in μmoles triethanolamine buffer, 40.0 (pH 7.8); magnesium acetate, 2.0; sodium pyrophosphate, 2.0; dithiothreitol, 1.0; 3-phosphoglycerate, 1.2; NADH, 0.24; UDP-Glc 0.4; 3.0 μl 3-phosphoglycerate kinase (10 mg/ml, Boehringer Mannheim); 1.5 μl of glyceraldehyde-3-phosphate dehydrogenase (2.0 mg/ml Boehringer Mannheim) and 50 μl high-speed supernatant (20–150 μg protein). The assay was started with the addition of the enzyme The rate of oxidation of NADH was recorded for 5–10 min with a Gilford Model 2000 recording spectrophotometer. The blank cuvettes contained water in place of UDP-Glc. The blanks had measurable amounts of NADH oxidation and this rate was subtracted from experimental cuvettes which contained the nucleoside diphosphate sugar. The results are calculated as μmoles of UTP formed/h per mg protein. Michaelis–Menten constant for UDP-Glc at 2.0 mM PP_i was determined by varying the concentration of UDP-Glc from 0.008 to 1.2 mM. The K_m for PP_i at 0.4 mM UDP-Glc was determined by varying concentrations of PP_i from 0.02 to 2.0 mM. The molar concentration of Mg^{2+} was always adjusted to be equal to half the molar concentrations of PP_i when PP_i concentrations were different.

Cell line	*Specific activity (μmoles UTP formed/h per mg protein)*	*K_m of UDP-Glc at 2.0 mM PP_i (mM)*	*Km of PP_i at 0.2 mM UDP-Glc (mM)*
Normal	0.4544 ± 0.0969 (18)	0.1395 ± 0.04614 (8)	0.1800 ± 0.02107 (3)
Galactosemic	0.4601 ± 0.0591 (16)	0.1782 ± 0.01363 (4)	0.204 ± 0.03459 (4)

TABLE II

BIOCHEMICAL PROPERTIES OF UDP-Gal PYROPHOSPHORYLASE OBTAINED FROM NORMAL AND GALACTOSEMIC FIBROBLASTS

Specific activity determinations for UDP-Gal pyrophosphorylase were carried out using the same system described in Table I, by substituting 0.8 μmole UDP-Gal for UDP-Glc and the results expressed as μmoles UTP formed/h per mg protein. K_m for UDP-Gal at 2.0 mM PP_i was determined by varying the concentrations of UDP-Gal from 0.2 to 6.0 mM. The K_m for PP_i was determined at 0.8 mM UDP-Gal concentration. The PP_i concentrations varied from 0.2 to 2.0 mM. The Mg^{2+} concentration was always adjusted to be equal to half the PP_i concentration.

Cell line	*Specific activity (μmoles UTP formed/h per mg protein)*	*K_m of UDP-Gal at 2.0 mM PP_i (mM)*	*Km of PP_i at 0.8 mM UDP-Gal (mM)*
Normal	0.1472 ± 0.030 (18)	0.3711 ± 0.1233 (6)	0.1797 ± 0.0459 (7)
Galactosemic	0.1648 ± 0.0223 (16)	0.474 ± 0.2363 (4)	0.222 ± 0.0485 (3)

Pyrophosphorylase assay

Pyrophosphorylase activity was assayed by a modification of the method of VERACHTERT *et al.*[12] substituting dithiothreitol for hydrazine. The amount of UTP formed by the reaction of pyrophosphorylase on UDP-Glc or UDP-Gal was coupled with excess of 3-phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase in presence of 3-phosphoglycerate and NADH. The reactions are summarized below.

$$\text{UDP-Glc} + PP_i \xrightarrow{\text{Pyrophosphorylase}} \text{Glc-1-}P + \text{UTP}$$

$$\text{UDP-Gal} + PP_i \xrightarrow{\text{Pyrophosphorylase}} \text{Gal-1-}P + \text{UTP}$$

$$\text{UTP} + \text{3-}P\text{-glycerate} \xrightleftharpoons{\text{3-phosphoglycerate kinase}} \text{1,3-diphosphoglycerate} + \text{UDP}$$

$$\text{1,3-Diphosphoglycerate} + \text{NADH} \xrightleftharpoons{\text{Glyceraldehyde-3-phosphate dehydrogenase}} \text{glyceraldehyde-3-}P + \text{NAD}^+ + \text{P}_\text{i}$$

The rate of oxidation of NADH is a quantitative measure of the UTP formed.

pH–activity studies

In order to establish the pH optimum of UDP-Glc pyrophosphorylase and UDP-Gal pyrophosphorylase, assays were carried out with both substrates as described in Tables I and II substituting 0.1 ml of 0.4 M triethanolamine buffer of pH values from 6.5 to 9.0.

Michaelis–Menten constants

The Michaelis–Menten constants of the enzymes for UDP-Glc and UDP-Gal were carried out at 2.0 mM pyrophosphate concentration. The K_m for pyrophosphate was determined at 0.4 mM UDP-Glc and 0.8 mM UDP-Gal concentrations.

Thermal stability

Thermal stability of UDP-Glc pyrophosphorylase and UDP-Gal pyrophosphorylase was carried out by incubating 0.4 ml of enzyme preparation (2.0 mg/ml protein) at 50 and 56° for 0, 5, 10 and 15 min. The samples were chilled in an ice bath immediately after heat inactivation and assays with UDP-Glc and UDP-Gal were performed on each sample as described in Tables I and II.

Starch gel electrophoresis

Electrophoreses of pyrophosphorylases were carried out with the vertical starch gel system of SMITHIES[13]. The gels were prepared in 0.05 M potassium phosphate buffer (pH 7.0). The buffer trays contained 0.1 M potassium phosphate buffer (pH 7.0). The gels were chilled for 3 h at 4° before the application of the sample. Approx. 200 μg protein in 50 μl each were applied to the slots, sealed with petrolatum and electrophoresis was carried out at 4° at 4 V/cm for 18 h.

RESULTS

In Tables I and II are shown the results of the comparative studies of both enzymes in normal and galactosemic cell lines with respect to specific activities and Michaelis–Menten constants. The specific activities of UDP-Glc pyrophosphorylase and UDP-Gal pyrophosphorylase are very similar in normal and galactosemic cell lines. The activities of both enzymes are not influenced by subculturing the fibroblasts up to 50 passages. The ratio of UDP-Glc pyrophosphorylase to UDP-Gal pyrophosphorylase is 3:1 in normal and galactosemic cells at all phases of growth and subculturing. In the growth experiment studies normal and galactosemic cell lines exhibited the same kind of growth patterns with respect to specific activity of both enzymes. There was no significant change in the specific activity of both enzymes from 0 to 168 h after seeding the cells. Daily change of the growth medium did not have any effect on the activity of either enzyme.

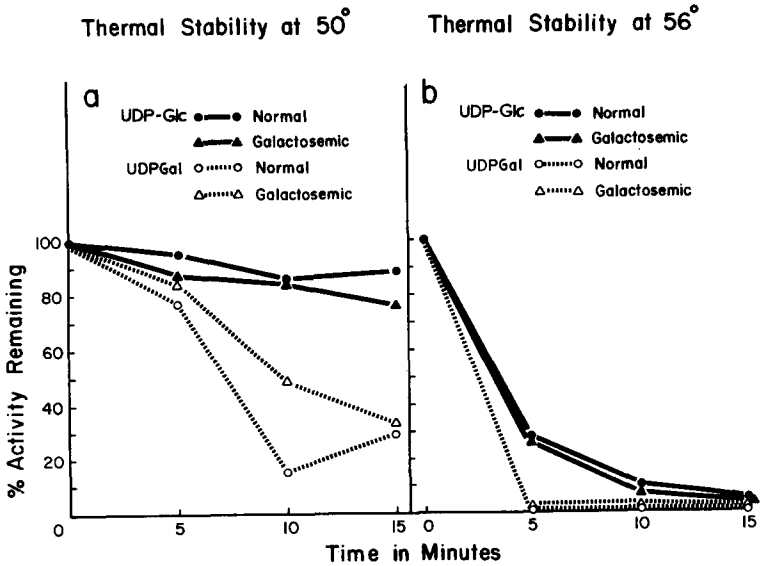

Fig. 1a and 1b. Thermal stability of UDP-Glc pyrophosphorylase and UDP-Gal pyrophosphorylase at 50 and 56°. The high-speed supernatant from normal and galactosemic fibroblasts were adjusted to contain 2.0 mg/ml protein. 0.4 ml of the preparation was then incubated at 50 or 56° for 0, 5, 10 and 15 min. The activity of these samples was then tested using UDP-Glc or UDP-Gal as substrate as described in Tables I and II. The percent activity remaining at the end of each incubation is plotted against the time of incubation. Each point represents the mean of three experiments performed in duplicate.

The Michaelis–Menten constants of UDP-Glc pyrophosphorylase obtained either by the Lineweaver–Burk plot or by Hanes plot for UDP-Glc at 2.0 mM PP_i and for PP_i at 0.4 mM UDP-Glc are similar for normal and galactosemic fibroblasts. The K_m values obtained for UDP-Gal pyrophosphorylase for UDP-Gal and for PP_i were also similar for both normal and galactosemic fibroblasts. Increasing the concentration of UDP-Glc to 4.0 mM and UDP-Gal to 8.0 mM did not have any appreciable inhibitory effect on their respective enzymes.

The results of heat inactivation studies of UDP-Glc pyrophosphorylase and UDP-Gal pyrophosphorylase of normal and galactosemic fibroblasts at 50 and 56° for various periods of time are shown in Figs. 1a and 1b. After 15 min incubation at 50° UDP-Glc pyrophosphorylase retained approx. 80–90% of its activity while UDP-Gal pyrophosphorylase retained only 30%. Heat treatment at 56° inactivated UDP-Gal pyrophosphorylase completely by the end of 5 min whereas UDP-Glc pyrophosphorylase retained about 25% activity. Continued heat treatment up to 15 min at 56° destroyed UDP-Glc pyrophosphorylase completely. Normal and galactosemic fibroblasts behaved in the same way to heat inactivation.

Specific activities of UDP-Glc pyrophosphorylase and UDP-Gal pyrophosphorylase of normal and galactosemic fibroblasts with respect to pH was performed in 0.4 M triethanolamine buffer from pH values 6.8–9.0. UDP-Glc pyrophosphorylase and UDP-Gal pyrophosphorylase of normal and galactosemic fibroblasts showed similar broad pH optima between pH 7.3 and 8.6 without any sharp peak.

Fig. 2 represents the electrophoretic mobility of UDP-Glc pyrophosphorylase and UDP-Gal pyrophosphorylase on starch gel at pH 7.0. Both normal and galac-

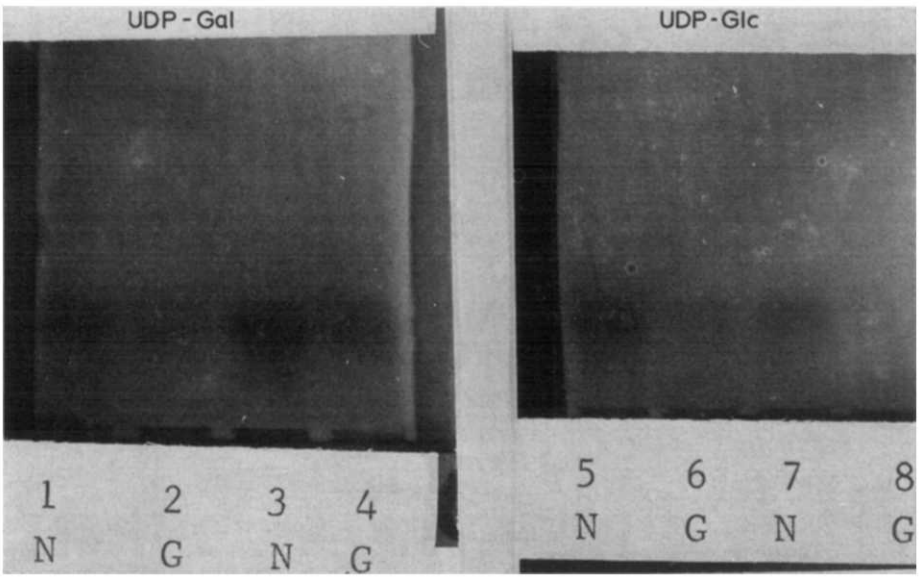

Fig. 2. Starch gel electrophoresis of UDP-Glc pyrophosphorylase and UDP-Gal pyrophosphorylase. Electrophoresis was carried out as described in MATERIALS AND METHODS. After electrophoresis the gels were sliced into two halves, one side stained for UDP-Glc pyrophosphorylase and the other side for UDP-Gal pyrophosphorylase. The staining mixture in a total volume of 10.0 ml contained 11.0 mg of UDP-Glc or UDP-Gal, 2.0 ml of 0.4 M triethanolamine buffer (pH 7.8), 17 mg PP_i, 1.9 mg NADH, 3.0 mg dithiothreitol, 4.3 mg magnesium acetate, 4.0 μg 3-phosphoglycerate, 0.1 ml of 3-phosphoglycerate kinase (10 mg/ml suspension) and 0.1 ml of glyceraldehyde-3-phosphate dehydrogenase (2.0 mg/ml suspension). This mixture was kept at 37°. 40 ml of 10% starch solution in 0.01 M triethanolamine buffer (pH 7.8) was brought to 45° and the reaction mixture poured and mixed into the starch, layered over the cut surface of the gel, and incubated at room temperature for 90 min. Nonfluorescent bands on a fluorescent background were visualised under long wave ultraviolet light. N, normal fibroblast supernatant; G, galactosemic fibroblast supernatant.

tosemic fibroblasts have one band each migrating toward the anode when UDP-Glc or UDP-Gal is used as a substrate. There was no difference in the distance migrated by UDP-Gal pyrophosphorylase or UDP-Glc pyrophosphorylase.

DISCUSSION

These data clearly demonstrate that fibroblasts derived from normal controls and galactosemic patients possess UDP-Glc pyrophosphorylase and UDP-Gal pyrophosphorylase. The specific activity of UDP-Glc pyrophosphorylase is approx. 3 times higher than UDP-Gal pyrophosphorylase.

Specific activity determinations after heat inactivation of the enzymes at 50 and 56° suggest that in cultivated fibroblasts these are two distinct enzymes. This interpretation is made despite the fact that the K_m values for UDP-Glc and UDP-Gal were of the same order of magnitude, the activity with respect to pH, and migration on starch gel at pH 7.0 were similar for both enzymes. Purification and separation of the two enzymes were not attempted because of the requirement for large quantities of fibroblasts. Attempts by other investigators[7,14,15] to separate UDP-Glc pyrophos-

phorylase and UDP-Gal pyrophosphorylase activity from purified preparations of the enzymes have not been very successful.

The demonstration of the presence of UDP-Gal pyrophosphorylase in normal and galactosemic fibroblasts and the fact that the UDP-Gal pyrophosphorylase can function with 30–35% efficiency with respect to UDP-Glc pyrophosphorylase supports the hypothesis that an alternate pathway of galactose metabolism exists in galactosemic patients. The Gal-1-*P* formed by the galactokinase can react with UTP in presence of UDP-Gal pyrophosphorylase to form UDP-Gal and PP_i and thus bypass the transferase step. The epimerase present in the fibroblasts will convert the UDP-Gal to UDP-Glc and thus the galactose moiety can enter the glucose pathway. The specific activity of UDP-Glc pyrophosphorylase is about twice that of the transferase enzyme in fibroblasts[16]. It has previously been shown that cultivated fibroblasts from galactosemics and normals contain appreciable amounts of UTP[17]. The observations that in fibroblasts UDP-Gal pyrophosphorylase has about one third of the activity of UDP-Glc pyrophosphorylase and that UDP-Glc pyrophosphorylase is twice more active than transferase is important because the ratios of these enzymes are very different in other tissues. For example, the ratio of UDP-Glc pyrophosphorylase to UDP-Gal pyrophosphorylase in calf liver has been shown to be 70:1 (ref. 15), while the ratio of transferase to UDP-Gal pyrophosphorylase is 100:1 in human liver[18] and about 1000:1 in rat liver[19].

Only a few reports of possible alternate pathways of galactose metabolism in man have been published. CUATRECASAS AND SEGAL[20,21] suggested that liver contains an enzyme, galactose dehydrogenase which catalyses the conversion of galactose directly to galactonic acid. A microsomal hexose-6-phosphate dehydrogenase which can convert galactose 6-phosphate to 6-phosphogalactonic acid has also been described[22,23]. Our studies of UDP-Gal pyrophosphorylase in fibroblasts indicate that the possible alternate operative pathway is by the UDP-Gal pyrophosphorylase reaction. Investigations are underway to isolate and compare the glucose derivatives on galactosemic and normal cells grown in galactose-containing medium.

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