Biochem. J. (1974) **143**, 19–27
Printed in Great Britain

A Study of the Kinetics and Mechanism of Rabbit Muscle L-Glycerol 3-Phosphate Dehydrogenase

By PHILIP BENTLEY* and F. MARK DICKINSON
Department of Biochemistry, University of Hull, Hull HU6 7RX, U.K.

(*Received* 14 *March* 1974)

1. The kinetics of oxidation of L-glycerol 3-phosphate by NAD^+ and of reduction of dihydroxyacetone phosphate by NADH catalysed by rabbit muscle glycerol 3-phosphate dehydrogenase were studied over the range pH 6–9. 2. The enzyme was found to catalyse the oxidation of glyoxylate by NAD^+ at pH 8.0 and the kinetics of this reaction were also studied. 3. The results are consistent with a compulsory mechanism of catalysis for glycerol 3-phosphate oxidation and dihydroxyacetone phosphate reduction in the intermediate regions of pH, but modifications to the basic mechanism are required to fully explain results at the extremes of the pH range, with these substrates and for glyoxylate oxidation at pH 8.0.

Kinetic studies on rabbit muscle glycerol 3-phosphate dehydrogenase (L-glycerol 3-phosphate–NAD^+ oxidoreductase, EC 1.1.1.8) have been performed by several groups of workers but with little agreement. Apitz-Castro *et al.* (1964) assuming three NADH binding sites/molecule proposed a complex random-order mechanism in which NADH formed several dead-end complexes with the enzyme. Black (1966) on the basis of a limited study of product inhibition suggested that a compulsory order of reaction is followed in which the coenzymes bind before the substrates. Again Keleti (1970) has proposed a rather complex random-order mechanism which involves thiol groups and enzyme-bound zinc atoms. Since it has now been shown that glycerol 3-phosphate dehydrogenase contains only two NADH binding sites/molecule (Holbrook *et al.*, 1972; Bentley *et al.*, 1973) and that the highly purified enzyme contains only trace amounts of zinc ($\leq$0.08 atom/molecule, Bentley *et al.*, 1973) at least two of the earlier investigations were based on incorrect assumptions. The studies of Black (1966) were also unsatisfactory because studies of glycerol phosphate oxidation and dihydroxyacetone phosphate reduction were made at different pH values so that usual tests of mechanism (Dalziel, 1957) could not be applied. The object of the present work was to perform a detailed study of the kinetics of the enzyme in both directions and over a wide range of pH values. It was expected that the information obtained, together with information about coenzyme binding given in the preceding paper (Bentley & Dickinson, 1974), would provide strong evidence about the mechanism of catalysis. A preliminary account of some of this work has been given (Bentley & Dickinson, 1973).

* Present address: Department of Pharmacology, Biozentrum der Universität Basel CH-4056, Basel, Klingelbergstrasse 70, Switzerland.

Experimental

Materials

Enzyme. Crystalline glycerol 3-phosphate dehydrogenase was prepared from rabbit muscle by the method of Bentley *et al.* (1973). Solutions of enzyme were dialysed against sodium phosphate buffer, pH 6.2, before use. The concentration of enzyme in solution was determined by using $E^{1\%}_{1cm} = 5.15$ at 280nm, which is based on dry-weight measurements (Bentley *et al.*, 1973).

Coenzymes. NAD^+ (grade II) was purchased from Boehringer Corp. (London) Ltd., London W.5, U.K., and was further purified before use by chromatography on DEAE-cellulose by the method of Dalziel (1963). Highly purified NADH was prepared from NAD^+ enzymically with yeast alcohol dehydrogenase by the method of Dalziel (1962*a*).

Substrates. DL-Glycerol 3-phosphate (sodium salt) and glyoxylic acid were of the highest purity available from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. L-Glycerol 3-phosphate (calcium salt) was purchased from Calbiochem Ltd., London W.1, U.K. Dihydroxyacetone phosphate hydrazone (barium salt) was a gift from Dr. C. I. Pogson, School of Biology, University of Kent, Canterbury, U.K.,

Solutions of L-glycerol 3-phosphate were obtained from the calcium salt by dissolving 100mg in 1ml of water with a few drops of 1M-HCl added. Ca^{2+} ions were removed by passage of the mixture through a small column of Chelex 100 (Na^+ form). This treatment also served to neutralize the solution.

Solutions of dihydroxyacetone phosphate were obtained from the barium salt of the hydrazone by adding the salt to a strong suspension of two equivalents of Dowex 50 resin (H^+ form). When the solid was dissolved, the resin was removed by filtration and washed with a small volume of water. The filtrate containing the dihydroxyacetone phosphate hydrazone was treated with three equivalents of H_2O_2 at room temperature for 5min. The solution was then adjusted to about pH6 by slow addition of 1M-$KHCO_3$, and excess of H_2O_2 was removed by adding a small amount of catalase. Solutions of dihydroxyacetone phosphate were adjusted to the required pH value before use or were immediately adjusted to pH2 and stored at −20°C.

Other chemicals. Glycerol 2-phosphate (sodium salt) was obtained from Sigma (London) Chemical Co. Chelex 100 (sodium form; 50–100 mesh) was purchased from Bio-Rad, St. Albans, Herts., U.K., and was washed with 0.5M-NaCl and distilled water before use. DEAE-cellulose (DE 11) and all other chemicals were obtained from Fisons Chemicals, Loughborough, Leics., U.K.

Water. Glass-distilled water was used throughout.

Buffers. All buffers contained 0.3mM-EDTA.

Determination of substrate and coenzyme concentrations

NAD^+ and NADH were assayed enzymically with yeast alcohol dehydrogenase by the procedures described by Dalziel (1962*a*, 1963*a*) and by using $\varepsilon = 6.22 \times 10^3$ litre·mol^{-1}·cm^{-1} for NADH at 340nm (Horecker & Kornberg, 1948).

L-Glycerol 3-phosphate was assayed by the enzymic method of Hohorst (1963). Dihydroxyacetone phosphate was assayed enzymically by following the decrease in NADH concentration at 340nm when glycerol 3-phosphate dehydrogenase was added to 3ml of a solution containing: dihydroxyacetone phosphate, approx. 240nmol; NADH, 480nmol; sodium phosphate buffer, pH7.0, 200μmol.

Initial-rate measurements

These were performed principally in a recording filter fluorimeter of similar design to that described by Dalziel (1962*b*).

All initial-rate measurements were performed at 25°C. At pH6, 7 and 8 sodium phosphate buffers, I 0.1, were used but at pH9 the buffer contained 20mM-glycine–NaOH buffer with sufficient disodium hydrogen phosphate added to achieve I 0.1. Solutions of purified NAD^+, which contained phosphate buffer (Dalziel, 1963*a*), were adjusted to the required pH value immediately before use and due allowance was then made for the phosphate content when making up assay mixtures.

The kinetic coefficients in the initial-rate equation:

$$\frac{e}{v_0} = \phi_0 + \frac{\phi_1}{[S_1]} + \frac{\phi_2}{[S_2]} + \frac{\phi_{12}}{[S_1][S_2]} \quad (1)$$

were obtained from primary and secondary plots of initial-rate measurements in the manner described by Dalziel (1957). In this equation e is the concentration of active sites, and $[S_1]$ and $[S_2]$ are coenzyme and substrate concentrations respectively. The quantity e was determined in titration experiments with NADH as described by Bentley *et al.* (1973). Unprimed symbols (ϕ_0, etc.) are used for the kinetic coefficients describing glycerol 3-phosphate oxidation by NAD^+ and primed symbols (ϕ'_0 etc.) for those describing dihydroxyacetone phosphate reduction by NADH. Initial-rate determinations were performed in duplicate and were reproducible to 5% in general and at worst 10% with the lowest concentrations of both substrate and coenzyme. At least two experiments were performed at each pH value and the initial-rate parameters were generally reproducible to within 25%.

Results

Initial-rate measurements for the glycerol 3-phosphate–NAD^+ reaction

The results of initial-rate measurements at 25°C in phosphate buffer, pH7.0, I 0.1, are shown in the double-reciprocal plot in Fig. 1(*a*). At relatively high concentrations of L-glycerol 3-phosphate substrate inhibition was observed, but linear plots were obtained by working with a wide range of substrate concentrations below those producing inhibition. Secondary plots of the intercepts and slopes of Fig. 1(*a*) are shown in Fig. 1(*b*). These are also linear within the limits of experimental error. The results of initial-rate measurements for L-glycerol 3-phosphate oxidation at pH6.0, 8.0 and 9.0 gave entirely similar plots to those given in Figs. 1(*a*) and 1(*b*). Substrate inhibition by glycerol 3-phosphate was observed in all cases but linear primary and secondary plots were obtained by working over wide ranges of glycerol 3-phosphate concentration below those causing inhibition. The initial-rate parameters ϕ_0, ϕ_1, ϕ_2 and ϕ_{12} in eqn. (1) were determined from the slopes and intercepts of secondary plots and the values are listed in Table 1 together with estimated values of the Michaelis constants for NAD^+ (ϕ_1/ϕ_0) and L-glycerol 3-phosphate (ϕ_2/ϕ_0).

The inhibition by glycerol 3-phosphate at high concentrations did not allow useful initial-rate measurements close to the apparent maximum rate and in some cases there were large extrapolations in the primary plots of e/v_0 versus 1/[glycerol 3-phosphate]. There were also large extrapolations of the

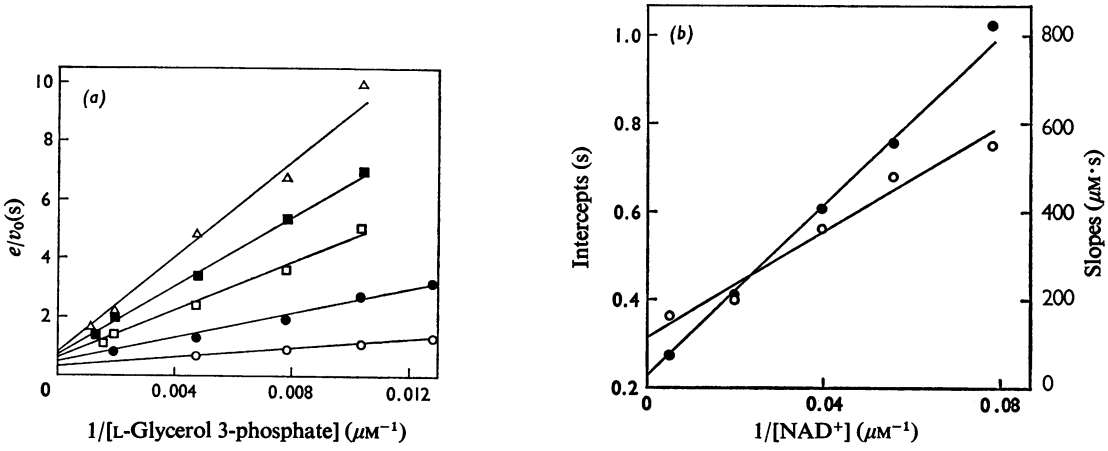


Fig. 1. *Initial-rate measurements for the glycerol 3-phosphate reaction*

(*a*) Primary plots showing the variation of the reciprocal of the specific initial rate at pH 7.0, 25°C, with the reciprocal of the L-glycerol 3-phosphate concentration at several constant NAD^+ concentrations. The NAD^+ concentrations (μM) were: ○, 202; ●, 50.5; □, 25.6; ■, 17.9; △, 12.8. (*b*) Secondary plots showing the variation of the intercepts (○) and the slopes (●) of Fig. 1(*a*) with the reciprocal of the NAD^+ concentration.

Table 1. *Kinetic coefficients describing the oxidation of L-glycerol 3-phosphate and glyoxylate by NAD^+ with glycerol 3-phosphate dehydrogenase at 25°C*

The kinetic coefficients are those in the initial rate equation:

$$\frac{e}{v_0} = \phi_0 + \phi_1/[S_1] + \phi_2/[S_2] + \phi_{12}/[S_1][S_2]$$

in which e is the concentration of enzyme active sites, $[S_1]$ is NAD^+ concentration and $[S_2]$ is either glycerol 3-phosphate or glyoxylate concentration. ϕ_1/ϕ_0 is the Michaelis constant for NAD^+ and ϕ_2/ϕ_0 is the Michaelis constant for the substrate.

Glycerol 3-phosphate oxidation

pH	ϕ_0 (s)	ϕ_1 (μM·s)	ϕ_2 (μM·s)	ϕ_{12} (μM^2·s)	ϕ_1/ϕ_0 (μM)	ϕ_2/ϕ_0 (μM)
6.0	46	29	3100	24000	0.63	68
7.0	0.320	5.94	30	9600	18.5	94
8.0	0.026	0.75	22	9000	29	850
9.0	0.016	0.50	8.6	6300	31	840

Glyoxylate oxidation

	ϕ_0 (s)	ϕ_1 (μM·s)	ϕ_2 (μM·s)	ϕ_{12} (μM^2·s)	ϕ_1/ϕ_0 (μM)	ϕ_2/ϕ_0 (μM)
8.0	43	6200	30000	15600000	144	700

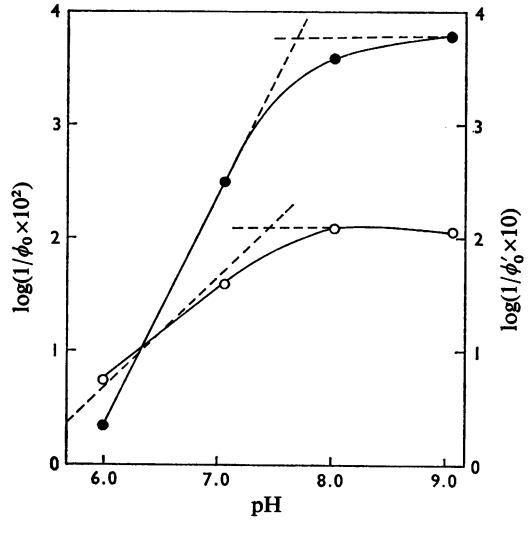


Fig. 2. *Variation of the logarithm of the maximum specific rate of L-glycerol 3-phosphate oxidation* ($1/\phi_0$) (●) *and of the logarithm of the maximum specific rate of dihydroxyacetone phosphate reduction* ($1/\phi'_0$) (○) *with pH at 25°C*

For details see the text. ----, Data from Tables 1 and 2.

secondary plots of slopes to obtain ϕ_2 at all pH values. These extrapolations undoubtedly increased the errors involved in the estimations of ϕ_0 (particularly at pH 6.0 and 7.0) and ϕ_2, although duplicate estimations of the kinetic coefficients usually agreed to within 25%. The low K_m values and the unfavourable equilibrium constant for the reaction added to the difficulties at the lower pH values.

Fig. 2 shows a plot of the logarithm of the maximum specific rate of glycerol 3-phosphate oxidation ($1/\phi_0$) versus pH. The results are consistent with two

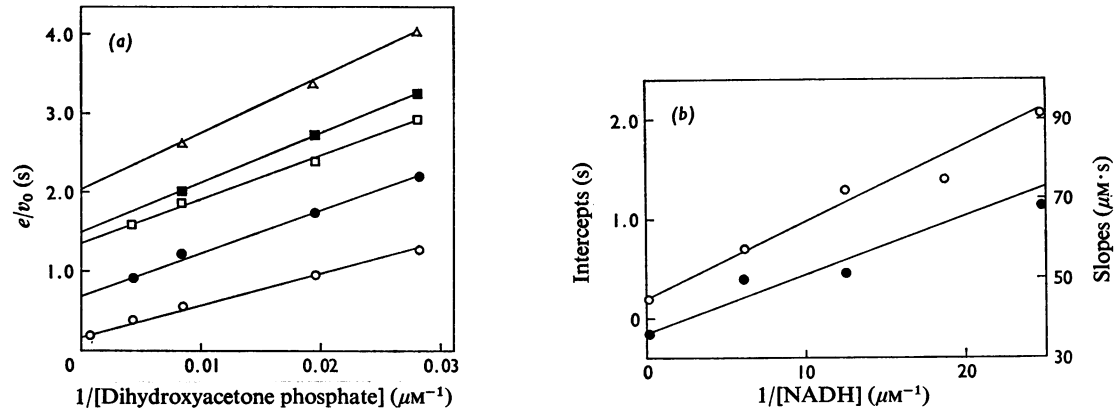


Fig. 3. *Initial-rate measurements for the dihydroxyacetone phosphate–NADH reaction*

(*a*) Primary plots showing the variation of the reciprocal of the specific initial rate at pH 6.0, 25°C, with the reciprocal of the dihydroxyacetone phosphate concentration at several constant NADH concentrations. The NADH concentrations (μM) were: ○, 9.35; ●, 0.15; □, 0.074; ■, 0.05; △, 0.04. (*b*) Secondary plots showing the variation of the intercepts (○) and slopes (●) of Fig. 3(*a*) with the reciprocal of the NADH concentration.

Table 2. *Kinetic coefficients describing the reduction of dihydroxyacetone phosphate by NADH with glycerol 3-phosphate dehydrogenase at 25°C*

The kinetic coefficients are those shown in the reciprocal initial-rate equation:

$$e/v_0 = \phi'_0 + \phi'_1/[S'_1] + \phi'_2/[S'_2] + \phi'_{12}/[S'_1][S'_2]$$

in which e is the concentration of enzyme active sites, $[S'_1]$ is NADH concentration and $[S'_2]$ is dihydroxyacetone phosphate concentration. ϕ'_1/ϕ'_0 is the Michaelis constant for NADH and ϕ'_2/ϕ'_0 that for dihydroxyacetone phosphate.

pH	ϕ'_0 (s)	ϕ'_1 (μM·s)	ϕ'_2 (μM·s)	ϕ'_{12} (μM²·s)	ϕ'_1/ϕ'_0 (μM)	ϕ'_2/ϕ'_0 (μM)
6.0	0.178	0.075	30	0.8	0.42	170
7.0	0.026	0.031	1.6	2.1	1.2	63
8.0	0.0079	0.064	1.83	11.9	8.1	230
9.0	0.0084	0.077	10	32.0	9.2	1200

straight lines of positive and zero slope intersecting at about pH 7.6. The data are clearly not sufficiently detailed to allow a precise determination of the pK of the controlling group(s). The most noteworthy feature of the data is that in the region pH 6.0–7.0 the plot exhibits a slope of +2.1 which is much higher than is normally expected (Dixon & Webb, 1964).

The substrate inhibition by glycerol 3-phosphate noted above has not been studied in detail, but certain significant points have been observed which merit description. At each pH value the concentration of glycerol 3-phosphate required to produce inhibition decreased as the concentration of NAD^+ increased. At any particular NAD^+ concentration the concentration of glycerol 3-phosphate required to produce inhibition decreased as the pH was decreased.

Initial-rate measurements of the oxidation of DL-glycerol 3-phosphate by NAD^+ at 25°C in phosphate buffers, pH 6.0 and 8.0, I 0.1, were also made. The results obtained were within the limits of experimental error the same as for the oxidation of L-glycerol 3-phosphate under these conditions, provided the results were plotted in terms of the concentration of L-glycerol 3-phosphate present. Studies in which glycerol 2-phosphate was added to assays containing L-glycerol 3-phosphate established that contamination of commercial preparations of DL-glycerol 3-phosphate by small amounts of glycerol 2-phosphate produced no significant effect.

Initial-rate measurements for the dihydroxyacetone phosphate–NADH reaction

The results of initial-rate measurements at 25°C in phosphate buffer, pH 6.0, I 0.1, are shown in Fig. 3(*a*). The secondary plot of the slopes and intercepts of Fig. 3(*a*) appears in Fig. 3(*b*). Similar results were obtained at pH 7.0, 8.0 and 9.0. Table 2 shows the initial-rate parameters ϕ'_0, ϕ'_1, ϕ'_2 and ϕ'_{12} obtained in the usual way, and also gives estimates for the Michaelis constants for dihydroxyacetone phosphate (ϕ'_2/ϕ'_0) and for NADH (ϕ'_1/ϕ'_0). Substrate inhibition was demonstrated with high concentrations of dihydroxyacetone phosphate at all pH values, but linear double-reciprocal plots were obtained by working over wide ranges of dihydroxyacetone phosphate concentrations below those producing inhibition. The

error involved in the estimation of ϕ'_{12} at pH 6.0 was rather large because of the difficulty in measuring initial rates at the exceedingly low NADH concentrations needed. Values of ϕ'_{12} obtained in duplicate experiments at pH 6.0 differed by a factor of two.

A plot of the logarithm of the maximum specific rate ($1/\phi'_0$) with pH is included in Fig. 2 and is consistent with two straight lines of positive and zero slope intersecting at pH 7.3. Again the data are not sufficiently detailed to allow an accurate estimation of the pK of the group controlling the reaction. In the region pH 6.0–7.0 the plot has a slope of approx. +0.7.

The substrate inhibition caused by dihydroxyacetone phosphate has not been studied in detail but the characteristics of the inhibition are quite different from those observed in the reverse reaction. The concentration of dihydroxyacetone phosphate required to cause inhibition at each pH value decreased as the NADH concentration decreased. This effect was most marked at pH 6.0. Fig. 3(*a*) shows the maximum dihydroxyacetone phosphate concentration which could be used at any particular NADH concentration and yet which did not cause inhibition. At the highest NADH concentration (9.4 μM) 1.03 mM-dihydroxyacetone phosphate caused no inhibitory effect whereas at the lowest NADH concentration (40 nM) there was marked inhibition with 0.25 mM-dihydroxyacetone phosphate.

Initial-rate measurements for the glyoxylate–NAD$^+$ reaction

It has been observed that rabbit muscle glycerol 3-phosphate dehydrogenase catalyses the oxidation of glyoxylate by NAD$^+$. The results of initial-rate measurements at 25°C in sodium phosphate buffer, pH 8.0, I 0.1, are shown in Fig. 4(*a*). The double-reciprocal plots are clearly linear within experimental error as are the secondary plots of slopes and intercepts (Fig. 4*b*). The estimated values for the initial-rate parameters and Michaelis constants for coenzyme (ϕ_1/ϕ_0) and substrate (ϕ_2/ϕ_0) are included in Table 1. No attempt was made to assay the reverse reaction.

Discussion

The initial-rate parameters in Tables 1 and 2 are based on total substrate concentrations and have not been corrected for the state of hydration of the dihydroxyacetone phosphate (Reynolds *et al.*, 1971) or the effect of pH on the state of ionization of the phosphate groups of the substrates (Dawson *et al.*, 1969). It may be that the active substrate concentration is less than the total concentration and that the given values of ϕ_2, ϕ'_2, ϕ_{12} and ϕ'_{12} and consequently ϕ_2/ϕ_0 and ϕ'_2/ϕ'_0 are too large. They may, however, be readily corrected when the appropriate correction factors are known. Errors here do not affect considerations of mechanism since for that purpose the ratios ϕ_{12}/ϕ_2, ϕ'_{12}/ϕ'_2 are required and any effect therefore cancels out.

It is clear from Tables 1 and 2 that the maximum specific rate of glycerol 3-phosphate oxidation ($1/\phi_0$) increased as the pH value was increased whereas the maximum specific rate for dihydroxyacetone phosphate reduction increased as the pH was increased from pH 6.0 to 8.0 but little further change occurred on raising the pH to 9.0. The Michaelis constants for

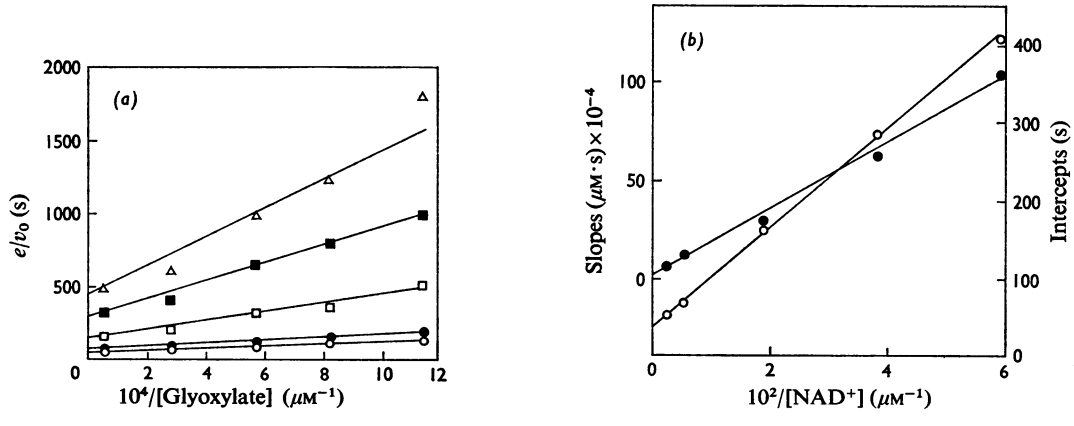


Fig. 4. *Initial-rate measurements for the glyoxylate–NAD$^+$ reaction*

(*a*) Primary plot showing the variation of the reciprocal of the specific initial rate at pH 8.0, 25°C with the reciprocal of the glyoxylate concentration at several constant NAD$^+$ concentrations. The NAD$^+$ concentrations (μM) were; ○, 525; ●, 210; □, 52.5; ■, 25.2; △, 16.8. (*b*) Secondary plots showing the variation of the intercepts (○) and slopes (●) of Fig. 4(*a*) with the reciprocal of the NAD$^+$ concentration.

Table 3. *Comparison of certain relationships between the kinetic coefficients of Tables* 1 *and* 2 *and some directly measured parameters for glycerol* 3*-phosphate dehydrogenase*

The approximate value for the equilibrium constant for the overall reaction ($K_{eq.}$) was calculated from the value estimated by Burton & Wilson (1953) at I 0.03 and by extending their extrapolation to I 0.1. The value given for $K_{eq.}$ at I 0.1 previously (Bentley & Dickinson, 1973) was incorrect. Values of $K_{E.NADH}$, $K_{E.NAD}$, k'_{+1} and k_{+1} are from Bentley & Dickinson (1974).

pH	$10^{12}\times K_{eq.}$ (M)	$10^{11}\times \phi'_{12}[H^+]/\phi_{12}$ (M)	$K_{E.NADH}$ (μM)	ϕ'_{12}/ϕ'_2 (μM)	$K_{E.NAD}$ (μM)	ϕ_{12}/ϕ_2 (μM)	$10^{-7}\times k'_{+1}$ ($M^{-1}\cdot s^{-1}$)	$10^{-7}\times 1/\phi'_1$ ($M^{-1}\cdot s^{-1}$)	$10^{-5}\times k_{+1}$ ($M^{-1}\cdot s^{-1}$)	$10^{-5}\times 1/\phi_1$ ($M^{-1}\cdot s^{-1}$)
6.0	7.0	3.33	0.02	0.026	9.2	7.8	2.5	1.33	5.6	0.35
7.0		2.2	0.63	1.3	260	320	2.6	3.2		1.68
8.0		1.32	1.82	6.5	325	410	5.2	1.6		13.3
9.0		0.52	2.05	3.2	460	730	9.4	1.3		20.0

the coenzyme and substrates in forward and reverse reactions tended to increase as the pH was increased. The K_m values obtained for glycerol 3-phosphate at pH9.0 and for dihydroxyacetone phosphate and NADH at pH8.0 agree with previous estimates (Black, 1966) but the K_m value for NAD^+ at pH9.0 is significantly lower. There are no other true K_m values available in the literature for comparison but the present work is consistent with earlier estimates of apparent Michaelis constants (Young & Pace, 1958; Telegdi & Keleti, 1968; Fondy *et al.*, 1969; Warkentin & Fondy, 1973).

Experiments showed that D-glycerol 3-phosphate and glycerol 2-phosphate are neither effective substrates nor inhibitors of rabbit muscle glycerol 3-phosphate dehydrogenase. The enzyme is not, however, absolutely specific for L-glycerol 3-phosphate since it will catalyse the oxidation of glyoxylate by NAD^+. The initial-rate parameters describing this reaction at pH8.0 are much larger than for L-glycerol 3-phosphate, the maximum specific rate being some 1600-fold slower. However, the K_m value for glyoxylate at pH8.0 is similar to that for L-glycerol 3-phosphate whereas the K_m value for NAD^+ in the glyoxylate reaction is only some fourfold larger than that for NAD^+ when glycerol 3-phosphate is the substrate. In solution glyoxylate exists entirely in a hydrated form and it is presumed that the reaction observed is the oxidation of the gem-diol to oxalate by NAD^+. The formation of oxalate has not, however, been confirmed.

The results of initial-rate studies and of the direct coenzyme-binding studies reported by Bentley & Dickinson (1974) may be used to deduce information about the mechanism of catalysis of glycerol 3-phosphate dehydrogenase. The following discussion will be in terms of a compulsory-order mechanism in which the coenzymes bind before the substrates and/or a rapid-equilibrium random-order mechanism. Table 3 shows that certain relationships exist between the kinetic coefficients of eqn. (1) for forward and reverse reactions and the equilibrium constant of the overall reaction ($K_{eq.}$), the dissociation constants of the enzyme–coenzyme complexes and the 'NADH-on'

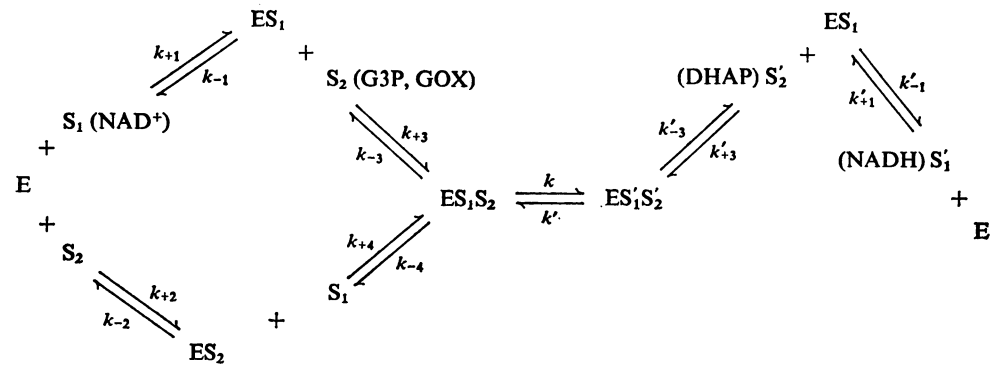
Table 4. *Relationships between the kinetic coefficients of glycerol* 3*-phosphate oxidation and dihydroxyacetone phosphate reduction*

pH	$\phi_1\phi_2/\phi_{12}\phi'_0$	$\phi'_1\phi'_2/\phi'_{12}\phi_0$
6.0	21	0.061
7.0	0.73	0.074
8.0	0.23	0.38
9.0	0.081	1.5

velocity constants. Thus within the limits of experimental error $\phi'_{12}[H^+]/\phi_{12} = K_{eq.}$, $\phi_{12}/\phi_2 = K_{E.NAD}$, $\phi'_{12}/\phi'_2 = K_{E.NADH}$ and at pH6.0, 7.0 and perhaps 8.0 $\phi'_1 = 1/k'_{+1}$. However, at pH9.0 $\phi'_1 > 1/k'_{+1}$ and at pH6.0 $\phi_1 \gg 1/k_{+1}$. In some cases the experimental error was considerable, and this was particularly true for ϕ'_{12} at pH6.0 and ϕ_2 at all pH values. Estimations of k'_{+1} were also rather difficult at pH8.0 and 9.0 (Bentley & Dickinson, 1974) and an error of up to 100% in these estimates is not improbable.

Certain relationships between the kinetic coefficients for glycerol 3-phosphate oxidation and dihydroxyacetone phosphate reduction are shown in Table 4. Except at pH6.0 and 9.0 where $\phi_1\phi_2/\phi_{12}\phi'_0$ and $\phi'_1\phi'_2/\phi'_{12}\phi_0 > 1$ the results are consistent with a compulsory-order mechanism (upper pathway in Scheme 1) in which coenzymes bind first and where the rate-limiting step is ternary-complex interconversion or the dissociation of the product ternary complex (Dalziel, 1957). The findings that $\phi_1\phi_2/\phi_{12}\phi'_0 < 1$ and $\phi'_1\phi'_2/\phi'_{12}\phi_0 < 1$ and $\phi'_1 = 1/k'_{+1}$ are not predicted by the rapid-equilibrium random-order mechanism and therefore the compulsory mechanism is preferred. In the cases where the compulsory mechanism is indicated, a minimum estimate of k_{+1}, the velocity constant describing the combination of enzyme and NAD^+, may be calculated from $1/\phi_1$. This clearly is not so at pH6.0 but it may be true at pH7.0, 8.0 and 9.0.

The suggestion that the principal rate-limiting step in the reaction is either ternary-complex interconversion or dissociation of the product ternary complex is substantiated in the case of glycerol 3-



Scheme 1. *Proposed mechanism for glycerol 3-phosphate dehydrogenase*

The pathways explain the kinetics at non-inhibitory substrate concentrations. G3P, Glycerol 3-phosphate; DHAP, dihydroxyacetone phosphate; GOX, glyoxylate.

Table 5. *Comparison of the maximum specific rates of glycerol 3-phosphate oxidation and dihydroxyacetone phosphate reduction and the velocity constant describing the dissociation of the enzyme–coenzyme binary complexes*

Value of k'_{-1} was calculated from values of k'_{+1} and $K_{E\cdot NADH}$. Estimations of k_{-1}, k'_{+1} and $K_{E\cdot NADH}$ are from Bentley & Dickinson (1974). The estimate of k_{-1} is a minimum one.

pH	V_m (glycerol 3-phosphate) (s^{-1})	k'_{-1} (s^{-1})	V_m (dihydroxyacetone phosphate) (s^{-1})	k_{-1} (s^{-1})
6.0	0.022	0.5	5.6	3.9
7.0	3.1	15.4		
8.0	38	95		
9.0	62	192		

phosphate oxidation by comparison of the 'NADH-off' velocity constant, k'_{-1} and the maximum specific rate (Table 5). In all cases the calculated velocity constant k'_{-1} exceeds the maximum rate. Confirmation of this sort is not at present available for dihydroxyacetone phosphate reduction at pH7.0, 8.0 and 9.0. At pH6.0, however, it appears that $k_{-1} \approx 1/\phi'_0$. This result implies a special case of the compulsory-order mechanism often called the Theorell–Chance mechanism. One would normally expect to find in this case the relationship $\phi_1\phi_2/\phi_{12}\phi'_0 = 1$ (Dalziel, 1957). The reason this is not found is that $\phi_1 \gg 1/k_{+1}$.

The cases where the data are not in accordance with the requirements of a strict compulsory-order mechanism may now be considered. The situation at pH6.0 arises because $\phi_1 \gg 1/k_{+1}$ and at pH9.0 because $\phi'_1 > 1/k_{+1}$. The study of the oxidation of glyoxylate at pH8.0 provides a third example. The parameter ϕ_1 in glyoxylate oxidation is some eightfold larger than the corresponding parameter in glycerol 3-phosphate oxidation at the same pH. Since $\phi_{1(\text{glycerol 3-phosphate})} \geqslant 1/k_{+1}$ it follows that $\phi_{1(\text{glyoxylate})} > 1/k_{+1}$. The oxidation of glyoxylate, therefore, cannot be described by a strict compulsory mechanism. We may note that in glyoxylate oxidation the ratio ϕ_{12}/ϕ_2 is very similar to the same ratio of parameters for glycerol 3-phosphate oxidation at pH8.0. Thus for glyoxylate oxidation, $\phi_{12}/\phi_2 = K_{E\cdot NAD+}$. This observation supports the view that the oxidation of glyoxylate is catalysed by glycerol 3-phosphate dehydrogenase and not by a trace amount of another enzyme present in our preparations.

It is logical to seek explanations for the data which do not fit our basic mechanism by introducing modifications which may apply in particular cases. There are two alternative simple modifications to a compulsory-order mechanism which lead to the expectation that the kinetics will obey eqn. (1) and that $\phi_1 > 1/k_{+1}$ or $\phi'_1 > 1/k'_{+1}$. These are (*a*) a mechanism involving significant dissociation of coenzyme from the ternary complex to yield an enzyme–substrate complex of the type enzyme–glycerol 3-phosphate (see Scheme 1) and (*b*) a mechanism involving isomeric enzyme–coenzyme complexes. The eightfold increase in ϕ_1 on passing from glycerol 3-phosphate to glyoxylate at pH8.0 effectively rules out possibility (*b*) above for glyoxylate oxidation (see for example Dickinson & Monger, 1973). These results are better explained by dissociation of coenzyme from the reactant ternary complex as in Scheme 1. This dissociation of coenzyme is probably significant in glyoxylate oxidation because the maximum rate of the reaction is so slow ($0.025\,s^{-1}$). It seems significant that oxidation of glycerol 3-phosphate at pH6.0 is also characterized by a very slow maximum rate ($0.025\,s^{-1}$) and much

slower than at any other pH value. Since the glyoxylate results appear to show that reactant ternary complexes can dissociate coenzyme, it seems most likely that the same process occurs to a marked extent in glycerol 3-phosphate oxidation at pH6.0. It is not suggested, by the way, that in dihydroxyacetone phosphate reduction a random order of product dissociation occurs. At pH6.0, for example, $\phi_0' \approx 1/k_{-1}$ implying a compulsory order. This is achieved if $k_{-3} \gg k_{-4}$.

Treatment of the basic mechanism in Scheme 1 by using the steady-state approximation leads to an equation which is not linear in double-reciprocal form. However, if the conditions: (i) $(k_{+4}/k_{+1})k_{+2}[S_1] \ll k_{-2} + k_{+4}[S_1]$ and (ii) $k_{+2}[S_2] \ll k_{+2} + k_{+4}[S_1]$ apply, then an equation of the form of eqn. (1) is obtained, namely:

$$\frac{e}{v_0} = \frac{1}{k'_{-1}} + \frac{1}{k'_{-3}} + \frac{A}{k} + \left(\frac{Ak_{-4}}{kk_{+4}} + \frac{1}{k_{+1}}\right)\frac{1}{[S_1]} + \frac{Ak_{-3}+k}{kk_{+3}[S_2]} + \frac{k_{-1}(Ak_{-3}+k)}{k_{+1}kk_{+3}[S_1][S_2]} \quad (2)$$

with

$$A = \frac{k' + k'_{-3}}{k'_{-3}}$$

This equation has been found to describe the kinetics of oxidation of secondary alcohols by horse liver alcohol dehydrogenase (Dalziel & Dickinson, 1966) and various primary and secondary alcohols by yeast alcohol dehydrogenase (Dickinson & Monger, 1973). The reciprocal rate equation for the reverse reaction of Scheme 1 with a compulsory order of product dissociation through ES_1 is as given by Dalziel (1957) which is also of the form of eqn. (1):

$$\frac{e}{v_0} = \frac{1}{k_{-1}} + \frac{1}{k_{-3}} + \frac{A'}{k'} + \frac{1}{k'_{+1}[S'_1]} + \frac{A'k'_{-3}+k'}{k'k'_{+3}[S'_2]} + \frac{k'_{-1}(A'k'_{-3}+k')}{k'_{+1}k'k'_{+3}[S'_1][S'_2]} \quad (3)$$

with

$$A' = \frac{k + k_{-3}}{k_{-3}}$$

The above equations provide a satisfactory explanation of the observed kinetics. Thus $\phi'_{12}[H^+]/\phi_{12} = K_{eq.}$, $\phi'_{12}/\phi'_2 = K_{E\cdot NADH}$ and $\phi_{12}/\phi_2 = K_{E\cdot NAD}$. The variation of ϕ_1 with the nature of the substrates is predicted and also that as in glycerol 3-phosphate oxidation at pH6.0 $\phi_1 > 1/k_{+1}$. On the other hand if $(k' + k'_{-3})k_{-4}/k'_{-3}kk_{+4} \ll 1/k_{+1}$ then $\phi_1 \simeq 1/k_{+1}$, which may be true in glycerol 3-phosphate oxidation at pH7.0, 8.0 and 9.0 where $\phi_1\phi_2/\phi_{12}\phi'_0 < 1$. A situation tending to bring this about is if $k \gg k_{-4}$ and it is noteworthy that the maximum rate of reaction is much faster at the other pH values than at pH6.0. Although the mechanism described is satisfactory it must be admitted that the results of glycerol 3-phosphate oxidation at pH6.0 and glyoxylate oxidation at pH8.0 could be also interpreted on the basis of a rapid-equilibrium random-order mechanism. The reverse reaction would still conform to a compulsory order if $k_{-3} \gg k_{-4}$. It is, however, possible to eliminate the special case of the rapid-equilibrium random-order mechanism in which the dissociation constants for NAD^+ from ternary and binary complexes are equal. In this case $\phi_1\phi_2/\phi_{12}\phi_0 = 1$ (Dalziel, 1957) and this relationship is not satisfied with the present results.

The results at pH9.0 where $\phi'_1\phi'_2/\phi'_{12}\phi_0 > 1$ and $\phi'_1 > 1/k'_{+1}$ could be explained by a dissociation of NADH from the enzyme–NADH ternary complex. However, at pH9.0, the maximum rate of dihydroxyacetone phosphate reduction is high compared with the maximum rate at pH6.0 and 7.0, and it seems more likely that there may be involvement of a kinetically significant isomeric enzyme–NADH complex. Mahler *et al.* (1962) have shown that isomeric enzyme–coenzyme complexes permit a compulsory-order mechanism to exhibit the relationship $\phi'_1\phi'_2/\phi'_{12}\phi_0 > 1$.

The substrate inhibition observed at all pH values with both glycerol 3-phosphate and dihydroxyacetone phosphate may now be considered. Black (1966) suggested that glycerol 3-phosphate at high concentrations inhibits because of the formation of an abortive enzyme–NADH–glycerol 3-phosphate complex. The observation that inhibition by glycerol 3-phosphate is most marked at high NAD^+ concentrations is consistent with this view. The fact that substrate inhibition by dihydroxyacetone phosphate is most marked at low NADH concentrations suggests that this inhibition process may be competitive with respect to NADH. Perhaps an alternative, less effective, pathway through an ES'_2 complex is available at high dihydroxyacetone phosphate concentrations.

In conclusion it is of interest to consider the variation of the maximum specific rate of glycerol 3-phosphate oxidation with pH. The positive slope of 2 units in Fig. 2 indicates that the maximum rate may be controlled by at least two groups with closely similar or perhaps equal pK values. In view of the fact that the phosphate group of the substrate has a pK at 6.6 (Dawson *et al.*, 1969) it is tempting to assign a controlling function to this group, the second group being in the active site. However, as reported (Bentley & Dickinson, 1974), there are large unexpected variations in $K_{E\cdot NADH}$ in the region pH6.0–7.0 due primarily to changes in k'_{-1}. Since variations in k'_{-1} with pH are clearly independent of glycerol 3-phosphate the large variations in ϕ_0 with pH may be solely due to the ionization of groups within the active site. The lack of effect of the phosphate groups may be because the enzyme only binds one ionic form of the substrates.

The very large decrease in ϕ_2' and particularly ϕ_2 on moving from pH6.0 to pH7.0 suggests that only those forms predominating on the alkaline side of the pK values are bound to the enzyme.

P. B. thanks the Science Research Council for a Research Studentship. We are also grateful to Dr. C. I. Pogson for gifts of substantial quantities of dihydroxyacetone phosphate hydrazone.

References

Apitz-Castro, R., Ryder, E. & Gaede, K. (1964) *Acta Cient. Venez.* **15**, 177–183
Bentley, P. & Dickinson, F. M. (1973) *Biochem. Soc. Trans.* **1**, 663–665
Bentley, P. & Dickinson, F. M. (1974) *Biochem. J.* **143**, 11–17
Bentley, P., Dickinson, F. M. & Jones, I. G. (1973) *Biochem. J.* **135**, 853–859
Black, W. J. (1966) *Can. J. Biochem.* **43**, 1301–1317
Burton, K. & Wilson, T. H. (1953) *Biochem. J.* **54**, 86–94
Dalziel, K. (1957) *Acta Chem. Scand.* **11**, 1706–1723
Dalziel, K. (1962*a*) *Biochem. J.* **84**, 240–244
Dalziel, K. (1962*b*) *Biochem. J.* **84**, 244–255
Dalziel, K. (1963) *J. Biol. Chem.* **238**, 1538–1543
Dalziel, K. & Dickinson, F. M. (1966) *Biochem. J.* **100**, 34–46
Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. (1969) *Data for Biochemical Research*, 2nd edn., Clarendon Press, Oxford
Dickinson, F. M. & Monger, G. P. (1973) *Biochem. J.* **131**, 261–270
Dixon, M. & Webb, E. C. (1964) *Enzymes*, 2nd edn., pp. 116–145, Longmans Green, London
Fondy, T. P., Ross, C. R. & Sollohub, S. J. (1969) *J. Biol. Chem.* **244**, 1631–1644
Hohorst, H. J. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 215–219, Academic Press, London
Holbrook, J. J., Yates, D. W., Reynolds, S. J., Evans, R. W., Greenwood, C. & Gore, M. G. (1972) *Biochem. J.* **128**, 933–940
Horecker, B. L. & Kornberg, A. (1948) *J. Biol. Chem.* **175**, 385–390
Keleti, T. (1970) in *Pyridine Nucleotide-Dependent Dehydrogenases* (Sund, H., ed.), pp. 103–120, Springer-Verlag, Berlin
Mahler, H. R., Baker, R. H. & Shiner, V. J. (1962) *Biochemistry* **1**, 47–52
Reynolds, S. J., Yates, D. W. & Pogson, C. I. (1971) *Biochem. J.* **122**, 285–297
Telegdi, M. & Keleti, T. (1968) *Acta Biochim. Biophys.* **3**, 131–140
Warkentin, D. L. & Fondy, T. P. (1973) *Eur. J. Biochem.* **36**, 97–109
Young, H. L. & Pace, N. (1958) *Arch. Biochem. Biophys.* **75**, 125–141