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KINETIC ANALYSIS OF PARTIALLY PURIFIED GLUCOSE-6-PHOSPHATE DEHYDROGENASES FROM RAT MUSCLE, ADIPOSE TISSUE AND LIVER, AND FROM BAKER'S YEAST

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SUMMARY

1. The major electrophoretic form of glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate–NADP oxidoreductase, EC 1.1.1.49) from rat muscle, liver and epididymal adipose tissue has been partially purified. The kinetic properties of these rat tissue enzymes have been studied and compared with those of crystalline baker's yeast.

2. Glucose-6-phosphate dehydrogenase from rat muscle, liver and adipose tissue had K_m values for glucose 6-phosphate of $3.4 \cdot 10^{-5}$–$4.8 \cdot 10^{-5}$ M and for $NADP^+$ of $2.0 \cdot 10^{-6}$ M.

3. When the supernatants from liver and muscle extracts were used as the enzyme sources, NAD^+ will not substitute for $NADP^+$. NAD^+ will, however, substitute for $NADP^+$ if purified liver glucose-6-phosphate dehydrogenase is used as the enzyme source. $NADP^+$ at concentrations greater than 0.01 M exhibits substrate inhibition.

4. NADPH is a competitive inhibitor of both glucose 6-phosphate and $NADP^+$ for the rat muscle enzymes, while NADH is without inhibitory effect.

INTRODUCTION

Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate–NADP oxidoreductase, EC 1.1.1.49) in mammalian tissues such as liver[1], mammary gland[2], adrenal gland[3], and adipose tissue[4] has been shown to be adaptive to changes in nutritional conditions and/or hormonal balance. Bakay and Nyhan[5] have shown that more than one electrophoretic form of glucose-6-phosphate dehydrogenase exists in many of the tissues they studied. It is, therefore, of interest to determine if the enzyme form(s) which are altered in the tissues studied to date have physical properties similar to each other. Highly purified glucose-6-phosphate dehydrogenase from rat mammary gland and bovine adrenal gland has been intensively studied[2,6].

The enzymes from these two tissues have similar K_m values for glucose 6-phosphate and $NADP^+$.

This report presents evidence indicating that the major forms of glucose-6-phosphate dehydrogenase in rat muscle, adipose tissue and liver are similar to each other with respect to their K_m values for glucose 6-phosphate, and $NADP^+$, and with respect to their electrophoretic mobility. In contrast to glucose-6-phosphate dehydrogenase from rat mammary gland and bovine adrenal gland, the enzyme in crude extracts of rat muscle and liver, cannot utilize NAD^+ in place of $NADP^+$. The rat enzymes used in this study are inhibited by NADPH and by high concentrations of the substrate $NADP^+$. Data are also presented for glucose-6-phosphate dehydrogenase from *Saccharomyces cerivisiae* (baker's yeast) for the sake of comparison with the mammalian enzymes.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (160–200 g) from Holtzman Laboratories of Madison, Wisc. were used as tissue donors. After an initial adjustment period, the rats were starved 72 h and then refed 72 h to maximize tissue levels of glucose-6-phosphate dehydrogenase at the time of sacrifice.

Chemicals and reagents

Glucose 6-phosphate, 6-phosphogluconic acid, and crystalline glucose-6-phosphate dehydrogenase from baker's yeast (containing 105 units/mg protein) were obtained from Sigma Chemical Company. NAD^+, $NADP^+$, NADH, and NADPH were obtained from P and L Laboratories.

Tissue extracts

Rats were decapitated and exanguinated. Epididymal adipose tissue was removed as described by Geisler and Hansen[4]. The muscles from the abdomen and hind limbs were dissected from the animal and separated from the bone and fat. 30% homogenates (w/v) were made of all tissues with standard phosphate buffer (0.01 M potassium phosphate, 0.005 M EDTA, 0.01 M glucose and 0.005 M 2-mercaptoethanol; pH 7.0). The liver and adipose tissue homogenates were centrifuged for 30 min at 34 000 $\times$ *g* at 0–4 °C; the muscle homogenate for 50 min at 12 000 $\times$ *g* at 0–4 °C. In each case the infranatant fluid was removed and served as the enzyme source.

Enzyme assay

The activity of the $NADP^+$-dependent form of glucose-6-phosphate dehydrogenase was measured by the method of Kornberg and Horecker[7], using glucose 6-phosphate and $NADP^+$ at concentrations of 0.02 M and 0.36 mM, respectively. Velocities were determined under steady-state conditions. The capacity of glucose-6-phosphate dehydrogenase to utilize NAD^+ was measured in the same system except $NADP^+$ was replaced with 0.01 M NAD^+ and the pH adjusted to 8.0, as suggested by Criss and McKerns[6]. One enzyme unit equals 1 μmole of NADPH or NADH formed per min.

Enzyme purification

The purification of the glucose-6-phosphate dehydrogenases from rat muscle, adipose tissue, and liver was accomplished as follows.

DEAE-cellulose chromatography. The supernatant fluid was adsorbed onto a DEAE-cellulose column (1.5 cm × 15 cm) which had been equilibrated in standard phosphate buffer. None of the 6-phosphogluconate dehydrogenase present in the supernate was adsorbed to the column. Glucose-6-phosphate dehydrogenase was eluted from the column with a linear KCl gradient (0–0.4 M; 300 ml total volume).

$(NH_4)_2SO_4$ fractionation I. The peak fractions containing glucose-6-phosphate dehydrogenase activity were pooled and fractionated with $(NH_4)_2SO_4$. Glucose-6-phosphate dehydrogenase precipitated in the protein fraction between 35 and 55% saturation in agreement with Kagawa *et al.*[8]. The protein fraction containing glucose-6-phosphate dehydrogenase activity was resuspended and dialyzed overnight against several changes of standard phosphate buffer.

DEAE-Sephadex chromatography. The dialyzed enzyme solution was adsorbed onto a DEAE-Sephadex column (1.5 cm × 15 cm). Glucose-6-phosphate dehydrogenase was eluted from the column with a linear KCl gradient (0–0.4 M; 250 ml total volume). The peak fractions containing glucose-6-phosphate dehydrogenase activity were pooled.

Ammonium sulfate fractionation II. The pooled fractions were fractionated with $(NH_4)_2SO_4$ as described above. The precipitated enzyme was resuspended and dialyzed overnight against standard phosphate buffer.

Hydroxylapatite chromatography. The enzyme sample was applied to a hydroxylapatite column (2 cm × 22 cm) and unadsorbed protein washed from the column with 50 ml of standard phosphate buffer. Glucose-6-phosphate dehydrogenase was eluted from the column with a linear phosphate gradient (0.01–0.25 M potassium phosphate; 200 ml total volume). The fractions containing glucose-6-phosphate dehydrogenase activity were pooled and the volume reduced by ultrafiltration. The concentrated enzyme was stored in the cold (0–4 °C) until used for kinetic studies. The results of the purification scheme are summarized in Table I.

Electrofocusing of yeast glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase was electrofocused on an analytical column (LKB 8101; 110 ml). A pH gradient from 3–10 was established with 1% Ampholine Carrier Ampholytes. 1000 units of yeast glucose-6-phosphate dehydrogenase (105 units/mg protein) were added to the column and subjected to electrofocusing for 60 h at 4 °C with a constant voltage of 300 V. Absorbance at 280 nm, pH and glucose-6-phosphate dehydrogenase activity of the 1-ml protein–Ampholyte samples were determined. Two peaks of enzymatic activity were obtained with apparent pI values of 5.4 and 7.4. The peak fractions of each were pooled, concentrated by ultrafiltration and dialyzed against three changes of standard phosphate buffer.

Disc gel electrophoresis

The procedure used for the electrophoretic studies was a modified method of Davis[9]. All solutions used in the gel preparations and the electrophoretic Tris–glycine buffer were prepared as described by Davis[9]. The enzymatic activity staining solution,

TABLE I

SUMMARY OF THE PURIFICATIONS OF GLUCOSE-6-PHOSPHATE DEHYDROGENASES FROM A VARIETY OF RAT TISSUES

Specific activity is defined as μmole/min per mg protein.

Step	*Enzyme source*					
	Adipose		*Muscle*		*Liver*	
	Total activity (units)	*Spec. act.*	*Total activity (units)*	*Spec. act.*	*Total activity (units)*	*Spec. act.*
1. Supernatant	23.0	0.079	51.6	0.0012	7.3	0.0018
2. DEAE-cellulose	8.0	0.127	29.6	0.012	7.4	0.020
3. $(NH_4)_2SO_4$ I	6.0	NT*	32.6	0.038	1.8	0.016
4. DEAE-Sephadex	4.0	0.644	24.2	0.090	2.0	0.028
5. $(NH_4)_2SO_4$ II	NT	NT	23.0	0.122	NT	NT
6. Hydroxylapatite	3.0	7.68	12.0	NT	0.8	NT
7. Concentration by Amicon ultrafiltration	3.0	7.68	11.6	1.13	1.1	0.186

* NT, not tested.

destaining of gels and their subsequent storage has been described by Bakay and Nyhan[5].

Purification of NADP+

NADP+ obtained from P and L Laboratories contains NADP+ *plus* three trace contaminants. In order to obtain NADP+ which was not contaminated, 75 mg of NADP+ (lot No. 19005) was dissolved in water and subjected to ascending single dimension paper chromatography using Whatman 3MM paper (47 cm × 57 cm) and the following solvent system: isobutyric acid–conc. NH_4OH–water, (66:1:33, by vol.). The developed chromatogram was air-dried overnight and the nucleotide spots located by the quenching of ultraviolet light. The R_F values obtained were 0.461, 0.336, 0.183, 0.106, corresponding to Trace 1, NADP+, Trace 2, and Trace 3, respectively. These results compare reasonably well with the Certificate of Analysis from P and L Biochemicals. The chromatogram was cut transversely into strips, each containing one of the isolated components. The spots were eluted from the paper by descending chromatography with water. The eluates were lyophilized to dryness; except for NADP+ which was precipitated from acidified water with 6 vol. of acetone at −10 °C. The precipitated NADP+ was washed several times with acetone and dried by passing a stream of N_2 over it. The amount of NADP+ collected was estimated by the method of Kornberg and Horecker[10] and appropriate dilutions made for the kinetic studies presented in the results section. None of the other isolated components exhibited NADP+ activity.

RESULTS

Electrophoresis of glucose-6-phosphate dehydrogenase

Electrophoretic studies of rat muscle, and adipose tissues indicate that more than one enzymatically active form of glucose-6-phosphate dehydrogenase is present

in these tissues (Table II), in agreement with the studies of other investigators[5]. Qualitative electrophoretic analysis (Table II) shows that the purification scheme used in this study purifies only the major form of this enzyme from these tissues. The R_M value for the major form of glucose-6-phosphate dehydrogenase from electrofocused baker's yeast was different from the major form from the rat tissues studied (Table II).

TABLE II

SUMMARY OF THE ELECTROPHORETIC MOBILITIES OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE FROM SEVERAL SOURCES

Enzyme source	R_M *values*
Rat adipose	0.61*, 0.74, 0.82
Rat muscle	0.61*, 0.73, 0.83, 0.90
Baker's yeast	0.56*, 0.74
Purified rat dipose	0.61

* Major form of enzyme.

Purification of glucose-6-phosphate dehydrogenase

A 100-fold purification of glucose-6-phosphate dehydrogenase from epididymal adipose tissue and liver and a 1000-fold purification from muscle were achieved through the use of the scheme described in detail in Materials and Methods. Typical purifications of the enzyme from each of the three tissues are presented in Table I. Hexokinase II cochromatographs with glucose-6-phosphate dehydrogenase except on hydroxylapatite columns, where a reasonable separation is achieved. The final purified forms of glucose-6-phosphate dehydrogenase described herein still have approximately 1% hexokinase II contamination.

Electrofocusing of yeast glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase activity and protein profiles, along with the linear pH gradient, were achieved by electrofocusing baker's yeast as described in Materials and Methods. Pool I represents those fractions between 20–35 with a pI of 5.4; pool III, those fractions between 50–65 with a pI of 7.4. This designation will be adhered to throughout the following discussion.

Kinetic properties of rat tissue glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenases from rat muscle, epididymal adipose tissue, and liver have similar kinetic properties (Table III). The kinetic data for muscle glucose-6-phosphate dehydrogenase (Fig. 1) are typical of those for liver and adipose tissue. Glucose-6-phosphate dehydrogenase from muscle tissue has a K_m for $NADP^+$ of $2.0 \cdot 10^{-6}$ M and for glucose 6-phosphate of $3.4 \cdot 10^{-5}$ M (Figs 1a and 1b). These figures agree well with the K_m values reported by Criss and McKerns[6] for glucose-6-phosphate dehydrogenase from bovine adrenals. A major difference between the bovine adrenal enzyme and the unpurified enzymes from rat muscle, and liver is that glucose-6-phosphate dehydrogenase obtained from these rat tissues cannot substitute NAD^+ for $NADP^+$. In contrast to the other rat tissues studied, the unpurified enzyme from adipose tissue can utilize NAD^+ and $NADP^+$. Partially purified

glucose-6-phosphate dehydrogenase from rat liver can substitute NAD^+ for $NADP^+$ (Table III). This phenomenon is presently under investigation to determine if the ability to use NAD^+ is a function of the purification process.

$NADP^+$ at concentrations higher than 0.01 M inhibit the enzyme from rat muscle (Fig. 1a), and from liver and adipose tissue (Table III). Fig. 1c shows that

TABLE III

SUMMARY OF KINETIC STUDIES OF PURIFIED GLUCOSE-6-PHOSPHATE DEHYDROGENASE FROM VARIOUS SOURCES

NR, no reaction; NT, not tested; +, NAD can substitute.

Kinetic parameter	*Enzyme source*			
	Adipose	*Muscle*	*Liver*	*Yeast*
K_m				
Glucose-6-*P* (μM)	35.0	34.0	48.0	35.0
$NADP^+$ (μM)	1.7	2.1	1.1	2.8
NAD^+ (μM)	+	NR	+	NR
Inhibition characteristics				
Glucose-6-*P* as variable substrate				
NADPH	Yes	Yes	Yes	Yes
NADH	No	No	No	Yes
6-Phosphogluconic acid	No	No	NT	No
$NADP^+$ as variable substrate				
$NADP^+$	Yes	Yes	Yes	Yes
NADPH	NT	Yes	NT	NT
K_i (calculated)				
NADPH (mM)	0.15	0.10	0.15	0.01
NADH (mM)	NR	NR	NR	0.10
pI	NT	5.6	5.8	5.4

NADPH is a competitive inhibitor of glucose 6-phosphate for muscle glucose-6-phosphate dehydrogenase while NADH (Fig. 1d) has no inhibitory effect. Fig. 2a shows that NADPH is also a competitive inhibitor of $NADP^+$ for muscle glucose-6-phosphate dehydrogenase. With increasing concentrations of glucose 6-phosphate, V is increased while the apparent K_m for $NADP^+$ decreases (Fig. 2b).

Kinetic properties of yeast glucose-6-phosphate dehydrogenase

Two activity bands were obtained by electrofocusing yeast glucose-6-phosphate dehydrogenase. The kinetic properties of the pool with a pI of 7.4 were identical to those of the pool with a pI of 5.4. Glucose-6-phosphate dehydrogenase from baker's yeast has a K_m for $NADP^+$ of $2.8 \cdot 10^{-6}$ M and for glucose 6-phosphate of $3.5 \cdot 10^{-5}$ M (Figs 3a and 3b and Table III). These figures agree well with the corresponding parameters of rat tissue glucose-6-phosphate dehydrogenase (Table III), and with those reported by others[6,11]. These characteristics are different from those presented by Sanwal[12] for the enzyme from *Escherischia coli*. Fig. 3 shows that yeast glucose-6-phosphate dehydrogenase is inhibited by high levels of $NADP^+$ in agreement with the enzymes from the rat tissues tested (Table III, Fig. 1a). NADPH is a competitive inhibitor of glucose 6-phosphate for yeast glucose-6-phosphate dehydrogenase (Fig. 3c). In contrast to the glucose-6-phosphate dehydrogenase from the rat tissues

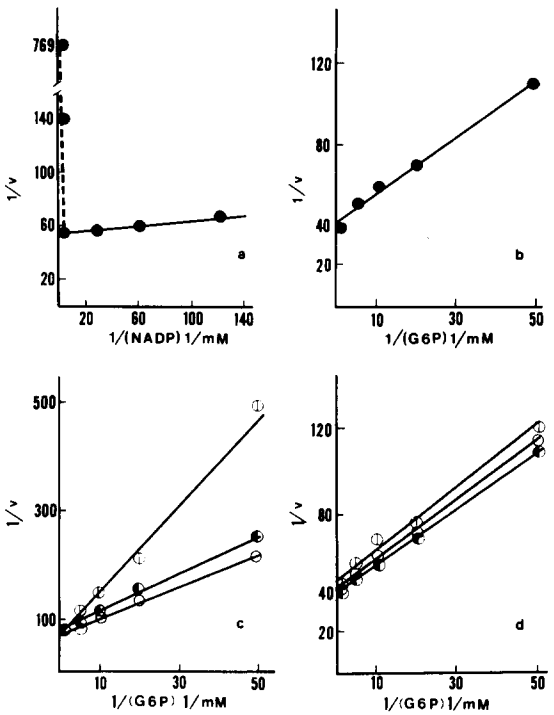

Fig. 1. Lineweaver–Burk plots of kinetic data obtained with partially purified glucose-6-phosphate dehydrogenase from rat muscle at 25 °C with various substrates. The assay mixture contained 0.018 M glycylglycine–HCl buffer at pH 7.5, 0.0036 M $MgCl_2$, enzyme, and various concentrations of substrates to a total volume of 3 ml. Each point represents the mean of several points and each line was determined by least squares method. The K_m was calculated from this line. (a) Glucose 6-phosphate concentration was 0.67 μM with various concentrations of $NADP^+$. (b) $NADP^+$ concentration was 0.36 mM with various concentrations of glucose 6-phosphate. (c) Inhibition of glucose-6-phosphate dehydrogenase by NADPH with glucose 6-phosphate as the variable substrate in the presence of 0.36 mM $NADP^+$. The concentrations of NADPH were: ○—○, no inhibitor; ◐—◐, 34 μM NADPH; ①—①, 167 μM NADPH. (d) The effect of NADH on glucose-6-phosphate dehydrogenase with glucose 6-phosphate as the variable substrate in the presence of 0.36 mM $NADP^+$. The concentratons of NADH were: ○—○, no inhibitor; ◐—◐, 3.7 μM NADH; ①—①, 7.4 μM NADH.

studied (Table III), the yeast enzyme is inhibited by NADH (Fig. 3d). This phenomenon agrees with that found by Sanwal[12] for glucose-6-phosphate dehydrogenase from *E. coli*.

Glucose-6-phosphate dehydrogenase kinetics with purified $NADP^+$

Since substrate inhibition had never been reported for this enzyme, it was possible that the inhibition could be due to a contaminant in the $NADP^+$. Therefore, $NADP^+$ was purified as presented in detail in the experimental procedures. Rat muscle glucose-6-phosphate dehydrogenase was used as the enzyme source to examine the purified $NADP^+$. The K_m for $NADP^+$ and the inhibitory properties of high concentrations of $NADP^+$ were similar to those obtained with chromatographically impure commercial $NADP^+$. The major contaminant of the commercial preparation of $NADP^+$ used was also isolated. It appeared to be a competitive inhibitor of

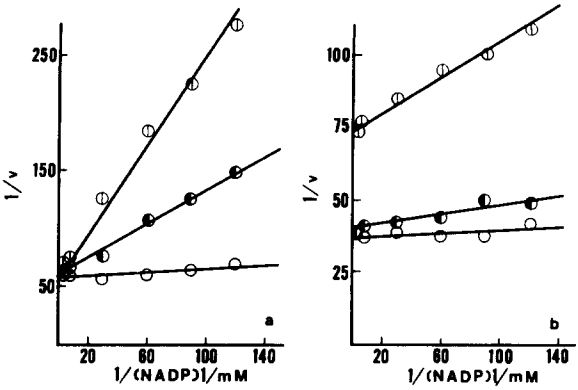

Fig. 2. Lineweaver–Burk plots of kinetic data obtained with partially purified glucose-6-phosphate dehydrogenase from rat muscle. All conditions are identical to those outlined in Fig. 1. (a) Inhibition of glucose-6-phosphate dehydrogenase by NADPH with $NADP^+$ as the variable substrate in the presence of 0.67 μM glucose 6-phosphate. The concentrations of NADPH were: ○—○, no inhibitor; ◐—◐, 34 μM NADPH; ⓘ—ⓘ, 167 μM NADPH. (b) Increase in V with increase in glucose 6-phosphate concentrations: ○—○, 1 mM glucose 6-phosphate; ◐—◐, 0.1 mM glucose 6-phosphate; ⓘ—ⓘ, 0.01 mM glucose 6-phosphate. Glucose 6-phosphate was held constant while $NADP^+$ was varied.

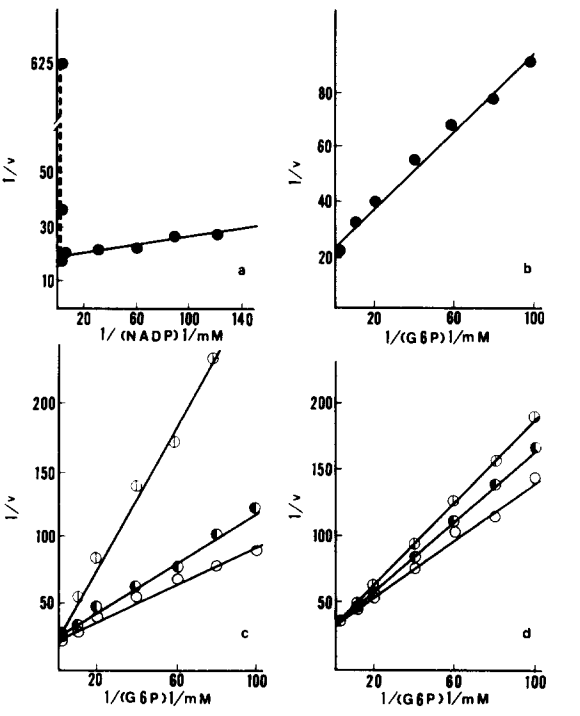

Fig. 3. Lineweaver–Burk plots of kinetic data obtained with glucose-6-phosphate dehydrogenase from crystalline baker's yeast following isoelectric focusing. All conditions and parameters are identical to those outlined in Fig. 1.

$NADP^+$ for glucose-6-phosphate dehydrogenase when added to assays containing purified $NADP^+$. The identity of this contaminant is not known, however, the preliminary results obtained with the contaminant warrant further study.

DISCUSSION

Glucose-6-phosphate dehydrogenase has been shown to reduce both NAD^+ and $NADP^+$ in some unicellular organisms[11,13] and also several mammalian tissues, mammary gland[14] and bovine adrenals[6]. In like manner, the unpurified isoenzymes of glucose-6-phosphate dehydrogenase from adipose tissue can utilize both NAD^+ and $NADP^+$. In contrast, the unpurified enzymes from liver and muscle can only utilize $NADP^+$. This report deals with the glucose-6-phosphate dehydrogenase isoenzyme which represents greater than 90% of the demonstrable enzymatic activity in the three tissues studied. Polyacrylamide gel electrophoresis shows that there are several forms of glucose-6-phosphate dehydrogenase present in these rat tissues, and that the purification scheme used here, purifies only the major form.

The K_m values reported here for glucose 6-phosphate and $NADP^+$ from these tissues agree well with those reported by others[6,11]. The competitive inhibition by NADPH also agrees with other reports[12]. The calculated K_i values for NADPH inhibition of rat muscle, liver, adipose tissue and yeast glucose-6-phosphate dehydrogenase are 0.1 mM, 0.15 mM, 0.15 mM, and 0.01 mM, respectively (Table III). The fact that the K_i for the yeast enzyme is 0.1 that of the rat enzymes suggests that NADPH may be a more effective inhibitor in yeast cells than in the rat tissues studied. Rat mammary gland glucose-6-phosphate dehydrogenase and the bovine adrenal gland enzyme utilize NAD^+ as a substrate and are inhibited by NADH. The fact that partially purified glucose-6-phosphate dehydrogenases isolated from rat liver or adipose tissue may be able to utilize NAD^+ as a substrate but are not inhibited by NADH, suggests that the major form of the enzyme in these tissues differs substantially from the major form of the enzyme in rat mammary gland or bovine adrenal gland. Yeast glucose-6-phosphate dehydrogenase, in contrast to the mammalian enzymes, is inhibited by NADH, but cannot utilize NAD^+. Sanwal[12] reports that glucose-6-phosphate dehydrogenase from *E. coli* is inhibited by NADH, and utilizes NAD^+ as a substrate but only at very high concentrations.

The inhibition of glucose-6-phosphate dehydrogenase from rat liver, muscle and adipose tissue by $NADP^+$ was unexpected. The possibility that inhibition of these enzymes by a contaminant in the commercial preparation of $NADP^+$ has been ruled out. Since NADPH is a competitive inhibitor of the enzyme it is possible that rapid formation of NADPH in the area surrounding the enzyme might be causing the enzymatic inhibition observed with high levels of $NADP^+$. This conclusion is partially ruled out by two factors: (1) the assays were continuously stirred during the reaction which should result in an even distribution of the product throughout the medium even if it is rapidly produced, provided the inhibitor is easily removed, and (2) if NADPH were formed, it should have been immediately detected spectrophotometrically. It, therefore, seems most likely that $NADP^+$ acts as an inhibitor of the reaction by competing with itself, in some undetermined manner, for proper binding to the enzyme.

Since inhibition of glucose-6-phosphate dehydrogenase from rat liver, muscle,

and adipose tissue by $NADP^+$ requires unphysiologically high amounts, and because the enzymes from these tissues are not inhibited by NADH, it is concluded that NADPH, but not NADH or $NADP^+$, functions as a physiologically important inhibitor of the enzyme in these tissues.

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