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The Kinetics and Reaction Mechanism of the Nicotinamide–Adenine Dinucleotide Phosphate-Specific Glycerol Dehydrogenase of Rat Skeletal Muscle

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The NADP-specific glycerol dehydrogenase of rat skeletal muscle has been partially purified by ammonium sulphate fractionation. The enzyme has been studied kinetically by initial-velocity analysis, product inhibition and inhibition by fluoride. The experimental results indicate that the reaction mechanism for the enzyme is ordered such that the first product leaves the enzyme before the addition of the second substrate.

The presence of an NADP-dependent glycerol dehydrogenase in skeletal muscle has been described (Toews, 1966). It is the purpose of this paper to report the kinetic properties of the enzyme and to determine its reaction mechanism. The kinetics and reaction mechanism of the glycerol dehydrogenase have been studied by initial-velocity analysis, product inhibition and inhibition with fluoride. The data indicate that the reaction mechanism for skeletal muscle glycerol dehydrogenase is probably an ordered mechanism, where the first product leaves the enzyme before the addition of the second substrate.

EXPERIMENTAL

Chemicals. D-Glyceraldehyde, NADPH and $NADP^+$ were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.) and were used without further purification. Glycerol, Na_2HPO_4 and KF were obtained from British Drug Houses Ltd. (Poole, Dorset).

Preparation of the enzyme. Skeletal muscle from the hind legs and the back of male hooded rats (Quebec Breeding Farms, St Eustache, Quebec, Canada) was removed and immediately homogenized in cold distilled water for 2 min. in a Waring Blendor. The homogenate was centrifuged at 100000*g* for 60 min. and $(NH_4)_2SO_4$ was slowly added to the supernatant. The supernatant with the added $(NH_4)_2SO_4$ was brought to pH 7·0 with aq. NH_3 soln., stirred for 5 min. and then centrifuged. Approx. 70% of the total enzyme activity originally present in the supernatant was precipitated at 50–70% saturation with $(NH_4)_2SO_4$. The precipitated enzyme was dissolved in a small volume of 0·01 M-EDTA–0·1 M-sodium phosphate buffer, pH 7·0. This enzyme preparation could be stored frozen for periods of up to 2 months without appreciable loss of activity. The enzyme preparation had specific activity 0·009 ΔE_{340}/min./mg. of protein under the conditions of the assay.

Determination of kinetic constants. The activity of the enzyme was determined by measuring the change in E_{340} in the presence of D-glyceraldehyde and NADPH. The incubation mixture, in a total volume of 3·0 ml., contained sodium phosphate buffer, pH 7·0 (50 mM). The concentrations of NADPH, D-glyceraldehyde and inhibitors are given in the legends describing the specific experiments. The control incubation mixture contained all the reagents except D-glyceraldehyde. All measurements of enzyme activity were made with a Unicam SP. 800 spectrophotometer equipped with a variable-speed slave recorder with a full-scale expansion 0–0·1 *E* unit. Before the actual measurements of enzyme activity, all reagents except enzyme were maintained at 30° in the same water bath that circulated water through the cuvette housing of the spectrophotometer. Incubations were carried out in quartz cuvettes of 1 cm. light-path. The enzyme reaction was started by the addition of 0·1 ml. of the enzyme preparation, and initial rates were measured for 5–10 min.

The regression lines were obtained by using the double-reciprocal (Lineweaver–Burk) form of the Michaelis–Menten equation. The regressions of $1/v$ against $1/[S]$ were obtained by the abbreviated Dolittle method (Ostle, 1954) with an I.B.M. model 360 computer and a Fortran IV programme (Queen's University Computing Centre, Kingston, Ont., Canada). Since each experiment was done in triplicate, the variance of the $1/v$ values could be experimentally obtained, and it was not necessary to assume equal variance of $1/v$ at different $1/[S]$ values (Wilkinson, 1961). In the kinetic data reported here the vertical bars represent the S.E.M. of the $1/v$ values in the Lineweaver–Burk plots and the standard errors of the slopes and intercepts in the replots of slopes and intercepts.

RESULTS

Initial-velocity analysis. When NADPH was the variable substrate and the D-glyceraldehyde concentration was held constant at different concentrations from 0·0167 mM to 0·33 mM, the double-reciprocal plots were linear and parallel to one another (Fig. 1). When the intercepts ($1/v$ at $1/[S] = 0$) from Fig. 1 were replotted against the

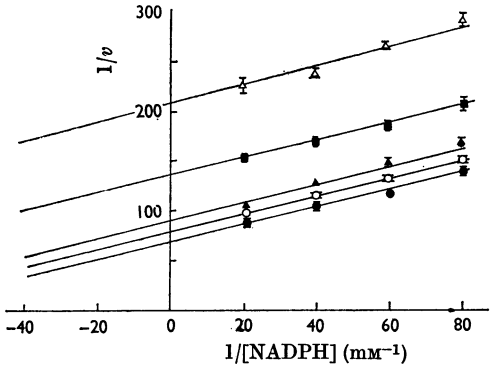


Fig. 1. Initial-velocity analysis of glycerol dehydrogenase with NADPH as the variable substrate. The D-glyceraldehyde concentration was held constant at 0·33 mM (●), 0·133 mM (○), 0·083 mM (▲), 0·033 mM (■) and 0·0167 mM (△). Further details are given in the Experimental section.

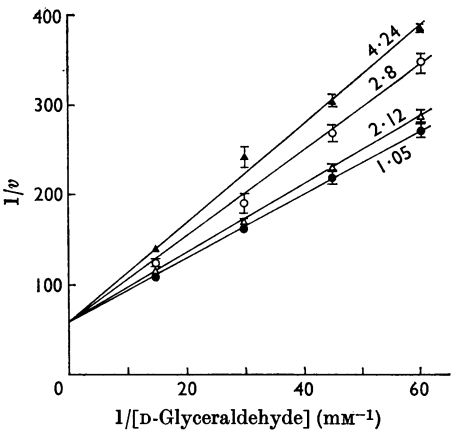


Fig. 2. Initial-velocity analysis of glycerol dehydrogenase with the concentrations of NADPH and D-glyceraldehyde held at a constant ratio. The molar ratios of D-glyceraldehyde: NADPH [1·05 (●), 2·12 (△), 2·8 (○) and 4·24 (▲)] are shown against each curve. Further details are given in the Experimental section.

reciprocal concentrations of D-glyceraldehyde (non-varied substrate), the points formed a straight line. The slopes of Fig. 1 do not vary significantly at different NADPH concentrations, indicating that the double-reciprocal plots are parallel. When the same type of initial-velocity experiments as described in Fig. 1 were done where NADPH, glycerol or $NADP^+$ was the variable substrate, linear double-reciprocal plots that were parallel to one another were obtained. In each case the replot of intercepts ($1/v$ at $1/[S]=0$) versus the reciprocal concentration of the non-varied substrate formed a straight line. The initial-velocity experiments where glycerol and $NADP^+$ were the substrates were made in 0·1 M-glycine and 0·5 M-hydrazine, pH 9·8 (C. J. Toews, unpublished work).

The rate equation describing the initial-velocity data is given by eqn. (1):

$$v = \frac{V_{max.}/[A][B]}{K_m^A[B]+K_m^B[A]+[A][B]} \quad (1)$$

where [A] and [B] are the concentrations of D-glyceraldehyde and NADPH and K_m^A and K_m^B are their respective K_m values. The initial-velocity data where glycerol and $NADP^+$ are the substrates are also described by eqn. (1), except that [A] and [B] are then the concentrations of glycerol and NADP.+

Eqn. (1) describes the initial-velocity pattern of all reaction mechanisms where the first product dissociates from the enzyme before the addition of the second substrate (Ping Pong Bi Bi mechanism) (eqn. 11 in Cleland, 1963*a*). Eqn. (1), however, does not distinguish between different types of Ping Pong Bi Bi mechanisms. Thus, it is not possible, on the basis of initial-velocity data alone, to determine

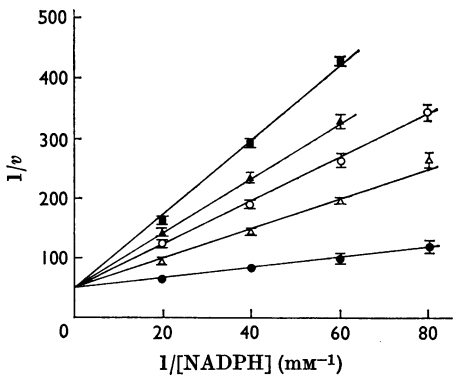


Fig. 3. Product inhibition of glycerol dehydrogenase by $NADP^+$ with NADPH as the variable substrate. The D-glyceraldehyde concentration was held constant at 0·067 mM. The concentration of the product inhibitor, $NADP^+$, was held constant at 0 (●), 0·037 mM (△), 0·074 mM (○), 0·111 mM (▲) and 0·148 mM (■). Further details are given in the Experimental section.

the order of addition and release of particular reactants or to detect the isomerization of the stable enzyme forms.

All Ordered Bi Bi, Theorell–Chance and Rapid-Equilibrium Random Bi Bi mechanisms yield initial-velocity data that, when plotted as double-reciprocal plots, intersect to the left of the $1/v$ axis. The rate equation describing these mechanisms, in the absence of products, would be identical with

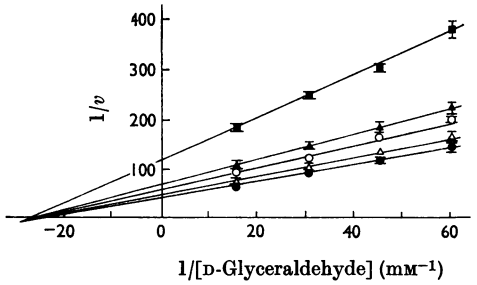


Fig. 4. Product inhibition of glycerol dehydrogenase by NADP+ with D-glyceraldehyde as the variable substrate. The NADPH concentration was held constant at 0·0475 mM. The concentration of the product inhibitor, NADP+, was held constant at 0 (●), 0·0148 mM (△), 0·037 mM (○), 0·074 mM (▲) and 0·148 mM (■). Further details are given in the Experimental section.

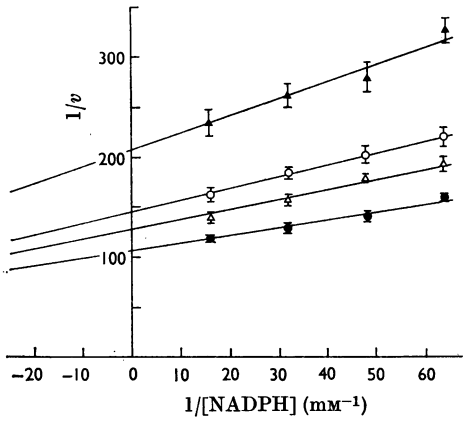


Fig. 5. Product inhibition of glycerol dehydrogenase by glycerol with NADPH as the variable substrate. The D-glyceraldehyde concentration was held constant at 0·067 mM. The glycerol concentration was held constant at 0 (●), 133 mM (△), 250 mM (○) and 500 mM (▲). Further details are given in the Experimental section.

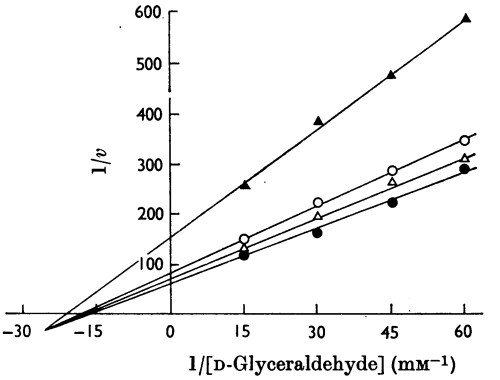


Fig. 6. Product inhibition of glycerol dehydrogenase by glycerol with D-glyceraldehyde as the variable substrate. The NADPH concentration was held constant at 0·067 mM. The glycerol concentration was held constant at 0 (●), 100 mM (△), 167 mM (○) and 500 mM (▲). Further details are given in the Experimental section.

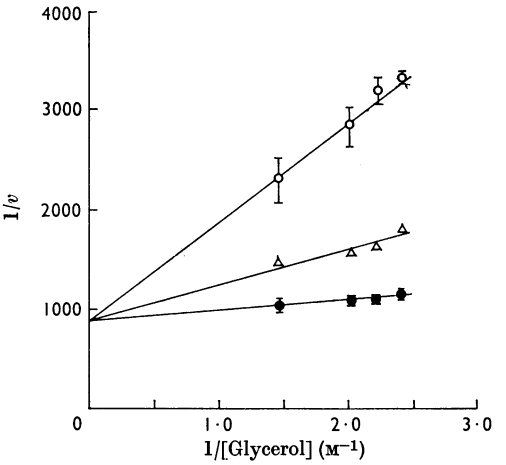


Fig. 7. Inhibition of glycerol dehydrogenase by fluoride with glycerol as the variable substrate. The assays were made with 100 mM-glycine, pH 9·8, and 0·074 mM-NADP+. The KF was present at a concentration of 0 (●), 3·3 mM (△) and 10 mM (○). Further details are given in the Experimental section.

eqn. (1) except that the denominator would contain an additional constant term, $K_i^A K_m^B$. If the term were very small then the initial-velocity pattern would approach the pattern described by eqn. (1). However, if [A] and [B] are varied together at a constant ratio, the presence of a small constant term is more readily detected. The double-reciprocal plots for eqn. (1) would then be straight lines, and the double-reciprocal plots in the presence of the constant term $K_i^A K_m^B$ would be a parabola. The result of such an experiment is shown in Fig. 2. The plots are linear, indicating that the constant term, $K_i^A K_m^B$, is not present in the rate equation describing the initial-velocity data. The enzyme mechanism therefore appears to be Ping Pong Bi Bi.

Product-inhibition studies. When NADPH was the varied substrate and the D-glyceraldehyde concentration was held constant at 0·067 mM, NADP+ was a competitive inhibitor (Fig. 3). The replot of slopes from Fig. 3 formed a straight line with an inhibition constant [$K_{i(slope)}$] (Cleland, 1963*b*) equal to $2{\cdot}5 \times 10^{-5}$ M. The intercepts ($1/v$ at 1/[NADPH] = 0)

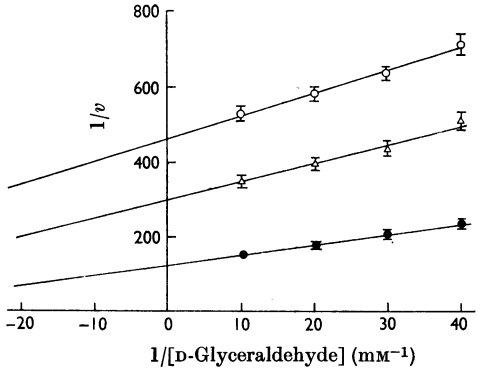


Fig. 8. Inhibition of glycerol dehydrogenase by fluoride with D-glyceraldehyde as the variable substrate. The NADPH concentration was 0·05 mM. The KF concentration was held constant at 0 (●), 1·0 mM (△) and 1·67 mM (○). Further details are given in the Experimental section.

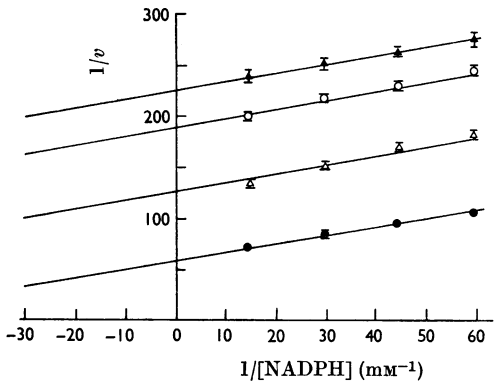
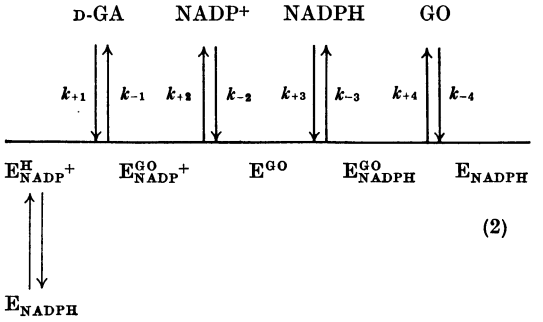


Fig. 9. Inhibition of glycerol dehydrogenase by fluoride with NADPH as the variable substrate. The D-glyceraldehyde concentration was 0·067 mM. The KF was present at a concentration of 0 (●), 0·67 mM (△), 1·33 mM (○) and 1·67 mM (▲). Further details are given in the Experimental section.

did not vary significantly (43·7 ± 9·2–50·5 ± 7·7). When D-glyceraldehyde was the varied substrate and the NADPH concentration was held constant at 0·0475 mM, $NADP^+$ was a non-competitive inhibitor (Fig. 4). The replots of slopes and intercepts versus the $NADP^+$ concentration from Fig. 4 were linear where $K_{i(\text{slope})}$ equalled $1{\cdot}02 \times 10^{-4}$ M and $K_{i(\text{intercept})}$ equalled $7{\cdot}5 \times 10^{-5}$ M. Glycerol was a non-competitive inhibitor of NADPH (Fig. 5) and of D-glyceraldehyde (Fig. 6). The replots of slopes and intercepts from Figs. 5 and 6 were linear.

Inhibition studies with fluoride. During investigation of possible inhibitors of skeletal muscle glycerol dehydrogenase it was found that fluoride was a potent inhibitor of the enzyme.



When the glycerol dehydrogenase was assayed in the direction glycerol to glyceraldehyde, and glycerol was the varied substrate, fluoride was a competitive inhibitor of glycerol (Fig. 7). This indicates that both glycerol and fluoride must react at the same active centre of the enzyme. Fluoride was a non-competitive inhibitor of D-glyceraldehyde (Fig. 8) and an uncompetitive inhibitor of NADPH (Fig. 9). The replots of slopes from Fig. 7, of slopes and intercepts from Fig. 8 and of intercepts from Fig. 9 all formed straight lines when plotted against the fluoride concentration.

DISCUSSION

The initial-velocity analysis and the product-inhibition experiments with glycerol and $NADP^+$ support an enzyme mechanism where the first product leaves the enzyme before the addition of the second substrate (Iso Ping Pong Bi Bi). Such a mechanism is shown in eqn. (2), where D-GA and GO represent D-glyceraldehyde and glycerol respectively. The stable enzyme form, E_{NADPH}, isomerizes before it reacts with D-glyceraldehyde. It is not possible to determine whether NADPH or D-glyceraldehyde is the first substrate to react with the enzyme, since the mechanism in eqn. (2) can also be written where NADPH is the starting substrate without changing the full rate equation for the mechanism.

The full rate equation for the mechanism in eqn. (2) has been derived by the method of King & Altman (1956), with the nomenclature of Cleland (1963*a*) and is shown in eqn. (3). [A], [B], [P] and [Q] are the concentrations of NADPH, D-glyceraldehyde, glycerol and $NADP^+$ respectively. K_m^A, K_m^B, K_m^P and K_m^Q are the K_m values for the respective reactants. V_1 is $V_{max.}$ in the direction D-glyceraldehyde to glycerol, and V_2 is $V_{max.}$ in the reverse direction. $K_{eq.}$ is the thermodynamic

$$v = \left\{ \frac{V_1([A][B]-[P][Q]/K_{eq.})}{K_m^B[A]+K_m^A[B]+[A][B]+\frac{K_i^A K_m^B[P]}{K_i^P}+\frac{K_m^P K_i^A K_m^B[Q]}{K_i^P K_m^Q}+\frac{K_i^A K_m^B[P][Q]}{K_i^P K_m^Q}+\frac{K_m^B[A][P]}{K_i^P} + \frac{K_i^A K_m^B[B][P]}{K_i^B K_i^P}+\frac{K_m^B[A][B][P]}{K_{ii}^B K_i^P}+\frac{K_m^A[B][Q]}{K_i^Q}+\frac{K_m^A[B][P][Q]}{K_{ii}^Q K_i^Q}} \right\} \quad (3)$$

equilibrium constant and equals $(k_{+1}k_{+2}k_{+3}k_{+4}k_{+5})/(k_{-1}k_{-2}k_{-3}k_{-4}k_{-5})$.

$K_i^A = k_{-3}/k_3$; $K_i^B = k_{-5}(k_{-1}+k_{+2})/k_{+1}k_{+2}$; $K_i^P = k_{+5}(k_{+5}+k_{-5})/k_{-4}k_{-5}$; $K_i^Q = k_{+2}/k_{-2}$; $K_{ii}^B = k_{-5}(k_{-1}+k_{+2})/k_{+1}k_{+2}$; $K_{ii}^Q = k_{+5}(k_{-3}+k_{+4})/k_{-3}k_{-4}$.

The full rate eqn. (3) describes the kinetic properties for the mechanism in eqn. (2). Thus if [A] and [B] or [P] and [Q] in eqn. (3) are set to zero, initial-velocity equations are obtained that are identical in form to eqn. (1). This equation predicts that the Lineweaver–Burk plots of the initial-velocity analysis should be parallel. These predictions are consistent with the data in Fig. 1.

The presence of the [P], [A][P], [B][P] and [A][B][P] terms in the denominator of eqn. (3) indicates that glycerol will be a non-competitive inhibitor of NADPH and D-glyceraldehyde. The absence of any $[P]^2$ terms in the denominator indicates that all the replots of slopes and intercepts where glycerol is a product inhibitor should be linear. The observed product-inhibition patterns of glycerol are in agreement with these predictions (Figs. 5 and 6).

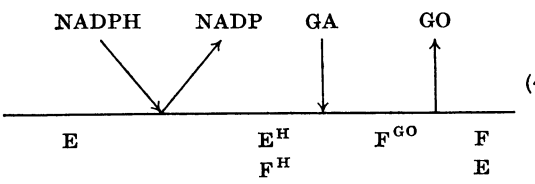
The observed non-competitive inhibition of NADP with respect to D-glyceraldehyde (Fig. 4) and the competitive inhibition of NADP with respect to NADPH (Fig. 3) confirm the presence of the [Q] and [B][Q] terms in eqn. (3). Some of the kinetic constants for the skeletal muscle NADP-dependent glycerol dehydrogenase are given in Table 1.

In analysing these kinetic data it is necessary to consider a large number of possible reaction mechanisms and to eliminate those mechanisms that do not fit the experimental results. The initial velocity analysis for skeletal muscle glycerol dehydrogenase suggests that the mechanism is Ping Pong Bi Bi. This conclusion rests largely on the observed parallel patterns obtained with the initial-velocity analysis. It is conceivable that, if the constant term in the denominator of eqn. (3) were very small, an intersecting initial velocity pattern might not be detected. However, it is possible to determine the value of the constant term, K_i^A, by other means. Thus if it is assumed, for the sake of these calculations, that the enzyme mechanism is ordered-sequential, then the value of K_i^A may be determined from the product-inhibition data of NADP. The K_i^A, determined from the data of Fig. 3, equals $6{\cdot}4 \times 10^{-6}$ M and $3{\cdot}6 \times 10^{-6}$ M respectively. This value of K_i^A is large enough to result in a detectable intersecting initial-velocity pattern. It is therefore unlikely that the observed parallel double-reciprocal plots of the initial-velocity data are masked intersecting patterns. It is thus possible to exclude, on the basis of the initial-velocity data alone, all Ordered Bi Bi, Theorell–Chance, Rapid Equilibrium Random Bi Bi and Random Bi Bi mechanisms.

Table 1. *Some kinetic constants of skeletal muscle glycerol dehydrogenase*

All the kinetic constants were determined in 0·1 M-sodium phosphate buffer, pH 7·0, except K_m values for glycerol and NADP, which were determined in 0·1 M-glycine–0·5 M-hydrazine, pH 9·8.

Constant	Concn. (M)
K_m of glyceraldehyde	4×10^{-5}
K_m of NADPH	$6{\cdot}7 \times 10^{-6}$
K_m of glycerol	1×10^{-1}
K_m of $NADP^+$	$1{\cdot}25 \times 10^{-5}$
K_i^P	$1{\cdot}05 \times 10^{-7}$
K_i^A	$2{\cdot}6 \times 10^{-7}$



(4)

The enzyme mechanism described by eqns. (2) and (3) is supported by all the initial-velocity and product-inhibition data. However, it would also be possible to explain the kinetic data by the enzyme mechanism (Ping Pong Bi Bi) of eqn. (4), where the stable enzyme forms, E and E^H, isomerize and where the E–NADPH binary complex concentration is very small. The full rate equation for mechanism (4) is identical with eqn. (3). This mechanism is therefore indistinguishable, on the basis of initial-velocity analysis and product-inhibition, from the Iso Ping Pong Bi Bi mechanism of eqn. (3). The fluoride-inhibition studies of the

enzyme, however, clearly distinguish between these two mechanisms.

Thus if the full rate equation, describing the dead-end inhibition of fluoride with the enzyme complex E_{NADPH} of eqn. (2), was derived, a rate expression was obtained which was identical with eqn. (3) except for the following additional terms in the denominator of the rate equation:

$$\frac{K_m^B[A][I]}{K_I} + \frac{[A][B][I]}{K_i^I} + \frac{K_m^P K_i^A K_m^B[Q][I]}{K_I K_i^P K_m^Q}$$

where $K_I = k_{-6}(k_{+5} + k_{-5})/k_{-5}k_{+6}$ and
$K_i^I = k_{-6}(k_{+2}k_{+5} + k_{+2}k_{+4} + k_{+4}k_{5+})/k_{+2}k_{+4}k_{+6}$
(where k_{-6}/k_{+6} equals the dissociation constant of the E–NADPH–fluoride complex).

When the full rate equation, including the inhibitory effect of fluoride, was simplified by setting [P] and [Q] equal to zero, eqns. (5) and (6) were obtained:

$$\frac{1}{v} = \frac{1}{V_1}\left[1 + \frac{K_m^A}{[A]} + \frac{[I]}{K_i^I}\right] + \frac{K_m^B}{V_1[B]}\left[1 + \frac{[I]}{K_I}\right] \quad (5)$$

$$\frac{1}{v} = \frac{1}{V_1}\left[1 + \frac{K_m^B}{[B]} + \frac{K_m^B[I]}{K_I[B]} + \frac{[I]}{K_i^I}\right] + \frac{K_m^A}{V_1[A]} \quad (6)$$

Eqn. (5) predicts that fluoride will be a non-competitive inhibitor of D-glyceraldehyde and eqn. (6) predicts that fluoride will be an uncompetitive inhibitor of NADPH. These predictions are supported by the experimental fluoride-inhibition data (Figs. 8 and 9). Similarly, the [Q][I] term indicates that fluoride will be a competitive inhibitor of glycerol. The fluoride-inhibition data therefore support the proposed Iso Ping Pong Bi Bi mechanism for skeletal muscle NADP–glycerol dehydrogenase.

The rate equation, describing the effect of fluoride combining with the enzyme complex, F, in eqn. (4), contains terms in [NADPH][D-GA][F^-], [$NADP^+$][F^-], [D-GA][F^-] and [$NADP^+$][D-GA][F^-]. Thus fluoride, for this enzyme mechanism, would be a non-competitive inhibitor of NADPH and an uncompetitive inhibitor of D-glyceraldehyde. Such an inhibition pattern is incompatible with the experimental findings and eqn. (4) is therefore excluded.

The [Q][I] term for eqn. (3) indicates that fluoride will remain an uncompetitive inhibitor of NADPH in the presence of glycerol, but that fluoride will become a non-competitive inhibitor of NADPH in the presence of NADP. That these predictions are, in fact, correct is indicated by the data in Fig. 10. In the absence of any products fluoride was an uncompetitive inhibitor of NADPH (Fig. 9). The presence of 0·33M-glycerol did not affect this uncompetitive pattern (C. J. Toews, unpublished results) but in the presence of $3{\cdot}7 \times 10^{-5}$M-$NADP^+$, fluoride became a non-competitive inhibitor of NADPH (Fig. 10).

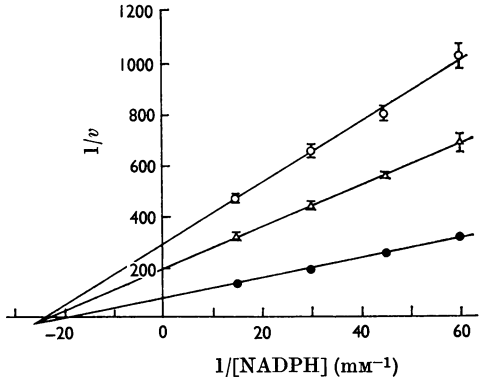


Fig. 10. Inhibition of glycerol dehydrogenase by fluoride in the presence of NADP. The D-glyceraldehyde (0·067mM) and NADP (0·037mM) concentrations were held constant. The KF concentration was 0 (●), 0·83mM (△) and 1·67mM (○). Further details are given in the Experimental section.

The NADP-dependent glycerol dehydrogenase of skeletal muscle described here and elsewhere (Toews, 1966) is similar in several ways to the NADP-dependent glycerol dehydrogenase that has been described in liver (Moore, 1959) and placenta (Hers, 1960). Thus all these enzymes are NADP-specific and of the various aldehydes that can serve as substrates, glyceraldehyde has the lowest K_m value. However, kinetically there are several major differences between the NADP-dependent glycerol dehydrogenases of different organs. Thus the K_m value for D-glyceraldehyde in the skeletal muscle and heart enzyme (C. J. Toews, unpublished work) is 4×10^{-5}M, whereas the K_m value for D-glyceraldehyde in the liver and kidney NADP-dependent glycerol dehydrogenase is $6{\cdot}25 \times 10^{-4}$M. The K_m value of glyceraldehyde in the placental enzyme is $5{\cdot}0 \times 10^{-4}$M (Hers, 1960). These enzymes can also be distinguished on the basis of inhibition by fluoride. The inhibition constant ($K_{i(\text{intercept})}$) for fluoride for the heart and skeletal muscle NADP-dependent glycerol dehydrogenase is $7{\cdot}0 \times 10^{-4}$M and the inhibition constants for the liver and kidney enzymes are $4{\cdot}5 \times 10^{-3}$M and $6{\cdot}5 \times 10^{-3}$M respectively. Therefore, on the basis of the K_m values for glyceraldehyde and of inhibition by fluoride, it appears that the NADP-dependent glycerol dehydrogenases from heart and skeletal muscle are very similar but differ markedly from the liver and kidney NADP-dependent glycerol dehydrogenases.

Unlike the NAD-dependent glycerol dehydrogenase, which appears to be present only in liver, the NADP-dependent glycerol dehydrogenases are

widely distributed in mammalian tissues. They have been found in placenta (Hers, 1960), liver (Moore, 1959), kidney, heart, skeletal muscle, small intestine, brain, lung and adipose tissue (C. J. Toews, unpublished work). The role of this enzyme in mammalian metabolism is not clear. It appears that the enzyme can act as the initiating step for glycerol metabolism in skeletal muscle and heart (Toews, 1966). However, in view of the high K_m for glycerol and the fact that the thermodynamic equilibrium constant for the reaction strongly favours glycerol formation, it would appear that the enzyme is more suited to effect the reduction of D-glyceraldehyde. If this does in fact occur, then the reduction of glyceraldehyde to glycerol by NADPH could well serve as a transhydrogenase system whereby the electrons from NADPH would be passed to NAD^+ either by the NAD-dependent glycerol dehydrogenase or the glycerol kinase and glycerol phosphate dehydrogenase in liver. This possibility, at the moment, is purely speculative.

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