Purification and Properties of Galactokinase from Pig Liver

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1. Galactokinase has been purified from the liver of young pigs by high-speed centrifugation, chromatography on Sephadex G-100 and DEAE-cellulose, and ammonium sulphate fractionation. 2. The enzyme preparation has a specific activity of 10–18 μmoles of galactose phosphorylated/mg. of protein/min. at 37° and has been purified 400-fold from the liver supernatant. 3. Purified liver galactokinase has Michaelis constants of 1×10^{-4}–3×10^{-4}M for galactose and 2×10^{-4}M for ATP–Mg^{2+}, and the enzyme reaction produces equimolar amounts of galactose 1-phosphate and ADP. 4. Galactokinase phosphorylates 2-deoxygalactose and galactosamine in addition to galactose, has a pH optimum of 7·8, a Q_{10} of 2, and is stimulated by cysteine and other thiols. 5. With the exception of substrate specificity, the properties of liver galactokinase are similar to galactokinase purified from yeast and *Escherichia coli*.

Kosterlitz (1943) isolated galactose 1-phosphate from the liver of rabbits that had been fed with galactose and on the basis of this result he postulated the existence of galactokinase (EC 2.7.1.6). The enzyme was detected in yeast (Caputto, Leloir & Trucco, 1948; Wilkinson, 1949) in liver and brain (Cardini & Leloir, 1953), in erythrocytes (Schwartz, Goldberg, Komrower & Holzel, 1956) and in *Escherichia coli* (Cardini, 1951). The enzyme catalyses the following reaction:

$$\text{ATP} + \text{galactose} \rightleftharpoons \text{ADP} + \text{galactose 1-phosphate}$$

Galactokinase has been partially purified from *E. coli* (Sherman & Adler, 1963) and from yeast (Heinrich, 1964).

In the present paper a 400-fold purification of galactokinase from young pig liver is described, and some of the properties of the purified enzyme are disclosed.

MATERIALS AND METHODS

Chemicals. ATP, ADP, NADH, phosphoenolpyruvate, *N*-acetyl-D-galactosamine, 6-deoxy-D-galactose, 6-deoxy-L-galactose, galactose 6-phosphate, galactose 1-phosphate and DEAE-cellulose (capacity 0·9 mg./g., fine mesh) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Pyruvate kinase, lactate dehydrogenase, 2-deoxygalactose, 2-deoxyglucose, galactosamine and dithiothreitol were purchased from Calbiochem, Los Angeles, Calif., U.S.A. Amberlite CG-400 (100–200 mesh) was obtained from Mallinckrodt Chemical Works, New York, N.Y., U.S.A.; Sephadex G-100 and G-200 (bead forms) were from Pharmacia, Uppsala, Sweden. [1-^{14}C]Galactose was from The Radiochemical Centre, Amersham, Bucks.

Radioactivity. Measurements were made with a Packard scintillation detector (model 314EX) with the solvent system of Herberg (1960).

Protein. Protein was measured by the u.v. method of Warburg & Christian (1941).

Galactokinase assay. ADP formed in the galactokinase reaction from ATP and galactose was measured spectrophotometrically at 340 mμ with phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase and NADH by the rate of oxidation of NADH. The assay is similar to that used by Heinrich (1964). Each cuvette contained 300 μmoles of triethanolamine (adjusted to pH 7·5 with 2N-HCl), 100 μmoles of KCl, 15 μmoles of cysteine hydrochloride (adjusted to pH 7 with 2N-NaOH), 1 unit each of pyruvate kinase and lactate dehydrogenase, 1 μmole of NADH, 2 μmoles of phosphoenolpyruvate, 2 μmoles of galactose, 2 μmoles of ATP (mixed with 2 μmoles of $MgCl_2$ and adjusted to pH 7·5 before addition) and enzyme. The total volume was 3·0 ml. and the reaction was measured at 37°; 50 μmoles of NaF were also added to the reaction mixture in all assays before DEAE-cellulose chromatography to inhibit an adenosine-triphosphatase activity in the extract. This activity was separated during Sephadex chromatography. In these assays the galactokinase activity was obtained by subtracting the blank activity in the absence of galactose. When this correction was made the extinction change was linear with time for at least 10 min. and proportional to the amount of enzyme preparation added. One unit of galactokinase activity is defined as the amount of enzyme that will catalyse the formation of 1 μmole of ADP/min. at 37°.

The assay shows a complete requirement for galactose, lactate dehydrogenase, pyruvate kinase, phosphoenolpyruvate, ATP, $MgCl_2$ and enzyme. The enzyme activity was stimulated by cysteine (see Table 2) and slightly increased by KCl. Assays of galactokinase with 1, 2 and 3 μg. of purified enzyme show linearity of reaction rate with enzyme concentration (Fig. 1).

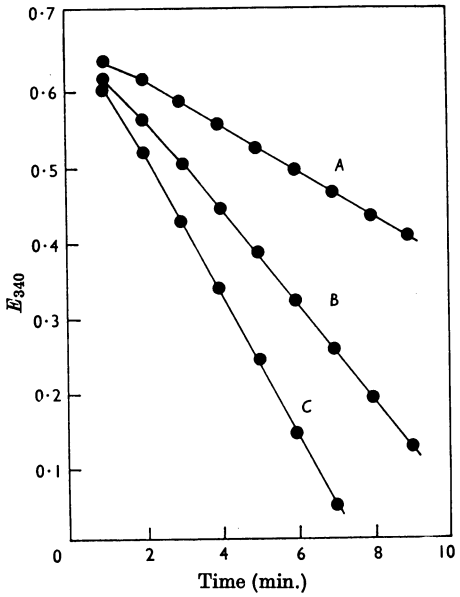

Fig. 1. Assay of galactokinase with the purified enzyme. The extinction change at 340mμ was followed spectrophotometrically. The extinction changes were 0·032, 0·063 and 0·095/min. for 1, 2 and 3μg. of enzyme (curves *A*, *B* and *C* respectively).

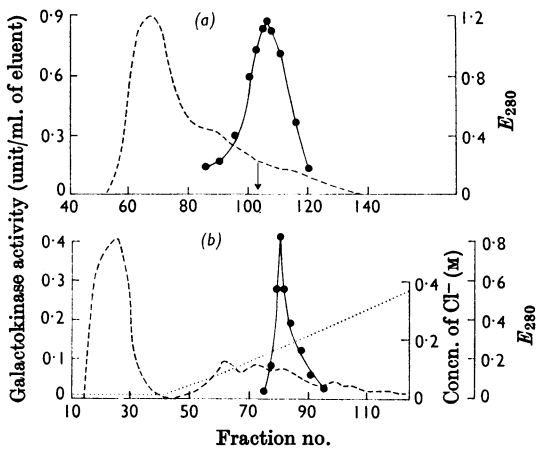

Fig. 2. (*a*) Chromatography of a pig-liver extract on a Sephadex G-100 column. The extinction at 280mμ (----) and the activity of galactokinase (●—●) are indicated. The arrow shows the elution volume of haemoglobin as determined by the extinction at 412mμ. (*b*) Chromatography of the eluate from Sephadex G-100 on DEAE-cellulose. The extinction at 280mμ (----), the activity of galactokinase (●—●) and the concentration of chloride (····) are indicated. In both experiments the fraction volume was 4·5ml. Additional experimental details are given under steps (3) and (4) of the purification procedure.

RESULTS

Purification of galactokinase

(1) *Choice of animals.* Preliminary experiments indicated that the activity of galactokinase was very low and difficult to measure in liver from adult rats. Both young rats and young pigs have higher liver galactokinase activity but pigs were chosen as more tissue could be obtained. The pigs (2 weeks old) were killed within 5hr. of the time they were taken from the sow.

(2) *Preparation of liver extract.* A pig was killed by decapitation and the liver placed in a solution containing: tris (50mM), potassium chloride (150mM), magnesium chloride (5mM) and β-mercaptoethanol (10mM) (adjusted to pH7·5 with 2N-hydrochloric acid) at 4°. All the following steps in the purification were carried out at 0–4°. A 50g. portion of liver was homogenized in 50ml. of the above solution with coaxial homogenizers. The homogenate was centrifuged at 105000*g* for 1hr. in a Spinco model L centrifuge. The supernatant (volume approx. 40ml.) was fraction I.

(3) *Chromatography on Sephadex G-100.* The Sephadex was suspended in a solution (solution A) containing: tris (20mM), galactose (1mM), EDTA (4mM), magnesium chloride (5mM) and β-mercaptoethanol (10mM) (adjusted to pH7·5 with 2N-hydrochloric acid) and poured into a column as described by Flodin (1961) to give a bed of 16cm.^{2} × 45cm. At least 1l. of solution A was passed through the column before the sample was applied. The supernatant from step (2) was passed on to the column and washed into the resin bed with solution A as described by Flodin (1961). The column was eluted with solution A, 5ml. fractions being collected at a flow rate of 1·5–2ml./min. Those fractions containing galactokinase activity (Fig. 2) were pooled to give fraction II. Sephadex G-200 may be used for this step but Sephadex G-100 is preferred since the flow rate is greater.

(4) *Chromatography on DEAE-cellulose.* The resin was suspended in solution A and poured in the same way as for Sephadex to give a column with bed dimensions 4·9cm.^{2} × 25cm. No pressure was applied to the column either in the preparation or in the chromatography. The column was washed with 1l. of 10mM-EDTA and then 1l. of solution A. The combined fractions from Sephadex G-100 chromatography were applied to the column and washed into the DEAE-cellulose with 100ml. of solution A. The galactokinase was eluted with an approximately linear gradient of solution A containing 0–1M-potassium chloride at a flow rate of 1·5–2ml./min. The gradient volume was 700ml. The enzyme was eluted between 0·2M- and 0·3M-

Table 1. *Purification of galactokinase from pig liver*

A summary of the purification procedure for galactokinase from 50g. of pig liver is shown. The details of the assay of galactokinase are given in the Materials and Methods section.

Fraction	Volume (ml.)	Total activity (units)	Total protein (mg.)	Specific activity (units/mg. of protein)	Purification factor	Recovery (%)
I: 105000*g* supernatant from pig liver	38	50·3	1420	0·035	1	100
II: combined fractions from Sephadex G-100	55	28·4	127	0·22	6·4	56
III: combined fractions from DEAE-cellulose	48	9·6	6·9	1·4	40	19
VI: 40–60% saturation $(NH_4)_2SO_4$ precipitate	2	7·6	0·5	15·2	435	15

Table 2. *Effect of thiols on galactokinase activity*

Enzyme activity was measured by ADP formation as described in the Materials and Methods section, except that cysteine was omitted and replaced by the additions mentioned below.

Addition	Concn. (mM)	Relative activity
None	–	1·0
Cysteine	5	3·4
	1	2·4
Glutathione	5	2·9
	1	2·3
β-Mercaptoethanol	5	2·8
	1	1·1
Ethanethiol	5	1·4
	1	1·1
Dithiothreitol	5	2·4
	1	1·9

potassium chloride (Fig. 2). Those fractions containing galactokinase activity were pooled to give fraction III.

(5) *Ammonium sulphate fractionation.* Solid ammonium sulphate was added to fraction III to bring the solution to 40% saturation. The precipitate was centrifuged off and discarded. The precipitate formed after bringing the solution to 60% saturation was collected and suspended in saturated ammonium sulphate (fraction IV). In suspension the enzyme loses about 20% of the enzyme activity in a week at 0–4°.

The purification procedure should not take longer than 24hr. if a reasonable yield of enzyme is required. The enzyme has been purified 250–500-fold and has a specific activity of 10–18 units/mg. of protein. A sample purification is shown in Table 1.

Properties of the purified galactokinase

Activation by thiols. Table 2 shows the activation of galactokinase by cysteine, glutathione, β-mercaptoethanol, ethanethiol and dithiothreitol. Activation was greatest with cysteine and least with

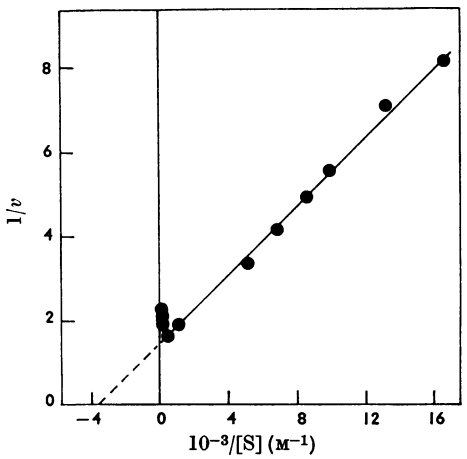

Fig. 3. Effect of galactose concentration on the activity of galactokinase. The data are plotted by the method of Lineweaver & Burk (1934). This experiment gives K_m $2·8 \times 10^{-4}$M for galactose. At a galactose concentration of 40mM, the activity is 0·7 of the extrapolated maximum velocity. The velocity units are arbitrary.

ethanethiol. All the thiol reagents gave greater stimulation at a concentration of 5mM than at 1mM.

Effect of pH *on activity.* The pH optimum for galactokinase in the triethanolamine buffer is about 7·8. Below pH 7·2 and above pH 8·7 the enzyme activity was considerably lower than at the optimum pH. In this experiment 10 units each of pyruvate kinase and lactate dehydrogenase were used in the assays to prevent these enzymes from becoming rate-limiting at extreme pH.

Effect of temperature on activity. The enzyme activity was measured over the temperature range 20–40°. From these experiments the energy of activation for the reaction was calculated to be 12·4kcal./mole and the Q_{10} for ADP production about 2.

Substrate specificity. The K_m for galactose phosphorylation was between 1×10^{-4} and 3×10^{-4}M (Fig. 3). Inhibition by substrate occurs at

galactose concentrations greater than 2mM. Of the other substrates tested only 2-deoxygalactose and galactosamine showed any activity with the enzyme preparation. Both these compounds, which are different structurally from galactose at C-2, have $V_{max.}$ greater than galactose and Michaelis constants about 7–9 times as large (Table 3). No enzyme activity could be detected with mannose, fructose, glucose, 2-deoxyglucose or 6-deoxy-D-galactose and 6-deoxy-L-galactose at concentrations of 30mM, or galactose 1-phosphate, galactose 6-phosphate, lactose and *N*-acetylgalactosamine at concentrations of 3mM as substrates.

The effect of ATP concentration at equimolar concentrations of magnesium chloride is shown in Fig. 4. From this Lineweaver–Burk plot the K_m for MgATP was calculated to be $1{\cdot}9 \times 10^{-4}$M.

Product of galactokinase reaction. To a galactokinase assay mixture as described in the Materials and Methods section but containing 0·45μmole of [1-^{14}C]galactose with no additional galactose, there was added 0·1 unit of purified galactokinase. The extinction was followed until the reaction had almost stopped, to measure the ADP formed, and the assay mixture added to 30μmoles of galactose 1-phosphate and 20μmoles of galactose 6-phosphate and immediately placed on a column of Amberlite CG-400 (100–200 mesh) prepared in the borate form with bed dimensions 1cm.^{2} × 35cm. as described by Diedrich & Anderson (1961). The column was eluted with 900ml. of 0·1M-sodium tetraborate, pH 9·3, followed by 250ml. of a linear pH gradient to 0·1M-sodium tetraborate adjusted to pH 8·5 with 2N-sulphuric acid, and finally with 300ml. of this solution at pH 8·5 (Diedrich & Anderson, 1961). Carbohydrate was determined by the cysteine–sulphuric acid method as described by Diedrich & Anderson (1961). This system separates galactose 1-phosphate from galactose, galactose 6-phosphate, glucose 1-phosphate, glucose 6-phosphate and fructose 6-phosphate. All the radioactivity originating in [^{14}C]galactose was found in galactose 1-phosphate (95%, equivalent to 0·43μmole) and in remaining galactose (Fig. 5).

Stoicheiometry of the galactokinase reaction. Different amounts (0·18, 0·22 and 0·36μmole) of galactose were added to assay mixtures together with 0·1 unit of galactokinase and the reactions followed spectrophotometrically until completion. As shown in Table 4, 1μmole of ADP was formed

Table 3. *Substrate specificity of galactokinase*

Enzyme activity was determined as described in the Materials and Methods section. The rates of galactokinase action on galactose, 2-deoxygalactose and galactosamine are compared at the maximum velocity determined by extrapolation of Lineweaver–Burk plots. This extrapolation was necessary because galactokinase shows substrate inhibition at high galactose concentrations.

	Relative rate	K_m (mM)
Galactose	100	0·28
2-Deoxygalactose	128	2·4
Galactosamine	124	1·9

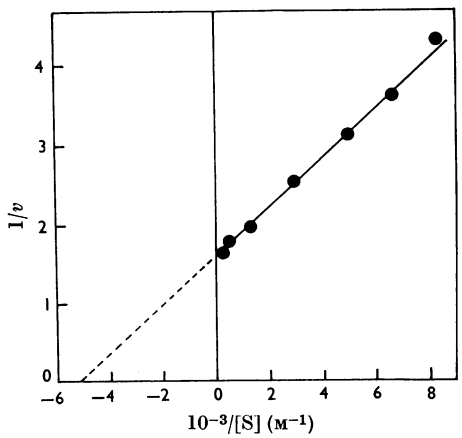

Fig. 4. Lineweaver–Burk plot of the effect of ATP concentration on galactokinase. This experiment gives K_m $1{\cdot}9 \times 10^{-4}$M for MgATP. The assay conditions are identical to those described in the Materials and Methods section except that the concentration of ATP and $MgCl_2$ were varied at a molar ratio of 1. The velocity units are arbitrary.

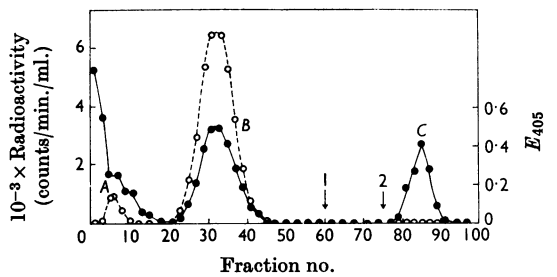

Fig. 5. Identification of galactose 1-phosphate as the product of the enzyme reaction. [1-^{14}C]Galactose was incubated in an assay system as described under 'Properties of the purified galactokinase'. After the reaction was nearly completed the reaction mixture was added to 30μmoles of galactose 1-phosphate and 20μmoles of galactose 6-phosphate and chromatographed on a column of Amberlite CG-400 (borate form). The column was eluted and the radioactivity (○) and carbohydrate (E_{405}, ●) were detected as described in the text. The pH gradient was started at arrow 1 and completed at arrow 2. Peak *A* is [^{14}C]galactose, *B* is galactose 1-phosphate and the ^{14}C-labelled product, and *C* is galactose 6-phosphate. Each fraction had a volume of 15ml.

Table 4. *Stoicheiometry of ADP formation*

In Expts. 1, 2 and 3, 0·18, 0·22 and 0·36 μmole of galactose, and in Expt. 4, 0·45 μmole of [1-^{14}C]galactose, were added to the assay system together with 0·1 unit of purified galactokinase. The amount of ADP formed was calculated from the extinction change at 340 mμ. The galactose 1-phosphate produced and the galactose remaining in Expt. 4 were determined by liquid-scintillation assays of the radioactivity eluted from a column of Amberlite CG-400 (see Fig. 5).

Expt. no.	Galactose added (μmole)	ADP formed (μmole)	Galactose 1-phosphate formed (μmole)	ADP/galactose ratio
1	0·18	0·19	—	1·06
2	0·22	0·21	—	0·95
3	0·36	0·37	—	1·03
4	0·45	0·43	0·43	1·00

for each μmole of galactose added. In addition, from the preceding experiment in which the phosphorylated product was found to be galactose 1-phosphate, 0·43 μmole of galactose 1-phosphate and 0·43 μmole of ADP were formed from 0·45 μmole of galactose and excess of ATP (Table 4).

DISCUSSION

The results in this paper with pig-liver galactokinase confirm and extend the work of Cardini & Leloir (1953) on crude preparations of galactokinase from rat liver. Although no evidence has been presented that the galactokinase is pure, the substrate-specificity studies and the omission of either pyruvate kinase or lactate dehydrogenase from the assay show that the preparation does not contain hexokinase, glucokinase, fructokinase, lactate dehydrogenase or pyruvate kinase, all of which are present in the liver extract.

The specific activity of galactokinase purified from liver (10–18 units/mg. of protein) is similar to the preparation from *E. coli* (14 units/mg.; Sherman & Adler, 1963) and greater than that purified from yeast by Heinrich (1964) or Howard & Heinrich (1965), namely 2·5–5 units/mg. Most of the properties of galactokinase purified from the three sources are similar. The pH optima, the Michaelis constant for ATP and the protection or stimulation by thiols are all alike. However, the substrate specificities of the preparations do differ. The Michaelis constant for galactose for the liver enzyme (1×10^{-4}–3×10^{-4}M) is lower than has been found for the *E. coli* enzyme (7×10^{-4}M; Sherman & Adler, 1963) or for the yeast enzyme ($1{\cdot}1 \times 10^{-3}$M; Heinrich, 1964). Although the liver enzyme reacts with both galactosamine and 2-deoxygalactose besides galactose, both of these compounds have only marginal activity with the yeast enzyme (Heinrich, 1964).

As liver galactokinase is eluted from Sephadex G-100 at approximately the same volume as haemoglobin (as determined by extinction at 412 mμ; see Fig. 2*a*), the molecular weight of galactokinase would be approx. 60000. This is larger than the reported value of 20000 determined by sedimentation methods for the *E. coli* enzyme (Sherman & Adler, 1963).

After this research had been completed, some studies on the development and adaptive characteristics of galactokinase in rat liver were published by Cuatrecasas & Segal (1965). These authors found that galactokinase occurs in all tissues tested, but it had highest activity in liver from young rats. They also presented some kinetic evidence for the occurrence of two galactokinase enzymes in rat liver. In my purification procedure, no evidence for heterogeneity of galactokinase on Sephadex G-100, Sephadex G-200 or DEAE-cellulose chromatography was obtained. This does not eliminate the possibility that two galactokinase enzymes are present, as the proteins may have similar molecular weights and behaviour towards ion-exchange resins, or may not be present together in liver of the pig at the age chosen for the purification.

As young animals obtain galactose from lactose in milk, it is not surprising to find that the liver at this stage contains more galactokinase than in the adult. This enzyme may limit the rate of galactose metabolism as the liver from young animals have higher rates of galactose oxidation, uptake (Segal, Roth & Bertoli, 1963) and conversion into glycogen (Ballard & Oliver, 1964, 1965) than adult liver.

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