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Rat Liver Cytoplasmic Glucose-6-phosphate Dehydrogenase. Steady-State Kinetic Properties and Circular Dichroism†

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ABSTRACT: Steady-state kinetic studies including initial velocity, NADPH product inhibition, dead-end inhibition, and combined dead-end and product inhibition measurements with purified rat liver glucose-6-phosphate dehydrogenase indicate a sequential and obligatory addition of substrates in the order of $NADP^+$, glucose-6-P for the catalytic pathway at pH 8.0. Although instability of 6-phosphoglucono-δ-lactone precluded product inhibition experiments which might directly exclude an enzyme-6-phosphoglucono-δ-lactone complex, the absence of an enzyme-glucose-6-P complex suggests that the enzyme-lactone product is unlikely and the release of products is also ordered, with NADPH released last. Consideration of the kinetic constants (K_a = 2.0 μM, K_{iq} = 13 μM) and cellular concentration of the substrates and products suggests extensive inhibition of the enzyme in vivo and control by the NADPH/$NADP^+$ ratios. Circular dichroism spectra of the enzyme in 20 mM phosphate buffer at pH 7.0 and 25 °C indicate 51% helix and 33% pleated sheet structures which is considerably different from results (14% helix) with yeast enzymes.

This paper reports steady-state kinetic and secondary structure analyses of rat liver glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:$NADP^+$ 1-oxidoreductase, EC 1.1.1.49) to further characterize the liver enzyme's physical properties and to provide estimates of catalytic activities with cellular substrate and product concentrations. Steady-state kinetic mechanisms have been reported for the enzymes from *Candida utilis* (Afolayan, 1972), human erythrocyte (Soldin and Balinsky, 1968), *Leuconostoc mesenteroides* (Olive et al., 1971), and human blood platelets (Kosow, 1974). Enzymes from these sources represent each of the three classes of nucleotide specificity (Olive et al., 1971). An ordered sequential mechanism with addition of coenzymes to the free enzyme forms is indicated in each case. In addition, the NAD^+-linked reaction with *L. mesenteroides* (Olive et al., 1971) appears to require an isomerization of free enzyme, and the $NADP^+$-linked reactions of *C. utilis* and erythrocyte enzymes (Afolayan, 1972; Afolayan and Luzzatto, 1971; Luzzatto and Afolayan, 1971) exhibit sigmoidal kinetics.

Although subunits of the enzyme are thought to be identical in all tissues of a given animal (Yoshida, 1966), considerable tissue differences are observed in both the polymerization (Schmukler, 1970) and the microheterogeneity of enzyme within a given molecular weight form.[1] Previous

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steady-state kinetic studies on rat liver cytoplasmic enzyme concerned themselves with the effect of substitution at C-2 of glucose-6-P on the reaction rate (Bessell and Thomas, 1973), nucleotide specificity (Metzger et al., 1972; Geisler et al., 1973), and substrate and product inhibition (Geisler et al., 1973). While the enzyme has been purified to near homogeneity by disc-gel electrophoresis and ultracentrifugation criteria in several laboratories giving specific activities of 134–210 μmol/(min mg) (U/mg) (Watanabe and Taketa, 1972, 1973; Matsuda and Yugari, 1967; Holten, 1972) previous steady-state kinetic measurements were limited to enzymes with a specific activity of less than 0.5 U/mg. Since Geisler et al. (1973) find specificity of enzyme to vary with purity and Bonsignore et al. (1968) describe three different glucose-6-P dehydrogenase inactivating proteins from rat liver, it is desirable to study the steady-state kinetic mechanism of purified enzyme. In this and previous reports on the enzymatic mechanisms, data on the reverse reaction and product inhibition by 6-phosphoglucono-δ-lactone are lacking due to the instability of the lactone (Horecker and Smyrniotis, 1953).

To our knowledge, circular dichroism of the liver enzyme has not been reported, though such data exist for yeast enzymes (Jirgensons, 1966; Domschke et al., 1970).

Experimental Section

Materials. D-Glucose-6-P (monosodium), D-glucosamine-6-P, nucleotides (highest purity), Tris (TRIZMA base), and 2-mercaptoethanol were from Sigma Chemical Co. $Na_2EDTA{\cdot}2H_2O$ was from Schwarz/Mann, and crystalline bovine serum albumin was from Pentex. Heavy metal contaminants were extracted from analytical reagent $MgCl_2$ with dithizone (Morrison and Uhr, 1966).

Enzyme Preparation and Assay. The enzyme was purified from Holtzman strain albino rats according to Holten's (1972) modification of the method by Matsuda and Yugari (1967) with minor alterations. The animals were fasted 2 days when fed a high carbohydrate (glucose), low fat diet (Rudack et al., 1971) for 3 days prior to sacrifice. The liver was homogenized in a Waring blendor. The enzyme was eluted from the CM-cellulose (Reeve-Angel) column with a linear gradient of 600 ml of 20 mM NH_4OAc buffer (pH 5.5) and 600 ml of 50 mM potassium phosphate buffer (pH 7.0), each containing 0.1 mM $NADP^+$. All buffers used contained 5 mM β-mercaptoethanol and 0.1 mM EDTA. The enzyme was concentrated by ultrafiltration through collodion membranes (Schleicher and Schuell). All enzyme storage buffers contained 0.1 mM $NADP^+$. Specific activities during enzyme purification were measured at 25 °C in 0.1 M Tris-HCl buffer (pH 8.0) with 4 mM $MgCl_2$, 1.0 mM glucose 6-phosphate, and 0.15 mM $NADP^+$. Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin standards whose concentrations were calculated from A_{280} measurements and an absorptivity of 0.67 $(mg\ cm/ml)^{-1}$.

Kinetics. Initial velocity measurements were made in 1.00-cm path-length cuvettes in a Coleman 124 spectrophotometer with a thermostated cell holder kept at 25 ± 0.1 °C. Linearity of absorbance with concentration was shown to be within 4% throughout the NADPH concentration range used in this study, demonstrating lack of stray light errors blamed for erroneous conclusions concerning NADH inhibition of the *Escherichia coli* enzyme (Cavalieri and Sable, 1973). Substrates and effectors were incubated with the assay buffer (0.1 M Tris-HCl–4 mM $MgCl_2$ (pH 8.0)) and reactions started by addition of enzyme. This buffer was initially chosen to permit comparison with literature data. Catalytic turnover of glucose and glucosamine 6-phosphate used in dead-end inhibitor experiments was tested and found negligible relative to turnover of glucose-6-P.

Cellular Concentrations and Redox Ratios. Cytoplasmic redox ratios, $r = (NADP^+)/(NADPH)$, and whole cell glucose-6-P concentrations were taken from Tables I and VI, respectively, of Greenbaum et al. (1971), and total nucleotide contents ($NADP^+ + NADPH$) were obtained from Williamson and Brosnan (1974) and Williamson and Corkey (1969). As discussed by Veech, et al. (1969), cytoplasmic content probably accounts for about 90% of the whole cell content of the metabolites considered here (100% of glucose-6-P and the dehydrogenase); thus we have equated whole cell and cytoplasmic contents. We further assume that the total coenzyme content does not vary significantly with the metabolic state; thus $NADP^+ \simeq r(NADP^+ + NADPH)$. Conversions to cytosolic concentrations were made with the factor 2.0 g of liver/ml of cytosolic water (Williamson, 1969). It should be noted, however, that the conclusions drawn from these concentrations depend almost exclusively (>99%) on the redox ratio and to a smaller extent on the glucose-6-P concentrations. The absolute concentrations of $NADP^+$ and NADPH may be in error by severalfold without significantly affecting the calculated enzyme fractional activity. Validity of the redox ratios is supported by independent methods of determining them (Greenbaum et al., 1971). Maximum catalytic capacity of rat liver was estimated as (enzyme units in crude extract per g of tissue)/(enzyme units per mg of crystalline enzyme) using Matsuda and Yugari's data (1967).

Circular Dichroism. Circular dichroism (CD) measurements were obtained with a Cary 61-CD spectropolarimeter and a 1-mm path-length cuvette while flushing the optical path with N_2. The CD instrument was calibrated with an aqueous solution of *d*-10-camphorsulfonic acid (Aldrich) (Cassim and Yang, 1969). Enzyme (50 μg/ml) was in 20 mM potassium phosphate buffer (pH 7.0) with 10 μM $NADP^+$. The background spectrum of 10 μM $NADP^+$ in buffer with virtually no CD activity was subtracted from the spectrum of enzyme solution. Ellipticities were not corrected to in vacuo conditions.

Data Analyses. Initial velocity, product inhibition, dead-end inhibition, and combined product and dead-end inhibition data were analyzed by equations 1–4, respectively. The combination of data from all of the above experiments was analyzed by eq 4. Each of these equations represents linear kinetic mechanisms.[2] A and B are substrate concentrations, and Q and I are product and dead-end inhibitor concentrations, respectively. Curve fitting to these equations was done by a Fortran program package VINIT which utilizes the nonlinear regression subroutine STEPIT (Chandler, 1971) to minimize a weighted sum of squares F defined by eq 5, where $Y_{i,exp}$ is the measured velocity, $Y_{i,calcd}$ is the computed velocity, N is the number of points, and σ_i is the standard deviation in measured velocity, found from repeated measurements to be about 7% of the measured velocity. With the VINIT package any parameter(s) may be simply fixed with a program switch to values determined from previous experiments designed specifically for the accurate determination of these parameters. This avoids the problem of

[2] Nomenclature of steady-state kinetics used in this article is that of Cleland (1970).

an inaccurately determined parameter assuming some physically unrealistic value in order to give an arbitrarily good fit. To permit combination of data from different days with different quantities of enzyme, separate V_m parameters (V_{m1}, V_{m2}, ...) were fitted to each day's experiment. This was done by defining F in eq 5 as the sum of F_1, F_2, ... with V_{m1}, V_{m2}, ... used in F_1, F_2, ..., respectively. All other kinetic constants were common to all F_1, F_2, ...

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \quad (1)$$

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB + (K_{ia}K_b + K_aB)Q/K_{iq}} \quad (2)$$

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA(1 + I/K_i) + AB} \quad (3)$$

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA(1 + I/K_i) + AB + (K_{ia}K_b + K_aB)Q/K_{iq}} \quad (4)$$

$$F = \sum_{i=1}^{N} \left[\frac{Y_{i,exp} - Y_{i,calcd}}{\sigma_i}\right]^2 \quad (5)$$

The program prints out the fitted parameter values, errors in these values based on the usual linear approximation (Bevington, 1969), the correlations among the parameters, and a chi-square estimate of the quality of the fit (Hamilton, 1969). A chi-square equal to the number of degrees of freedom (data points minus adjustable parameters) corresponds to a 50% probability of obtaining a worse fit (chi-square probability = 0.5). A chi-square probability greater than approximately 0.1 is considered an acceptable fit by this criterion. Circular dichroism data were analyzed according to the method of Chen, et al. (Chen et al., 1972) using their published values for $[\theta]_{H,\lambda}$, $[\theta]_{\beta,\lambda}$, and $[\theta]_{R,\lambda}$ determined from proteins. However, the curve fitting was done using the STEPIT subroutine (Chandler, 1971) in a Fortran program HELIX. The constraints of the Chen algorithm were employed, namely $0 \leq f_H, f_\beta \leq 1$. However, as f_H and f_β were optimized they were further constrained within the interval [0, 1]. That is, if f_H assumed some value greater than 0, then f_β assumed some value less than $1 - f_H$. This constraint was implemented by optimizing the parameters f_H and $f_\beta/(1 - f_H)$ within the intervals [0, 1]. Error estimates for f_H and f_β were by the usual linear approximation (Bevington, 1969). The weighted sum of squares minimized was the function F as above with $Y_{i,exp}$ and $Y_{i,calcd}$ equal to measured and calculated $[\theta]_i$ values, respectively, N equal to the number of wavelengths i (N = 14), and σ_i equal to the S.D. in the mean residue ellipticity, approximated as 470 deg cm^2/dmol (2% of instrument range setting) at each wavelength. A mean residue molecular weight of 118 was used in these calculations. Initial estimates for f_H were made from data at 222 nm according to the formula of Chen and Yang (1971) and the initial f_β values were arbitrarily estimated at 0.5.

Results

Enzyme Purity. Enzyme specific activities (μmol min^{-1} mg^{-1}) at 25 °C in the Tris-HCl buffer at pH 8.0 were 100–150 for our kinetic studies and 200 for enzyme used in the circular dichroism measurements. This criterion indicates high enzyme purity when compared with literature specific activities for homogeneous enzyme assayed in the same buffer: 142 at 37 °C (Watanabe and Taketa, 1972) and 210 at 30 °C (Holten, 1972).

Initial Velocities. Measurements shown in Figure 1A indicate an intersecting initial velocity, double reciprocal pattern, but since K_a was low and data below 10 μM NADP$^+$ are impractical in a 1-cm cell, a parallel pattern is not convincingly excluded by these data. Initial velocity experiments at lower NADP$^+$ concentrations in a 10-cm path-length cell were unsuccessful, however, due we believe to instability of enzyme at these low NADP$^+$ and enzyme concentrations.

Product Inhibitions. Inhibition by NADPH appears linear competitive with respect to NADP$^+$ and linear noncompetitive with respect to glucose-6-P (Figure 1C and D). Although accuracy in the K_a value determined from initial velocity data in a 1-cm path-length cell alone is poor (50% S.D.), NADPH inhibition raises the apparent K_a (K_a') to easily measured values and K_a is more accurately determined from the dependence of K_a' on the concentrations of B and Q (see below for results).

Instability of 6-phosphogluconol-δ-lactone (Horecker and Smyrniotis, 1953) prevents its use in product inhibition measurements. Thus, neither the qualitative features of the NADPH inhibition pattern nor the initial velocity data are sufficient to exclude a ping-pong[2] mechanism. This mechanism can be convincingly excluded, however, by quantitatively examining fits to the combined NADPH inhibition data of Figure 1C and D. Fits of eq 2 with K_{ia} first fitted and then fixed at zero were computed; in the latter case both alternative identities of A and B were used. Whereas a good fit was obtained with finite K_{ia} (chi-square probability of 0.5 with a S.D. of 8.4% in individual velocity measurements) the best fit obtained with $K_{ia} = 0$ showed that a ping-pong mechanism was unacceptable (chi-square probability $\leq 10^{-4}$).

Dead-End Inhibitors. Glucosamine-6-P gives linear competitive inhibition with respect to glucose-6-P and linear uncompetitive inhibition with respect to NADP$^+$ (Figure 1E and F). In the latter case, data below 10 μM NADP$^+$ are impractical in a 1-cm path-length cell and a small slope variation cannot be excluded in Figure 1E. But such a term is not needed to give a good fit. Another substrate analogue, glucose, gives competitive inhibition with respect to glucose-6-P as expected from observation of Metzger et al. (1972) that glucose is a poor alternate substrate of the enzyme. Glucose concentrations required for significant inhibitions, however, were so high (0.25–1.0 M) that some of the other kinetic constants were also altered and hence these data are not shown.

Double Inhibition. Data with varying concentrations of product inhibitor, NADPH, and dead-end inhibitor, glucosamine-6-P, at fixed substrate concentrations (Figure 1B) conform to the parallel pattern predicted by eq 4 with a high quality fit.

Quantitative Results. Data from each steady-state pattern gave least-squares fits with 50% chi-square probabilities assuming 5–7% standard deviations in measured velocities, in good agreement with the frequently measured velocity with reference concentrations. A more stringent test of the proposed mechanism is the quality of fit to all data in Figure 1 with a single set of kinetic constants. The precision in kinetic constants and chi-square probability of this overall fit summarized in Table I are quite satisfactory. These data cover several months and different enzyme prepara-

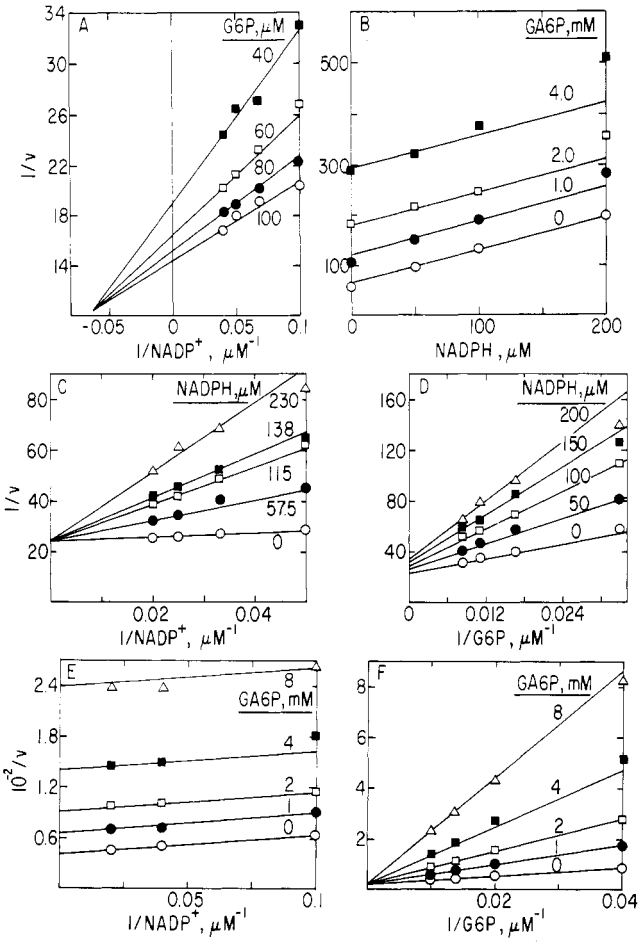


FIGURE 1: Steady-state kinetic data at 25 °C in 0.1 M Tris-HCl-4 mM $MgCl_2$ at pH 8.0. Double reciprocal plots with velocity v in units of μmol/(min ml). Nonstandard abbreviations: G6P, glucose-6-P; GA6P, glucosamine-6-P. Experiments are shown in the order of initial velocity (A), double inhibition (B), NADPH inhibition (C and D), and glucosamine-6-P (dead-end) inhibition (E and F). Concentrations of fixed substrates and inhibitors are shown beside the lines except for B with 50 μM $NADP^+$ and 50 μM G6P, C with 400 μM G6P, D with 60 μM $NADP^+$, E with 100 μM G6P, and F with 50 μM $NADP^+$. Straight lines are those calculated from the overall best constants in Table I, and experimental data are represented by symbols, which are averages of duplicate measurements. Best fit V_m's (enzyme concentrations) in units of μmol of product/(min ml) are 0.0471 (A), 0.0267 (B), 0.0437 (C), 0.0422 (D), 0.0306 (E), and 0.0309 (F), where figure designations are in parentheses.

tions. A reference velocity was determined for each experiment by repetitive assays with near saturating substrates. These velocities were corrected (15%) to saturating substrate concentrations with the best fit constants in Table I to give experimental V_m values. Best fit V_m's were not significantly different (1–9%) from these experimental values except possibly for the double inhibition data, where fitted V_m was 16% higher. Nevertheless, fixing all V_m's to their experimental values caused a tenfold increase in chi-square and an unacceptable fit. Fixing only the V_m of the double inhibition data to its experimental value gave a good fit (only a 10% increase in chi-square) and did not significantly alter any kinetic constants.

Circular Dichroism. A far-ultraviolet dichroic spectrum of the enzyme in 20 mM potassium phosphate buffer (pH 7.0) with 10 μM $NADP^+$ is shown in Figure 2. A good fit to the HELIX program was obtained (chi-square probability of 0.5 with a reasonable S.D. in [θ]) and best fit values of 51 ± 1% helix and 33 ± 5% pleated sheet conformations.

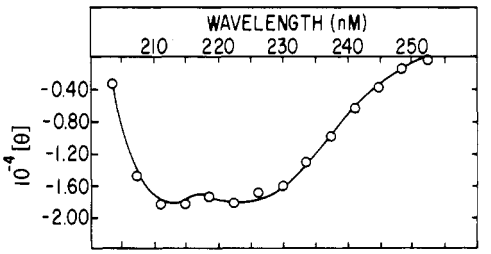


FIGURE 2: Circular dichroism of enzyme at 50 μg/ml in 20 mM potassium phosphate (pH 7.0) with 10 μM $NADP^+$. Mean residue molar ellipticities, [θ], are those of enzyme solution minus 10 μM $NADP^+$ in the buffer.

Table I: Kinetic Constants (μM) and Quality of Fit.[a]

K_a	K_b	K_{ia}	K_{iq}	K_i
2.0 ± 0.4	29 ± 3	16 ± 2	13 ± 2	390 ± 30

[a] Least squares fit of eq 4 to all kinetic data in Figure 1 with 96 degrees of freedom. A chi-square probability of 0.5 (maximum likely fit) is given by assuming 8.8% S.D. in each velocity datum (6.2% S.D. in average of duplicate values), in good agreement with the experimentally estimated S.D. Subscripts a, b, q, and i refer to $NADP^+$, glucose-6-P, NADPH, and glucosamine-6-P, respectively.

Table II: Expected Steady-State Patterns[a] of Random and Ordered Mechanisms with Substrate Analogues.

Substrate Analogue of	A		B	
Variable substrate	A	B	A	B
Rapid equilibrium random	C	C(NC)[b]	C(NC)[b]	C
Ordered	C	NC	UC	C(NC)[c]

[a] C, NC, and UC are competitive, noncompetitive, and uncompetitive, respectively. [b] Patterns in parentheses occur, if in the random mechanism, ternary complexes EBI_a and EAI_b form in addition to EI_a and EI_b, where I_a and I_b are inhibitor analogues of A and B, respectively. [c] NC if an EQI_b complex forms.

Discussion

Steady-State Mechanism. Intersecting velocity patterns indicate a sequential[2] mechanism as expected for a nicotinamide dehydrogenase. The necessity of a K_{ia} term to represent product inhibition data, as presented under Results, confirmed the sequential pattern. Since measurements with 6-phosphoglucono-δ-lactone are not practical, product inhibition data do not distinguish between a random or ordered addition of substrates and products. Dead-end inhibition studies can reveal the mechanism of substrate addition, however, since a B substrate analogue is expected to combine with both the free enzyme (E), and the enzyme-A (EA) forms in a random mechanism, but only to the EA form in an ordered pathway. Thus, a diagnostic uncompetitive inhibition results with a B substrate analogue and variable A substrate in an ordered mechanism as summarized in Table II. Consideration of the rapid equilibrium type of random mechanism is considered adequate since (a) linear double reciprocal plots were obtained, and (b) steady-state random mechanisms with intersecting initial velocity patterns often appear the same in practice as rapid equilibrium ones (Cleland, 1970). Uncompetitive inhibition by glucosamine-6-P (I_b) with variable $NADP^+$ (Figure 1E) is compatible, therefore, only with an ordered addition of sub-

Table III: Fractional Activities of Rat Liver Glucose-6-P Dehydrogenase in Various Metabolic States.[a]

	Control	Starved	Starved and Fat	Starved and Carboh.	Diabetic	Diabetic and Insulin
$10^3 \times$ (NADP$^+$)/(NADPH)	9.09	5.71	4.93	22.2	5.38	9.35
(NADP$^+$), μM	6.6	4.2	3.6	16.2	3.9	6.8
(NADPH), μM	720	726	726	714	726	723
f_2[b]	0.35	0.57	0.51	0.49	0.48	0.35
v/V_m	0.037	0.015	0.015	0.068	0.018	0.038

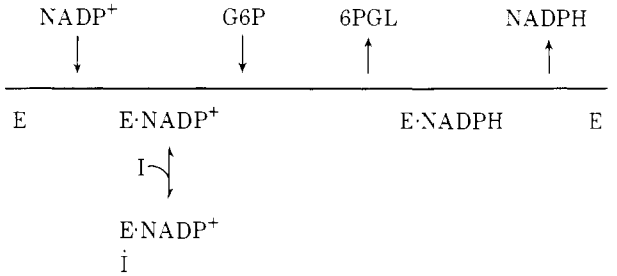
[a] Fractional activities were calculated from equation 2 and cellular concentrations and ratios estimated as explained in Experimental Section. The metabolic states of rats are defined as starved 3 days, starved 3 days, and refed 3 days high fat or carbohydrate, and alloxin diabetic with and without 2 units of insulin injections. [b] Fraction of v/V_m contributed by term proportional to glucose-6-P (see equation 6).

strates and identifies NADP$^+$ and glucose-6-P as the first and second added substrates, A and B, respectively. This pattern specifically excludes significant concentrations of an enzyme-I_b complex (an argument used below).

Formation of the analogous dead-end complex with glucosamine-6-P inhibitor (I_b) and NADPH is also excluded by both the competitive pattern of this inhibition with respect to glucose-6-P (Figure 1F) and the double inhibition data (Figure 1B). The latter experiment is more quantitatively significant since the presence of NADPH increases the fraction of enzyme present in an enzyme-NADPH form during the steady state. The presence of a significant fraction of enzyme-NADPH-I_b complex would require an additional coefficient, $(1 + I_b/K)$, of the NADPH (Q) term in the denominator of equation 4, and hence an intersecting, rather than the parallel pattern observed in Figure 1B. Cleland (1970) and Beytia et al. (1970) give additional description and examples of double inhibition experiments.

An ordered release of products is also likely, since if neither glucose-6-P nor glucosamine-6-P can bind to the catalytic site of the free enzyme form, 6-phosphoglucono-δ-lactone is not likely to bind to it either. Thus an ordered release of products in the sequence 6-phosphoglucono-δ-lactone, NADPH is indicated. The NADPH product inhibition patterns in Figure 1B and C are consistent with this argument, although none of our data directly exclude a random sequence of product release, since experiments with 6-phosphoglucono-δ-lactone are not practical.

The following scheme summarizes the conclusions drawn above and is the model of eq 1-4:



where E = enzyme; G6P = glucose 6-P; 6PGL = 6-phosphoglucono-δ-lactone; and I = glucosamine 6-P.

The steady-state kinetic mechanism of the rat liver enzyme, therefore, appears to be of the simplest type. The sigmoidal kinetics observed with common variants of the human erythrocyte enzyme at fractional velocities, $v/V_m \simeq$ 0.1-0.5 (Afolayan and Luzzatto, 1971; Luzzatto, 1967), are not observed in our data on rat liver enzyme. Data extending to 5 μM NADP$^+$ (not shown) reveal this difference more dramatically. Since we know of no rat erythrocyte enzyme kinetic data, we cannot say whether this represents species differences or post-translational tissue differences.

Estimates of Fractional Activity in Vivo. Kinetic constants of this study indicate that extensive NADPH product inhibition may exist in vivo. The extent of this inhibition is estimated in Table III for various metabolic states from literature data on metabolite concentrations and cytoplasmic redox ratios (see the Experimental Section for references and calculation methods). Only the product inhibition term of eq 2 is significant in any of the metabolic states giving the enzyme fractional activity:

$$f = v/V_m \simeq (1 + 4.31B\ [\text{mM}]A/Q \qquad (6)$$

Thus f is proportional to the coenzyme redox ratio, (NADP$^+$)/(NADPH) = $A/Q = r$, in agreement with its previous postulated importance in controlling pentose phosphate shunt dehydrogenase activity (Greenbaum et al., 1971, Sapag-Hagar et al., 1973). This ratio is also effective in controlling gluconate-6-P dehydrogenase (Procsal and Holten, 1972). The fractional activity of glucose-6-P dehydrogenase is also linear in glucose-6-P concentration (B), though this term in eq 6 contributes an approximately constant fraction (35-57%) to f in the various metabolic states. Effects of pH and buffer composition should be considered. With near saturating substrate concentrations (0.1 mM NADP$^+$-0.2 mM glucose-6-P), however, we found no significant differences in the enzyme's activity or NADPH inhibition in the buffers, 0.1 M Tris-Cl at pH 8.0 and 0.01 M phosphate at pH 7.0 (data not shown) and Yugari and Matsuda (1967) found essentially constant activity and fatty acid inhibition between pH 6.0 and 8.5.

Such extensive inhibition (93-98%) of the dehydrogenase in all states listed in Table III may at first seem unlikely, but it is well to remember that the V_m reference state is quite unphysiological. All products are present in vivo. Data on the erythrocyte enzyme (Yoshida, 1973) indicate that it functions in vivo at about 0.2% of V_m, and estimates of flux through this enzymatic reaction in liver (Katz and Rognstad, 1967) indicate that the catalytic capacity of liver is ample at 1% of V_m.

Far-Ultraviolet Circular Dichroism. A surprisingly large apparent helix content (51%) is indicated for the liver enzyme from the calculations of secondary structure. Optical rotation data on *C. utilis* enzymes indicate 13-34% helix content, the higher value corresponding to the AS95 isoenzyme with 0.6 mM NADP$^+$ (Jirgensons, 1966; Domschke et al., 1970). The possibility that enzyme-bound NADP$^+$ might contribute CD activity and significant error in the secondary structure analysis needs consideration, although differences between enzymes, both of which contain bound NADP$^+$ represent some type of structural differences. We suspect that the quality of fit obtained with the secondary

structure model (chi-square probability = 0.5) would not have been possible with contributions from absorption bands as different as nucleotide and peptide bonds. The inexactness of the reference data set for protein structures, however, makes it difficult for us to assess this argument without further analysis. Perhaps a stronger argument is based on the fact that no dichroic activity is observed in Figure 2 at 252 nm, which is well within the 260-nm absorption band of $NADP^+$ ($\epsilon_{252}/\epsilon_{260} = 0.88$).

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