A Product-Inhibition Study of Bovine Liver Glutamate Dehydrogenase

By PAUL C. ENGEL and SOO-SE CHEN
*Department of Biochemistry, University of Sheffield,
Western Bank, Sheffield S10 2TN, U.K.*

(Received 22 April 1975)

1. Initial rates of oxidative deamination of L-glutamate with NAD^+ as coenzyme, and of reductive amination of 2-oxoglutarate with NADH as coenzyme, catalysed by bovine liver glutamate dehydrogenase were measured in 0.111 M-sodium phosphate buffer, pH 7, at 25°C, in the absence and presence of product inhibitors. All 12 possible combinations of variable substrate and product inhibitor were used. 2. Strict competition was observed between NAD^+ and NADH, and between glutamate and 2-oxoglutarate. All other inhibition patterns were clearly non-competitive, except for inhibition by NH_4^+ with NAD^+ as variable substrate. Here the extrapolation did not permit a clear distinction between competitive and non-competitive inhibition. 3. Mutually non-competitive behaviour between glutamate and NH_4^+ indicates that these substrates can be bound at the active site simultaneously. 4. Primary Lineweaver–Burk plots and derived secondary plots of slopes and intercepts against inhibitor concentration were linear, with one exception: with 2-oxoglutarate as variable substrate, the replot of primary intercepts against inhibitory NAD^+ concentration was curved. 5. Separate K_i values were evaluated for the effect of each product inhibitor on the individual terms in the reciprocal initial-rate equations. With this information it is possible to calculate rates for any combination of substrate concentrations within the experimental range with any concentration of a single product inhibitor. 6. The inhibition patterns are consistent with neither a simple compulsory-order mechanism nor a rapid-equilibrium random-order mechanism without modification. They can, however, be reconciled with either type of mechanism by postulating appropriate abortive complexes. Of the two compulsory sequences that have been proposed, one, that in which the order of binding is NADH, NH_4^+ , 2-oxoglutarate, requires an implausible pattern of abortive complex-formation to account for the results. 7. On the basis of a rapid-equilibrium random-order mechanism, dissociation constants can be calculated from the K_i values. Where these can be compared with independent estimates from the kinetics of the uninhibited reaction or from direct measurements of substrate binding, the agreement is reasonably good. On balance, therefore, the results provide further support for the rapid-equilibrium random-order mechanism under these conditions.

The reactions catalysed by L-glutamate dehydrogenase (EC 1.4.1.3) involve, in addition to the proton and water, two substrates in the direction of oxidative deamination, and three in the direction of reductive amination (eqn. 1):

It was initially suggested that in both directions the reaction follows a compulsory sequence of substrate binding in which the coenzymes are the first reactants to be bound at the active site and the last to be released (Frieden, 1959*b*; Fisher, 1960; Corman *et al.*, 1967; Fahien & Strmecki, 1969). Systematic analysis of the initial-rate behaviour of the enzyme from bovine liver showed clearly, however, that the

three-substrate reaction does not follow a compulsory order (Engel & Dalziel, 1970). The results were compatible with random-order binding of all three substrates with interconversion of the central complexes as the rate-limiting step. When this mechanism was originally proposed, the available evidence from studies of substrate binding did not support it (Fisher, 1960). Subsequently studies of the protection of the enzyme by substrates against inactivation (Eisenkraft & Veeger, 1968; Malcolm & Radda, 1970; Wallis & Holbrook, 1973; Chen & Engel, 1974, 1975) and careful spectrophotometric studies of substrate binding (Cross *et al.*, 1972; Prough *et al.*, 1972; di Franco, 1971) have provided direct evidence of the existence of some of the binary and ternary complexes required by a random-order mechanism. Binding

studies are not wholly conclusive, however, since it can never be certain that the measured binding occurs specifically at the active site, especially for an enzyme known to possess auxiliary regulatory coenzyme-binding sites (for review see Goldin & Frieden, 1972). Further evidence against a compulsory-order mechanism and in support of a random-order mechanism has been provided by the elegant experiments of Silverstein & Sulebele (1973) on isotope exchange at equilibrium. Despite the accumulating corroborative evidence, Fisher (1973) has challenged the basis of the original kinetic argument.

The sequence of substrate addition is less certain in the two-substrate direction than in the three-substrate direction, even though initial-rate studies have been performed with two coenzymes, each at two pH values, and also with an alternative amino acid substrate, norvaline (Engel & Dalziel, 1969). There are two reasons for this uncertainty. First, a compulsory order of binding in a two-substrate reaction does not affect the number of terms in the reciprocal initial-rate equation, as it does in a three-substrate reaction (Frieden, 1959*b*; Dalziel, 1969). Secondly, bovine glutamate dehydrogenase displays a complex pattern of allosteric behaviour with glutamate as the substrate (Engel & Dalziel, 1969). The Lineweaver-Burk plots are linear only within restricted ranges of coenzyme concentration, for reasons discussed elsewhere (Dalziel & Engel, 1968; Engel & Dalziel, 1969; Dalziel & Egan, 1972; Engel & Ferdinand, 1973). Normally (Dalziel & Dickinson, 1966; Dickinson & Monger, 1973), a comparison of kinetic parameters obtained with different substrates affords an excellent test of kinetic mechanism. For glutamate dehydrogenase, although kinetic parameters for each linear region of the Lineweaver-Burk plot (Engel & Dalziel, 1969) may reflect the properties of a distinct conformational state, it is clearly difficult to select appropriate paired sets of kinetic parameters for comparison. It has not been possible on the basis of available evidence from steady-state kinetics to exclude completely a compulsory-order mechanism for glutamate oxidation in which NAD(P)^+ is the leading substrate. Although an enzyme-glutamate binary complex has now been detected (Prough *et al.*, 1972), and isotope-exchange studies support a random-order mechanism for the two-substrate reaction, there is clearly a need for further evidence.

In the present paper the results of a product-inhibition study (Alberty, 1958; Cleland, 1963*a,b*) of glutamate dehydrogenase are given. The theoretical treatment may be of some general interest; a somewhat similar approach to product inhibition in a presumed rapid-equilibrium random-order mechanism has been adopted by Morrison & James (1965) for the two-substrate reaction catalysed by creatine kinase, but the present treatment is more exhaustive.

Experimental

Sources of materials and techniques used in fluorimetric studies of bovine glutamate dehydrogenase have been described elsewhere (Engel & Dalziel, 1969, 1970). In the present work, reactions with the coenzyme couple NAD^+/NADH and the substrate couple glutamate/2-oxoglutarate were studied with ammonium phosphate as the source of ammonia when required, and 0.111 M-sodium phosphate, pH 7 (10.25 mol/l) as the buffer, at 25°C, with enzyme concentrations of 0.25–6.4 $\mu\text{g/ml}$.

Experiments with product inhibitors pose special problems, since the inhibitor not only lowers the initial rate, but also may increase the curvature of the recorded time-course of reaction. In meeting these difficulties, most acute in experiments with NADH as an inhibitor, the great sensitivity of fluorimetry and the possibility of 'backing off' a large part of the signal were invaluable.

Inhibition of the reaction in each direction was studied with all the possible combinations of inhibitor and variable substrate. In each experiment the concentration of one substrate was varied with several fixed concentrations of a single product, the other substrate concentration(s) being kept constant. In a few cases experiments were performed with two concentrations of the 'fixed' substrate for reasons elaborated in the Discussion section.

The initial rate under each set of conditions was determined at least in duplicate. In the double-reciprocal plots best-fit lines were drawn 'by eye'.

Results

The primary Lineweaver-Burk plots appeared to be linear within experimental error in all cases, and yielded for each substrate-inhibitor combination a set of slopes and ordinate intercepts corresponding to the several inhibitor concentrations used. These slopes and intercepts were replotted against inhibitor concentration. Ordinate intercepts of such secondary plots give parameters corresponding to the uninhibited reaction, and the slopes define the nature of the inhibition and the effectiveness of the inhibitor. With a single clear exception, the secondary plots were linear, and their slopes are given with relevant experimental details in Table 1.

Clear-cut competitive inhibition was observed in three cases, between NAD^+ , either as substrate or inhibitor, and NADH (Figs. 1*a* and 2*a*), and between glutamate as inhibitor and 2-oxoglutarate as substrate (Fig. 3*a*). 2-Oxoglutarate as inhibitor is probably competitive with glutamate as substrate; the extrapolation in Fig. 4(*a*) is too great to allow a definite decision on the basis of this experiment alone, but subsidiary experiments designed to test this specific point produced no clear evidence of

Table 1. Summary of product-inhibition experiments on glutamate dehydrogenase in 0.111 M-sodium phosphate, pH7, at 25°C

The abbreviations C and NC denote competitive and non-competitive inhibition respectively. An asterisk indicates that the slope or intercept in question could not be reliably estimated from these results.

Product inhibitor	Highest inhibitor concentration	Varied substrate	Substrate concentration range	Fixed substrates	Slopes of secondary plots against inhibitor concentration		Type of inhibition	Corresponding Figure
					Primary slopes	Primary intercepts		
2-Oxoglutarate	2 mM	NAD ⁺	13.8–69 μM	5 mM-glutamate	2.5 ms	40 s M ⁻¹	NC	—
2-Oxoglutarate	1 mM	Glutamate	2–40 mM	49 μM-NAD ⁺	0.50 s	—	C	Fig. 4(a) and 4(b)
NADH	14.4 μM	NAD ⁺	14.2–71 μM	5 mM-glutamate	0.47 s	—	C	Figs. 2(a) and 2(b)
NADH	8.16 μM	Glutamate	2–40 mM	49 μM-NAD ⁺	1.22 ms	6400 s M ⁻¹	NC	—
NH ₄ ⁺	25 mM	NAD ⁺	13.8–69 μM	5 mM-glutamate	0.151 s	*	NC/C	Figs. 5(a) and 5(b)
NH ₄ ⁺	25 mM	Glutamate	2–40 mM	29.4 μM-NAD ⁺	0.080 s	2.52 s M ⁻¹	NC	Figs. 8(a) and 8(b)
NH ₄ ⁺	25 mM	Glutamate	2–40 mM	58.8 μM-NAD ⁺	0.24 ms	2.16 s M ⁻¹	NC	Fig. 8(b)
Glutamate	40 mM	2-Oxoglutarate	0.1–1 mM	5.04 μM-NADH, 5 mM-NH ₄ ⁺	0.66 μs	—	C	Figs. 3(a) and 3(b)
Glutamate	40 mM	NADH	1–10 μM	0.5 mM-2-oxoglutarate, 5 mM-NH ₄ ⁺	2.5 ms	0.33 s M ⁻¹	NC	Figs. 10(a) and 10(b)
Glutamate	40 mM	NH ₄ ⁺	2.5–20 mM	1.02 mM-2-oxoglutarate, 5 mM-NH ₄ ⁺	1.1 ms	0.47 s M ⁻¹	NC	Fig. 7(b)
				5.04 μM-NADH, 0.5 mM-2-oxoglutarate			NC	Figs. 7(a) and 7(b)
NAD ⁺	1.68 mM	2-Oxoglutarate	0.1–1 mM	1.03 μM-NADH, 5 mM-NH ₄ ⁺	6.7 ms	Curve	NC	Fig. 9(b)
NAD ⁺	1.68 mM	2-Oxoglutarate	0.1–1 mM	1.03 μM-NADH, 15 mM-NH ₄ ⁺	3.5 ms	Curve	NC	Figs. 9(a) and 9(b)
NAD ⁺	1.07 mM	NADH	1–10 μM	0.5 mM-2-oxoglutarate, 5 mM-NH ₄ ⁺	0.136 ms	—	C	Figs. 1(a) and 1(b)
NAD ⁺	2.05 mM	NH ₄ ⁺	2.5–20 mM	0.845 μM-NADH, 0.5 mM-2-oxoglutarate	0.318 s	640 s M ⁻¹	NC	Figs. 6(a) and 6(b)

intercept variation. Another case where the length of the extrapolation did not allow a clear decision between competitive inhibition and non-competitive inhibition was that of inhibition by NH₄⁺ with NAD⁺ as the variable substrate (Fig. 5a). In the converse experiment, however, NAD⁺ is clearly not a competitive inhibitor with respect to NH₄⁺ (Fig. 6a).

All other inhibition patterns were non-competitive in the sense defined by Cleland (1963a), which requires alteration of the slopes and the intercepts of Lineweaver-Burk plots.

For inhibition by glutamate with varied NH₄⁺ concentration, two experiments were performed with identical 2-oxoglutarate concentrations but a five-fold difference in NADH concentrations (Figs. 7a and 7b). The absence of strict competition (Fig. 7a) indicates that NH₄⁺ and glutamate may be bound to the enzyme simultaneously, a surprising finding at first sight, which conflicts with the conclusions of Fisher & McGregor (1960). Moreover, as shown in the secondary plots (Fig. 7b), the intercept change over the same range of inhibitor concentration is much smaller with the lower NADH concentration. This clearly indicates the existence of a complex in which NADH, NH₄⁺ and glutamate are all simultaneously bound to the enzyme. If these conclusions are correct, NH₄⁺ should give non-competitive inhibition with glutamate as the varied substrate. This was indeed the observed pattern (Figs. 8a and 8b).

NAD⁺ as a product inhibitor gave the only clear departure from linearity in the plots. With a varied concentration of 2-oxoglutarate, the individual primary plots were linear (Fig. 9a), but the replot of intercepts against NAD⁺ concentration was a gentle curve (Fig. 9b). The experiment was repeated to confirm this. It should be noted that a very high maximal NAD⁺ concentration, 2.05 mM, was used in this experiment. The converse experiment with 2-oxoglutarate as the inhibitor showed linear non-competitive behaviour within the narrow NAD⁺ concentration range used.

In all cases the non-competitive plots have been drawn without the third and fourth quadrants shown. Each line has thus been drawn to fit the experimental points without reference to a possible common point of convergence. This appears to be the most objective procedure for plotting inhibition results by hand.

Discussion

Qualitative conclusions from the inhibition patterns

Several qualitative conclusions may be drawn from the results without prejudgement of the reaction pathway. The competition between NAD⁺ and NADH (Figs. 1a and 2a) indicates mutually exclusive binding of these two coenzymes to glutamate dehydrogenase. This is to be expected and is in

Fig. 1. Product inhibition by NAD^+ with $NADH$ as varied substrate

The primary Lineweaver-Burk plots are shown in (a) (conditions are given in Table 1). Inhibitor (NAD^+) concentrations are 0 ($\blacktriangle$), 213 ($\square$), 426 ($\bullet$), 745 ($\triangle$) and 1065 ($\blacksquare$) μM . In the secondary plot (b) slopes from (a) are plotted against inhibitor concentration. Enzyme concentration was 0.25 $\mu g/ml$.

Fig. 2. Product inhibition by $NADH$ with NAD^+ as varied substrate

(a) Primary Lineweaver-Burk plots (conditions are given in Table 1) for the following inhibitor concentrations: 0 ($\blacktriangle$), 1.44 ($\square$), 2.88 ($\bullet$), 7.2 ($\triangle$) and 14.4 ($\blacksquare$) μM - $NADH$. (b) Primary slopes are plotted against inhibitor concentration. Enzyme concentration was 0.25–6.4 $\mu g/ml$.

agreement with the findings of Frieden (1959a) with $NADP^+$ and $NADPH$. Similarly glutamate and 2-oxoglutarate clearly cannot be bound at the same active site simultaneously (Figs. 3a and 4a), in agree-

ment with the results of Caughey *et al.* (1957) and Eisenkraft & Veeger (1968), but contrary to results obtained with porcine glutamate dehydrogenase (Younes *et al.*, 1973). On the other hand, glutamate

Fig. 3. Product inhibition by glutamate with 2-oxoglutarate as varied substrate

(a) Primary Lineweaver-Burk plots (conditions are given in Table 1) for the following inhibitor concentrations: 0 (Δ), 4 ($\square$), 8 ($\bullet$), 20 ($\triangle$) and 40 ($\blacksquare$) mM-glutamate. (b) Primary slopes are plotted against inhibitor concentration. Enzyme concentration was $0.54 \mu\text{g/ml}$.

Fig. 4. Product inhibition by 2-oxoglutarate with glutamate as varied substrate

(a) Primary Lineweaver-Burk plots (conditions as given in Table 1) for the following inhibitor concentrations: 0 (Δ), 0.1 ($\square$), 0.2 ($\bullet$), 0.4 ($\triangle$) and 1.0 ($\blacksquare$) mM-2-oxoglutarate. (b) Secondary plot of slopes against inhibitor concentration. Enzyme concentration was $0.25 \mu\text{g/ml}$.

and NH_4^+ do not compete; neither as substrate can completely displace the other as inhibitor (Figs. 7a and 8a). The α -amino group of glutamate represents the potential product NH_4^+ ion, and must

presumably be able to interact with the same part of the active site (Fisher & McGregor, 1960). Thus, even though the potent inhibitory effect of glutamate (Caughey *et al.*, 1957; Chen & Engel, 1974) indicates

Fig. 5. Product inhibition by NH_4^+ with NAD^+ as varied substrate

(a) Primary plots (conditions are given in Table 1) for the following NH_4^+ concentrations: 0 ($\blacktriangle$), 1 ($\square$), 5 ($\bullet$), 10 ($\triangle$) and 25 ($\blacksquare$) mM. (b) Secondary plot of slopes against inhibitor concentration. Enzyme concentration was 1.3 $\mu g/ml$.

Fig. 6. Production inhibition by NAD^+ with NH_4^+ as varied substrate

Primary plots (a) (conditions are given in Table 1) are for the following NAD^+ concentrations: 0 ($\blacktriangle$), 103 ($\square$), 205 ($\bullet$), 410 ($\triangle$), 718 ($\blacksquare$), 1025 ($\circ$) and 2050 ($\blacktriangledown$) μM . (b) Secondary plots of slopes ($\bullet$) and intercepts ($\blacktriangle$). Enzyme concentration was 0.25 $\mu g/ml$.

that the α -amino group contributes little to the strength of binding of glutamate by glutamate dehydrogenase, it is still somewhat surprising that occupation of the NH_4^+ site should be compatible

with simultaneous binding of glutamate. Presumably this indicates two modes of binding of the glutamate molecule, differing in the orientation of the α -amino group.

Fig. 7. Product inhibition by glutamate with NH_4^+ as varied substrate

(a) Primary plots ($5.04 \mu\text{M-NADH}$, other conditions as given in Table 1) for the following glutamate concentrations 0: (▲), 4 (□), 8 (●), 20 (△) and 40 (■) mM. (b) Secondary plot against inhibitor concentration of slopes (●, ○) and intercepts (▲, △) from (a) (filled symbols) and from a similar experiment with $1.02 \mu\text{M-NADH}$ (open symbols). Enzyme concentration was $0.54 \mu\text{g/ml}$.

Fig. 8. Product inhibition by NH_4^+ with glutamate as varied substrate

(a) Primary plots ($29.4 \mu\text{M-NAD}^+$, other conditions as given in Table 1) for the following NH_4^+ concentrations: 0 (▲), 2 (□), 5 (●), 15 (△) and 25 (■) mM. (b) Secondary plots against inhibitor concentration of slopes (●, ○) and intercepts (▲, △) from (a) (open symbols) and from a similar experiment with $58.8 \mu\text{M-NAD}^+$ (filled symbols).

Derivation of K_i values for inhibition of three-substrate reaction

The results of inhibition experiments are customarily described in terms of K_i values. For non-competitive inhibition it becomes necessary to define separate slope and intercept K_i values (Cleland,

1963a). It is frequently assumed that a K_i represents the dissociation constant for dissociation of the complex between inhibitor and free enzyme, but this is seldom justified. One may consider as an example the three-substrate reaction catalysed by glutamate dehydrogenase, for which the linear

Fig. 9. Product inhibition by NAD^+ with 2-oxoglutarate as varied substrate

(a) Primary plots (15 mM-NH_4^+ , other conditions as in Table 1) for the following NAD^+ concentrations: 0 ($\blacktriangle$), 164 ($\square$), 410 ($\bullet$), 737 ($\triangle$), 1065 ($\blacksquare$) and 1683 ($\circ$) μM . (b) Secondary plot against inhibitor concentration of slopes ($\bullet, \circ$) and intercepts ($\blacktriangle, \triangle$) from (a) (filled symbols) and from a similar experiment with 5 mM-NH_4^+ (open symbols). Enzyme concentration was $0.25 \mu\text{g/ml}$.

reciprocal initial-rate equation contains eight terms (Engel & Dalziel, 1970):

$$\frac{e_0}{v} = \phi_0 + \frac{\phi_A}{[A]} + \frac{\phi_B}{[B]} + \frac{\phi_C}{[C]} + \frac{\phi_{AB}}{[A][B]} + \frac{\phi_{AC}}{[A][C]} + \frac{\phi_{BC}}{[B][C]} + \frac{\phi_{ABC}}{[A][B][C]} \quad (2)$$

In this equation, e_0 is the total enzyme concentration, v is the initial rate, $[A]$, $[B]$ and $[C]$ are the concentrations of 2-oxoglutarate, NH_4^+ and NADH , and ϕ_0 , ϕ_A etc. are constants. A compound may combine with several of the enzyme-containing complexes in the reaction mechanism, in each case with a different dissociation constant. Such an inhibitor would alter the coefficients of several terms in the reciprocal initial-rate equation, so that an experimentally determined slope K_i or intercept K_i would be valid only with the fixed substrate concentrations for which it was determined. Provided that the inhibition is linear, however, a general description of the inhibition, valid for all substrate concentrations, may be achieved by evaluating the individual K_i associated with each term in the reciprocal initial-rate equation. Ideally this would involve determining the full

equation several times with different fixed inhibitor concentrations. This laborious procedure has been circumvented in the present study by means of a few assumptions and the solution of simultaneous equations. This inevitably entails greater uncertainty than the more exhaustive method, but various internal comparisons may be made as a check on the validity of the derived constants. The method is developed below, and is exemplified by the treatment of product inhibition by glutamate.

The assumption is made that, since glutamate appears to be strictly competitive with 2-oxoglutarate (Fig. 3a), it does not affect the ϕ_0 , ϕ_B , ϕ_C and ϕ_{BC} terms in eqn. (2), these four terms being independent of 2-oxoglutarate concentration. If one now considers the Lineweaver-Burk plots obtained with glutamate as inhibitor and NH_4^+ as varied substrate (Figs. 7a and 7b), it can be seen from eqn. (2) that

$$\text{Slope} = \phi_B + \frac{\phi_{BC}}{[\text{NADH}]} + \frac{\phi_{AB}}{[\text{2-oxoglutarate}]} + \frac{\phi_{ABC}}{[\text{NADH}][\text{2-oxoglutarate}]} \quad (3)$$

Of the four terms in eqn. (3), however, only the last two are affected by glutamate inhibition. One may

therefore rewrite eqn. (3) for the inhibited reaction as follows:

Slope

$$= \phi_B + \frac{\phi_{BC}}{[\text{NADH}]} + \frac{\phi_{AB}}{[\text{2-oxoglutarate}]} \left[1 + \frac{[\text{glutamate}]}{K_{i,\text{Glu,AB}}} \right] + \frac{\phi_{ABC}}{[\text{NADH}][\text{2-oxoglutarate}]} \left[1 + \frac{[\text{glutamate}]}{K_{i,\text{Glu,ABC}}} \right]$$

= Constant term

$$+ [\text{glutamate}] \left[\frac{\phi_{AB}}{[\text{2-oxoglutarate}] K_{i,\text{Glu,AB}}} + \frac{\phi_{ABC}}{[\text{2-oxoglutarate}][\text{NADH}] K_{i,\text{Glu,ABC}}} \right] \quad (4)$$

In this equation $K_{i,\text{Glu,AB}}$ and $K_{i,\text{Glu,ABC}}$ are the K_i values for glutamate inhibition affecting the ϕ_{AB} and ϕ_{ABC} terms respectively. Thus, when the primary slopes are plotted in a secondary plot (Fig. 7b) against glutamate concentration, one obtains for each combination of fixed substrate concentrations a straight line with its slope given by the bracketed expression in eqn. (4) with the appropriate concentrations substituted in the denominators of the two terms. The only unknowns in the resulting expression are $K_{i,\text{Glu,AB}}$ and $K_{i,\text{Glu,ABC}}$, since the values of ϕ_{AB} and ϕ_{ABC} are available from the study of the uninhibited reaction (Engel & Dalziel, 1970). By performing the experiment with two combinations of fixed substrate concentrations, it is possible to evaluate the unknown constants by solving two simultaneous equations.

Since glutamate does not affect the ϕ_0 or the $\phi_c/[\text{NADH}]$ terms, an analogous procedure adopted with the intercepts of the primary plots gives values for $K_{i,\text{Glu,A}}$ and $K_{i,\text{Glu,AC}}$.

When analysed in this way the results shown in Figs. 7(a) and 7(b) yield the following K_i values: $K_{i,\text{Glu,A}} = 5 \text{ mM}$; $K_{i,\text{Glu,AC}} = 54 \text{ mM}$; $K_{i,\text{Glu,AB}} = 7.8 \text{ mM}$; $K_{i,\text{Glu,ABC}} = 28 \text{ mM}$. The validity of these constants can be tested by using them to predict the slopes of the secondary plots of results obtained with 2-oxoglutarate or NADH as variable substrate and glutamate as inhibitor. If either too many or too few initial-rate parameters have been assumed to be affected by glutamate inhibition, or if the inherent accuracy of the experiments is insufficient, such predictions cannot be expected to be accurate. For the plot against glutamate concentration (Fig. 10b) of the intercepts of primary plots (Fig. 10a) against $[\text{NADH}]^{-1}$ the predicted slope is:

$$\frac{\phi_A}{[\text{2-oxoglutarate}] K_{i,\text{Glu,A}}} + \frac{\phi_{AB}}{[\text{2-oxoglutarate}][\text{NH}_4^+] K_{i,\text{Glu,AB}}}$$

With the reactant concentrations used here the calculated value of this function is $3.3 \times 10^{-1} \text{ s} \cdot \text{M}^{-1}$. This corresponds exactly to the measured value (Fig. 10b). Similarly the predicted and measured slopes of the secondary plot of slopes (Fig. 10b) are $6.5 \times 10^{-7} \text{ s}$ and $6.6 \times 10^{-7} \text{ s}$ respectively.

Fig. 10. Product inhibition by glutamate with NADH as varied substrate

Primary plots (a) (conditions given in Table 1) are for the following glutamate concentrations: 0 (▲), 4 (□), 8 (●), 20 (Δ) and 40 (■) mM. (b) Secondary plot of slopes (●) and intercepts (▲). Enzyme concentration was $0.54 \mu\text{g/ml}$.

With 2-oxoglutarate as varied substrate, all the four terms affected by glutamate inhibition are in the slope of the primary plot, since the inhibition is strictly competitive in this case. The predicted and measured slopes of the secondary plot (Fig. 3b) are 2.3×10^{-4} s and 2.4×10^{-4} s respectively.

These agreements, testing various combinations of the evaluated constants, indicate that, in conjunction with the previously determined kinetic parameters (Engel & Dalziel, 1970) for the uninhibited reaction, the four K_i values accurately describe product inhibition by L-glutamate. If, however, the estimate of any individual ϕ parameter is in error, the estimate of the corresponding K_i will inevitably be in error by the same factor, since the experiments yield estimates of the ratios of ϕ and K_i values, rather than their separate magnitudes.

The results of experiments with NAD^+ as a product inhibitor were treated in a similar fashion. Since NAD^+ was found to be strictly competitive with NADH (Fig. 2a), it was assumed that it does not affect the ϕ_0 , ϕ_A , ϕ_B or ϕ_{AB} terms in eqn. (2). Of the remaining four terms that are or may be affected, two occur in the expression for the slopes and two in the expression for the intercepts of Lineweaver-Burk plots of e_0/v against $[\text{2-oxoglutarate}]^{-1}$. Thus, if two combinations of fixed substrate concentrations are used, it again becomes possible to solve the equations for the relevant K_i values (Figs. 9a and 9b).

As noted above, however, the interpretation of experiments with NAD^+ as an inhibitor and 2-oxoglutarate as the varied substrate is complicated by the fact that the replot of the primary intercepts against $[\text{NAD}^+]$ is non-linear (Fig. 9b). Since this curvature becomes obvious only when very high concentrations of NAD^+ are used, and since it is apparent in the replot of intercepts, corresponding to saturation with 2-oxoglutarate, rather than the slopes, it may be an allosteric effect analogous to that seen with NAD^+ as a coenzyme in the two-substrate reaction. The latter effect also is manifest only with high concentrations of dicarboxylic acid substrates or substrate analogues (Engel & Dalziel, 1969; Dalziel & Egan, 1972; Chen & Engel, 1974).

The curvature precluded estimation of K_i values affecting the ϕ_C and ϕ_{BC} terms. The replots of slopes, however, were linear (Fig. 9b), and analysis similar to that adopted for glutamate inhibition yielded the following estimates of the two other K_i values describing inhibition by NAD^+ :

$$K_{i,\text{NAD},\text{AC}} = 4.2 \text{ mM}; K_{i,\text{NAD},\text{ABC}} = 1.0 \text{ mM}$$

Values of kinetic parameters for the uninhibited reaction were taken for this calculation from Engel & Dalziel (1969). These two K_i values are likely to be only approximate, since the slope variations from which they are derived are small.

Derivation of K_i values for inhibition of the two-substrate reaction

The reciprocal initial-rate equation for the two-substrate reaction within a 'linear region' (Engel & Dalziel, 1969) contains only four terms:

$$\frac{e_0}{v} = \phi'_0 + \frac{\phi'_D}{[D]} + \frac{\phi'_E}{[E]} + \frac{\phi'_{DE}}{[D][E]} \quad (5)$$

In this equation, e_0 is the total enzyme concentration, v is the initial rate, $[D]$ and $[E]$ are the concentrations of glutamate and NAD^+ respectively and ϕ'_0 , ϕ'_D etc. are constants. As in eqn. (1), the alphabetical order of the subscripts is not intended to imply the order of substrate addition to the enzyme.

The individual K_i values affecting these terms can be evaluated similarly to those for the three-substrate reaction by making appropriate assumptions. For inhibition by NADH and by 2-oxoglutarate this process is simplified, since there are only two unknowns. Thus 2-oxoglutarate, which is competitive with glutamate (Fig. 4a), can only affect the ϕ'_D and ϕ'_{DE} terms. Using the primary plots (not shown) against $[\text{NAD}^+]^{-1}$, one obtains by calculations similar to those above: from the slopes (Table 1) $K_{i,\text{Og},\text{DE}} = 1.27 \text{ mM}$; from the intercepts (Table 1) $K_{i,\text{Og},\text{D}} = 0.25 \text{ mM}$. The assignment of subscripts is similar to that used for the three-substrate reaction and Og refers to constants for product inhibition by 2-oxoglutarate. From these K_i values one may predict the slope of the secondary plot (Fig. 4b) against $[\text{2-oxoglutarate}]$ of the slopes of primary plots (Fig. 4a) against $[\text{glutamate}]^{-1}$ obtained with different concentrations of 2-oxoglutarate as inhibitor. The prediction, 0.42 s, may be compared with a measured value of 0.50 s.

Similarly, on the basis of the results shown in Fig. 1(a), it is assumed that NADH as a product inhibitor does not affect the ϕ'_0 or ϕ'_D terms. The effects on the ϕ'_E and ϕ'_{DE} terms may be separately examined by analysing experiments with NADH as the inhibitor and glutamate as the variable substrate. Replots (not shown) against $[\text{NADH}]$ of the slopes and intercepts of Lineweaver-Burk plots (not shown) yield the following K_i values: $K_{i,\text{NADH},\text{E}} = 3 \mu\text{M}$; $K_{i,\text{NADH},\text{DE}} = 10.5 \mu\text{M}$. As before, if the ratios of the K_i and ϕ' parameters have been correctly assigned, it should be possible to predict the results of NADH inhibition when NAD^+ is the varied substrate and the two inhibited terms appear together in the expression for the slopes of the Lineweaver-Burk plots. The secondary plot of these slopes against $[\text{NADH}]$ (Fig. 1b) has a slope of 0.47 s as compared with a predicted value of 0.52 s.

The analysis of product inhibition by NH_4^+ requires more experimental information, since this product is not strictly competitive with either glutamate or NAD^+ , and accordingly may affect all

Table 2. *Predicted patterns of product inhibition for a strictly compulsory-order mechanism without abortive complexes (Mechanism 1)*

The symbols S_1 , P_1 etc. are those of Mechanism 1, and denote the order of substrate addition. The abbreviations C, NC and UC indicate competitive, non-competitive and uncompetitive patterns of inhibition respectively.

Product inhibitor	Varied substrate	Inhibition pattern
P_1	S_1	C
P_1	S_2	NC
P_1	S_3	NC
P_2	S_1	NC
P_2	S_2	NC
P_2	S_3	NC
S_1	P_1	C
S_1	P_2	NC
S_2	P_1	UC
S_2	P_2	UC
S_3	P_1	NC
S_3	P_2	NC

four terms in eqn. (5). Product inhibition by NH_4^+ was therefore studied, with two fixed concentrations of NAD^+ , and glutamate as the varied substrate. It is evident from Fig. 8(a) that, with the glutamate concentrations used, the estimates of the intercepts and their variation can only be approximate. From the slopes of the Lineweaver-Burk plots (Fig. 8b) it is possible to obtain as previously $K_{i,\text{NH}_4^+,\text{DE}} = 3.8\text{ mm}$; $K_{i,\text{NH}_4^+,\text{D}} = 5.3\text{ mm}$; from the intercepts: $K_{i,\text{NH}_4^+,\text{O}} = 32\text{ mm}$; $K_{i,\text{NH}_4^+,\text{E}} = 14\text{ mm}$. The subscript 0 denotes inhibition affecting the ϕ'_0 term in eqn. (5).

Conclusions about the mechanism of glutamate dehydrogenase

In considering the mechanistic implications of the observed patterns of product inhibition, the qualitative rules given by Cleland (1963b) are helpful. One may consider first of all a strictly compulsory-order mechanism of the type proposed for glutamate dehydrogenase from bovine (Frieden, 1959b; Fisher, 1960; Fahien & Strmecki, 1969) and dogfish (Corman *et al.*, 1967) liver (Mechanism 1).

Table 2 shows the predictions for this mechanism. Strict competition should only be observed between S_1 and P_1 , the two substrates that bind the free enzyme. This clearly does not match the experimental findings, since not only NAD^+ and NADH , but also glutamate and 2-oxoglutarate, form a competitive pair. Moreover, substrate S_2 as a product inhibitor

should be uncompetitive (i.e. give rise to parallel Lineweaver-Burk plots) with respect to both P_1 and P_2 . None of the substrates fits this requirement for S_2 . If the mechanism were of the Theorell-Chance type, without kinetically significant central complexes, competition would be expected between S_3 and P_2 as well as between S_1 and P_1 , but uncompetitive inhibition by S_2 would still be predicted.

The second obvious case to consider is the rapid-equilibrium random-order mechanism (Engel & Dalziel, 1969, 1970). In this mechanism (Mechanism 2) the interconversion of central complexes before product release is rate-limiting, and all the reactants are capable of forming productive complexes with free enzyme. If product inhibitors combine exclusively with free enzyme, all inhibition patterns must be strictly competitive (Cleland, 1963b). The experimental results are clearly inconsistent with this prediction.

Both mechanisms considered above are, however, misleadingly oversimplified by the omission of abortive complexes. In Mechanism 1, for example, the prediction that S_3 and P_2 should be mutually non-competitive rests on the assumption that P_2 may not combine with ES_1S_2 , nor S_3 with EP_1 . If the abortive complexes $ES_1S_2P_2$ and EP_1S_3 are introduced, S_3 and P_2 should be competitive. Similarly, in the simple rapid-equilibrium random-order mechanism, NADH , for example, is expected to compete with glutamate on the assumption that NADH can combine with free enzyme and with no other complex in the sequence leading to the rate-limiting step. On this basis saturation with glutamate would remove the only enzyme species with which the product inhibitor can combine. If, however, an abortive complex, enzyme-glutamate- NADH , may be formed, the inhibition by NADH should not be strictly competitive with glutamate.

There is, in fact, abundant evidence that glutamate dehydrogenase forms abortive complexes (e.g. Winer & Schwert, 1958; Pantaloni & Iwatsubo, 1967; Egan & Dalziel, 1971; di Franco, 1971; di Franco & Iwatsubo, 1972; Cross *et al.*, 1972). Indeed the formation of such complexes is perhaps to be expected; glutamate and 2-oxoglutarate, for instance, must share a common binding site, and if 2-oxoglutarate can form a productive ternary complex with enzyme- NADH , it is not surprising that glutamate should form an analogous abortive complex.

It is thus necessary to re-examine the basic mechanisms considered above, taking full account of possible abortive complex-formation. The inclusion of such complexes, giving rise to what Cleland (1963a,b) terms 'mixed dead-end and product inhibition' makes the task of distinguishing between various mechanisms much more difficult. Observed alterations of slope or intercept, if not predicted by

Table 3. Comparison of substrate dissociation constants calculated from the product-inhibition results on the assumption of a rapid-equilibrium random-order mechanism with independent estimates

In the symbol for each dissociation constant the first subscript, Glu, NAD⁺ etc. denotes the dissociating substrate. Thus K_{NAD^+} , for example, is the dissociation constant for the binary enzyme–NAD⁺ complex. Where there are further subscripts in parentheses, these denote other substrates present in the dissociating complex. Thus, for example, $K_{\text{NH}_4^+(\text{NAD}^+, \text{Glu})}$ is the constant governing dissociation of NH₄⁺ from the quaternary complex enzyme–NH₄⁺–NAD⁺–glutamate. Abbreviations: Og, 2-oxoglutarate; Glu, L-glutamate. References are: a, Engel & Dalziel (1969); b, Engel & Dalziel (1970); c, Prough *et al.* (1972); d, Kubo *et al.* (1957); e, Dalziel & Egan (1972); f, Cross *et al.* (1972). *Denotes 'Region 1' of Lineweaver–Burk plots against [NAD⁺]^{–1} (Engel & Dalziel, 1969).

Constant	Estimate from product inhibition	Estimate from uninhibited kinetics	Direct measurement
K_{Glu}	28 mM	57 mM ^a	48 mM ^c
$K_{\text{Glu}(\text{NADH})}$	7.8 mM		
$K_{\text{Glu}(\text{NH}_4^+)}$	54 mM		
$K_{\text{Glu}(\text{NADH}, \text{NH}_4^+)}$	5 mM		
K_{NAD^+}	1 mM	0.43 mM ^a	0.3–0.4 mM ^d 0.47 mM ^e
$K_{\text{NAD}^+(\text{NH}_4^+)}$	4.2 mM		
K_{Og}	1.27 mM	1.49 mM ^b	2.8 mM ^f
$K_{\text{Og}(\text{NAD}^+)}$	0.25 mM		
K_{NADH}	10.5 μM	8.6 μM ^b	
$K_{\text{NADH}(\text{Glu})}$	3 μM		
$K_{\text{NH}_4^+}$	3.8 mM	3.1 mM ^b	
$K_{\text{NH}_4^+(\text{NAD}^+)}$	5.3 mM		
$K_{\text{NH}_4^+(\text{Glu})}$	32 mM		
$K_{\text{NH}_4^+(\text{NAD}^+, \text{Glu})}$	14 mM		

the basic mechanism, may simply be accounted for by postulating suitable abortive complexes.

In one of the compulsory-order mechanisms proposed for glutamate dehydrogenase (Frieden, 1959b; Fisher, 1960; Corman *et al.*, 1967), S₁, S₂, S₃, P₁ and P₂ in Mechanism 1 are respectively NADH, NH₄⁺, 2-oxoglutarate, NAD⁺ and glutamate. If this is the basic linear reaction sequence, it is necessary (Table 2) to explain the competitive behaviour observed with 2-oxoglutarate as inhibitor and glutamate as varied substrate. Such behaviour requires (Cleland, 1963b) that glutamate should be able to combine with all those complexes in the mechanism with which 2-oxoglutarate can combine, and therefore requires the existence of the abortive complex enzyme–NADH–NH₄⁺–glutamate. Likewise, competition by glutamate with 2-oxoglutarate as variable substrate may be explained by postulating the complex enzyme–NAD⁺–2-oxoglutarate. It would, however, seem surprising that 2-oxoglutarate should be unable to bind to enzyme–NADH, as required by the mechanism, if it is able to bind to enzyme–NAD⁺. Although the forbidden complex enzyme–NADH–2-oxoglutarate has not been directly demonstrated, the corresponding NADPH-containing complex has been characterized by Cross (1972). It is also necessary to account for the ability of NH₄⁺ to vary the slopes of

Lineweaver–Burk plots against [glutamate]^{–1} and [NAD⁺]^{–1}, but this may be explained by postulating the complex enzyme–NAD⁺–NH₄⁺ and/or enzyme–NAD⁺–glutamate–NH₄⁺.

Another proposed compulsory sequence (Fahien & Strmecki, 1969) differs from the first in that 2-oxoglutarate and NH₄⁺ are interchanged. On the basis of this mechanism, the mutual competition between 2-oxoglutarate and glutamate requires the existence of the abortive complexes enzyme–NADH–glutamate and enzyme–NAD⁺–2-oxoglutarate. The latter complex appears a reasonable postulate on the basis of this mechanism, which already includes an enzyme–NADH–2-oxoglutarate complex. The existence of the abortive complex enzyme–NAD⁺–2-oxoglutarate would also account for non-competitive behaviour, rather than uncompetitive as predicted, by 2-oxoglutarate with respect to NAD⁺.

Of the two proposed versions of Mechanism 1, the second requires fewer and more reasonable additional postulates to reconcile it with the observed patterns of product inhibition.

In Mechanism 2, product interactions beyond the central complex cannot affect the initial rate. Any effects of product inhibitors on the intercepts of Lineweaver–Burk plots can therefore only be explained by abortive complex-formation. In a

random-order system, however, the formation of a wide range of abortive complexes presents little conceptual difficulty. The only slightly surprising requirement, as discussed above, is for complexes containing both glutamate and NH_4^+ .

It may be argued that the linear kinetics observed in the presence and absence of product inhibitors points to a rapid-equilibrium mechanism, since, in a steady-state system, combination of an inhibitor at two or more reversibly connected points in the reaction sequence leads to non-linearity (Cleland, 1963*a,b*). The inhibition by NAD^+ , however, is not entirely linear; the kinetics of the uninhibited reaction shows complex behaviour as the NAD^+ concentration is varied (Engel & Dalziel, 1969), and departure from linearity is also produced by NADH, glutamate and 2-oxoglutarate as substrates. Although it is possible to find explanations for all these observations, they nevertheless diminish the weight of the argument based on linearity.

Clearly the product-inhibition patterns alone are not very helpful in distinguishing realistically formulated mechanisms, since the wide possibilities for abortive complex-formation negate the qualitative distinctions that might otherwise be made. Taken in conjunction with the results of other experiments, however, the product inhibition studies do provide some evidence in favour of Mechanism 2, as shown below.

In the reciprocal initial-rate equation for a system following Mechanism 2, each term, in the absence of inhibitors, is proportional to the fractional contribution of a single enzyme-containing species to the total. For example, in eqn. 2, the ϕ_0 , ϕ_{ABC} and ϕ_{AB} terms are proportional respectively to the fractional concentrations of quaternary complex, free enzyme and enzyme- NAD^+ complex. The K_i values evaluated for individual terms can therefore be identified (Engel, 1968) with the dissociation constants for the corresponding enzyme-inhibitor complexes. Thus, for instance, $K_{i,\text{Glu,AB}}$ is the constant for dissociation of glutamate from the abortive complex enzyme-glutamate- NADH . Interpretation of the results according to Mechanism 2, therefore, predicts values (Table 3) of a large number of dissociation constants, some of which may be compared with independent estimates. The kinetics of the uninhibited reaction (Engel & Dalziel, 1969, 1970) provides estimates for the constants governing binding of substrates and coenzymes to free enzyme, and these are directly comparable with the predictions from the product-inhibition experiments, since identical conditions of temperature and buffering were used for the two studies. Dissociation constants for the abortive complexes are only obtainable by direct measurement, and unfortunately many of the reported values of such constants have been determined under conditions different from those used in the present study.

Several constants were estimated, for example, by di Franco (1971), but the buffer used was 0.1M-Tris-acetate, pH 7.5, and the temperature in some cases was 10°C. Substitution of Tris for phosphate markedly alters the kinetic parameters (Engel & Dalziel, 1969), and presumably also the individual dissociation constants. In Table 3, therefore, only estimates made under conditions roughly comparable with our own are included.

The overall agreement between the independent estimates of dissociation constants and prediction based on Mechanism 2 is reasonably good in view of the possibilities of cumulative error. The observed mutual enhancement of binding by NADH and glutamate is in accord with experimental observation (Pantaloni & Iwatsubo, 1967; di Franco & Iwatsubo, 1972) and the same enhancement factor is obtained from the $K_{i,\text{Glu}}$ values and the $K_{i,\text{NADH}}$ values. The values suggest relatively little mutual interaction in binding between glutamate and NH_4^+ , although they are equivocal as to whether the interaction is one of hindrance or assistance. There is a more marked internal discrepancy in the results for the mutual effects of NH_4^+ and NAD^+ , but this almost certainly stems from the imprecision of the estimate of ϕ'_D (ϕ'_2 of Engel & Dalziel, 1969) which is used in the calculation of $K_{\text{NH}_4^+(\text{NAD}^+)}$.

On balance, the product-inhibition results appear to give some further support to the rapid-equilibrium random-order mechanism, modified by the inclusion of several abortive complexes, for both directions of reaction. The prediction of a large number of dissociation constants (Table 3) offers an opportunity for further tests of this mechanism. The delineation of the range of active and abortive complexes available to glutamate dehydrogenase should provide a useful framework for any future attempt to arrive at a formal chemical and structural account of events at the catalytic site.

Much of this work is taken from a D.Phil. Thesis (Engel, 1968), although some experiments have been repeated or extended, and the theoretical treatment and interpretation has been substantially revised. The support of the Medical Research Council (1965–1968) and the current support of the Science Research Council are gratefully acknowledged.

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