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Kinetic properties of hexose-monophosphate dehydrogenases. II. Isolation and partial purification of 6-phosphogluconate dehydrogenase from rat liver and kidney cortex*

F. Javier Corpas[1], Leticia García-Salguero[2], Juan B. Barroso[2], Fermín Aranda[3] and José A. Lupiáñez[2]

[1]*Unit of Vegetable Biochemistry, Zaidín Experimental Station [Consejo Superior de Investigaciones Científicas (CSIC)], Granada;* [2]*Department of Biochemistry and Molecular Biology, Centre of Biological Sciences. University of Granada, Granada;* [3]*Department of Biochemistry, Faculty of Sciences, University of Jaén, Jaén, Spain*

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Abstract

6-Phosphogluconate dehydrogenase (6PGDH) from rat-liver and kidney-cortex cytosol has been partially purified and almost completely isolated (more than 95%) from glucose-6-phosphate dehydrogenase activity. The purification and isolation procedures included high-speed centrifugation, 60–75% ammonium-sulphate fractionation, by which both hexose-monophosphate dehydrogenases activities were separated, and finally the protein fraction was applied to a chromatographic 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 the isolated partially purified liver and renal 6PGDH were examined. The saturation curves of this enzyme in both rat tissues showed a typical Michaelis-Menten kinetic, with no evidence of co-operativity. The optimum pH for both liver and kidney-cortex 6PGDH was 8.0. The Km values of liver 6PGDH for 6-phosphogluconate (6PG) and for NADP were 157 μM and 258 μM respectively, while the specific activity measured at optimum conditions (pH 8.0 and 37°C) was 424.2 mU/mg of protein. NADPH caused a competitive inhibition against NADP with an inhibition constant (K_i) of 21 μM. The Km values for 6PG and NADP from kidney-cortex 6PGDH were 49 μM and 56 μM respectively. The specific activity at pH 8.0 and 37°C was 120.7 mU/mg of protein. NADPH also competitively inhibited 6PGDH activity, with a K_i of 41 μM. This paper describes a quick, easy and reliable method for the separation of the two dehydrogenases present in the oxidative segment of the pentose-phosphate pathway in animal tissues, eliminating interference in the measurements of their activities. (Mol Cell Biochem **144:** 97–104, 1995)

Key words: hexose-monophosphate dehydrogenases, 6-phosphogluconate dehydrogenase, pentose-phosphate cycle, rat-kidney cortex, rat liver

Introduction

6-Phosphogluconate dehydrogenase, 6PGDH (6-phospho-D-gluconate:NADP 2-oxidoreductase, decarboxylating, EC 1.1.1.44) is an oxidative decarboxylase of the hexose monophosphate shunt which catalyzes the oxidative decarboxylation of 6-phosphogluconate to ribulose 5-phosphate and CO_2 in the presence of NADP. This enzyme is considered to be a key regulatory lipogenic enzyme and to act in the oxidative segment of the pentose-phosphate cycle following the action

*Publication No 170 from 'Drugs, Environmental Toxics and Cell Metabolism' research group. Department of Biochemistry and Molecular Biology, University of Granada, Granada, Spain

Address for offprints: J.A. Lupiáñez, Departamento de Bioquímica y Biología Molecular, Centro de Ciencias Biológicas, Avenida de Fuentenueva s/n, 18001 Granada, Spain

of glucose-6-phosphate dehydrogenase, the other key enzyme in the cycle. Both dehydrogenases provide reducing equivalents in the form of NADPH for a variety of metabolic and cellular processes, and ribose 5-phosphate for nucleotide synthesis [1, 2].

This cytosolic enzyme is widely distributed in different isoforms in many biological sources and tissues [3–8], although it is particularly active in the liver, where substantial quantities of NADPH and deoxyriboses are needed to support the *de novo* fatty-acid and cholesterol system [9, 10] and nucleotide metabolism [11] for growth and cell proliferation [4, 12]. In addition, NADPH serves as a reducing agent, via the glutathione-peroxidase-reductase system, of free oxygen radicals and other related oxygen species which may play a role in inducing cell damage [5, 13].

It is well known that 6PGDH activity is controlled by several dietary and hormonal factors [14–16] which function at the enzyme synthesis level [17, 18] via changes in the cell concentration of the specific translatable mRNA of 6PGDH [18–20]. Several studies have shown that when lipogenesis is stimulated in animals that have been fasted and refed on a high-carbohydrate diet hepatic hexose-monophosphate dehydrogenase activities increase 10–30 fold [9, 14, 18], while in animals which have been fasted alone or fasted and subsequently refed on a high-fat diet these enzyme activities drop significantly [9, 16].

In this paper we describe the partial purification of rat-liver and kidney-cortex 6PGDH and its complete isolation from the other dehydrogenase in the cycle, G6PDH. The result is a quick and easy method of separating 6PGDH and G6PDH activity, thus eliminating any possible interferences, mainly by glutathione reductase activity, in their measurement and significantly improving upon previous works [16, 21]. We have also taken the opportunity to study the main kinetic properties of this enzyme from rat liver and kidney cortex, two kinds of tissue, the role of which in the use of NADPH is clearly different.

Materials and methods

Chemicals

Substrates, glucose-6-phosphate and 6-phosphogluconate, and coenzymes, NAD, NADP, NADH and NADPH were supplied by Boehringer (Mannheim, Germany). Bovine-serum albumin came from the Sigma Chemical Co. (St. Louis, MO, USA) and Sephadex G-25 was from Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals used for buffers were of analytical grade obtained from readily available sources.

Animals

Male rats of the Wistar strain (*Rattus novergicus*) weighing 200–250 g were maintained under controlled temperature (22 ± 1°C) and lighting (8:00 to 19:00 h) conditions. They were adapted to a standard diet (Sandermus DGPA 14685) containing 54.5% carbohydrates, 18% protein and 4.5% olive oil for at least 10 days and had free access to food and water.

Preparation of tissues for enzyme assays

The rats were killed by cervical dislocation. Their kidneys and several liver lobules were quickly removed and placed in ice-cold saline solution. The kidneys were decapsulated and the renal cortex isolated and sliced with a stadie Riggs microtome, as were the liver pieces. The tissues were homogenized in a proportion of 400 mg/ml in a homogenization buffer containing 10 mM Tris-HCl, 1 mM EDTA and 0.1 mM NADP, pH 7.6. The homogenates were centrifuged at 105,000 × g for 60 min at 4°C. The supernatant was dialyzed against a buffer containing 10 mM Tris-HCl and 0.025 mM saccharose, pH 7.6, (1/50: v/v) for 3 h.

Enzyme assays and protein determination

Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were assayed in routine experiments at 25°C, as described by Lupiáñez *et al.* [22] with some modifications [7, 23]. 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 glucose-6-phosphate (G6P) and/or 6-phosphogluconate (6PG), to a total volume of 1 ml. The amount of protein from the dialyzed supernatant used was 0.600 mg in routine experiments and 0.150 mg in kinetic studies. The concentrations of G6P and 6PG ranged from 0.005 mM to 2 mM. During the partial purification and isolation procedure, the activities of both enzymes, 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 Lowry *et al.* [24]. One mUnit activity is defined as being the amount of enzyme required to reduce 1 nmol NADP × min^{-1}. Enzyme activity is expressed as mUnits per mg of protein.

Isolation and partial purification of 6PGDH

Isolation and partial purification of rat-liver and kidney-cor-

tex 6PGDH were carried out as outlined below. Unless otherwise stated all operations were carried out between 0 and 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 × 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 60% saturation and kept at 0°C for about 30 min. The supernatant obtained by centrifugation at 10,000 × g for 10 min was then saturated up to 75% 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 × 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 × 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 6PGDH 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 $E_{1\%} = 12.7$ (25).

Kinetic parameters

The kinetic data were analysed by the non-linear-regression method described by the Michaelis-Menten equation $V = V\max [S] / Km + [S]$. This non-linear plot was constructed with the aid of a computer programme. Catalytic efficiency, defined as the ratio between enzyme activity and Km, was determined at saturated substrate concentrations, which indicates the relation between the total enzyme concentration and the degree of interaction between the enzyme and the substrate. The inhibition constant (K_i) values during the competitive inhibition of NADPH on 6PGDH activity were calculated by the followed equation $V = V\max [6PG] / Km_{(ap)} + [6PG]$, where apparent Km (Km_{ap}) for 6PG in the presence of NADPH is $Km_{(ap)} = Km (1 + [NADPH]K_i)$. Data are also presented as double-reciprocal plots for illustrative and comparative purposes.

Data analysis

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

Results and discussion

6-Phosphogluconate dehydrogenase from rat liver and kidney cortex was partially purified by the process summarized in Table 1. The activity of this cytosolic enzyme in both tissues was almost completely separated (more than 95%) from glucose-6-phosphate dehydrogenase activity after fractionation with a 60–75% saturated ammonium-sulphate solution, as shown in Fig. 1. The partial purification of the enzyme activities at pH 7.6 were 10- and 16-fold for the liver and kidney cortex enzymes respectively. The recovery of the two forms was about 10 and 20% of the total initial activity.

During this purification procedure the activity of the other dehydrogenase, G6PDH, changed from 13.86 mU/mg in crude extracts to 0.21 mU/mg after the dialyzed protein solution was applied to a Sephadex G-25 column. This accounted for a drop in G6PDH activity of almost 99%. Similar behaviour was found in the kidney-cortex enzyme, the activity of which fell from 8.19 to 0.09 mU/mg.

The effects of temperature and pH are depicted in Fig. 2. 6PGDH from liver and kidney cortex was incubated for 10 min at temperatures from 10 to 65°C. In general terms a Q_{10} factor was found in both isoenzymes to show a maximum activity at 55°C. At 37°C 6PGDH activity in the liver was 67% of its maximum, and the activity of kidney-cortex 6PGDH was at 43% (Fig. 2 left). These results coincide with those obtained for G6PDH activity in the same tissues [26] and other biological sources [27].

The effects of a pH range from 5.4 to 11.4 on liver and kidney-cortex 6PGDH are shown in Fig. 2 (right). The activity curves of 6PGDH in both types of tissue were similar, reaching a single pH optimum of 8.0 in the presence of EDTA. It has been shown that EDTA, like histidine and citrate, shifts the optimum pH from the high alkaline to low alkaline or neutral range [28]. The high optimum pH clearly indicates the need for negative charges to be present at the enzyme's active site, aspartic and glutamic acids and histidine being possible candidates. Topham and Dalziel [29] have in fact demonstrated the presence of a histidine-242 molecule at the active site as well as recognizing its role in the catalysis of 6-phosphogluconate dehydrogenase in sheep liver.

We studied the kinetic behaviour of both forms of 6PGDH by measuring the formation of NADPH via the oxidative decarboxylation of 6-phosphogluconate. The saturation

Table 1. Partial purification of 6-phosphogluconate dehydrogenase from rat liver and kidney cortex

Step	Volume (ml)	Total protein (mg)	Total activity (Units)	Specific activity (mUnits/mg)	Recovery (%)	Purification -fold
LIVER						
Homogenate	50.0	2340.5	44.92	20.50	100	1.0
High-speed supernatant	40.0	971.6	28.94	29.79	64	1.5
60–75% $(NH_4)_2SO_4$	8.0	30.6	5.75	187.76	13	9.2
Sephadex G-25	16.0	17.8	3.67	206.62	8	10.1
KIDNEY CORTEX						
Homogenate	13.0	480.0	1.67	3.48	100	1.0
High-speed supernatant	11.0	177.7	1.45	8.15	87	2.3
60–75% $(NH_4)^2SO_4$	4.0	8.9	0.48	53.11	29	15.3
Sephadex	8.5	6.1	0.34	56.00	20	16.1

Liver and kidney cortex were homogenized in a medium containing Tris (10 mM), EDTA (1 mM) and NADP (0.1 mM) pH 7.6 in a relation 1:2.5 (p/v). The supernatant from 105,000 × g was dialyzed in a Tris (10 mM) and saccharose (0.025 mM) buffer, pH 7.6, (v/v, 1:50). The total wet weight of the liver tissue and kidney cortex were 20 g and 5.2 g respectively.

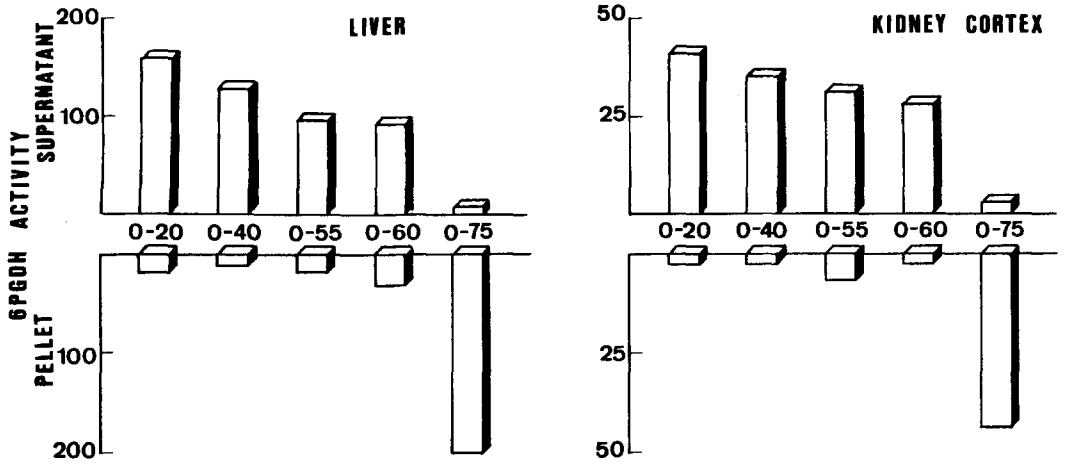

Fig. 1. Ammonium-sulphate fractionation for rat-liver and kidney-cortex 6-phosphogluconate dehydrogenase. The activity of this enzyme present in the supernatant and/or pellet at different fractions of $(NH_4)_2SO_4$ is expressed as mUnits at pH 7.4 and 25°C. Results are means of at least 10 observations. The SEM was always less than 10%.

curves and double-reciprocal plots under different enzyme conditions are depicted in Figs 3 and 4. Enzyme activity was measured at 37°C and optimum conditions. The enzyme kinetics always showed hyperbolic curves with no evidence of sigmoidicity (Fig. 3). This was confirmed by the Hill coefficient values (h), which were 1.00 ± 0.01 for the liver enzyme and 0.99 ± 0.01 for kidney-cortex 6PGDH. Furthermore, the strict linearity found in the double-reciprocal plots of the variation in the initial velocity as a function of substrate (G6P and NADP) concentrations also exclude any significant cooperative effects and suggests that it is unlikely that any random-order mechanism operates under steady-state conditions [30].

Dalziel's group, in a fine work [31], studied and the steady-state kinetics of 6-phosphogluconate dehydrogenase from sheep liver in several buffered media and their results were consistent with an assymetric sequencial mechanism. In addition, these authors show that in the pre-steady-state study the hydride transfer of the oxidative decarboxylation reaction at pH 6.0–8.0 was higher than 900 s^{-1} and that the rate of NADPH formation was equal to the steady-state rate, the fast formation of NADPH being equivalent to amounts of about half of the enzyme-active-centre concentration and therefore the fast dissociation of NADPH cannot be the rate-limiting step in the oxidative decarboxylation of 6-phosphogluconate.

With regard to this, Dallochio *et al.* [32] have previously reported that one molecule of the dimeric 6PGDH from human erythrocytes binds two molecules of NADP and only one molecule of NADPH and that the bindings of NADP and NADPH are mutually exclusive. These authors also proposed, from initial-rate kinetics and inhibition studies, a sequencial

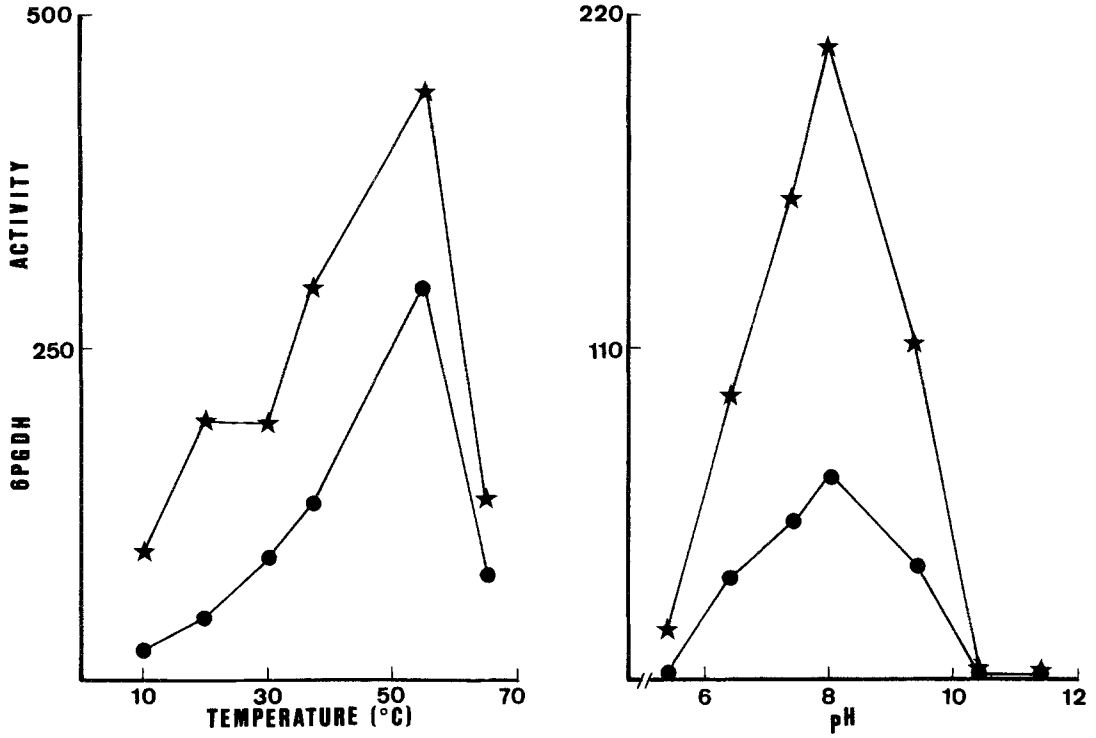

Fig. 2. Effect of temperature and pH on the activity of partially purified 6-phosphogluconate dehydrogenase from rat liver (★) and kidney cortex (●). 6PGDH activity is expressed as mU/mg of protein at pH 7.4 for temperature effect and 25°C for pH effect. Results are means of at least 10 observations. The SEM was always less than 10%.

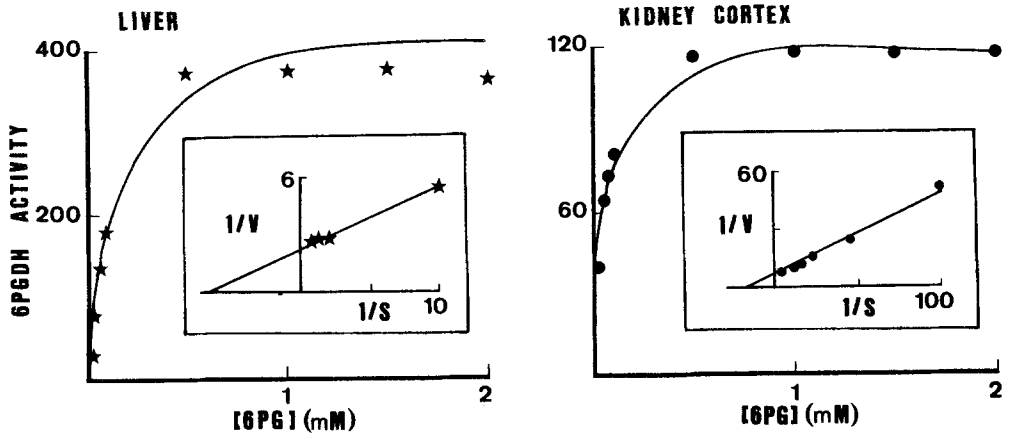

Fig. 3. Effects of the variation of 6-phosphogluconate concentration on 6-phosphogluconate activity from rat liver (★) and kidney cortex (●). The 6PGDH activity is expressed as mU/mg of protein at pH 8.0 and 37°C. Inset graphs show the Lineweaver-Burk plot of the kinetic data. Results are means of at least 10 different experiments. The SEM values were always less than 10% and have been omitted to clarify the figure. The kinetic parameters, Vmax and Km, obtained from these plots were for liver 446.4 mU/mg of protein and 146 μM respectively; and for kidney cortex 128.2 mU/mg of protein and 57 μM.

random-order mechanism. In addition, the downward curve in the Lineweaver-Burk plot with NADP at various concentrations indicated a negative co-operativity and that this could be explained as a result of the half-site reactivity for the NADPH. The differences in the stoichiometrics for oxidized and reduced coenzyme produced a non-linear relationship between the apparent dissociation constant for the NADPH and the NADP concentrations, and this kinetic behaviour explains the existence of a regulatory mechanism that responds in a very highly sensitive way to the changes in the NADP/NADPH ratio [9].

The effects of a fixed NADP concentration and variable 6PG and of a fixed 6PG concentration and variable NADP concentrations on the kinetic parameters are shown in Fig.

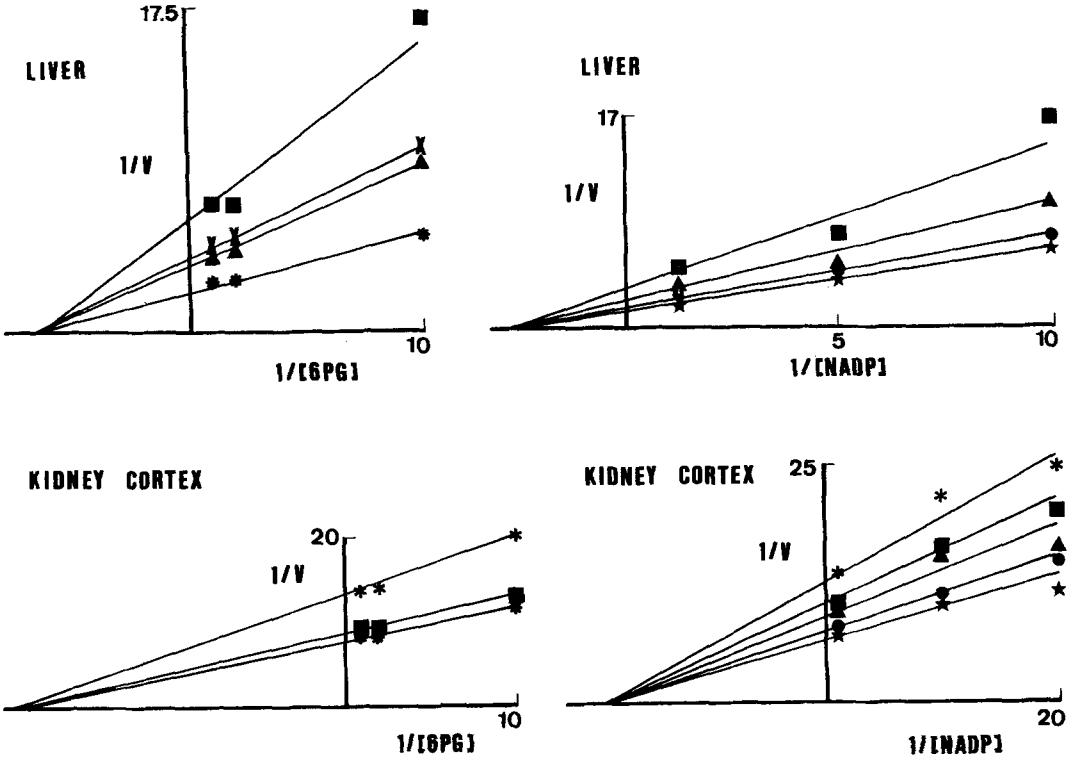

Fig. 4. Effects of variations in NADP concentrations at fixed concentrations of 6PG (right) and of the variations in 6PG concentrations at fixed concentrations of NADPH (left) on the 6-phosphogluconate dehydrogenase activity of rat liver (above) and kidney-cortex (below). The graphs show the double-reciprocal plots of the kinetic data. 6PGDH activity is expressed as mU/mg of protein at pH 8.0 and 37°C. Results are means of at least 10 observations. The SEM values were always less than 10%. Symbols indicate the follow concentrations: (✳) 0.05, (■) 0.10, (×) 0.15, (▲) 0.20, (●) 0.50, (✸) 0.80 and (★) 1.00 mM. The Km values obtained were: for liver Km (6PG) 153 μM and Km (NADP) 252 μM; for kidney cortex Km (6PG) 51 μM and Km (NADP) 50 μM.

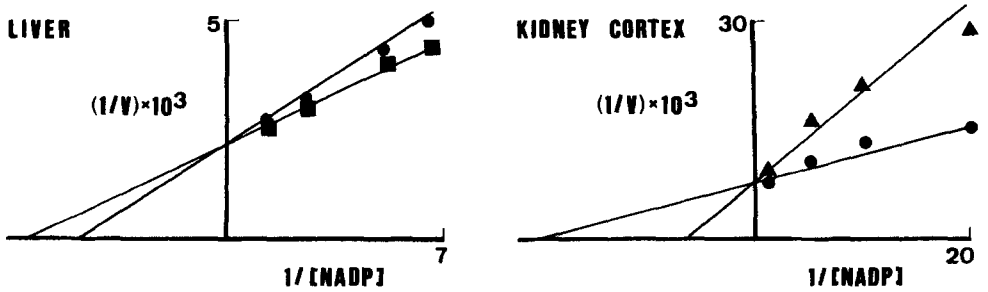

Fig. 5. Competitive inhibition of NADPH on 6-phosphogluconate dehydrogenase activity from rat liver and kidney cortex. The graphs show the double-reciprocal plots of the kinetic data. 6PGDH activity is expressed as mU/mg of protein at pH 8.0 and 37°C. Results are means of at least 10 observations. The SEM values were always less than 10%. Symbols indicate the follow NADPH concentrations: (●) for 0 mM, (■) for 0.01 mM and (▲) for 0.10 mM. The Km and apparent Km ($Km_{(ap)}$) values for NADP were: for liver the Km at 0 mM NADPH was 0.250 mM and $Km_{(ap)}$ at 0.01mM NADPH was 0.356; for kidney cortex the Km at 0 mM NADPH was 0.050 and $Km_{(ap)}$ at 0.10 mM NADPH was 0.162 mM. The values of K_i obtained from these data were: for liver, K_i of NADPH 24 μM; for kidney-cortex K_i of NADPH 45 μM.

4. Double-reciprocal plots were always linear, suggesting that 6PGDH displays a Michaelian behaviour with both sub strates, and were used to determine the kinetic parameters, Km for 6PG (Km_{6PG}) and NADP (Km_{NADP}) and maximum velocity, Vmax. The values of these parameters are shown in the captions to Figs 3 and 4. Our results with rat liver and kidney cortex differ significantly from those obtained in human erythrocytes where the double-reciprocal plots with variable concentrations of NADP were non-linear, indicating a negative co-operativity [32]. Tophan *et al.* [31] found that at lower substrate concentration the 6PGDH activity in sheep liver is inhibited at higher NADP concentrations, whereas at higher 6PG concentrations there is no inhibition over a wide range of coenzyme concentrations. This inhibition could be overcome by increasing the 6PG, as occurs in 6PGDH from *Candida utilis* [3].

Table 2. Kinetic parameters of partially purified 6-phosphogluconate dehydrogenase from rat liver and kidney cortex

Kinetic parameters	Liver 6PGDH	Kidney cortex 6PGDH
Km (6PG)	0.157 ± 0.005	0.019 ± 0.001
Km (NADP)	0.258 ± 0.008	0.056 ± 0.002
K_i (NADPH)	0.021 ± 0.0005	0.041 ± 0.001
Vmax	108.3 ± 3.1	30.40 ± 0.48
Catalytic efficiency	0.690 ± 0.02	0.620 ± 0.016
Specific activity	424.2 ± 12.3	120.7 ± 3.5

Results are means ± SEM at least 10 different experiments. The kinetic parameters Km, K_i, Vmax and specific activity were determined from a simple least-square fitting of the untransformed data to a rectangular hyperbola and are expressed in terms of mM for Km and K_i, mUnits for Vmax, mUnits/μM for catalytic efficiency and mU/mg of protein for specific activity.

The inactivation of 6PGDH by NADPH is shown in Fig. 5. As with G6PDH activity, the presence of NADPH produced a competitive inhibition of 6PGDH activity. Double-reciprocal plots in the presence of NADPH at concentrations of 0.01 mM and 0.1 mM in both kinds of tissue were used to determine the inhibition constants (K_i) for NADPH. Our results showed that K_i in the kidney-cortex enzyme was double that in the liver, suggesting that in the former tissue the inhibitory capacity of NADPH is lower. It is generally accepted that the activity of 6PGDH is metabolically controlled by the cytosolic ratio between free NADP and NADPH [3, 31, 32].

In mammalian tissues and microorganisms the concentration of NADPH is similar or higher than NADP concentrations [33–35]. Under these conditions all the enzyme should be bound to the NADPH, the dissociation constant of which is significantly lower than the concentration of free enzyme, and thus the enzyme should be totally inhibited. In the presence of 6PG the dissociation constant for NADP diminishes facilitating the release of the NADPH and thus the inhibition by NADPH, could be removed [3].

It is well known that a double-reciprocal plot tends to emphasise the weight of the data points obtained at low substrate concentrations, precisely where the degree of error is highest [36]. For this reason, the kinetic data from the experiments were analyzed by a single least-square fitting of the untransformed data to a rectangular hyperbola as an additional and comparative check. This non-linear plot was constructed with the aid of a computer programme. Kinetic data for 6PGDH from rat liver and kidney cortex are shown in Table 2.

Significant differences were found in the kinetic parameters of 6PGDH from rat liver and kidney cortex. The specific activity of liver 6PGDH was almost 4-fold higher and the Km values for 6PG and NADP were three and five times higher respectively. This kinetic behaviour would explain the higher catalytic efficiency of the hepatic enzyme. The inhibition constants for NADPH were also significantly different. In the kidney-cortex enzyme this K_i was almost double that of the value for the liver enzyme, which indicates a higher protection in the renal isoform against NADPH inhibition.

As far as the specific properties of this enzyme is concerned, several studies have been made to find out more about the principal kinetic parameters of this dehydrogenase from several biological sources [16, 22, 27, 37–42]. In general terms our kinetic values agree fairly well with those reported by other authors using similar tissues [4, 7, 16, 43]. Nevertheless, some of 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, the buffer medium and/or the ionic strength.

It is interesting to note that while in rat-liver and kidney-cortex 6PGDH the values of Km for NADP were 1.6 and 3 times higher respectively than the Km for 6PG, the opposite being the case in the livers of other types of mammals [31]. This important kinetic difference probably does not essentially modify the asymmetric sequencial mechanism for this enzyme reaction, in which 6PG and NADP bind in a random form and the products, CO_2, ribulose 5-phosphate and NADPH, are released in a ordered form, although it is possible that the pathway through which the enzyme complexes or binds 6PG and NADP is different in the case of rat-liver and kidney-cortex 6PGDH.

It was concluded from these results that the procedures used for isolating G6PDH and 6PGDH activities from animal tissues are highly reproducible and can be completed in a few hours, completely avoiding any interferences in the measurement.

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