THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 250, No. 10, Issue of May 25, pp. 3595-3601, 1975
Printed in U.S.A.

Galactose-6-phosphate Dehydrogenase

PURIFICATION AND PARTIAL CHARACTERIZATION

(Received for publication, November 15, 1974)

MANJU RAY AND AMAR BHADURI

From the Division of Biochemistry, Department of Pharmacy, Jadavpur University, Calcutta-700032, India

SUMMARY

A new enzyme, galactose-6-phosphate dehydrogenase has been purified about 50-fold from goat liver. The enzyme can be distinguished from the nonspecific hexose-6-phosphate dehydrogenase and glucose-6-phosphate dehydrogenase by its high substrate specificity and absolute pyridine nucleotide requirement. In contrast to the hexose-6-phosphate dehydrogenase, this enzyme is located exclusively in the cytoplasmic fraction of the cell. The enzyme is a metalloprotein and is highly sensitive to mercurials. The product of the reaction is possibly a ketoaldose, phosphorylated at the primary alcoholic group.

We have recently reported the presence of a new enzyme in mammalian liver that specifically oxidizes galactose 6-phosphate in the presence of NAD (1, 2), galactose 6-phosphate has not yet been shown as a normal metabolite for any tissue or organism. Inouye *et al.* (3) claimed that the compound accumulated in red blood cells under galactosemic conditions but this has been disputed by Ng (4). In 1966, Ohno *et al.* (5) and Shaw (6) detected the presence of a dehydrogenase in mammalian liver which could oxidize both glucose 6-phosphate and galactose 6-phosphate quite efficiently in the presence of both NAD and NADP. This enzyme was later found to be identical with the well known microsomal glucose dehydrogenase (7). Because of the much lower K_m values for both galactose 6-phosphate and glucose 6-phosphate in comparison to glucose, glucose dehydrogenase was renamed as hexose-6-phosphate dehydrogenase. Because both hexose-6-phosphate dehydrogenase and galactose-6-phosphate dehydrogenase could utilize galactose 6-phosphate as a substrate in the presence of NAD, a detailed study regarding the differences in property and the separate identity of the two enzymes was in order. Along with the above aspect of the problem, this paper is also concerned with a general characterization of the new enzyme, galactose-6-phosphate dehydrogenase.

MATERIALS AND METHODS

All of the biochemicals used for this work were purchased from Sigma Chemical Co. The mixed bed resin, Rexyn 300 was purchased from Fisher Scientific Co. The sodium salt of galactose 6-phosphate was assayed for any possible contamination of alcohol with alcohol dehydrogenase. The compound was found to be virtually free of alcohol. This precaution was taken to avoid any possible confusion that might arise because of the presence of alcohol dehydrogenase in our enzyme preparations (8, 9). Calcium phosphate gel was prepared according to the method of Keilin and Hartree (10). The reducing value of the sugars was determined by the method of Nelson (11). Estimation of inorganic phosphate was carried out following the method of Lowry and Lopez (12). In purified enzyme fractions, protein was determined by the method of Warburg and Christian (13). For some other samples the method of Lowry *et al.* (14) was used.

Galactose-6-phosphate dehydrogenase was routinely assayed spectrophotometrically. The usual assay mixture contained in a total volume of 1 ml, 100 μmol of potassium phosphate buffer, pH 7.6, and 0.5 μmol of NAD. The reaction was started by the addition of 1 μmol of galactose 6-phosphate. The rate remained linear for more than 5 min and could be very conveniently measured by following the increase in absorbance at 340 nm. One unit of the enzyme was defined as the amount of enzyme needed for the formation of 1 μmol of NADH per min. The optimum pH of the enzyme was found to be around 8.4, when the enzyme was assayed with glycylglycine buffer. But at this pH there was often a nonspecific increase in absorbance which complicated the accurate determination of the rate. For some experiments however, the phosphate buffer for the routine assay was replaced either by 100 μmol of glycylglycine buffer, pH 8.4, or by glycine buffer, pH 9.6, which is mentioned in the appropriate places in the text.

For the assay of the nonspecific hexose-6-phosphate dehydrogenase, galactose 6-phosphate was not normally used as the substrate. The enzyme was usually assayed in a total volume of 1 ml, containing 100 μmol of potassium phosphate buffer, pH 7.4, and 1 μmol of NAD. The reaction was started by the addition of 500 μmol of glucose and the rate of increase in absorbance at 340 nm was followed over a period of 5 min. Control blanks without glucose were simultaneously run. Assay of the enzyme at pH values over 9 often posed some problems because of a rapid increase in absorbance even in the absence of the substrate. This was also noted by Beutler and Morrison (7). In such cases, the enzyme could be conveniently assayed in the presence of NADP because the nonspecific increase in absorbance was much smaller when NADP was used instead of NAD. Lactic dehydrogenase and glucose-6-phosphate dehydrogenase were assayed following the standard methods described in Refs. 15 and 16. Glyoxalase I was assayed according to the method of Racker (17).

RESULTS

Purification of Enzyme—Since our preliminary communication on this enzyme (1), the purification procedure has been somewhat modified and extended. The enzyme being highly sensitive to the presence of ethylenediaminetetraacetate, it was omitted from the extraction medium. Unless otherwise stated all of the operations were carried out at 5–6°. A typical purification procedure with goat liver proceeded as follows. Twelve grams of freshly frozen goat liver were taken into 36 ml of 0.02 M sodium phosphate buffer, pH 7.4. The liver was homogenized in a Waring Blendor

for 60 s. The homogenized suspension was centrifuged at 12,000 × *g* for 30 min. The supernatant was now treated with a solution of protamine sulfate (2% w/v) until the volume of added protamine sulfate was 15% of the original volume of the supernatant. After standing for 10 min the precipitate was centrifuged at 12,000 × *g*. The protamine sulfate-treated supernatant was now subjected to fractionation with the increasing ammonium sulfate concentration. Solid, recrystallized ammonium sulfate was slowly added to the supernatant with constant stirring until the amount of the ammonium sulfate present, as determined at 0°, was 35% of its saturating concentration. The accumulated precipitate was rejected and to the supernatant another quantity of ammonium sulfate was slowly added until 55% saturation was obtained. The centrifuged precipitate was collected and redissolved in 5 ml of 0.02 M sodium phosphate buffer, pH 7.4. The solution was dialyzed for 6 hours with one change against the same buffer. Two milliliters of the dialyzed solution containing approximately 40 mg/ml of protein was then treated with calcium phosphate gel. Two milliliters of the gel (16 mg dry weight per ml of solution) was lightly centrifuged and the centrifuged gel was added to 2 ml of the dialyzed enzyme solution. The gel was mixed with stirring for 3 min and then centrifuged at 2,000 × *g*. The enzyme was not absorbed on the gel and was further purified on a DEAE-cellulose column.

Coarse mesh DEAE-cellulose, purchased from Sigma Co. was thoroughly washed. About 2 g of washed DEAE-cellulose was used to prepare a column of about 22 cm in height with diameter of 1.6 cm. The column was first equilibrated with 0.02 M sodium phosphate buffer, pH 7.4 One milliliter of enzyme solution containing 0.35 unit of galactose-6-phosphate dehydrogenase activity and 20 mg of protein was now absorbed on the column and the column was then eluted with increasing concentrations of sodium phosphate buffer of the same pH. The rate of flow was maintained at 15 drops/min and 2.5 ml of the eluted sample was collected in each tube. The column was eluted with 40 ml of each of 0.02 M, 0.04 M, 0.07 M, and 0.1 M of sodium phosphate buffer, pH 7.4. The enzyme which was unabsorbed in the column usually came out between tubes 7 to 10. Two to three tubes containing about 80% of the total eluted activity were now combined and the enzyme was reprecipitated with the addition of ammonium sulfate. The enzyme which precipitated between 40 to 60% of saturating ammonium sulfate concentration was redissolved in 1 ml of 0.02 M sodium phosphate buffer, pH 7.4. When kept frozen at −10° the enzyme was found to be stable for 3 to 4 days. Because the spectrophotometric assay could not be used on the crude extract, the yield and purification at this stage is expressed in terms of the total activity present in the supernatant fraction after protamine sulfate treatment. The final yield over the protamine sulfate fraction was usually between 50 to 55% and the enzyme was purified about 30-fold over this fraction. The respective yield and purification during the various stages of purification are presented in Table I.

Separation from Hexose-6-phosphate Dehydrogenase—During the purification of the enzyme, the hexose-6-phosphate dehydrogenase activity was also assayed in the various fractions. More than 60% of the total activity of this enzyme was precipitated between 0 to 35% of the saturating concentration of ammonium sulfate. The remaining activity was ultimately completely separated from the galactose-6-phosphate dehydrogenase on the DEAE-cellulose column (Fig. 1). Hexose-6-phosphate dehydrogenase which was measured in terms of the glucose dehydrogenase activity was eluted with 0.07 M sodium phosphate buffer between tubes 38 to 42. This enzyme fraction, as expected, also showed activity when assayed with galactose 6-phosphate as substrate and NAD or NADP as the hydrogen acceptor. The rate of oxidation of galactose 6-phosphate however was considerably slower than in case of glucose indicating that in goat liver at least glucose is much more efficiently oxidized than the hexose 6-phosphates by this enzyme. In contrast to this enzyme fraction, the fraction containing galactose-6-phosphate dehydrogenase activity failed to catalyze oxidation of glucose under different conditions of pH and glucose concentrations.

Substrate Specificity—The purified enzyme from the goat liver obtained after the second ammonium sulfate treatment was found to be completely free of glucose-6-phosphate dehydrogenase and hexose-6-phosphate dehydrogenase. The enzyme was found to be absolutely specific both for galactose 6-phosphate and for NAD. Our previous report that NADP could partially replace NAD (1) was shown not to be correct with this purified enzyme. A large number of sugars and sugar phosphates including glucose, galactose, fructose, mannose, glucose 6-phosphate, mannose 6-phosphate, glucosamine 6-phosphate, glucose 1-phosphate, galactose 1-phosphate, and fructose 6-phosphate were tested both at pH 7.4 and 9.6 and with NAD and NADP as the potential electron acceptors. No significant increase in absorbance at 340 nm was detected with any of these compounds as the substrate.

The clear difference between galactose-6-phosphate dehydrogenase and the nonspecific hexose-6-phosphate dehydrogenase was also evident when the enzyme preparations were obtained from rat liver. The two enzymes from the rat liver could be separated by basically following the same procedure as that

TABLE I

Purification of galactose-6-phosphate dehydrogenase from goat liver

Step	Total activity	Total protein	Yield	Specific activity
	units	*mg*		*units/mg protein* × 10^3
Crude				
Protamine sulfate	2.43	600	100	0.40
Ammonium sulfate	2.33	280	96	0.83
Calcium phosphate gel	2.18	126	90	1.73
DEAE-cellulose column	1.41	14	58	10.10
Second ammonium sulfate	1.24	8.4	51	14.5

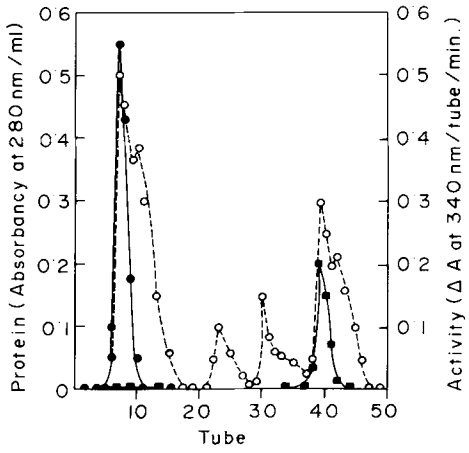

FIG. 1. Separation of galactose-6-phosphate dehydrogenase and hexose-6-phosphate dehydrogenase on DEAE-cellulose column. O- - -O indicates the elution pattern of the proteins from the column. ●——●, denotes the activity of galactose-6-phosphate dehydrogenase in various tubes. The activity of hexose-6-phosphate dehydrogenase is indicated ■——■.

developed for the goat liver. Ten grams of freshly excised rat liver were homogenized in 30 ml of 0.02 M potassium phosphate buffer, pH 7.4. The suspension was centrifuged at 12,000 × *g* for 30 min. The crude homogenate was treated with protamine sulfate exactly in the same manner as described for goat liver. The supernatant obtained after the protamine sulfate treatment was separated into two fractions of 0 to 40% and 40 to 55% of saturating concentration of ammonium sulfate. The precipitates obtained from both these fractions after centrifugation were separately redissolved in 5 ml each of 0.02 M sodium phosphate buffer pH 7.4. Hexose-6-phosphate dehydrogenase was precipitated completely in the 0 to 40% fraction whereas galactose-6-phosphate dehydrogenase was obtained almost exclusively in the 40 to 55% fraction. The difference in substrate specificity between these two fractions at two different pH values and using both NAD and NADP as the potential oxidants is shown in Table II. The substrate specificity of the 0 to 40% fraction followed very closely the substrate specificity obtained for the hexose-6-phosphate dehydrogenase in rat liver by Beutler and Morrison (7). In contrast, the other fraction containing galactose-6-phosphate dehydrogenase could oxidize galactose 6-phosphate alone only in presence of NAD, thus clearly indicating the separate identity of galactose-6-phosphate dehydrogenase from the nonspecific hexose-6-phosphate dehydrogenase in the rat liver system.

pH Optimum—Galactose-6-phosphate dehydrogenase was found to be active over a wide range of pH. The enzyme could be conveniently assayed at pH 7.4 in 0.1 M sodium phosphate buffer or around pH 9.2 in glycine-sodium hydroxide buffer. Using glycylglycine buffer of varying pH and saturating concentration of the substrate, the optimum pH of the enzyme was determined to be 8.4 (Fig. 2). When glycylglycine buffer was replaced by Tris-hydrochloric acid buffer, the rate was found to be considerably lower though the nature of the pH curve remained basically the same.

Determination of K_m—The Michaelis constant for galactose 6-phosphate was determined for the enzyme at pH 7.4 with phosphate buffer and at pH 8.4 with glycylglycine buffer. The substrate saturation curve followed the usual Michaelis-Menten kinetics at both the pH values. The K_m when plotted according to the method of Lineweaver and Burk came out to be 1.5 mM at pH 7.4 when the concentration of NAD was kept at a fixed level of 1 mM (Fig. 3). No significant variation in the value of K_m was observed with the change of pH. At a fixed concentration of galactose 6-phosphate (1 mM), the K_m for NAD was calculated to be 0.6 mM at pH 7.4 (Fig. 4).

Localization of Enzyme—For the determination of the localization of the enzyme, freshly excised goat liver was homogenized and fractionated according to a modified method of Schneider and Hogeboom (18). The excised liver was first cut into small pieces. About 2 g of the liver were suspended in 14 ml of 0.25 M sucrose containing 0.02 M sodium phosphate buffer, pH 7.4. The cells were homogenized in a Potter-Elvehjem type glass homogenizer with a glass pestle. The operation was repeated four times, so that a total of 8 g of liver were homogenized in a total volume of about 60 ml. The nuclear fraction consisting of nuclear material, whole cells, and cell debris was obtained by centrifuging the homogenate for 20 min at 600 × *g*. For washing, the precipitated mass was resuspended in 5 ml of the same medium and

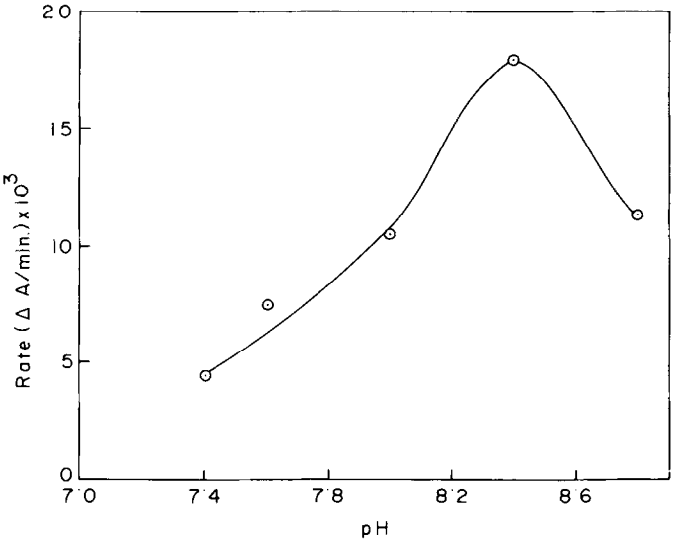

FIG. 2. Galactose-6-phosphate dehydrogenase activity as a function of pH. Enzyme activities were determined in 0.1 M glycylglycine buffer of varying pH containing 2.8 mM of galactose 6-phosphate, 1 mM of NAD, and requisite amount of galactose-6-phosphate dehydrogenase.

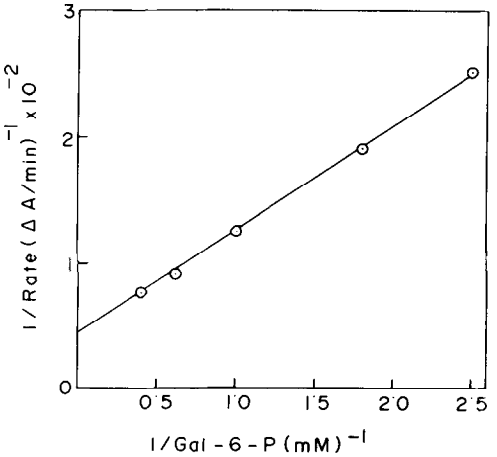

FIG. 3. Determination of apparent Michaelis constant for galactose 6-phosphate. Each assay mixture contained in a total volume of 1 ml, 100 μmol of sodium phosphate buffer, pH 7.4, 1 μmol of NAD, requisite amount of purified galactose-6-phosphate dehydrogenase, and varying amounts of galactose 6-phosphate. Reciprocal of the rate was plotted against the reciprocal of galactose 6-phosphate concentration.

TABLE II

Differences between galactose-6-phosphate dehydrogenase and hexose-6-phosphate dehydrogenase from rat liver in relation to specificity towards substrates

The method for obtaining the two fractions are described in the text.

Substrate	Concentration	Coenzyme	pH	Activity (ΔA/min) × 10³	
				Galactose-6-phosphate dehydrogenase	Hexose-6-phosphate dehydrogenase
	mM				
Galactose 6-phosphate..	1.0	NAD	7.4	22.5	4.3
Galactose 6-phosphate..	1.0	NAD	9.6	20.0	6.0
Galactose 6-phosphate..	1.0	NADP	7.4	0.0	9.8
Galactose 6-phosphate..	1.0	NADP	9.6	0.0	15.0
Glucose.....	500.0	NAD	7.4	0.0	20.0
Glucose.....	500.0	NAD	9.6	1.2	18.0

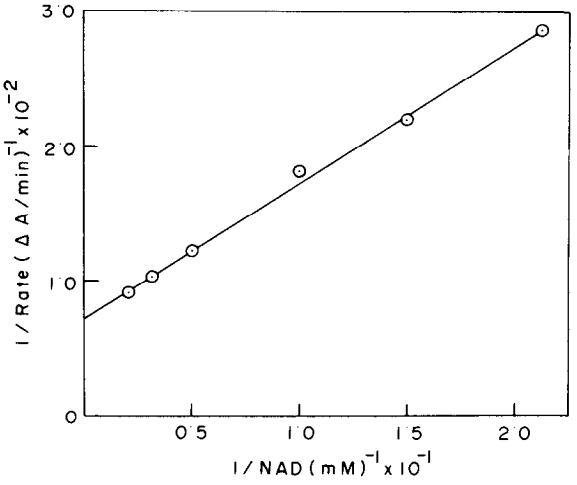

FIG. 4. Determination of apparent Michaelis constant for NAD. Each assay mixture contained in a total volume of 1 ml, 100 μmol of sodium phosphate buffer, pH 7.4, 1 μmol of galactose 6-phosphate, requisite amount of purified galactose-6-phosphate dehydrogenase and varying amounts of NAD. Reciprocal of the rate was plotted against the reciprocal of NAD concentration.

recentrifuged at the same speed. The supernatant, after the separation of the nuclear fraction, was centrifuged at 1000 × *g* for 20 min and the precipitate collected at this stage was rejected. The supernatant of 1000 × *g* was then centrifuged for 10 min at 9000 × *g*. The precipitate collected at this stage was washed twice in the same medium and at the same speed. The washed fraction was designated as the mitochondrial fraction. The supernatant of 9000 × *g* was further centrifuged at 20,000 × *g* for 20 min to remove any unwanted mitochondria still remaining in the supernatant. The supernatant from this treatment was centrifuged at 100,000 × *g* for 40 min. The pellet collected at this stage was washed once in the same medium and was presumed to contain the microsomal fraction. The supernatant after 100,000 × *g* centrifugation was designated as the soluble supernatant. The soluble supernatant was fractionated with ammonium sulfate in two portions. Both of the precipitates obtained with 0 to 38% and 38 to 55% saturation of ammonium sulfate were redissolved in 3 ml of 0.02 M sodium phosphate buffer, pH 7.4. The washed nuclear, mitochondrial, and microsomal fractions were finally disrupted by freezing and thawing four times in 5 ml of 0.02 M sodium phosphate buffer containing 0.5% sodium cholate. The disrupted nuclear and mitochondrial fractions were finally centrifuged at 40,000 × *g* for 20 min. The microsomal fraction was centrifuged at 100,000 × *g* for 20 min. Both galactose-6-phosphate dehydrogenase and the hexose-6-phosphate dehydrogenase were assayed in the clear supernatants of the various fractions. The hexose-6-phosphate dehydrogenase activity was assayed with glucose (500 mM) and NAD (1 mM) as substrates at pH 7.4. The results of such an experiment are shown in Table III. Galactose-6-phosphate dehydrogenase appears to be almost exclusively a cytoplasmic enzyme as almost 90% of the total activity is present in the soluble supernatant fraction. The localization of the hexose-6-phosphate dehydrogenase is generally consistent with the earlier report of the localization of this enzyme (7).

Metal Ion Involvement—During the purification of galactose-6-phosphate dehydrogenase, we observed that the presence of even low concentrations of ethylenediaminetetraacetate rapidly destroyed the enzyme activity. On addition of ethylenediaminetetraacetate at 5 mM concentration, the enzyme was almost

TABLE III

Localization of galactose-6-phosphate dehydrogenase and hexose-6-phosphate dehydrogenase in subcellular fractions from goat liver

Fraction	Galactose-6-phosphate dehydrogenase			Hexose-6-phosphate dehydrogenase		
	Specific activity	Total activity	Per cent of total activity	Specific activity	Total activity	Per cent of total activity
	(*unit/mg*) × 10^2	*units*		(*unit/mg*) × 10^2	*units*	
Nuclear	0.11	0.10	2.3	0.41	0.37	1.55
Mitochondrial	0.10	0.10	2.3	0.24	0.27	1.13
Microsomal	0.18	0.31	7.2	12.40	20.60	86.20
Soluble supernatant						
35% ammonium sulfate	0.19	0.30	7.2	1.64	1.94	8.2
35–55% ammonium sulfate	1.53	3.4	81.0	0.40	0.72	3.0

immediately deactivated by about 40%. When the enzyme was preincubated for 10 min in presence of ethylenediaminetetraacetate at 10 mM concentration, the activity was completely lost (Table IV, A). To check if the inactivated enzyme could be reactivated in presence of specific metal ions, the inactivated enzyme was precipitated with 70% saturation of ammonium sulfate and redissolved in 0.02 M glycine-sodium hydroxide buffer, pH 8.4. The inactive enzyme was then dialyzed against the same buffer for 4 hours and then tested for reactivation with various metal ions. A large number of metal ions were used between 5 mM to 50 mM concentrations but except for Ca^{+} none of the metal ions were found to restore the activity even partially (Table IV, B). Ca^{2+} ion at a concentration of 10 mM restored about one-third of the original activity. Increasing the concentration of Ca^{2+} however did not significantly further increase the activity of the inactivated enzyme. Addition of Zn^{2+} at 10 mM concentration led to some nonspecific precipitation of proteins. These were removed by centrifugation. The clean supernatant was found to be devoid of any activity. Fresh addition of Zn^{2+} (10 mM) in the assay medium failed to restore any enzyme activity.

Involvement of Sulfhydryl Group—The enzyme was also found to be highly sensitive to the treatment of *p*-chloromercuribenzoate (Table V). The enzyme could also be inactivated with iodoacetate but the concentration of iodoacetate needed was considerably higher than in the case of *p*-chloromercuribenzoate. Incubation of the *p*-chloromercuribenzoate-treated inactive enzyme with mercaptoethanol for 10 min could fully restore the activity of the enzyme. Total inactivation at very low concentrations of *p*-chloromercuribenzoate and complete reversal of this effect with mercaptoethanol suggest that one or more specific sulfhydryl groups might be involved in the active site.

Partial Characterization of Products—The enzymatic reaction catalyzed by galactose-6-phosphate dehydrogenase is not accompanied by any cleavage of the ester linkage of galactose 6-phosphate. Thus, when the enzymatic reaction was allowed to proceed for over 30 min in glycine buffer, pH 8.8, until about 25% of the initial galactose 6-phosphate was converted to its oxidized product, assay of an aliquot at that stage revealed that the value of the total organic phosphate remained constant. Moreover, release of no inorganic phosphate could be detected in the incubation medium.

Formation of 1:4 NADH during the course of this reaction was confirmed by reoxidizing completely and quantitatively the

TABLE IV

Involvement of metal ion for catalytic activity of enzyme

For this experiment, the enzyme was prepared by replacing the usual sodium phosphate buffer with 0.02 M glycylglycine buffer, pH 7.4. The complete system for A contained in a total volume of 1 ml, 100 μmol of glycine buffer, pH 8.8, 0.5 μmol of NAD, and the requisite amount of the enzyme. The reaction was started with the addition of 1 μmol of galactose 6-phosphate. For experiments in B, 0.15 unit of the enzyme was incubated for 10 min in a total volume of 1 ml containing 30 μmol of glycylglycine buffer, 7.4, and 10 μmol of ethylenediaminetetraacetate. The complete inactivation of the enzyme was checked by assaying an aliquot. To remove excess ethylenediaminetetraacetate, the inactive enzyme was then precipitated with 70% saturation of ammonium sulfate and the precipitate was redissolved in 1 ml of 0.02 M glycylglycine buffer, pH 7.4. This process was repeated once more. The complete (inactive) system for this set contained in a total volume of 1 ml, 100 μmol of glycine buffer, pH 8.8, 0.5 μmol of NAD, and the requisite amount of the ethylenediaminetetraacetate-free inactive enzyme. A control was run simultaneously.

Addition	Activity
	%
A. Complete	100
+ Ethylenediaminetetraacetate (5 mM)	59
+ Ethylenediaminetetraacetate (10 mM)	35
+ Ethylenediaminetetraacetate (10 mM) After 10 min	0
B. Control	100
Complete (inactive)	0
+ Mg^{2+} (10 mM)	0
+ Mn^{2+} (10 mM)	0
+ Na^{+} (10 mM)	0
+ K^{+} (10 mM)	0
+ Fe^{3+} (10 mM)	0
+ Cu^{2+} (10 mM)	0
+ Zn^{2+} (10 mM)	0
+ Ca^{2+} (5 mM)	20
+ Ca^{2+} (10 mM)	30
+ Ca^{2+} (50 mM)	35
+ Ca^{2+} (100 mM)	42

TABLE V

Involvement of sulfhydryl group for enzyme activity

The complete system contained in a total volume of 1 ml, 100 μmol of sodium phosphate buffer, pH 7.4, 0.5 μmol of NAD, and the requisite amount of the enzyme. The reaction was started with the addition of 1 μmol of galactose 6-phosphate.

Additions	Activity
	%
Complete	100
+ *p*-Chloromercuribenzoate	
(5×10^{-3} mM)	74
(2×10^{-2} mM)	30
(5×10^{-2} mM)	0
(5×10^{-2} mM)	100
+ Mercaptoethanol (10 mM)	
Complete	100
+ Iodoacetate	
(1 mM)	83
(2 mM)	65

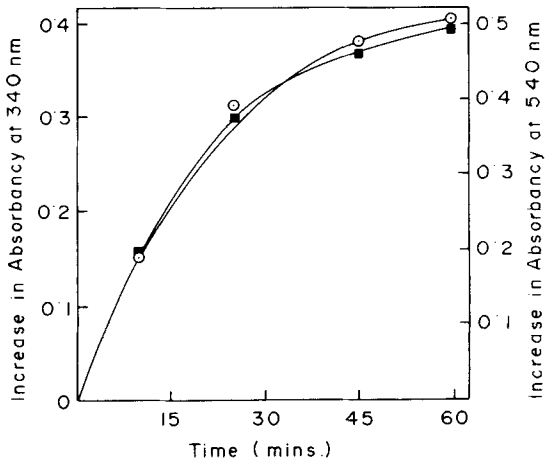

FIG. 5. Correspondence between NADH formation and increased reducing value. For this experiment, the change in absorbance at 340 nm was measured in a cuvette that contained 100 μmol of sodium phosphate buffer, pH 7.4, 1 μmol of NAD, and 1 μmol of galactose 6-phosphate. The readings were taken at fixed intervals of time as is indicated in the figure. For the determination of reducing value several tubes were set up with the same composition. The enzymatic reactions were stopped in each tube at different times with alkaline copper sulfate solution and put on a water bath. ⊙——⊙ and ■——■ indicate increase in absorbance at 340 nm and absorbance at 540 nm.

NADH formed with pyruvate and the lactic dehydrogenase system (15).

During the oxidation of galactose 6-phosphate, the free aldehydic group remained unaffected as was evident by the increased reducing value of the total incubation medium. The formation of NADH over a period of time closely corresponded with the increase in reducing value (Fig. 5). A control for this experiment was simultaneously run with the glucose-6-phosphate dehydrogenase system which showed, as expected, a progressive decrease in the reducing value with increasing time. Because the enzymatic oxidation led to an increase in the reducing value, the formation of an additional center of oxidation should be assumed. Further, because there was no liberation of inorganic phosphate, it may be presumed that a ketoaldose phosphorylated at C_6 position is the product of the reaction. Confirmation of the generation of a product having the properties of a reducing sugar containing a keto group was obtained by treatment of the product with alkaline phosphatase and subsequent characterization by paper chromatography and by color reactions.

For the chromatographic characterization of the product, the following procedure was adopted. 0.03 unit of purified galactose-6-phosphate dehydrogenase was added to a total volume of 1 ml, containing 100 μmol of sodium phosphate buffer, pH 7.4, 2 μmol of galactose 6-phosphate, and 1 μmol of NAD. About 0.75 μmol of the product was allowed to form as measured by the increase in absorbance at 340 nm. Contents of five such tubes were pooled. Along with these incubation mixtures, five control tubes were run which contained all of the reagents except NAD. Contents of these control tubes were collected together and were subsequently treated in the same manner as the experimental sample. After adjusting the pH of the solution to 9, 30 units of alkaline phosphatase were added and the reaction was allowed to proceed for 2 hours at 37°. The protein was removed by trichloroacetic acid precipitation and centrifugation. Mixed bed resin was added batchwise to the clear supernatant until the pH of the supernatant corresponded to the pH of the distilled water. The solution was evaporated to dryness under reduced pressure at room temperature. The dried material was dissolved in 0.1 ml of water. Ten microliters of the solution were now spotted on What-

man No. 1 paper. In separate lanes the following were simultaneously spotted, (*a*) 10 μl of the control sample, (*b*) 10 μl of a mixture containing 15 μg each of glucose, galactose, and fructose, (*c*) all of the reference sugars individually in separate lanes. Several chromatograms were developed with two solvent systems. Solvent A consisted of ethylacetate-pyridine-water in 8:2:1 proportions (v/v) and Solvent B consisted of glacial acetic acid-isopropyl alcohol-water in 3:1:1 proportions (v/v). After 18 to 20 hours of descending chromatography, chromatograms were developed in each case with the following spraying reagents, (*a*) alkaline silver nitrate (19), (*b*) *p*-aminobenzoic acid (20), and (*c*) α-napthoresorcinol (21). Assuming the distance travelled by glucose to be 1.00 (R_{Glc}), both the experimental and the control sample showed with both the solvents one spot which corresponded with known galactose. The experimental sample however, showed a faster moving spot with both of the solvents which was absent in the case of the control sample. The R_{Glc} value of this faster moving spot was 2.0 and 1.2 for Solvent A and Solvent B, respectively. Under identical conditions, the R_{Glc} values for galactose and fructose were found to be 0.81 and 1.37 in Solvent A and 0.93 and 1.08 in Solvent B. *p*-Aminobenzoic acid and α-napthoresorcinol are known to be generally specific for reducing sugars. Moreover, oxidation of the sugar at C_1 position would have resulted in the adsorption of the product on the resin.

Color reaction tests specific for ketoses were also carried out. For this, the product of the enzymatic reaction was formed in the same way as for chromatographic separation except that 0.3 μmol of galactose 6-phosphate was taken in each tube to ensure complete conversion of galactose 6-phosphate into the product. Control samples without NAD were also incubated in this case. After treatment with alkaline phosphatase and the resin, aliquots from the experimental and control tubes, each containing about 25 to 40 μg of reducing sugar were subjected to cysteine-carbazole (22) and resorcinol (23) color reactions. Control reactions were also carried out for tubes containing separately, 100 μg of galactose, 50 μg of fructose, and 100 μg of methylglyoxal. After 10 min of color development with cysteine-carbazole reaction, only tubes containing fructose and the product showed the typical pink colors for the keto group. Methylglyoxal developed blue color whereas tubes containing galactose and the control showed the color of the blank. The absorbance at 560 nm with 1-cm light path for fructose and the product were 0.650 and 0.095, respectively. The nature of the spectrum for the two samples were also identical between 500 and 600 nm. When similar color reactions were carried out with resorcinol (23), galactose and the control failed to develop any color but the tubes containing fructose, the product, and methylglyoxal developed pink colors. The absorbance at 520 nm for these tubes was 0.26, 0.09, and 0.04, respectively. In this case, also the nature of the spectrum for fructose and the product were almost identical. The positive color tests with cysteine-carbazole and resorcinol indicated the possible presence of a keto group in the molecule.

The ketoaldose nature of the phosphorylated product raised the possibility that this compound could serve as the substrate for the glyoxalase enzyme system. This was tested with glyoxalase I alone, following the method of Racker (17). About 0.2 μmol of the product was first formed in a total volume of 1 ml containing 100 μmol of sodium phosphate buffer, pH 7.4, 0.4 μmol of galactose 6-phosphate, excess galactose-6-phosphate dehydrogenase, and limiting amounts (0.2 μmol) of NAD. Two-tenths milliliter of this aliquot containing about 0.04 μmol of the product was now transferred to an assay mixture of 1 ml containing 100 μmol of sodium phosphate buffer, pH 7.0, and 3.5 μmol of glutathione. Absorbance of this incubation medium at 240 nm was 1.030. On addition of 2 μl of glyoxalase I, containing 2 units of activity, no increase in absorbance could be observed for 4 min. When 0.2 μmol of methylglyoxal was however added to the system, a very rapid increase in absorbance was noted. The product therefore apparently failed to react with glutathione in the presence of glyoxalase I. Further, addition of excess glyoxalase I and glyoxalase II to the product in the presence of catalytic amounts of glutathione failed to show any decrease in the total reducing value. Under identical conditions, with methylglyoxal as the substrate, a significant decrease in the reducing value was observed at the end of a 30-min incubation period.

DISCUSSION

Galactose has been shown to be uniformly catabolized in a large variety of cell types through the Leloir pathway described in Ref. 24. The only other route of galactose metabolism that has been characterized in detail, is the one operative in *Pseudomonas saccharophilia* (25, 26). There is, however, evidence in literature that suggests that an alternate weaker pathway may also be operative in the mammalian systems. Thus, certain galactosemic individuals were shown to have the ability to metabolize considerable quantities of galactose (27, 28). Further, Segal *et al.* (29) reported that some galactosemic individuals were able to convert carbon 1 of intravenously administered galactose to carbon dioxide to quite a large extent. Using [1-^{14}C]galactose, these workers also showed that galactose is metabolized to a significant extent in galactosemic liver when compared to a normal liver control (30). More recently, Petricciani *et al.* (31) have shown that galactosemic fibroblasts produce $^{14}CO_2$ from [1-^{14}C]galactose, but this does not begin until after 4 days of incubation. Radioautography of cultured, human galactosemic, and normal cells also suggests the possible existence of an alternate pathway for galactose metabolism (32). The existence of UDP-galactose pyrophosphorylase discovered by Isselbacher (33) is not adequate enough to explain these data as the rate of conversion of galactose 1-phosphate to UDP-galactose by this enzyme is not very significant (34). The possibility that hexose-6-phosphate dehydrogenase might be involved in the supposed auxiliary pathway was initially suggested by Beutler and Morrison (7) but has subsequently been ruled out by these authors on the basis of both physiological (35) and biochemical (36) experiments. Thus, the product of hexose-6-phosphate dehydrogenase reaction with galactose 6-phosphate as substrate is 6-phosphogalactonic acid which is a dead-end product of metabolism (36). As for galactose-6-phosphate dehydrogenase, there is no absolutely direct evidence at the moment to suggest that this enzyme might be involved in an auxiliary pathway of galactose metabolism. However, we have observed that UDP-glucose or UDP-galactose specifically inhibits the enzyme at low concentrations of the substrate (2). In any case, if the product is a phosphorylated ketoaldose and not 6-phosphogalactonic acid, the possibility cannot be excluded that the enzyme lies on an alternate pathway for the catabolism of galactose.

We have already pointed out that the presence of galactose 6-phosphate as a normal metabolite has not yet been unequivocally demonstrated (3, 4). Galactose 6-phosphate has been shown to be formed from galactose 1-phosphate by phosphoglucomutase in *in vitro* systems by Posternak and Resselet (37) and also by Lowry and Passonneau (38). The rate of formation of galactose 6-phosphate in such artificial systems is however too small to

suggest any possible metabolic role for the enzyme in synthesizing galactose 6-phosphate in cellular systems. It is therefore not unlikely that a specific enzyme may be present in mammalian liver for phosphorylating galactose in the C_6 position. Whether galactose-6-phosphate dehydrogenase along with other undetected enzymes leads to an alternate path in mammalian liver remains to be established.

REFERENCES

1. Ray, M., Pal, D. K., and Bhaduri, A. (1972) *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **25,** 239
2. Ray, M., and Bhaduri, A. (1973) *Ninth Int. Congr. Biochem. Abstr.* p. 55
3. Inouye, T., Tannenbaum, M., and Hsia, D. Y.-Y. (1962) *Nature* **193,** 67
4. Ng, W. G. (1964) Ph.D. thesis, University of Southern California, University Microfilm Incorporated, Los Angeles
5. Ohno, S., Morrison, M., and Beutler, E. (1966) *Science* **153,** 1015
6. Shaw, C. R. (1966) *Science* **153,** 1013
7. Beutler, E., and Morrison, M. (1967) *J. Biol. Chem.* **242,** 5289
8. Beutler, E. (1967) *Science* **156,** 1516
9. Shaw, C. R., and Koen, A. L. (1965) *J. Histochem. Cytochem.* **13,** 431
10. Keilin, D., and Hartree, E. F. (1938) *Proc. Roy. Soc. London Biol. Sci.* **124,** 397
11. Nelson, N. (1944) *J. Biol. Chem.* **153,** 375
12. Lowry, O. H., and Lopez, J. A. (1946) *J. Biol. Chem.* **162,** 421
13. Warburg, O., and Christian, W. (1940) *Biochem. Z.* **310,** 384
14. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265
15. Kornberg, A. (1955) *Methods Enzymol.* **1,** 441
16. Horecker, B. L., and Smyrniotis, P. Z. (1955) *Methods Enzymol.* **1,** 323
17. Racker, E. (1951) *J. Biol. Chem.* **190,** 685
18. Schneider, W. C., and Hogeboom, G. H. (1950) *J. Biol. Chem.* **183,** 123
19. Trevelyan, W. E., Procter, D. P., and Harrison, J. S. (1950) *Nature* **166,** 444
20. Heftmann, E. (1962) *Chromatography,* Reinhold Publishing Corporation, p. 507
21. Isherwood, F. A., and Jermyn, M. A. (1951) *Biochem. J.* **48,** 515
22. Dische, Z., and Borenfreund, E. (1951) *J. Biol. Chem.* **192,** 583
23. Roe, J. H., Epstein, J. H., and Goldstein, N. P. (1949) *J. Biol. Chem.* **178,** 839
24. Kalckar, H. M. (1958) *Physiol. Rev.* **20,** 111
25. De Ley, J., and Duodoroff, M. (1957) *J. Biol. Chem.* **227,** 745
26. Ornston, L. N. (1971) *Bacteriol. Rev.* **35,** 87
27. Cusworth, D. C., Dent, C. E., and Flynn, F. V. (1955) *Arch. Dis. Childhood* **30,** 155
28. Komrower, G. M., Schwarz, V., Holzel, A., and Goldberg, L. (1956) *Arch. Dis. Childhood* **31,** 254
29. Segal, S., Blair, A., and Topper, Y. J. (1962) *Science* **136,** 150
30. Topper, Y. J., Laster, L., and Segal, S. (1962) *Nature* **196,** 1006
31. Petricciani, J. C., Binder, M. K., Merril, C. R., and Geier, M. R. (1972) *Science* **175,** 1368
32. Hill, H. Z., and Puck, T. T. (1973) *Science* **179,** 1136
33. Isselbacher, K. J. (1958) *J. Biol. Chem.* **232,** 429
34. Abraham, H. D., and Howell, R. R. (1969) *J. Biol. Chem.* **244,** 546
35. Mandula, B., Srivastava, S. K., and Beutler, E. (1970) *Arch. Biochem. Biophys.* **141,** 155
36. Srivastava, S. K., and Beutler, E. (1969) *J. Biol. Chem.* **244,** 6377
37. Posternak, T., and Resselet, J. P. (1954) *Helv. Chim. Acta* **37,** 246
38. Lowry, O. H., and Passonneau, J. V. (1969) *J. Biol. Chem.* **244,** 910