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KINETIC PROPERTIES OF HEXOSE-MONOPHOSPHATE DEHYDROGENASES. I. ISOLATION AND PARTIAL PURIFICATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE FROM RAT LIVER AND KIDNEY CORTEX [1]

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Summary

Glucose-6-phosphate dehydrogenase (G6PDH) from rat-liver and kidney-cortex cytosol has been partially purified and almost completely separated from 6-phosphogluconate dehydrogenase activity. The purification and isolation procedures included high-speed centrifugation, 40-55% ammonium sulphate fractionation, by which both enzyme activities were separated, and finally, the application of the protein fraction to a column of Sephadex G-25 equilibrated with 10 mM Tris-EDTA-NADP buffer, pH 7.6, to eliminate any contaminating metabolites. The kinetic properties of isolated liver and renal G6PDH were examined. Both enzymes showed a typical Michaelis-Menten kinetic saturation curve with no evidence of co-operativity. The optimum pH of both liver and kidney cortex G6PDH was 9.4. The Km values for glucose-6-phosphate (G6P) and for NADP were 3.29 x 10^{-4} M and 1.00 x 10^{-4} M respectively. The specific activity measured at 37°C and optimun pH was 327.1 mU/ mg of protein. NADPH caused a competitive inhibition with a K_i of 10 µM. The K_m values for the G6P and NADP of kidney-cortex G6PDH were 2.06 x 10^{-4} and 0.25 x 10^{-4} M respectively. The specific activity at pH 9.4 and 37°C was 76.55 mU/ mg of protein. The K_i value for NADPH inhibition was 4 µM. This work describes an easy, rapid and reliable method for the separation of the two dehydrogenases involved in the hexose-monophosphate shunt in animal tissues.

Key Words: G6PDH, kinetics, liver, kidney cortex

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Glucose-6-phosphate dehydrogenase, G6PDH (D-glucose-6-phosphate: NADP 1-oxidoreductase, EC 1.1.1.49) catalyzes the oxidation of D-glucose-6-phosphate to D-glucose-O-lactone 6-phosphate in the presence of NADP. It is known that this enzyme is the first in the oxidative segment of the hexose-monophosphate shunt and precedes 6-phosphogluconate dehydrogenase activity. This shunt generates two important metabolites: reducing equivalent in the form of NADPH, and pentose phosphates, the most important of these being ribose-5-phosphate (1,2).

This cytosolic enzyme occurs in many different species of animals, plants and microorganisms (3-6). In animals G6PDH is distributed throughout the different tissues (4,7-19) and data on several features, such as its structural, catalytic and regulatory properties are widely available (2,11-14).The step catalyzed by G6PDH is an important control point in the metabolic, nutritive and hormonal regulation of the pentose-phosphate pathway. It has been well established that the activity of this rat-liver hexose-monophosphate dehydrogenase changes significantly according to hormonal and nutritional conditions (15-19).

The main purposes of the hexose monophosphate shunt are: to generate ribose-5-phosphate for incorporation into nucleotides and also to supply reducing equivalents such as NADPH, which serves as a hydrogen and electron donor for a variety of reductive reactions, including fatty-acid and cholesterol synthesis (20); mixed-function oxidations involved in detoxifying cells of xenobiotics (11,20); and its participation in the protection of cells against oxidation via the glutathione-reductase systems (11,21).

One of the problems concerning the measurement of these enzyme activities in cell homogenates is the interference that takes place between both enzymes in the cycle, glucose-6-phosphate and 6-phosphogluconate dehydrogenases, because they both catalyze the formation of NADPH. Thus until now it has been necesary to use an indirect and coordinated method (5,10,22), although interference exists even at subsaturating substrate concentrations. Bearing these problems in mind, the aim of our work has been to develop a quick and easy method to separate the activities of G6PDH and 6PGDH and to study the main kinetic properties of the partially purified G6PDH from rat liver and kidney cortex.

Materials and Methods.

Chemical. Bovine-serum albumin was obtained from the Sigma Chemical Co. (USA). Substrates and coenzymes, NADP, NADPH, NAD, NADH, glucose 6-phosphate (G6P) and 6-phosphogluconate (6PG), came from Böehringer (Manheim, Germany) and Sephadex G-25 was from Pharmacia Fine Chemicals, Upsala, Sweden. Other chemical compounds used in buffer preparations were of analytic grade.

Animals. All experiments were performed using Wistar rats (*Rattus novergicus*) weighing 200-250 g. They were maintained under controlled temperature (22 ±1 °C) and lighting (light from 8:00 to 19:00 hr.) conditions. They were adapted to a standard diet (Sandermus DGPA 14685: 54.5% carbohydrates, 18% protein, 4.5% olive oil) for at least 10 days, having free access to food and water.

Preparation of tissues for enzyme assays. The rats were killed by cervical dislocation and

their kidneys and several liver lobes quickly removed and placed in ice-cold saline solution. After decapsulation, the renal cortices of the kidneys were sliced with a Stadie Riggs microtome. The hepatic and renal cortex tissues (400 mg/ml) were homogenized in a medium containing Tris (10 mM), EDTA (1 mM) and NADP (0.1 mM), pH 7.6. The homogenates were centrifuged at 105,000 x *g* for 60 min at 4°C. The supernatant was dialyzed against Tris (10 mM) and saccharose (0.025 mM) buffer (1/50, v/v), pH 7.6.

Enzyme assays. G6PDH was assayed at 25°C as described by Lupiáñez *et al.* (5) with some modifications (10,20). G6PDH activity was corrected for 6-phosphogluconate dehydrogenase activity. G6PDH assays were carried out in a medium containing 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 0.6 mM NADP and G6P, to a total volume of 1 ml. The concentrations of glucose 6-phosphate were from 0.005 mM to 2 mM. 6PGDH was measured in the same medium. The concentrations of 6PG ranged from 0.005 mM to 2 mM. During the purification process the activities of both G6PDH and 6PGDH were measured at pH 7.4 and 25°C, whereas for kinetic studies enzyme activity was measured at pH 9.4 and 37°C for G6PDH, and pH 8.0 and 37°C for 6PGDH. Protein was measured according to the method used by Lowry *et al.* (23). One mUnit activity is defined as the amount of enzyme required to reduce 1 nmol NADP x min^{-1}. Enzyme activity is expressed as mU x mg of $protein^{-1}$.

Isolation and partial purification of G6PDH. Isolation and partial purification of the rat-liver and kidney-cortex G6PDH were carried out as outlined below. Unless otherwise stated all operations were carried out at 0-4°C:

Step 1: High-speed supernatant.- Fresh rat-liver and kidney cortex were weighed, minced and homogenised with 1:2.5 vol of the homogenate medium. The homogenate was then centrifuged at 105,000 x *g* for 60 min. The supernatant thus obtained, termed high-speed supernatant, was used for further processing.

Step 2: Ammonium-sulphate fractionation.- Solid ammonium sulphate was added slowly to the high-speed supernatant to a quantity of 40% saturation and kept at 0°C for about 30 min. The supernatant obtained by centrifugation at 10,000 x *g* for 10 min was then saturated up to 55% by the slow addition of solid ammonium sulphate. The pellet obtained after being kept at 0°C for 20-30 min, followed by centrifugation at 10,000 x *g* for 10 min, was dissolved in 8 ml of a buffer medium containing 10 mM Tris, 1 mM EDTA and 0.1 mM NADP (pH 7.6).

Step 3: Sephadex G-25 column chromatography.- The dissolved ammonium-sulphate fraction was then loaded onto a Sephadex G-25 column (1 x 21 cm), which had been previously equilibrated with the homogenization buffer. The sample was passed through the column with a flux of 25 ml/h. Fractions of 1 ml were collected every 2.4 min. The fractions showing G6PDH activity were identified and those with the highest activity were pooled and stored frozen. Enzyme activities (G6PDH and 6PGDH) were measured in these fractions as discussed below. Protein concentrations were also estimated by absorption at 280 nm using an $E_{1\%} = 12.7$ (24).

Kinetic parameters. The kinetic data were analysed by the non-linear-regression method described by the Michaelis-Menten equation V = Vmax [S] / Km + [S]. This non-linear plot was contructed with the aid of a computer program. Catalytic efficiency, defined as the ratio between enzyme activity and Km, was determined at saturated substrate

concentrations, which indicate the relationship between the total enzyme concentration and the interaction of the substrate with the enzyme. The K_i values during the competitive inhibition of NADPH on G6PDH activity were calculated by the equation V = Vmax [G6P] / Km(ap) + [G6P], where Km(ap) = Km (1 + [NADPH]/K_i). Data are also presented as double-reciprocal plots for illustrative and comparative purposes.

Analysis of data. Results are expressed as mean ± SEM. Statistical significance was determined using the paired or unpaired two-tailed Student's *t* test as appropiate. The differences were considered significant from $P < 0.05$.

Results and Discussion

The partial purification processes of liver and kidney-cortex cytosol G6PDH are summarized in Table 1. After high-speed centrifugation to obtain the cytosolic fraction, the enzyme in both tissues was almost completely separated (99%) from 6-phosphogluconate dehydrogenase activity by subjecting the supernatant to a 40-55% saturated ammonium sulphate fractionation (Fig. 1). G6PDH activity is present almost exclusively (99%) in the resulting pellet, which was dissolved and applied to a Sephadex G-25 column equilibrated with 10 mM Tris, 1 mM EDTA and 0.1 mM NADP buffer, pH 7.6, to eliminate any contaminating substances. The purification process showed a recovery of 35% for the enzyme from both liver and kidney cortex and a partial purification of 12-fold and 7-fold for each tissue respectively. The specific activity of liver 6PGDH in the first purification step was 20.50 mU/mg of protein, whereas in the last step it was 0.19 mU/ mg of protein, indicating a 99 % elimination of enzyme activity. Similar results were obtained with the kidney-cortex enzyme; in this case 6PGDH activity fell from 3.48 to 0.08 mU/mg of protein.

TABLE I

Isolation and partial purification of glucose-6-phosphate dehydrogenase from rat liver and kidney cortex.

Step	Volume (ml)	Total protein (mg)	Total activity (Units)	Specific activity (mU/mg)	Recovery (%)	Purification n-fold
LIVER						
Homogenate	50.0	2340.5	32.44	13.86	100	1.0
High-speed supernatant	40.0	971.6	20.36	20.96	63	1.5
40-55% $(NH_4)_2SO_4$ fraction	8.0	140.3	13.89	99.01	43	7.2
Sephadex G-25	17.5	69.4	11.23	161.80	35	11.7
KIDNEY CORTEX						
Homogenate	13.0	480.0	3.93	8.19	100	1.0
High-speed supernatant	11.0	177.7	2.77	15.60	70	1.9
40-55% $(NH_4)_2SO_4$ fraction	4.0	46.7	1.72	36.85	44	4.5
Sephadex G-25	9.0	22.8	1.37	60.26	35	7.4

Liver and kidney cortex were homogenized in a medium containing Tris (10 mM), EDTA (1 mM) and NADP (0.1 mM), pH 7.6. The supernatant from 105,000 x g was dialyzed in a buffer of Tris (10 mM), saccharose (0.025 mM) pH 7.6 (v/v 1:50). The total wet weight of the livers and kidney-cortex were 20 g and 5.2 g respectively.

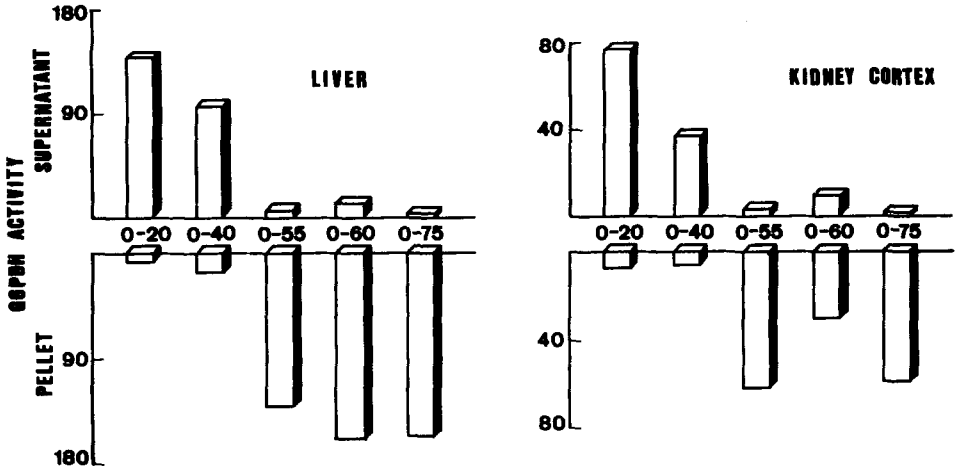

FIG. 1

Ammonium-sulphate fractionation for rat liver and kidney-cortex G6PDH. The activity of the enzyme present in the supernatant and/or pellet at different fractions of $(NH_4)_2SO_4$ is expressed as mUnits at pH 7.4 and 25°C. Results are means of at least 10 observations. The SEM was always less than 10%.

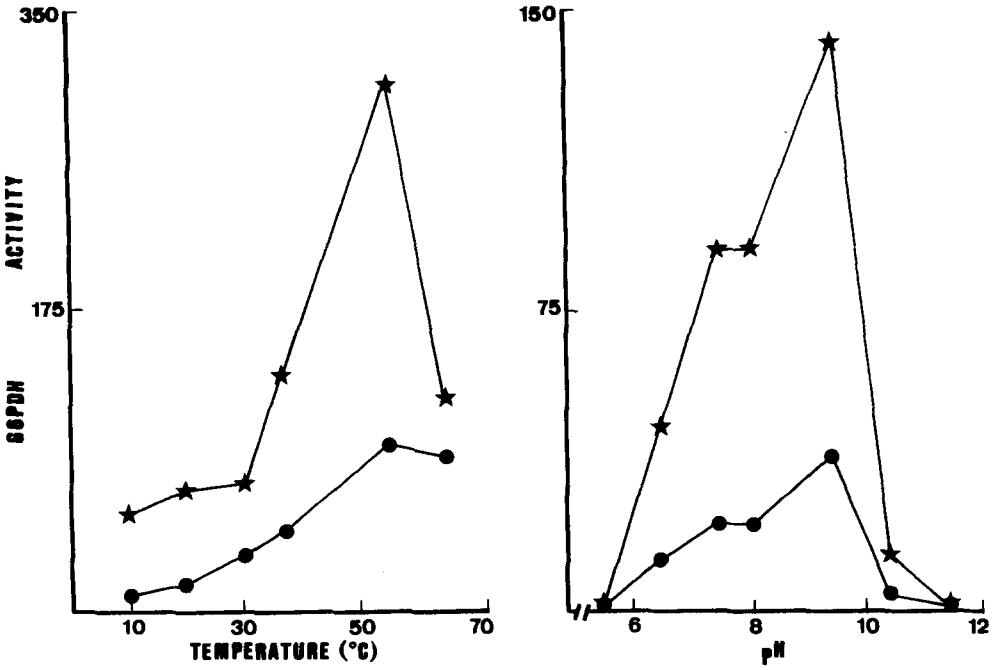

FIG. 2

Effect of temperature and pH on the activity of partially purified G6PDH from rat liver (★) and kidney cortex (●). G6PDH activity is expressed as mU/mg of protein at pH 7.4 for temperature effect and 25°C for pH effect. Results are means of at least 10 observations. The SEM was always less than 10%.

Figure 2 depicts the effects of temperature and pH on G6PDH activity in both tissues. The two enzyme forms were incubated for 10 min at temperatures from 10°C to 65°C, showing maximun activity at 55°C, after which the liver enzyme activity fell sharply at 65°C, while only a slight fall was found in the renal enzyme. At 37°C the activity of G6PDH in both tissues was 45% of its maximum. These results agree with those obtained with this enzyme from other sources (25,26).

The activity of this enzyme in both tissues was measured within a range of pH values from 5.4 to 11.4. The activity curves at different pH's were qualitatively similar, 9.4 being the optimun pH for both forms of the enzyme. This pH differs from that obtained in other animal tissues (27).

Kinetic experiments on G6PDH from both tissues were performed by measuring the oxidation of different G6P concentrations to form NADPH and the saturation curves are depicted in Figures 3 and 4. Typical hyperbolic kinetics were obtained for both enzyme activities, with no evidence of sigmoidicity. This was confirmed by the Hill's plots of the kinetic data, which gave interaction coefficient values (h) of 1.02 ± 0.01 for the liver enzyme and 1.08 ± 0.01 for the kidney-cortex G6PDH. Double-reciprocal plots of the variation in the initial velocity of both enzyme activities as a function of the concentrations of their substrates, G6P and NADP were completely linear.

This also excludes any significant co-operative effects and suggests that a random-order mechanism operating under steady-state conditions is unlikely, although, as pointed out by Petterson (28), a departure from Michaelis-Menten behaviour may not always be apparent with such a mechanism.

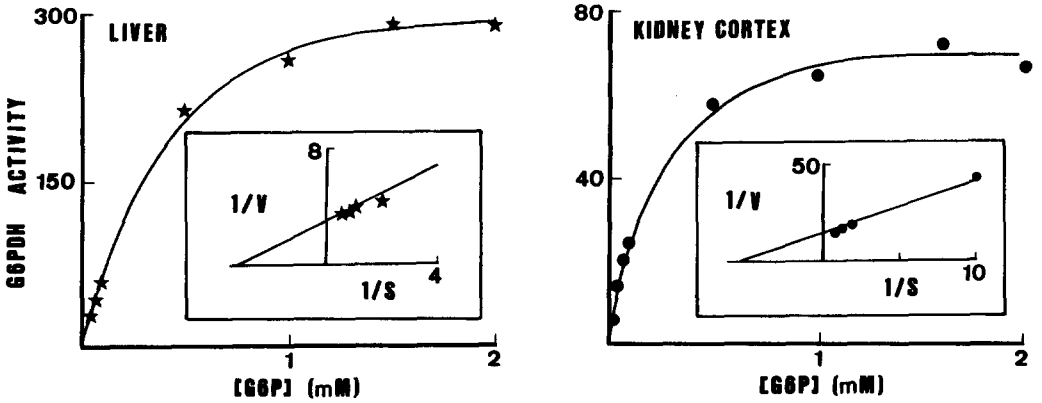

FIG. 3

Effects of the variations of glucose-6-phosphate concentrations on G6PDH activity from rat liver (★) and kidney cortex (●). G6PDH activity is expressed as mU/mg of protein at pH 9.4 and 37°C. Inset graphs show the Lineweaver-Burk plot of the kinetic data. Results are means of at least 10 different experiments. The SEM was always less than 10% and these values have been omitted in order to clarify the figure. The kinetic parameters Vmax and Km obtained from these plots were 421.3 mU/mg of protein and 0.326 mM respectively for liver; and 71.4 mU/mg of protein and 0.178 mM for kidney cortex.

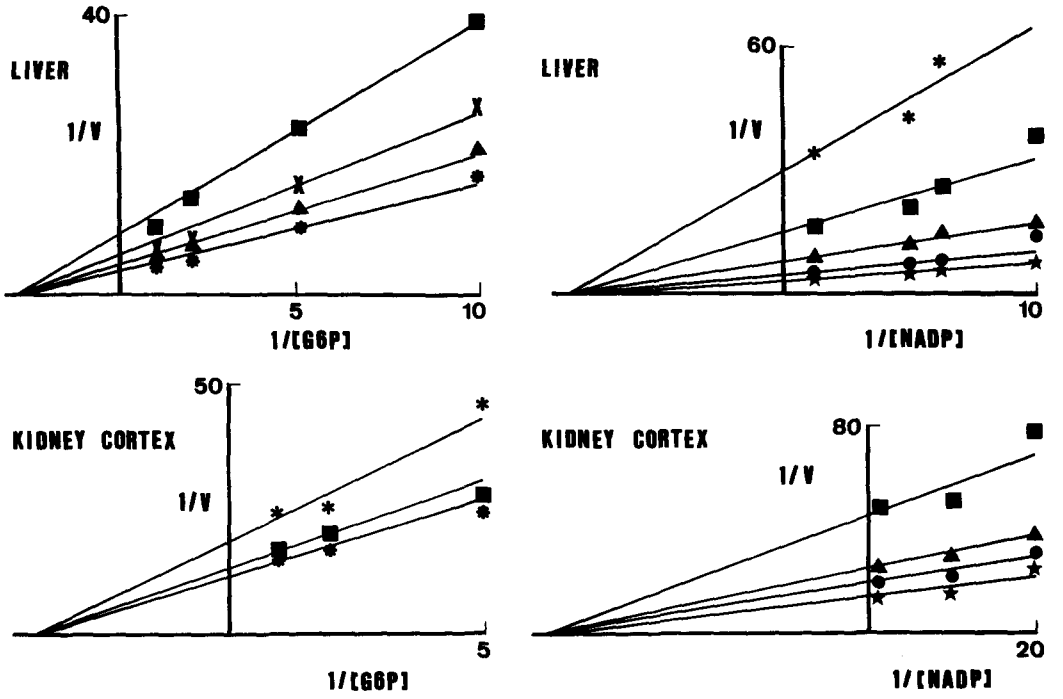

FIG. 4

Effects of variations in NADP concentrations at fixed concentrations of G6P (right) and of G6P concentrations at fixed concentrations of NADPH (left) on the G6PDH activity of rat liver (above) and kidney cortex (below). The graphs show the double-reciprocal plots of the kinetic data. G6PDH activity is expresed as mU/mg of protein at pH 9.4 and 37°C. Results are means of at least 10 observations. The SME was always less than 10%. Symbols indicate the following concentrations: (✱) 0.05, (■) 0.1, (✖) 0.15, (▲) 0.2, (●) 0.5, (✸) 0.8 and (★) 1.0 mM. The Km values obtained were: for liver Km (G6P) 0.326mM and Km (NADP) 0.108 mM; for kidney cortex Km (G6P) 0.178 mM and Km (NADP) 0.033 mM.

Graphs of the slopes and intercepts of the lines against the reciprocal concentrations of the fixed substrates, which were always linear, were used to determine the kinetic parameters, Michaelis-Menten constants (Km) for G6P and NADP and maximun velocity (Vmax) for the enzyme activities. The values of these kinetic parameters are shown in the captions to Figures 3 and 4.

The competitive inhibition of liver and kidney-cortex G6PDH by NADPH is shown in Figure 5. Double-reciprocal plots in the absence and presence of NADPH in concentrations of 0.01 mM and 0.1 mM in both tissues were used to determine the inhibition constants (K_i) for NADPH.

Our results show that K_i for kidney-cortex is greater than that for liver tissues and suggest that in kidney cortex NADPH inhibits G6PDH activity to a lesser extent. Inhibition of G6PDH by NADPH was first described by Negelein and Hass (29) using the enzyme from yeast, and later confirmed in the enzyme from other sources (1,3,11,14,26,30). It is generally accepted nowadays that the activation of this enzyme is metabolically controlled by the cytosolic ratio between free NADP and NADPH (11,17,30).

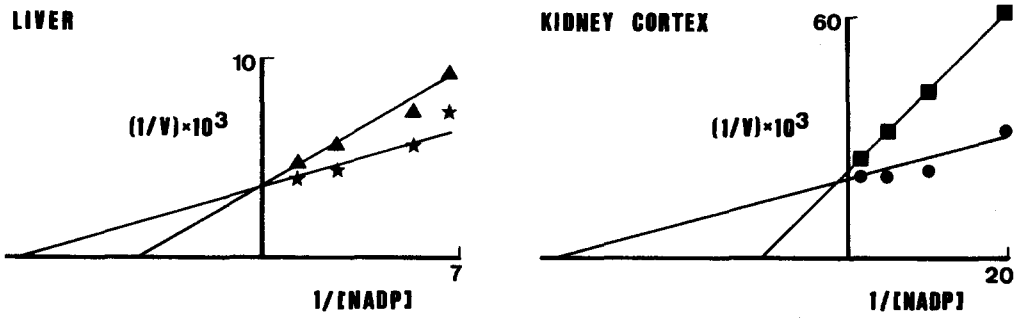

FIG. 5

Competitive inhibition of NADPH on G6PDH activity from rat liver and kidney cortex. The graphs show the double-reciprocal plots of the kinetic data. G6PDH activity is expressed as mU/mg of protein at pH 9.4 and 37°C. Results are means of at least 10 observations. The SEM values were always less than 10%. Symbols indicate the following NADPH concentrations: (★) and (●) for 0 mM and (▲) and (■) for 0.01 mM. The Km and apparent Km ($Km_{(ap)}$) values for NADP were: for liver, Km at 0 mM NADPH 0.117 mM and $Km_{(ap)}$ at 0.01 mM NADPH 0.228; for kidney cortex, Km at 0 mM NADPH 0.027 and $Km_{(ap)}$ at 0.01 mM NADPH 0.092 mM. According to the equation $Km_{(ap)} = Km\ (1 + [I]/K_i)$, the values of K_i obtained from these data were: for liver, K_i of NADPH 0.011 mM; for kidney cortex, K_i of NADPH 0.004 mM.

Since the double-reciprocal plots tend to emphasize the data points obtained at low substrate concentrations, where the degree of error is likely to be greatest (31), as an additional and comparative check the data from the experiments were analyzed in a simple least-square fitting of the untransformed data to a rectangular hyperbola (32,33). This non-linear plot was constructed with the aid of a computer program. Kinetic data obtained for G6PDH from rat liver and kidney cortex are shown in Table 2.

TABLE II

Kinetic parameters of the partially purified glucose-6-phosphate dehydrogenase from rat liver and kidney cortex.

Kinetic parameters	Liver G6PDH	Kidney cortex G6PDH
Km (G6P)	0.329 ± 0.007	0.206 ± 0.003
Km (NADP)	0.100 ± 0.003	0.025 ± 0.001
K_i (NADPH)	0.010 ± 0.0002	0.004 ±0.00008
Vmax	83.41 ± 1.70	19.52 ± 0.35
Catalytic efficiency	0.254 ± 0.005	0.095 ± 0.002
Specific activity	327.1 ± 9.30	76.55 ± 2.11

Data are means ± SEM of at least 10 experiments. The kinetic parameters Km, K_i, Vmax, and specific activities were determined from a simple least-squares fitting of the untransformed data to a hyperbola and are expressed in terms of mM for Km and K_i; mUnits for Vmax, mUnits / $M \cdot 10^{-6}$ for catalytic efficiency and mU/mg of protein for specific activity. Significant differences between G6PDH from liver tissue and kidney cortex were in all parameters of at least $P<0.0001$.

Glucose 6-phosphate dehydrogenase from liver and kidney cortex shows clear differences in its kinetic parameters. The specific activity of the partially purified enzyme was 4-5 times higher in liver tissues whereas the Km values for G6P and NADP were significantly lower in kidney cortex, especially for NADP. These results demonstrate that, although the activity is higher in the liver, the affinity of the enzyme for the substrates, G6P and NADP is higher in the kidney-cortex. In addition the K_i values for NADPH also differed: in kidney cortex they were almost 3-fold higher than in liver tissue, which concurs with a lower Km value for NADP.

As far as the specific properties of these enzymes is concerned, several studies have been made to find out more about the principal kinetic features of this dehydrogenase from several biological sources (34,35). In general terms our kinetic values agree fairly well with those reported by other authors using similar tissues (36,37); the differences found are in fact probably due to different reaction conditions, such as the kind of enzyme preparation used, and physical-chemical measurement conditions, such as the pH and/or ionic strength.

In this sense, Levy (35) and Levy and Christoff (36) have previously reported that the specific activities of various different preparations of purified G6PDH obtained from rat-liver homogenates ranged from between 9 and 16 units/mg. Their liver preparations, however, gave an almost five-fold increment on this activity, which we put down to the fact that they were 130 times purer. Nevertheless, Louie *et al.* (38) obtained a G6PDH activity from centrifuged and sonicated extract of rat-adipose tissue very similar to the activity of the rat-liver tissue that we have found, which is in accordance with the similar roles of the liver and adipose tissues in reductive biosynthetic processes.

In addition, Luzzato *et al.* have recently published (39,40) results on some of the kinetic properties of recombinant human G6PDH generated in E. coli and human red-cell G6PDH. The specific activities of both these enzyme preparations were also higher than those obtained with rat liver and kidney cortex, indicating both significant catalytic differences between red-cell and liver enzymes and also a higher degree of purity in the human enzymes. Furthermore, the Km values for both substrates, G6P and NADP, were lower with the red cells (41), probably due to the fact that the red-cell enzyme needs NADPH for the maintenance and protection of the red-cell membrane. Nevertheless, the Ki values for NADPH reported by these authors were very similar to ours for both rat-liver and kidney-cortex enzymes.

Finally, the results of our study go to support the hypothesis that G6PDH from liver and kidneycortex may be isoenzymes, although other molecular and physico-chemical aspects still need to be investigated to confirm this hypothesis and we are continuing with our research into the subject.

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