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DIFFERENTIAL EFFECTS OF THE NADPH/NADP$^+$ RATIO ON THE ACTIVITIES OF HEXOSE-6-PHOSPHATE DEHYDROGENASE AND GLUCOSE-6-PHOSPHATE DEHYDROGENASE

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The steady-state kinetics of rat liver hexose-6-phosphate dehydrogenase (β-D-glucose: NAD(P)$^+$ 1-oxidoreductase, EC 1.1.1.47) using glucose 6-phosphate and NADP$^+$ as substrates is studied. NADPH has been found to inhibit the enzyme noncompetitively with respect to NADP$^+$, and uncompetitively with respect to glucose 6-phosphate. At a given concentration of glucose 6-phosphate, the reaction follows the basic inhibition equation. This suggests the presence of the enzyme-NADP$^+$-NADPH complex, and contrasts with the NADPH inhibition of glucose-6-phosphate dehydrogenase which is competitive with respect to NADP$^+$. An attempt was made to estimate the in vivo activities of the two enzymes in rat liver in the presence of NADPH at various NADPH/NADP$^+$ ratios. The results show that the two enzymes appear to be at about the same level of activity in normal rat liver where the coenzyme redox ratio is 110 and the glucose 6-phosphate concentration is 217 μM. Under the same conditions, but with 50 μM dehydroepiandrosterone, a potent inhibitor of glucose-6-phosphate dehydrogenase, but not of hexose-6-phosphate dehydrogenase, the latter enzyme is estimated to be 1.6-times as active as the former. Such differential effects of NADPH and steroids on the two enzymes may support our notion that hexose-6-phosphate dehydrogenase may have advantages over glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP$^+$ 1-oxidoreductase, EC 1.1.1.49) in steroid-metabolizing tissues (the activity of hexose-6-phosphate dehydrogenase is not, or less, affected by steroids or NADPH).

Glucose dehydrogenase (β-D-glucose: NAD(P)$^+$ 1-oxidoreductase, EC 1.1.1.47) was first described by Harrison [1], and its properties have been studied by several workers [2–6]. This enzyme is mainly located in microsomes and is active on hexose 6-phosphate as well as on glucose and xylose. Hence, it is also called hexose-6-phosphate dehydrogenase [2,7]. In contrast to the very high K_m value for glucose, the K_m for glucose 6-phosphate in the presence of NADP$^+$ and the K_m values for NADP$^+$ and NAD$^+$ in the presence of glucose 6-phosphate are well within the physiological range [8]. Thus, it appears likely that this enzyme may be functioning as a glucose-6-phosphate dehydrogenase rather than as a glucose dehydrogenase in vivo [9]. If this is valid, it can be said that vertebrates and echinoderms as well [10,11], possess two isozymes of glucose-6-phosphate dehydrogenase.

The physiological implications of the presence of two glucose-6-phosphate dehydrogenase isozymes have not been fully explored, although it is probable that hexose-6-phosphate dehydrogenase might be involved in drug and steroid metabolism [12–14]. As one of a series of studies for solving this problem, attempts were made in this study to compare the two enzymes with respect to their sensitivity to NADPH inhibition in the presence of NADP$^+$ and glucose 6-phosphate, and to estimate their in vivo activities in normal rat livers. The sensitivity to NADPH inhibition was chosen as a parameter, because NADPH has been known to inhibit glucose-6-phosphate dehydrogenase competitively to NADP$^+$ [15–21], thus being

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important for regulating the enzyme activity in vivo [22,23], whereas its effect on the glucose-6-phosphate dehydrogenase activity of hexose-6-phosphate dehydrogenase has not been reported.

Kinetic studies on glucose-6-phosphate dehydrogenase

For initial velocity studies Eqn. 1 [3,19] was used:

$$\frac{V}{v} = \frac{K_{ia} \cdot K_b}{A \cdot B} + \frac{K_a}{A} + \frac{K_b}{B} + 1 \qquad (1)$$

where V is the maximal velocity, A and B are concentrations of $NADP^+$ and glucose 6-phosphate, respectively, K_a and K_b are Michaelis constants for $NADP^+$ and glucose 6-phosphate, respectively, and K_{ia} is the dissociation constant for $NADP^+$. The Lineweaver-Burk plots of $1/v$ vs $1/NADP^+$, and $1/v$ vs. 1/glucose 6-phosphate similarly gave a family of lines which intersected at a common point, thus indicating a sequential mechanism. The K_a, K_b and K_{ia} are 3.0, 18.1 and 4.2 μM, respectively. The inhibition patterns obtained with varied concentrations of $NADP^+$, NADPH and glucose 6-phosphate suggest that the NADPH inhibition is competitive with respect to $NADP^+$ and noncompetitive with respect to glucose 6-phosphate. Dead-end inhibition studies using glucosamine 6-phosphate as a substrate analogue for glucose 6-phosphate indicated that the glucosamine 6-phosphate inhibition is uncompetitive to $NADP^+$ and competitive to glucose 6-phosphate. Using Eqn. 2, [3,19] where Q is the NADPH concentration and K_{iq} is the dissociation constant for NADPH, K_{iq} is estimated to be 4.2 μM.

$$\frac{V}{v} = \frac{K_a}{A}\left(\frac{K_{ia} \cdot K_b}{K_a \cdot B} + 1\right)\left(\frac{Q}{K_{iq}} + 1\right) + \frac{K_b}{B} + 1 \qquad (2)$$

These findings are comparable to those reported by others with glucose-6-phosphate dehydrogenases from various sources [15–20].

Kinetic studies on hexose-6-phosphate dehydrogenase

As shown in Fig. 1, initial velocity studies indicate a sequential mechanism. The data fits well to Eqn. 1. The K_a, K_b and K_{ia} are 1.2, 1.3 and 5.4 μM, respectively. Figs. 2 and 3 show the NADPH inhibition patterns obtained when $NADP^+$ and NADPH concentrations were varied in the presence of 217 and 55 μM glucose 6-phosphate, respectively. Figs. 4 and 5 show the NADPH inhibition patterns obtained when glucose 6-phosphate and NADPH concentrations were varied in the presence of 5.8 μM and 0.5 mM $NADP^+$, respectively. The data show that the inhibition by NADPH is noncompetitive with respect to $NADP^+$, and uncompetitive with respect to glucose 6-phosphate (noncompetitive when the enzyme is not saturated with $NADP^+$), and that the binding of NADP and NADPH to the enzyme may be independent of each other, since the K_a does not differ significantly at different concentrations of NADPH. This suggests the presence of the ternary complex, enzyme-$NADP^+$-NADPH. The nature of this complex is extremely interesting, and thus merits further elaboration. In any case, at a given concentration of glucose 6-phosphate, the reaction follows the basic inhibition equation (Eqn. 3; Ref. 24):

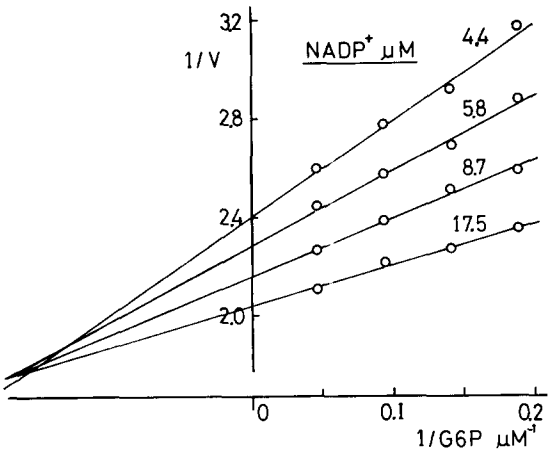

Fig. 1. Initial velocity pattern of hexose-6-phosphate dehydrogenase with glucose 6-phosphate (G6P) as the varied substrate. $NADP^+$ varied from 4.4 to 17.5 μM, and glucose 6-phosphate varied from 5.3 to 21.1 μM. The enzyme was purified as described previously [36]. $1/v$ = [nmol $NADP^+$ reduced/min]$^{-1}$. The straight lines are the regression curves calculated from four sets of data.

$$\frac{V}{v} = \frac{K_a}{A}\left(\frac{Q}{K_{iq}} + 1\right) + \frac{Q}{K_{iq'}} + 1 \qquad (3)$$

where K'_{iq} is the dissociation constant of the enzyme-$NADP^+$-NADPH complex.

Estimation of in vivo activities of the two enzymes

The above inhibition studies yielded the fol-

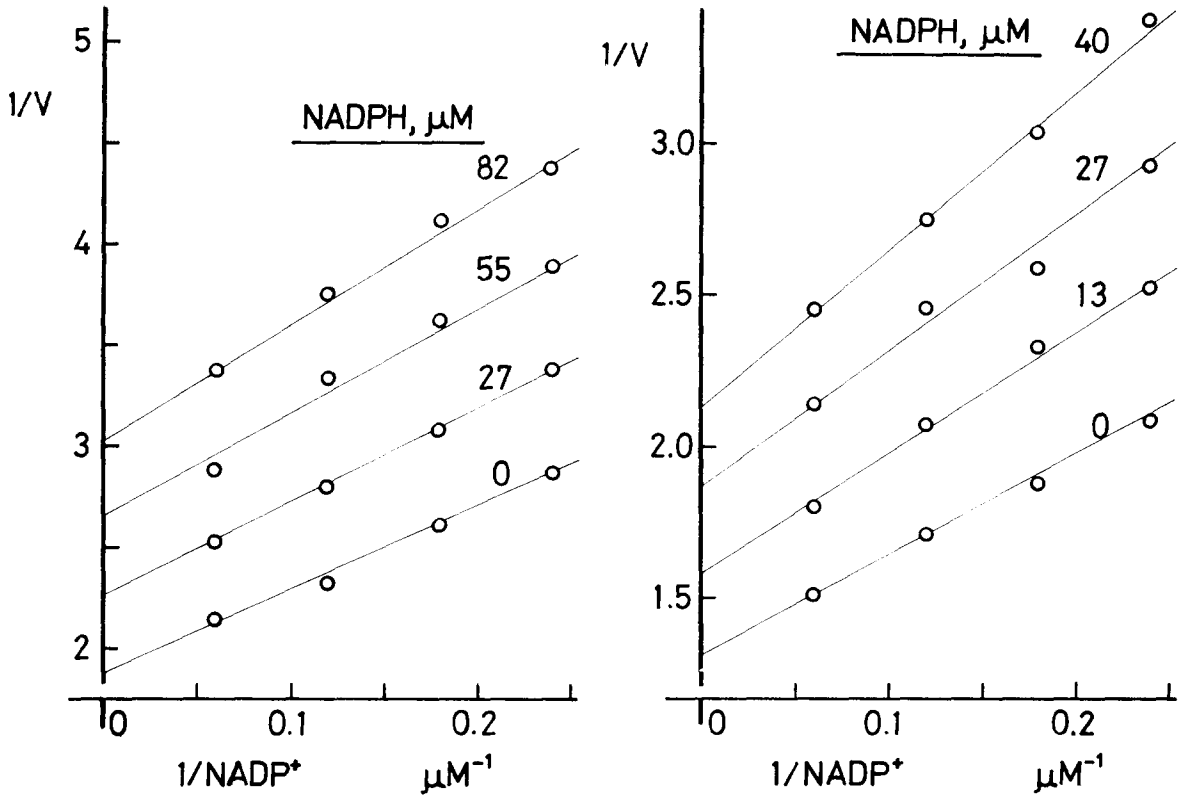

Fig. 2. NADPH inhibition plot of hexose-6-phosphate dehydrogenase with $NADP^+$ as the varied substrate. Glucose 6-phosphate is 217 μM, $NADP^+$ varied from 4.2 to 16.7 μM, and NADPH varied from 27 to 82 μM. $1/v$ = [nmol $NADP^+$ reduced/min]$^{-1}$. Regression curves obtained from four sets of data clearly indicated that the NADPH inhibition is noncompetitive with respect to $NADP^+$. Therefore, the straight lines fitting to Eqn. 3 are computed in such a way as to minimize a sum of squares F defined by:

$$F = \sum_{i=1}^{16} (V_{i,\mathrm{exp}} - V_{i,\mathrm{calc}})^2 ,$$

where $V_{i,\mathrm{exp}}$ is the measured velocity and $V_{i,\mathrm{calc}}$ is the computed velocity, and drawn on the figure.

Fig. 3. NADPH inhibition plot of hexose-6-phosphate dehydrogenase with $NADP^+$ as the varied substrate. Glucose 6-phosphate is 55 μM, $NADP^+$ varied from 4.2 to 16.7 μM, and NADPH varied from 13 to 40 μM. $1/v$ = [nmol $NADP^+$ reduced/min]$^{-1}$. The method for obtaining the straight lines is given in the legend for Fig. 2.

lowing results which may be of physiological importance: glucose-6-phosphate dehydrogenase is more susceptible to NADPH inhibition than hexose-6-phosphate dehydrogenase at a given concentration of glucose 6-phosphate, whereas the latter enzyme is more affected by glucose 6-phosphate concentration than the former at a given concentration of NADPH. This is clearly shown in Table I. Based on these findings, an attempt was next made to estimate the in vivo activities of the two enzymes at various concentrations of substrates and NADPH. The estimation was made as follows: (1) maximal in vitro enzyme activities (V) of microsomal (hexose-6-phosphate dehydrogenase) and cytosol (glucose-6-phosphate dehydrogenase) fractions of rat liver under various conditions were assayed with excess substrates at pH 7.5 and 37°C (Table II); (2) the K_{iq} values of the two enzymes are determined graphically from plots of $1/v$ vs. Q (Dixon plots), and the K'_{iq} of hexose-6-phosphate dehydrogenase from plots of s/v versus Q

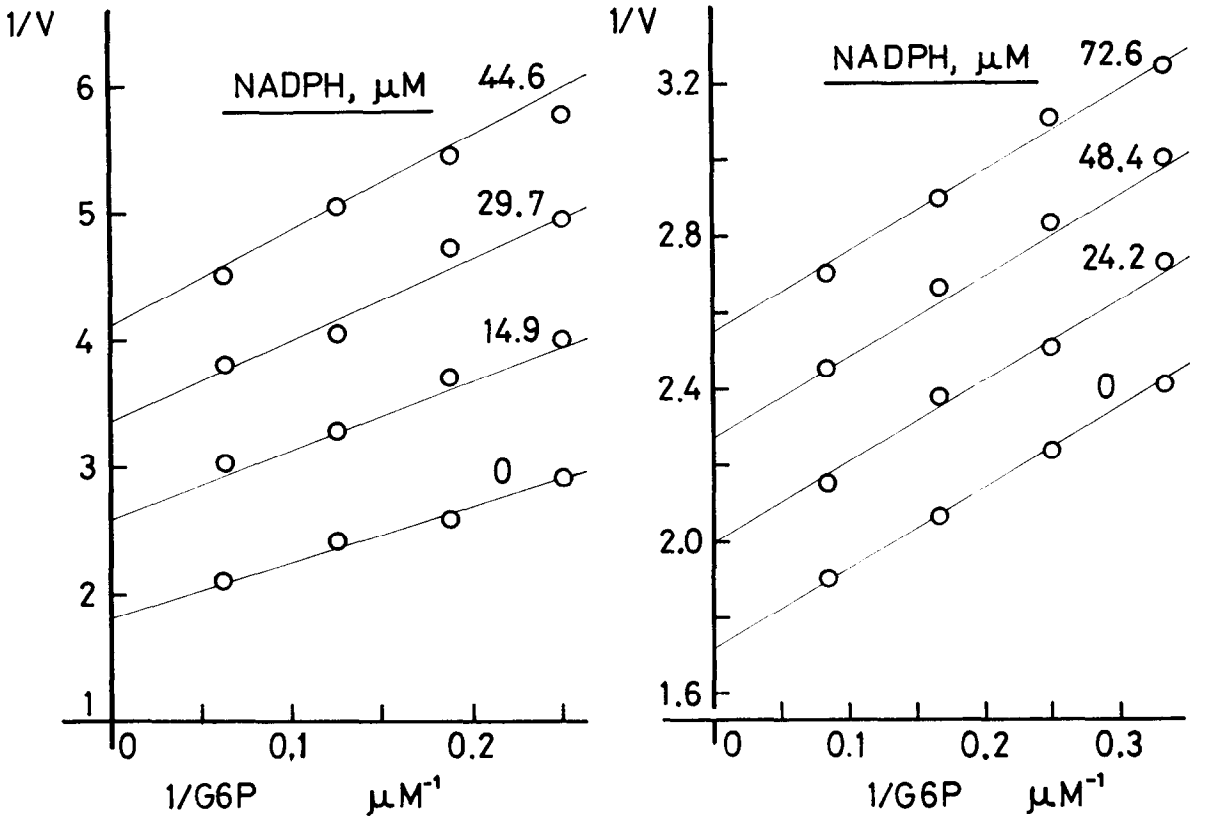

Fig. 4. NADPH inhibition plot of hexose-6-phosphate dehydrogenase with glucose 6-phosphate (G6P) as the varied substrate. $NADP^+$ is 5.8 μM, glucose 6-phosphate varied from 4.0 to 16.0 μM, and NADPH varied from 14.9 to 44.6 μM. $1/v$ = [nmol $NADP^+$ reduced/min]$^{-1}$. The method for obtaining the straight lines is given in the legend for Fig. 2.

Fig. 5. NADPH inhibition plot of hexose-6-phosphate dehydrogenase with glucose 6-phosphate (G6P) as the varied substrate. $NADP^+$ is 0.5 mM, glucose 6-phosphate varied from 3.1 to 12.4 μM, and NADPH varied from 24.2 to 72.6 μM. $1/v$ = [nmol $NADP^+$ reduced/min]$^{-1}$. The method for obtaining the straight lines is given in the legend for Fig. 2. Regression curves obtained from four sets of data indicated in this case that the NADPH inhibition is uncompetitive with respect to glucose 6-phosphate.

(Cornish-Bowden plots); (3) fractional activity (activity at given concentrations of substrates and NADPH relative to $V = v/V$) is calculated from Eqn. 3 using the K_{iq} and K'_{iq} estimated as above and the data of Greenbaum et al. [25] and Thompson et al. [20] on the in vivo concentrations of glucose 6-phosphate, $NADP^+$ and NADPH in rat livers under various conditions (Table III); and (4) the in vivo activity is then calculated from: $V \times$ fractional activity. The results are given in Table III, and may be summarized as follows: (1) although the V of glucose-6-phosphate dehydrogenase is 20-times greater than that of hexose-6-phosphate dehydrogenase in normal rat liver, the two enzymes appear to be at the same level of activity under the normal in vivo conditions (Table II); (2) hexose-6-phosphate dehydrogenase may be more active than glucose-6-phosphate dehydrogenase in fasted rats before and after refeeding of a high fat diet, whereas the latter enzyme becomes 22-times as active as the former enzyme upon refeeding of a high carbohydrate diet. This is explained in terms of the induction of glucose-6-phosphate dehydrogenase and a marked decrease of redox ratio in high carbohydrate-fed rats.

Besides the substrate concentrations there are many other factors which must be taken into account when the in vivo activities of these enzymes are to be estimated as precisely as possible. Among them,

TABLE I

EFFECTS OF GLUCOSE 6-PHOSPHATE CONCENTRATION AND COENZYME REDOX RATIO ON THE ACTIVITIES OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND HEXOSE-6-PHOSPHATE DEHYDROGENASE

The activities at three coenzyme redox ratios and at two glucose 6-phosphate concentrations were estimated from (maximal in vitro activity) × (fractional activity), which was calculated from Eqn. 3 as described in the text. NADPH used in this study (Kyowa Hakko Kogyo Co.) was substantially free of $NADP^+$ and gave the same results before and after purification. Its concentration was determined using $\epsilon_{340} = 6.22 \cdot 10^3$. Concentrations of $NADP^+$ and glucose 6-phosphate were determined enzymatically using purified rat liver glucose-6-phosphate dehydrogenase. Differences between 1 and 2, 3 and 4, and 5 and 6 are statistically significant at the 2.5% level, while others (1 vs. 3 or 5; 2 vs. 4 or 6; 7 vs. 8; 9 vs. 10; 11 vs. 12; 7 vs. 9 or 11; 8 vs. 10 or 12) are at the 1% level or less.

NADPH/$NADP^+$ μM (ratio)	Glucose-6-phosphate dehydrogenase (munits ± S.E./g liver)		Hexose-6-phosphate dehydrogenase (munits ± S.E./g liver)	
Glu-6-*P*	86 μM	217 μM	86 μM	217 μM
714/16 (45)	30.7 ± 1.64[1]	37.5 ± 2.00[2]	12.0 ± 0.34[7]	18.6 ± 0.53[8]
720/6.6 (110)	12.7 ± 0.68[3]	15.5 ± 0.82[4]	10.2 ± 0.29[9]	16.4 ± 0.47[10]
726/3.6 (203)	6.9 ± 0.37[5]	8.4 ± 0.45[6]	8.5 ± 0.24[11]	14.2 ± 0.41[12]
0/600 (maximal in vitro activity)	2620 ± 140 (n = 8) [a]		129 ± 3.7 (n = 8) [a]	

[a] Cited from Table II.

bivalent cations and steroids are thought to be particularly important, since these two classes of materials have diffferential effects on the two enzymes; i.e., Mg^{2+} and Ca^{2+} slightly stimulate glucose-6-phosphate dehydrogenase, but inhibit hexose-6-phosphate dehydrogenase, while steroids are inhibitory to the former [26–28], but without effect on the latter [11,29–31]. Therefore, we examined effects of these substances on the two enzymes at the coenzyme redox ratio of 110 and at a glucose 6-phos-

TABLE II

MAXIMAL IN VITRO ACTIVITIES OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND HEXOSE-6-PHOSPHATE DEHYDROGENASE IN CONTROL AND TREATED RAT LIVERS (MEAN ± S.E.)

The cytosol glucose-6-phosphate dehydrogenase activity and microsomal hexose-6-phosphate dehydrogenase activity were assayed spectrophotometrically with the 144 000 × g supernatant and sediment of 1 : 10 0.25 M sucrose homogenates, respectively. The assay mixture contained in a total of 1 ml : 50 μmol Tris/6 μmol glucose 6-phosphate/0.6 μmol $NADP^+$/enzyme (pH 7.5, 37°C). In the case of hexose-6-phosphate dehydrogenase assay, an aliquot of the microsomal fraction was first treated with excess anti-glucose-6-phosphate dehydrogenase antiserum [39] at 21°C for 30 min and then the enzyme activity was assayed as above. Treatment with the antiserum completely removed contaminated glucose-6-phosphate dehydrogenase activity as judged by polyacrylamide electrophoresis. In the case of glucose-6-phosphate dehydrogenase assay, contamination of hexose-6-phosphate dehydrogenase in the cytosol fraction was so low in comparison with glucose-6-phosphate dehydrogenase that the fraction was not treated with anti-hexose-6-phosphate dehydrogenase antiserum. High fat diet = 57% salad oil, 28% casein, 9% cellulose powder, 5% salt mixture, 1% saccharose. High carbohydrate diet = 60% saccharose, 30% casein, 4% salt mixture, 4% salad oil, 2% dry yeast.

	Glucose-6-phosphate dehydrogenase units/g liver (no. of rats)	Hexose-6-phosphate dehydrogenase munits/g liver (no. of rats)
Control	2.62 ± 0.14 (8)	129 ± 3.7 (8)
3-day fasting	2.27 ± 0.11 (4)	155 ± 5.3 (8)
3-day fasting, 3-day fat feeding	2.33 ± 0.11 (3)	109 ± 7.6 (3)
3-day fasting, 3-day feeding of high carbohydrate diet	22.2 ± 1.35 (3)	115 ± 9.7 (10)

TABLE III

ESTIMATION OF IN VIVO ACTIVITIES OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND HEXOSE-6-PHOSPHATE DEHYDROGENASE UNDER VARIOUS CONDITIONS

Enzyme activities were calculated as described in the text using the data shown in Table II and Figs. 1–5.

	NADPH [a] (μM)	$NADP^+$ [a] (μM)	Redox [a] ratio	Glucose-6-phosphate [a] (μM)	Glucose-6-phosphate dehydrogenase (munits/g liver)	Hexose-6-phosphate dehydrogenase (munits/g liver)
Control	720	6.6	110	217	15.5	16.4
3-day fasting	726	4.2	175	86	7.0	10.8
3-day fasting, 3-day fat feeding	726	3.6	203	111	6.6	8.5
3-day fasting, 3-day high carbohydrate diet	714	16.2	45	120	286	12.9

[a] Cited from Thompson et al. [20] and Greenbaum et al. [24].

phate concentration of 217 μM. For this purpose, enzyme activities were assayed in the presence of these substances at various concentrations of substrates, and the K_a, K_{iq}, K'_{iq} and fractional activities were determined by the same methods as described above. The results are shown in Table IV, which may be summarized as follows: both bivalent cations and steroids at the concentrations tested partially nullify

TABLE IV

EFFECTS OF BIVALENT CATIONS AND STEROIDS ON THE ACTIVITIES OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND HEXOSE-6-PHOSPHATE DEHYDROGENASE

	none	$CaCl_2$ 5 mM	$MgCl_2$ 5 mM	Dehydroepiandrosterone 50 μM	Androsterone 50 μM	Progesterone 50 μM
Glucose-6-phosphate dehydrogenase						
K_a (μM) [a]	5.66	4.51	6.73	3.66	3.05	3.75
K_{iq} (μM) [a]	4.03	5.70	9.79	6.02	5.43	6.05
$10^2 \times v/V$	0.59	1.13	1.29	1.46	1.58	1.43
V [b] (units/g liver)	2.62	2.80	2.75	0.705	1.70	1.90
V in vivo [c] (munits/g liver)	15.5	31.7	35.5	10.2	26.8	27.2
Hexose-6-phosphate dehydrogenase						
K_a (μM) [a]	2.20	1.46	1.48	same as none		
K_{iq} (μM) [a]	220	219	231			
K_{iq}' (μM) [a]	134	284	312			
$10^2 \times v/V$	12.7	22.3	23.6			
V [b] (munits/g liver)	129	76.6	88.5			
V in vivo [c] (munits/g liver)	16.4	17.1	20.9			

[a] Kinetic parameters were determined using the enzymes purified as described previously [36,38] at a glucose 6-phosphate concentration of 217 μM and at varied concentrations of $NADP^+$ and NADPH (pH 7.5, 37°C). Using these parameters, fractional activity (v/V) at a coenzyme redox ratio of 110 was estimated from Eqn. 3 as described in the text.

[b] Maximal in vitro activity with 217 μM glucose 6-phosphate and 0.6 mM $NADP^+$ in the presence of bivalent cations or steroids (pH 7.5, 37°C).

[c] In vivo activity in the presence of 217 μM glucose 6-phosphate and at a coenzyme redox ratio of 110 was estimated from $V \times$ fractional activity (v/V) as described in the text.

the inhibitory effect of NADPH on the two enzymes. Due to such effects, the activity in vivo doubles in the presence of bivalent cations in the case of glucose-6-phosphate dehydrogenase, and elevates 27% in the presence of Mg^{2+} in the case of hexose-6-phosphate dehydrogenase. We set the test concentration of bivalent cations at 5 mM in order to exaggerate the effects, but the physiological concentration of free Mg^{2+} in the liver cytosol is reported to be 0.4–0.8 mM [32–33]. Within such a range, therefore, the bivalent cations would probably have only an insignificant, if any, effect on the activities of the two enzymes. On the other hand, dehydroepiandrosterone is extremely inhibitory to glucose-6-phosphate dehydrogenase only. Although we have no information on the actual concentration of this steroid in rat tissues, available data indicate that the normal level of this steroid in human blood is about 0.4–1.4 μg/ml (1.4–4.9 μM; Refs. 34 and 35). It appears reasonable to assume therefore that the in vivo concentration of this steroid would not be so different from the tested concentration, 50 μM, within steroid-metabolizing cells. If so, then it may be said that it is advantageous for such cells to have hexose-6-phosphate dehydrogenase as an NADPH generator, since its activity is not or less affected by steroids or NADPH.

For the sake of simplicity, the above discussion is based on an assumption that hexose-6-phosphate dehydrogenase would exist in a fully active form. However, our previous data indicate that this enzyme is located in the luminal surface of the microsomal membrane, and its activity is latent probably because of the impermeability of the membranes to $NADP^+$ [36–38]. Physiological substances which have influence on such enzyme latency are not known. Accordingly, further studies on the latency are badly needed for an accurate estimation on the in vivo activity of this enzyme.

Carper et al. [3] studied the kinetics of pig liver glucose dehydrogenase with NAD^+, $NADP^+$, glucose and xylose as substrates, and reported that NADPH had no inhibitory effect on the enzyme, and that ethylenediaminetetraacetate completely removed the glucose 6-phosphate activity without affecting the glucose activity. However, this is in contrast to our finding that the glucose 6-phosphate activity of hexose-6-phosphate dehydrogenase is not affected by the chelating agent, irrespective of the source of the enzyme (rat, frog, fish and starfish [29–31]). It remains to be seen, therefore, if Carper et al. [3] and we are dealing with homologous enzymes.

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