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THE ISOLATION AND CHARACTERIZATION OF SORBITOL-6-PHOSPHATE DEHYDROGENASE FROM *CLOSTRIDIUM PASTEURIANUM*

P. J. DU TOIT AND J. P. KOTZÉ*

National Institute for Water Research of the South African Council for Scientific and Industrial Research, P.O. Box 395, Pretoria (South Africa)

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SUMMARY

The enzyme sorbitol-6-*P* dehydrogenase was isolated from *Clostridium pasteurianum*. The isolation procedure included $(NH_4)_2SO_4$ fractionation, DEAE-cellulose chromatography (twice), Biogel P60 gel filtration and Sephadex G-200 gel filtration techniques. The purified protein fraction, sorbitol-6-*p* dehydrogenase purified 176.5-fold, carried a negative charge at pH 8.6 and, according to electrophoretic mobility, was predominantly of one species. The molecular weight of the enzyme was found to be between 74 000 and 94 000 and separation on Sephadex G-200 revealed a strong resemblance between the elution of sorbitol-6-*P* dehydrogenase (determined by spectrophotometric assay) and the total protein in the fraction.

Sorbitol-6-*P* dehydrogenase from *Clostridium pasteurianum* was found to be specific for cofactors NAD^+ and NADH, and substrates sorbitol-6-*P* and fructose-6-*P*. The enzyme also reacted very slowly with mannitol-1-*P* as substrate in place of sorbitol-6-*P*. For oxidation of sorbitol-6-*P* an optimum pH of 8.5 was found for the enzyme in Tris–HCl buffer and an optimum value of pH 10 in glycine–NaOH buffer. Catalytic activity of the enzyme was at its highest when Tris–HCl buffer was used. Reduction of fructose-6-*P* was optimal at pH 6.5–6.7 in K_2HPO_4–KH_2PO_4 buffer.

Michaelis–Menten constants (K_m) and inhibitor constants (K_i) were determined for all reactants of sorbitol-6-*P* dehydrogenase. Kinetic constants for sorbitol-6-*P* and NAD^+ had lower numerical values when determined in Tris–HCl buffer (pH 8.5) compared to the values obtained when glycine–NaOH buffer (pH 9.8) was used. For sorbitol-6-*P* K_m as well as K_i values were observed at two concentration levels of NAD^+. NAD^+ on the other hand displayed two K_m values, being dependent upon NAD^+ concentration alone and remaining unaffected by sorbitol-6-*P* concentration. The v_{max} of the reaction was only dependent on the concentration level of NAD^+. In K_2HPO_4–KH_2PO_4 buffer (pH 6.45) the K_m and K_i values for NADH were found to be unchanged by increased fructose-6-*P* concentrations, whereas the K_m value of fructose-6-*P* was dependent upon the concentration level of NADH. The v_{max} of this reaction was influenced by fructose-6-*P* concentration. The reduced reactants, NADH

* Present address: National Nutrition Research Institute of the S.A. CSIR, P.O. Box 395, Pretoria, South Africa.

and sorbitol-6-*P* thus had K_m as well as K_i values in contrast to the oxidized reactants, fructose-6-*P* and NAD^+ which displayed only K_m values. The v_{max} values of both reactions were influenced by the concentration levels of the oxidized reactants.

The apparent equilibrium constant for the reaction as catalyzed by sorbitol-6-*P* dehydrogenase (fructose-6-*P* + NADH ⇋ sorbitol-6-*P* + NAD^+) at pH 7.0, was $3.81 \cdot 10^8\ M^{-1}$. This indicates that the equilibrium lies heavily on the side of sorbitol-6-*P* formation.

INTRODUCTION

Mannitol-1-*P* dehydrogenase was reported to be a constitutive enzyme in *Aerobacter aerogenes*, ATCC 8724, whilst sorbitol-6-*P* dehydrogenase was only present in cultures grown with D-sorbitol as carbon source[1]. Similar results were obtained for the obligate anaerobe *Clostridium pasteurianum*, ATCC 6013, with regard to these two enzymes[2].

The isolation and characterization of sorbitol-6-*P* dehydrogenase from *C. pasteurianum* were carried out to determine the properties of this enzyme and to compare it with the same enzyme from a different bacterial source.

EXPERIMENTAL

Materials

Purified enzymes, NAD^+ and NADH (sodium salts), the calcium salt of fructose-6-*P* and the sodium salt of glucose-6-*P* were obtained from C. F. Boehringer and Soehne, Mannheim, Germany. Sorbitol-6-*P* and mannitol-1-*P* were prepared from the hexose phosphate by $NaBH_4$ reduction according to the method of WOLFF AND KAPLAN[3].

Clostridium pasteurianum, ATCC 6013, was obtained from the American Type Culture Collection.

DEAE-cellulose and Biogel P60 were obtained from Biorad and Sephadex G-200 from Pharmacia Fine Chemicals, Uppsala, Sweden.

Methods

Cultivation of C. pasteurianum

C. pasteurianum, ATCC 6013, was cultivated anaerobically according to the method of CARNAHAN AND CASTLE[4], with D-sorbitol as the carbon source. Cultures were incubated at 30° for 48 h. Cells were harvested by centrifugation at 25 000 × *g* for 30 min at 5°. The collected cell-paste was stored at −13 to −18°.

Assay of sorbitol-6-P dehydrogenase activity

Activity of sorbitol-6-*P* dehydrogenase was routinely assayed at 366 mμ and 25° by measuring the rate of increase in absorbance over the interval 30–90 sec after the reaction was started. The assay solution contained 244 μmoles Tris–HCl buffer (pH 9.0), 1 μmole NAD^+, 0.05–0.10 unit* sorbitol-6-*P* dehydrogenase and 7 μmoles sorbitol-6-*P* in a final volume of 3.3 ml.

* A unit of activity is the amount of enzyme capable of transforming 1 μmole substrate in 1 min.

Protein

Protein was determined according to the modified method of LAYNE[5].

Electrophoresis

A Beckman Microzone electrophoretic apparatus was used for determination of electrophoretic homogeneity. Electrophoresis was carried out at room temperature for 20 min in a Veronal buffer (pH 8.6) at 200 V and 5–7 mA. Cellulose acetate membranes were used and developed according to the manufacturers procedure.

A Beckman Analytrol was used for determination of distribution of protein after electrophoresis.

Determination of molecular weight

The method of ANDREWS[7] was used. A mixture of six enzymes was prepared consisting of 1 mg each of fumarase (pig heart), aldolase (rabbit muscle), alcohol dehydrogenase (yeast), D-glyceraldehyde-3-*P* dehydrogenase (rabbit muscle), malate dehydrogenase (pig heart) and sorbitol-6-*P* dehydrogenase in a final volume of 2 ml. Conditions were similar to that of ANDREWS[7] and fractions of 5 ml each were collected. Positions of enzymes were determined by spectrophotometric assays[8].

Determination of optimum pH of purified sorbitol-6-P dehydrogenase

The standard assay procedure for sorbitol-6-*P* dehydrogenase activity was employed. The effects of Tris–HCl, glycine–NaOH and Na_2CO_3–$NaHCO_3$ buffers, were evaluated.

The optimum pH for the reduction of fructose-6-*P* was determined in K_2HPO_4–KH_2PO_4 buffer. These assay solutions contained in a final volume of 3.3 ml, 244 μmoles buffer, 1.5 μmoles NADH, 0.03–0.05 unit sorbitol-6-*P* dehydrogenase and 7 μmoles fructose-6-*P*.

Determination of kinetic parameters

The standard assay procedure was employed and kinetic studies were carried out in both Tris–HCl buffer (pH 8.5) and glycine–NaOH buffer (pH 9.8). In the assay solutions the concentration of sorbitol-6-*P* was varied from 0.48 to 7.75 mM at two levels (0.45 and 1.13 mM) of NAD^+ and the concentration of NAD^+ was varied from 0.11 to 4.50 mM at two levels (0.97 and 1.94 mM) of sorbitol-6-*P*. Each assay solution contained 0.08 unit sorbitol-6-*P* dehydrogenase.

Kinetic studies on the reduction of fructose-6-*P* were carried out in K_2HPO_4–KH_2PO_4 buffer (pH 6.45). In these assay solutions the concentration of fructose-6-*P* was varied from 0.21 to 3.12 mM at two levels (0.43 and 1.08 mM) of NADH and the concentration of NADH was varied from 0.11 to 1.08 mM at two levels (0.42 and 1.04 mM) of fructose-6-*P*. Each assay solution contained 0.04 unit sorbitol-6-*P* dehydrogenase.

Determination of apparent equilibrium constant (K_e) for the reaction catalyzed by sorbitol-6-P dehydrogenase

The assay solutions contained in a final volume of 3 ml; 222 μmoles buffer, 3.55 μmoles NADH, 2.65 μmoles fructose-6-*P* and 0.15 unit sorbitol-6-*P* dehydrogenase. Three buffers were used. Tris–HCl (pH 7.38), glycine–NaOH (pH 9.8) and K_2HPO_4–KH_2PO_4 (pH 6.45). Reaction mixtures were incubated for 2 h at 25° and NADH concentration determined spectrophotometrically.

Preparation of chromatographic columns

A batch of 40 g of DEAE-cellulose was washed as described by PETERSON AND SOBER[6], adjusted to pH 7 and equilibrated for 24 h against 0.02 M K_2HPO_4–KH_2PO_4

buffer (pH 7.0). Columns were poured in one section and equilibrated for at least 4 h with buffer passing through at flow rates of 20–30 ml/h.

Biogel P60 was washed with distilled water and equilibrated over 24 h against distilled water.

Sephadex G-200 was prepared according to the procedure of ANDREWS[7].

All chromatographic columns were operated at 2° and fractions of 5 ml each were collected.

Isolation procedure

All work during the isolation procedure was carried out at 0–5°. Activity of sorbitol-6-*P* dehydrogenase and protein were determined at each step.

(1) Preparation of cell-free extract. Frozen cell-paste (40 g) was suspended in 70 ml 0.1 M Tris–HCl buffer (pH 7.6) and treated ultrasonically (Branson Sonifier S125) for 10 min. This treatment was repeated after 30 min during which the suspension was cooled in a salt–ice solution. Insoluble material was removed by centrifugation at 30 000 × *g* for 30 min and the supernatant fluid decanted.

(2) Fractionation with $(NH_4)_2SO_4$. $(NH_4)_2SO_4$ fractionation was carried out at pH 7.6 by addition of finely powdered $(NH_4)_2SO_4$ to the cell-free extract from Step 1. Protein fractions were collected by centrifugation at 50 000 × *g* for 20 min after standing 30 min. Sorbitol-6-*P* dehydrogenase was precipitated in the protein fraction obtained after increasing $(NH_4)_2SO_4$ saturation from 30 to 75%. This fraction was dissolved in distilled water.

(3) Centrifugation. Centrifugation at 100 000 × *g* for 35 min was carried out on the protein fraction from Step 2. The supernatant fluid was decanted, dialyzed against three changes of distilled water (18 h) and lyophylized. The fraction was redissolved in a minimum volume of 0.02 M K_2HPO_4–KH_2PO_4 buffer (pH 8.0) and dialyzed against three changes of the same buffer for 18 h. Insoluble protein was removed by centrifugation at 25 000 × *g* for 20 min.

(4) Chromatography on DEAE-cellulose. The first chromatography step was carried out on a DEAE-cellulose column (2.5 cm × 35 cm) prepared as described previously. The protein fraction from Step 3 was applied to the column and eluted with 500 ml of a linear gradient, 0–0.2 M NaCl in 0.02 M K_2HPO_4–KH_2PO_4 buffer (pH 7.0). The collected fractions were monitored at 280 mμ and the fractions containing sorbitol-6-*P* dehydrogenase were combined, dialyzed against two changes of distilled water (12 h) and lyophylized. This fraction was then redissolved in a minimum volume of distilled water.

(5) Gel filtration on Biogel P60. Gel filtration on Biogel P60 was carried out on a column (2.5 cm × 30 cm). The column was developed with distilled water after application of the sample from Step 4. The eluted protein fraction containing sorbitol-6-*P* dehydrogenase was lyophylized, dissolved in a minimum volume of 0.02 M K_2HPO_4–KH_2PO_4 buffer (pH 7.0) and dialyzed against three changes the same of buffer for 18 h.

(6) Rechromatography on DEAE-cellulose. The second chromatography step on DEAE-cellulose was carried out on a column (2 cm × 20 cm) with all operating conditions similar to those in Step 4. The dialyzed sample from Step 5 was applied to the column and the collected fractions monitored at 280 mμ. The sorbitol-6-*P* dehydrogenase fraction was dialyzed against three changes of distilled water for 18 h, lyophylized and dissolved in a minimum volume of 0.05 M Tris–HCl buffer (pH 7.5) containing 0.1 M KCl.

TABLE I

SUMMARY OF THE RESULTS OF THE ISOLATION PROCEDURE FOR SORBITOL-6-*P* DEHYDROGENASE FROM *C. pasteurianum*

Step	*Vol. (ml)*	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg protein)*	*Total yield (%)*
1. Cell-free extract	89	3100	760	0.255	100.0
2. $(NH_4)_2SO_4$ fractionation	25	1800	740	0.41	97.5
3. Centrifugation	33	585	708	1.21	93.0
4. Chromatography on DEAE-cellulose	100	58.2	416	7.14	54.7
5. Gel filtration on Biogel P60	30	32.4	320	9.88	42.0
6. Rechromatography on DEAE-cellulose	55	16.5	330	20.00	43.4
7. Gel filtration on Sephadex G-200	50	7.0	302	43.20	39.7

(7) Gel filtration on Sephadex G-200. Gel filtration on Sephadex G-200 on a column (2.5 cm × 50 cm) was carried out as the final purification step. The sample from Step 6 was applied to the column and the column developed by passing 0.1 M KCl in 0.05 M Tris–HCl buffer (pH 7.5) through the column. The purified sorbitol-6-*P* dehydrogenase fraction was dialyzed against distilled water, lyophylized and dissolved in a small volume of 0.02 M K_2HPO_4–KH_2PO_4 buffer (pH 7.0). The results of the isolation procedure are summarized in Table I.

RESULTS AND DISCUSSION

Purity

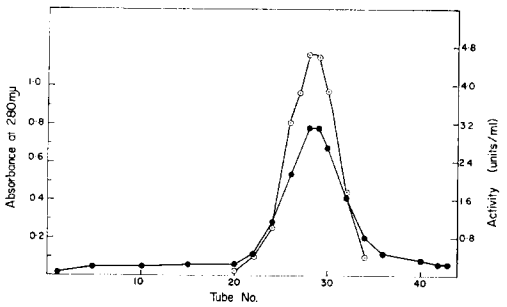
Sorbitol-6-*P* dehydrogenase was purified 176.5-fold during the isolation procedure (Table I). The specific activity (expressed as international units/mg of protein) of the purified enzyme from *C. pasteurianum* was 43.2 while that of the same enzyme from *Aerobacter aerogenes* was 21.3 (ref. 1).

Results of the final purification step of sorbitol-6-*P* dehydrogenase from *C. pasteurianum* (gel filtration on Sephadex G-200), are shown in Fig. 1 and indicate an elution pattern for the protein fraction which corresponded closely to the elution pattern for sorbitol-6-*P* dehydrogenase as determined by spectrophotometric assay.

Electrophoresis of this purified fraction (Fig. 2) indicated that the protein carried a negative charge at pH 8.6 and was predominantly of one species according to electrophoretic mobility.

Molecular weight

The molecular weight of sorbitol-6-*P* dehydrogenase from *C. pasteurianum* was found to be 84 000 ± 10 000 according to the method of ANDREWS[7], if the mean molecular weight values of the different enzymes (Table II) were used in the determination. The average value of two determinations was taken. Results are depicted in



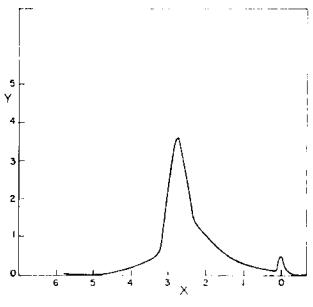


Fig. 1. Final purification of sorbitol-6-*P* dehydrogenase on a Sephadex G-200 column (2.5 cm × 50 cm). Conditions as described in isolation procedure. Description of curves: ●—●, absorbance at 280 mμ; ⊙—⊙, sorbitol-6-*P* dehydrogenase activity.

Fig. 2. Densitometric analysis of distribution of protein in purified sorbitol-6-*P* dehydrogenase fraction after electrophoresis and development of cellulose acetate membrane. Conditions as described in *Methods*. Y = distribution of protein as a function of colour intensity as determined with Analytrol; X = distance moved (cm) from point of application (o) during electrophoresis.

Table II. Liss *et al.*[1] did not determine the molecular weight of sorbitol-6-*P* dehydrogenase from *A. aerogenes*, but reported a corrected sedimentation coefficient (s_{20}) of $6.0 \cdot 10^{-13}$. The two enzymes thus seem to be rather similar with respect to molecular size.

TABLE II

RESULTS OF MOLECULAR WEIGHT DETERMINATION OF SORBITOL-6-*P* DEHYDROGENASE ON SEPHADEX G-200

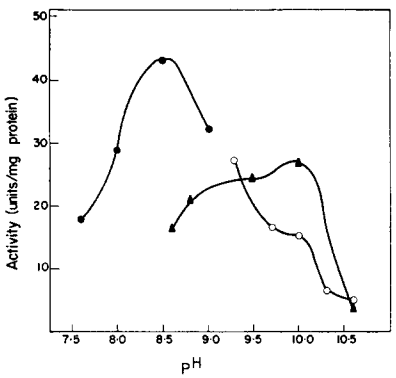
Enzyme	*Source*	*Elution vol. (ml)*	*Literature mol. wt.* × 10^{-3}
Fumarase	Pig heart	100	185–225
Aldolase	Rabbit muscle	115	140–150
Alcohol dehydrogenase	Yeast	120	125–155
D-Glyceraldehyde-3-*P* dehydrogenase	Rabbit muscle	135	115–145
Sorbitol-6-*P* dehydrogenase	*C. pasteurianum*	140	—
Malate dehydrogenase	Pig heart	155	55– 70

Substrate specificity

Sorbitol-6-*P* dehydrogenase from *C. pasteurianum* displayed no activity with $NADP^+$ as cofactor and was specific for NAD^+ and NADH. Fructose-6-*P* and sorbitol-6-*P* were active as substrates for the enzyme which also reacted slowly with mannitol-1-*P* as substrate. Activity with mannitol-1-*P* as substrate was, however, low (1–5%) as compared with the activity with an equal concentration of sorbitol-6-*P*.

Optimum pH

The optimum pH for sorbitol-6-*P* dehydrogenase from *C. pasteurianum* was found to be pH 8.5 in Tris–HCl buffer (Fig. 3). Liss *et al.*[1] reported a value of pH 9 under similar conditions for the enzyme from *A. aerogenes*. The kind of buffer used in



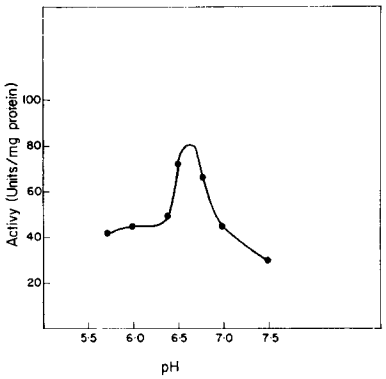


Fig. 3. Influence of different buffers, Tris–HCl (●—●), glycine–NaOH (▲—▲) and Na_2CO_3–$NaHCO_3$ (○—○) on the catalytic activity of sorbitol-6-*p* dehydrogenase over the range from pH 7.6 to 10.6. Assay conditions as described in *Methods*.

Fig. 4. Optimum pH of reduction of fructose-6-*P* by sorbitol-6-*P* dehydrogenase in K_2HPO_4–KH_2PO_4 buffer. Assay conditions as described in *Methods*.

the assay influenced the catalytic activity of sorbitol-6-*P* dehydrogenase. In glycine–NaOH buffer a maximum value was observed at pH 10, and catalytic activity was lower than in Tris–HCl buffer (Fig. 3). Liss *et al.*[1] reported similar results.

Reduction of fructose-6-*P* was optimal at pH 6.5–6.7 in K_2HPO_4–KH_2PO_4 buffer (Fig. 4).

Kinetic parameters of reactants

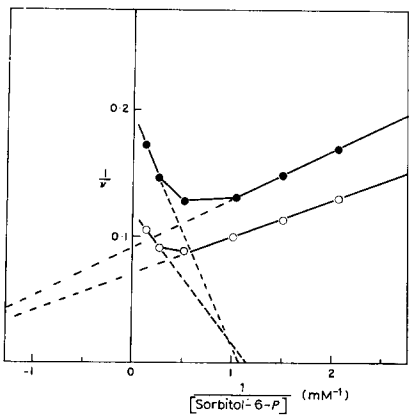
Michaelis–Menten constants (K_m) and inhibitor constants (K_i) were determined for all reactants of sorbitol-6-*P* dehydrogenase. The Lineweaver–Burk graphical method of plotting $1/v$ against $1/[S]$ was used and the different plots are presented in Figs. 5–8. The kinetic constants are given in Table III.

For sorbitol-6-*P* K_m as well as K_i values were observed in glycine–NaOH buffer at the tested concentrations of NAD^+ (0.45 and 1.13 mM) (Fig. 5, Table III). Two v_{max} values were also observed at the different levels of NAD^+ concentration, being

ABLE III

ICHAELIS-MENTEN CONSTANTS OF REACTANTS IN SORBITOL-6-*P* DEHYDROGENASE

uffer	*Reactant*	*Concn. range (mM)*	*2nd reactant concn. (mM)*		*Kinetic constants of reactant*	
					$K_m \times 10^3$ *(M)*	$K_i \times 10^3$ *(M)*
lycine–NaOH	Sorbitol-6-*P*	0.48–7.75	NAD^+	0.45, 1.13	0.40–0.44	0.83–1.00
ris–HCl	Sorbitol-6-*P*	0.48–7.75	NAD^+	0.45	0.04–0.05	0.32–0.37
lycine–NaOH	NAD^+	0.11–0.45	Sorbitol-6-*P*	0.97, 1.94	0.19–0.21	—
	NAD^+	0.45–4.50	Sorbitol-6-*P*	0.97, 1.94	0.74–0.84	—
ris–HCl	NAD^+	0.11–0.45	Sorbitol-6-*P*	0.97	0.07–0.08	—
	NAD^+	0.45–4.50	Sorbitol-6-*P*	0.97	0.20–0.25	—
$_2HPO_4$–KH_2PO_4	NADH	0.11–1.08	Fructose-6-*P*	0.42, 1.04	0.02–0.03	0.33–0.35
	Fructose-6-*P*	0.21–3.12	NADH	0.43	2.80–3.00	—
	Fructose-6-*P*	0.21–3.12	NADH	1.08	7.50–7.80	—



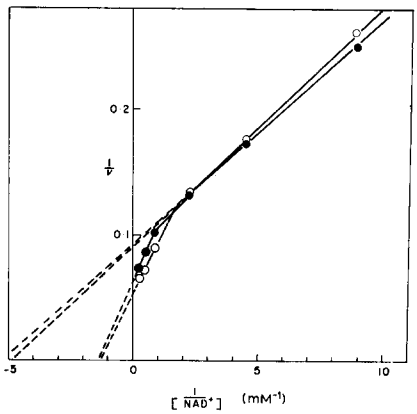
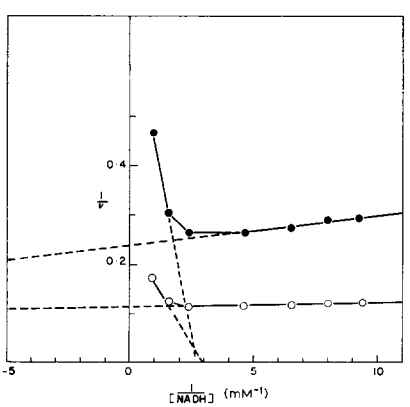


Fig. 5. Lineweaver–Burk plot for evaluation of kinetic parameters of sorbitol-6-*p*. Studies carried out in glycine–NaOH buffer (pH 9.8). ●—●, 0.45 mM NAD^+; ○—○, 1.13 mM NAD^+. v = reaction rate in μmoles/sec.

Fig. 6. Lineweaver–Burk plot for evaluation of kinetic parameters of NAD^+. Studies carried out in glycine-NaOH buffer (pH 9.8). ●—●, 0.97 mM sorbitol-6-*p*; ○—○, 1.94 mM sorbitol-6-*P*. v = reaction rate in μmoles/sec.

respectively 1.0–1.1 μmoles/sec and 1.4–1.6 μmoles/sec. Numerical values of K_m and K_i values were decreased when Tris–HCl buffer was used (Table III).

NAD^+ on the other hand displayed more complicated behaviour. The K_m value for NAD^+ was altered by increased NAD^+ concentrations (0.45 mM and above), but was not influenced by the sorbitol-6-*P* concentration (Fig. 6, Table III). Two v_{max} values were observed being dependent on NAD^+ concentration, *i.e.* 1.0–1.2 μmoles/sec for NAD^+ concentrations below 0.45 mM, and 1.6–1.7μmoles/sec at NAD^+ concen-



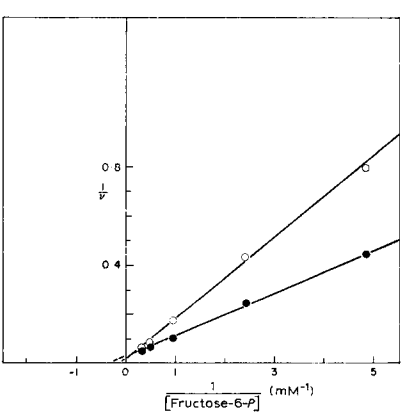


Fig. 7. Lineweaver–Burk plot for evaluation of kinetic parameters of NADH. Studies carried out in K_2HPO_4–KH_2PO_4 buffer (pH 6.45). ●—●, 0.42 mM fructose-6-*P*; ○—○, 1.04 mM fructose-6-*P*. v = reaction rate in μmoles/sec.

Fig. 8. Lineweaver–Burk plot for evaluation of kinetic parameters of fructose-6-*P*. Studies carried out in K_2HPO_4–KH_2PO_4 buffer (pH 6.45). ●—●, 0.43 mM NADH; ○—○, 1.08 mM NADH. v = reaction rate in μmoles/sec.

trations above 0.45 mM. Increasing the concentration level of sorbitol-6-*P* from 0.97 to 1.94 mM did not influence v_{max} values. Smaller K_m and K_i values were observed when Tris–HCl buffer was used (Table III).

High concentrations of NADH inhibited sorbitol-6-*P* dehydrogenase and the K_m and K_i values for NADH were not influenced by increasing fructose-6-*P* concentration from 0.42 to 1.04 mM (Fig. 7, Table III). The v_{max} value was however increased from 0.4–0.5 to 0.9–1.0 μmoles/sec upon increasing fructose-6-*P* concentration as indicated.

The K_m value of fructose-6-*P* seemed to be altered by increased NADH concentrations (Fig. 8, Table III), and two v_{max} values were also observed at the two concentration levels of NADH (0.43 and 1.08 mM) being 3.2–3.4 and 4.8–5.0 μmoles/sec, respectively.

The reduced reactants, NADH and sorbitol-6-*P* thus had K_m as well as K_i values in contrast to the oxidized reactants (fructose-6-*P*, NAD^+) which displayed only K_m values. The activity of sorbitol-6-*P* dehydrogenase thus seemed to controlled by the reduced reactants, whilst the actual activity level at which this control took place was dictated by the concentration of the oxidized reactants, as displayed in the increased v_{max} values upon increasing NAD^+ and especially fructose-6-*P* concentrations.

Apparent equilibrium constant (K_e) for the reaction catalyzed by sorbitol-6-P dehydrogenase

By using equation:

$$K_e = \frac{[\text{sorbitol-6-}P]\ [NAD^+]}{[\text{fructose-6-}P]\ [NADH]\ [H^+]}$$

values were calculated for the reaction written as: Fructose-6-*P* + NADH + $H^+ \leftrightharpoons$ sorbitol-6-*P* + NAD^+. Results are shown in Table IV. Concentrations were converted to percentages, with initial NADH concentration taken as 100.

The equilibrium lies heavily on the side of sorbitol-6-*P* formation at pH 7. The average K_e value was $3.81 \cdot 10^8\ M^{-1}$. Comparison of reaction rates as determined also indicated that reduction of fructose-6-*P* was more rapid than oxidation of sorbitol-6-*P* for an equal amount of enzyme.

ABLE IV

SULTS OF STUDIES FOR DETERMINATION OF K_e FOR THE REACTION CATALYZED BY SORBITOL-6-*P* DEHYDROGENASE

ffer (0.074M)	*pH*	*Determined*			*Calculated*			
		Initial NADH (%)	*Initial fructose-6-P (%)*	*Equilibrium NADH (%)*	*Equilibrium fructose-6-P (%)*	*Equilibrium sorbitol-6-P (%)*	*Equilibrium NAD^+ (%)*	$K_e \times 10^{-8}$ *(M^{-1})*
HPO_4–KH_2PO_4	6.45	100	74.6	26.8	1.4	73.2	73.2	4.04
is–HCl	7.38	100	74.6	35.1	9.7	64.9	64.9	2.98
ycine–NaOH	9.80	100	74.6	82.0	56.6	18.0	18.0	4.40

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