A steady-state random-order mechanism for the oxidative deamination of norvaline by glutamate dehydrogenase

Charles LiMUTI and J. Ellis BELL

Department of Biochemistry, University of Rochester Medical Center, Rochester, NY 14642, U.S.A.

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The kinetic mechanism of glutamate dehydrogenase with the monocarboxylic substrate norvaline was examined by using initial-rate steady-state kinetics and inhibition kinetics. To a first approximation the reaction mechanism can be described as a rapid-equilibrium random-order one. Binding synergism between the monocarboxylic substrate and coenzyme is not observed. Dissociation constants for NAD^+ and 2-oxoglutarate calculated from the kinetic data assuming a rapid-equilibrium random-order model are in good agreement with independently obtained estimates. Lineweaver–Burk plots with varied norvaline concentration are not strictly linear, and it is concluded that a steady-state random-order model more accurately reflects the observed kinetics with norvaline as substrate.

Glutamate dehydrogenase (EC 1.4.1.3) catalyses the reversible oxidative deamination of L-glutamate as well as other L-amino acids (Struck & Sizer, 1960). Numerous kinetic investigations have been performed in order to try to characterize the kinetic mechanism. Early studies of the reaction in both directions suggested that the forward reaction with glutamate and the reverse reaction with 2-oxoglutarate could be described as compulsory ordered, with coenzyme being the obligatory first substrate (Frieden, 1959; Fisher, 1960; Fahien & Stremcki, 1969). A pre-steady-state kinetic investigation (Colen *et al.*, 1972) and more extensive steady-state kinetic experiments (Engel & Dalziel, 1969) of the forward reaction, however, were in agreement with a random-order model, but could not rule out completely a compulsory-order mechanism. The reverse reaction with 2-oxoglutarate as substrate appears to be random order (Engel & Dalziel, 1970).

Studies of isotope exchange at equilibrium of the forward reaction with glutamate at pH 8.0 are consistent with a random-order mechanism (Silverstein & Sulebele, 1973). Similar studies at pH 8.8, however, are consistent with a compulsory-order addition of substrates, where coenzyme is the leading substrate (Silverstein, 1974). Lineweaver–Burk plots with varied NAD(P)^+ concentration at a fixed glutamate concentration are not linear, but are concave down, exhibiting several abrupt changes in slope and intercept as the coenzyme concentration is varied (Engel & Dalziel, 1969). These effects have been attributed to negative co-operativity in the hexamer of the enzyme, and subsequent studies (Bell

& Dalziel, 1973; Alex & Bell, 1980) have confirmed this suggestion. Linear double-reciprocal plots were obtained, however, with varied coenzyme concentration when the monocarboxylic substrate norvaline was used (Engel & Dalziel, 1969). It was concluded either that the negative interactions do not occur with the monocarboxylic substrate or that they involve steps that are not rate-limiting with norvaline as substrate.

Initial-rate kinetic studies and inhibition studies were performed with norvaline as substrate in order to characterize further the kinetic mechanism with the monocarboxylic substrate and to explore the nature of the differences in the mechanism of glutamate dehydrogenase with the monocarboxylic and dicarboxylic substrates.

Materials and methods

Reagents

Bovine liver glutamate dehydrogenase (type IV) was obtained from Sigma Chemical Co. Before use portions of the suspension in $(\text{NH}_4)_2\text{SO}_4$ solution were dialysed at 4°C against 0.1 M-sodium phosphate buffer, pH 7.0, containing $10\ \mu\text{M}$ -EDTA. The dialysed enzyme solution was centrifuged or passed through $0.45\ \mu\text{m}$ Millipore filters to clarify the solution. Protein concentrations were determined from the absorbance of the solution at 280 nm by using a specific absorption coefficient of $0.93\ \text{litre}\cdot\text{g}^{-1}\cdot\text{cm}^{-1}$ (Dalziel & Egan, 1972). NADH, NAD^+ , L-norvaline, 2-oxo-2-oxovaleric acid and 2-oxoglutaric acid were obtained from Sigma Chemical

Co. Valeric acid was obtained from Eastman Kodak Co. All reagents were used as obtained without further purification.

Kinetics

Initial rates of NADH formation were followed by monitoring the increase in fluorescence emission at 470nm on a Varian fluorimeter, with excitation at 340nm. Experiments were performed at 30°C in 0.1M-sodium phosphate buffer containing 10 μ M-EDTA, adjusted to pH8.0 with NaOH. All solutions were adjusted to pH8.0 if the pH changed after the addition of reagent. The reaction was initiated by the addition of 20 μ l of the solution containing enzyme to 3ml of the preincubated reagents. Reaction rates were linear for at least 1 min. All rates were measured at least in triplicate.

The fluorescence of a standard stock solution of quinine in the pH8.0 buffer was obtained in each experiment. A standard curve was obtained with NADH in order to convert fluorescence measurements into concentration of reduced coenzyme. The intention was to determine the ratio of the measured coenzyme fluorescence to the fluorescence of the quinine standard in each experiment, to compensate for changes that may occur in time (photomultiplier, lamp intensity etc.) that would alter the signal of the emission. It was found that the emission of the quinine standard did not significantly change in any of the experiments.

Regression analysis

Linear regression was conducted by using the University of Pennsylvania Minitab Statistics Package. Standardized residual plots were obtained from the Minitab regression analysis. Standardized residuals are the residuals normalized by the estimated standard error. The 95%-confidence intervals for slopes and intercepts were calculated by using a Student's *t* distribution with $\alpha=0.025$ and the appropriate degrees of freedom. Non-linear curve-fitting was performed by using the National Institutes of Health Modelling Laboratory Library (MLAB). The MLAB program uses a least-squares fitting method, employing a gradient search routine.

Results

Experiments with norvaline concentration varied

Lineweaver-Burk plots at different fixed concentrations of the coenzyme NAD⁺ (between 0.1 and 1.0mM) and varied norvaline concentrations are not linear. In the experiment shown in Fig. 1 the norvaline concentration was varied from 1 to 100mM at a fixed NAD⁺ concentration of 0.5mM. Initial-rate studies made by Engel & Dalziel (1969) with norvaline as the substrate with varied concentration were described as being linear. However,

Fig. 1. Lineweaver-Burk plot for the oxidative deamination of norvaline by glutamate dehydrogenase over a wide norvaline concentration range

The final enzyme concentration was approx. 20 μ g/ml. The NAD⁺ concentration was 0.5mM, and the L-norvaline concentration was varied from 1 to 100mM at pH8.0, in 0.1M-sodium phosphate buffer containing 10 μ M-EDTA at 30°C. The inset shows the standardized residual plot with norvaline concentration varied between 10 and 100mM at 0.5mM-NAD⁺.

the norvaline concentration in those experiments was varied only between 5 and 25mM.

With the concentration range of norvaline used in the inhibition and ϕ -parameter experiments described below (10–100mM) the non-linearity is not easily detected in Lineweaver-Burk plots. However, in all experiments in which norvaline was the substrate with varied concentration, plots of the standardized residuals exhibited a consistent pattern (see inset to Fig. 1), suggesting systematic deviation from the linear reciprocal velocity-[substrate] model used to fit the data points.

Experiments with NAD⁺ concentration varied

The coenzyme concentration was varied between 0.1 and 1.0mM in the inhibition and ϕ -parameter experiments described below. The fixed norvaline concentration ranged between 10 and 100mM. In view of the non-linearity demonstrated above with norvaline as the substrate with varied concentration, the results obtained with NAD⁺ as the substrate with varied concentration are presented as an Eadie (1942) plot, which would detect any

Fig. 2. Eadie plot for the oxidative deamination of norvaline by glutamate dehydrogenase with NAD^+ concentration varied

NAD^+ concentration was varied from 0.1 to 1 mM at a fixed concentration of 50 mM-norvaline. Enzyme concentration was approx. $12 \mu\text{g}/\text{ml}$. Other conditions were as indicated in Fig. 1 legend.

non-linearity more readily. The data are shown in Fig. 2. Residual analysis showed no consistent pattern, suggesting that within the concentration ranges of substrate used and within the precision of estimation of the initial rates these data can be adequately described by a linear model.

Determination of ϕ parameters

As described above, reciprocal velocity-[substrate] plots with coenzyme as the substrate with varied concentration are linear. With norvaline as the substrate with varied concentration (between 10 and 100 mM), the deviation from linearity in the reciprocal velocity-[substrate] plots is small, and a linear reciprocal velocity-[substrate] approximation was made in order to calculate coefficients. Initial rates were obtained at five different concentrations of the varied substrate and at five different con-

Table 1. ϕ parameters for the oxidative deamination of norvaline by glutamate dehydrogenase

The ϕ parameters were estimated as explained in the text and in accordance with Dalziel (1957).

ϕ parameter	Estimated value (+ S.D.)*	Literature value (Engel & Dalziel, 1969)†
ϕ_0	1.48 (± 0.10) s	3.57 s
ϕ_{NAD^+}	0.84 (± 0.02) mM · s	0.96 mM · s
ϕ_{Nva}	76.2 (± 4.5) mM · s	44.6 mM · s
$\phi_{\text{NAD}^+, \text{Nva}}$	50.9 (± 0.87) mM ² · s	35.7 mM ² · s

* At pH 7.0 at 30°C.

† At pH 7.0 at 25°C; values corrected to an active-centre concentration with a molecular weight equivalent to 56 000.

centrations of the fixed substrate. The estimates of the slopes and intercepts from primary reciprocal regressions were regressed to obtain estimates for the ϕ parameters (Dalziel, 1957). Estimates for the ϕ parameters are shown in Table 1.

Inhibition experiments with NAD^+ as the substrate with varied concentration

Inhibition by glutarate. The estimated slopes and intercepts obtained at a fixed concentration of norvaline (50 mM) and varied NAD^+ concentrations at various fixed concentrations of glutarate are listed in Table 2. The intercepts are clearly different in the presence and in the absence of the inhibitor. The 95%-confidence intervals about the values for the slopes overlap, indicating that there is no statistically significant difference in the values of the slopes in the presence and in the absence of the inhibitor glutarate. Glutarate is strictly an uncompetitive inhibitor of NAD^+ . These data indicate that glutarate does not detectably combine with the same form of the enzyme that binds the coenzyme.

Inhibition by 2-oxoglutarate, valerate and 2-oxovalerate. Table 3 shows results obtained with NAD^+ as the substrate with varied concentration at a fixed norvaline concentration of 50 mM. Experiments were performed at several concentrations of 2-oxoglutarate, valerate or 2-oxovalerate. In all cases the inhibition was non-competitive.

Inhibition experiments with norvaline as the substrate with varied concentration

The reciprocal velocity-[substrate] plots with varied norvaline concentration at a fixed concentration of NAD^+ (0.5 mM) and different fixed concentrations of the inhibitors are shown in Figs. 3(a)–3(d). Linear-regression analysis of these data suggests that the inhibition patterns vary from competitive at low concentrations of inhibitor to non-competitive at higher concentrations of inhibi-

Table 2. *Inhibition by glutarate of the oxidative deamination of norvaline by glutamate dehydrogenase, with NAD⁺ concentration varied: regression analysis and statistics*

The NAD⁺ concentration was varied between 0.1 and 1.0 mM. The norvaline concentration was fixed at 50 mM. In the inhibition experiments the final enzyme concentration ranged from 7 to 12 μ g/ml. All experiments were performed at pH 8.0 at 30°C in 0.1 M-sodium phosphate buffer containing 10 μ M-EDTA. The 95%-confidence intervals were obtained as described in the Materials and methods section. Regressions are based on unweighted data. The standard deviations (s.d.) indicated reflect the imprecision obtained from replication within a single experiment. The intercepts in these experiments in the absence of inhibitor are expected to be the same. These experiments were performed over several months with different enzyme preparations reagents etc. The observed intercept differences in the absence of inhibitor (Table 2, 3.12 s; Table 3, 2.45 s; Table 5, 3.58 s) are due to batch-to-batch differences in the specific activity of the enzyme, differences in reagent composition etc. over the period of time during which these experiments were performed.

[Glutarate] (mM)	Slope (mM · s)	95%- confidence interval (mM · s)	Intercept (+s.d.) (s)	Degrees of freedom
0	1.36	0.02	3.12 (± 0.05)	24
0.125	1.44	0.06	5.30 (± 0.12)	24
0.25	1.44	0.06	7.48 (± 0.16)	25
0.50	1.42	0.08	11.60 (± 0.22)	25

tor. As already noted above, the reciprocal velocity-[substrate] plots with varied norvaline concentration are not strictly linear, and imprecision in the fit could explain the lack of convergence. Other potential explanations are elaborated upon in the Discussion section.

Discussion

As mentioned in the Results section, over a limited norvaline concentration range (10–100 mM) the initial-velocity data may be adequately fitted to a linear double-reciprocal velocity-[substrate] model. Determination of the ϕ parameters (Dalziel, 1957) and analysis of the results was based on such a linear approximation, justification of which is discussed below. Various relations exist among the ϕ coefficients, depending on the particular kinetic mechanism that is followed (Dalziel, 1957). These relations must be obeyed within experimental error if a given mechanism is followed, but of course do not constitute proof for a given mechanism.

Studies have shown that the K_m for coenzyme is greater with the monocarboxylic substrate than with glutamate as substrate (Engel & Dalziel, 1969; Rife & Cleland, 1980; Bailey *et al.*, 1982). It is known from binding studies that glutamate and the dicarboxylic analogue glutarate bind to the enzyme synergistically with coenzyme (Dalziel & Egan, 1972; Koberstein & Sund, 1973). The relationship $\phi_0 = \phi_{\text{NAD}^+} \cdot \phi_{\text{Nva}} / \phi_{\text{NAD}^+ \cdot \text{Nva}}$ is expected to be observed for a rapid-equilibrium random-order bisubstrate

Table 3. *Inhibition by 2-oxoglutarate, 2-oxovalerate and valerate of the oxidative deamination of norvaline by glutamate dehydrogenase, with NAD⁺ concentration varied: regression analysis and statistics*

Details are similar to those indicated in Table 2 legend.

[2-Oxoglutarate] (mM)	Slope (mM · s)	95%-confidence interval (mM · s)	Intercept ($\pm$ s.d.) (s)	Degrees of freedom
0	1.59	0.02	2.45 (± 0.06)	25
0.25	1.71	0.02	5.03 (± 0.06)	25
0.50	1.95	0.06	6.90 (± 0.16)	25
[2-Oxovalerate] (mM)	Slope ($\pm$ s.d.) (mM · s)	Intercept (s)	95%-confidence interval (s)	Degrees of freedom
0	1.94 (± 0.03)	3.58	0.2	22
5	2.46 (± 0.04)	4.27	0.3	21
10	3.20 (± 0.04)	4.96	0.2	21
15	4.13 (± 0.12)	5.70	0.7	24
[Valerate] (mM)	Slope ($\pm$ s.d.) (mM · s/ μ M-NADH)*	Intercept (s/ μ M-NADH)	95%-confidence interval (s/ μ M-NADH)	Degrees of freedom
0	80.2 (± 1.2)	14.6	1.3	19
20	89.6 (± 1.5)	20.4	1.7	19
40	105.5 (± 3.2)	25.8	3.8	16
80	198.5 (± 3.0)	37.0	3.4	19

* In this experiment the enzyme concentration was unknown. These values then are enzyme-concentration-dependent.

Fig. 3. Lineweaver-Burk plots for the oxidative deamination of norvaline by glutamate dehydrogenase: inhibition plots with norvaline concentration varied

The lines represent non-weighted least-squares fits to the data points assuming a linear double-reciprocal velocity-[substrate] model. In all the inhibition experiments norvaline concentration was varied between 10 and 100 mM and the NAD^+ concentration was fixed at 0.5 mM. The enzyme concentration in different experiments ranged between 7 and 12 $\mu\text{g}/\text{ml}$. Other conditions were as indicated in Fig. 1 legend. (a) Inhibition by glutarate. Curve 1, no inhibitor; curve 2, 0.25 mM-glutarate; curve 3, 0.5 mM-glutarate. (b) Inhibition by 2-oxoglutarate. Curve 1, no inhibitor; curve 2, 0.25 mM-2-oxoglutarate; curve 3, 0.5 mM-2-oxoglutarate. (c) Inhibition by valerate. Curve 1, no inhibitor; curve 2, 2 mM-valerate; curve 3, 4 mM-valerate; curve 4, 8 mM-valerate. (d) Inhibition by 2-oxovalerate. Curve 1, no inhibitor; curve 2, 5 mM-2-oxovalerate; curve 3, 10 mM-2-oxovalerate; curve 4, 20 mM-2-oxovalerate.

reaction in which the substrates bind independently of each other. The calculated value ($\phi_{\text{NAD}^+} \cdot \phi_{\text{Nva}} / \phi_{\text{NAD}^+, \text{Nva}} = 1.25$) is, within experimental error, equal to the estimated value for ϕ_0 (1.48). This suggests that a rapid-equilibrium random-order model is followed with norvaline and NAD^+ . The values for ϕ parameters obtained here are compared with the values obtained by Engel & Dalziel (1969) in Table 1. These values are in reasonable agreement, considering the temperature differences between the experiments. However, the value of ϕ_0 (3.57) appears to be significantly different from the calculated value (1.20) obtained by using the above expression and the values reported by Engel & Dalziel (1969). The dissociation constant for binding of oxidized coenzyme to the enzyme or enzyme-norvaline binary complex is then given by $\phi_{\text{NAD}^+} / \phi_0$. This is calculated to be 0.52 mM, which compares favourably with an estimate obtained from a direct binding study (Dalziel & Egan, 1972), which was calculated to be 0.47 mM at pH 7.0 at 23°C.

The inhibition patterns obtained with varied coenzyme concentration at a fixed concentration of norvaline are also consistent with a random-order kinetic model for norvaline and NAD^+ . A summary of the various dissociation constants calculated from

the inhibition experiments is given in Table 4. These dissociation constants are calculated assuming a rapid-equilibrium random-order kinetic model.

The calculated dissociation constant for 2-oxoglutarate dissociation from the enzyme-oxoglutarate binary complex, 1.5 mM, is in reasonable agreement with 2.8 mM obtained by Cross *et al.* (1972) with a spectrophotometric perturbation assay at pH 7.6. Assuming a rapid-equilibrium random-order mechanism for the reverse reaction, values of 1.49 mM (pH 7.0) and 0.79 mM (pH 8.0) could be calculated from previously published ϕ parameters (Engel & Dalziel, 1970). The estimated dissociation constant for 2-oxoglutarate dissociation from the enzyme- NAD^+ -oxoglutarate ternary complex is 0.35 mM, which is in reasonable agreement with 0.25 mM estimated from a product-inhibition study with glutamate as the substrate with fixed concentration at pH 7.0 at 25°C (Engel & Chen, 1975).

The calculated dissociation constants for 2-oxovalerate binding to form a binary enzyme-keto acid complex (11 mM) and ternary enzyme-coenzyme-keto acid complex (7 mM) are quite similar, as expected if the monocarboxylic keto acid and coenzyme cannot synergistically bind to the enzyme. With the exception of the experiment involving inhibition by valerate, ϕ parameters calculated from

Table 4. Dissociation constants calculated from ϕ parameters and from inhibition experiments, assuming a rapid-equilibrium random-order kinetic model for the oxidative deamination of norvaline by glutamate dehydrogenase, compared with independent estimates

The first subscript in the symbol for the dissociation constant represents the dissociating species. The additional subscripts in parentheses represent other species that are also bound to the enzyme. Abbreviations: OGI, 2-oxoglutarate; OVa, oxo-valerate; Nva, norvaline; Va, valeric acid; Gl, glutarate. Key to references: ^aDalziel & Egan (1972); ^bCross *et al.* (1972); ^cEngel & Dalziel (1970); ^dEngel & Chen (1975).

Constant	Estimate from ϕ parameters or inhibition (mM)	Independent estimates (mM)
$K_{\text{NAD}^+} = K_{\text{NAD}^+(\text{Nva})}$	0.52	0.47 (direct) ^a
$K_{\text{Nva}} = K_{\text{Nva}(\text{NAD}^+)}$	51.5	
K_{OGI}	1.5	2.8 (direct) ^b 1.49 (kinetic, pH 7.0) ^c 0.79 (kinetic, pH 8.0) ^c 0.25 (kinetic) ^d
$K_{\text{OGI}(\text{NAD}^+)}$	0.35	
K_{OVa}	11	
$K_{\text{OVa}(\text{NAD}^+)}$	7	
K_{Va}	25*	
$K_{\text{Va}(\text{NAD}^+)}$	3.2*	
$K_{\text{Gl}(\text{NAD}^+)}$	0.09	

* As explained in the text, estimates of ϕ_0 and ϕ_{NAD^+} can be obtained from the inhibition experiments and compared with the values obtained from the independent experiments performed to determine the values of the ϕ coefficients. In all cases except in the inhibition experiment with valerate these values were in excellent agreement.

the inhibition data, assuming a rapid-equilibrium random-order mechanism, agree within experimental error with the directly determined values. However, in the experiment involving inhibition by valerate this is not the case, suggesting that the dissociation constants for valerate in Table 4 must be considered as questionable.

It should be noted that the inhibition patterns are also consistent with a compulsory-order model where the monocarboxylic compounds can bind to the free enzyme to form binary enzyme-inhibitor complexes and where norvaline can bind to the free enzyme to form a dead-end complex. Since with norvaline as substrate no subunit interactions have been observed, the non-Michaelis-Menten kinetics with norvaline rule out a strict compulsory-order model under these assay conditions.

The strict uncompetitive inhibition with glutarate (Table 2) shows that the dicarboxylic analogue does not detectably combine with the free enzyme, but binds tightly to the enzyme-coenzyme binary complex with a calculated dissociation constant of 90 μM . It can be calculated, assuming the rapid-

equilibrium random-order model and assuming a standard error of approx. 0.04 mM·s in the determination of the value of the slope from the double-reciprocal regression, that the dissociation constant for binding of glutarate is greater than 10 mM (if glutarate binds at all to the free enzyme), indicating at least a 100-fold increase in the affinity of the dicarboxylic analogue for the enzyme-coenzyme complex. Additional inhibition experiments at higher concentrations of the inhibitor are required in order to determine if binding to the free enzyme can be detected. Prough *et al.* (1972), using u.v. difference spectrophotometry, observed evidence for an enzyme-glutarate binary complex and estimated a dissociation constant of approx. 38 mM at pH 7.6 at 25°C.

Studies of the effects of isotope substitution on the kinetic parameters with the use of deuterated norvaline and glutamate (Rife & Cleland, 1980) were consistent with a random-order mechanism with norvaline as a substrate and NADP⁺ as coenzyme (but not rapid equilibrium). Because a significant isotope effect could not be observed with glutamate, no conclusion concerning the order of addition could be made with this substrate. Transient-state kinetic studies (Colen *et al.*, 1972) support a random-order binding of glutamate and coenzyme. Studies of isotope exchange at equilibrium support a random-order model with glutamate at pH 8.0 (Silverstein & Sulebele, 1973); however, similar studies at pH 8.8 were in agreement with a compulsory-order model (Silverstein, 1974).

After an extensive kinetic investigation with both norvaline and glutamate as substrates, Engel & Dalziel (1969) concluded that the reaction was rapid-equilibrium random-order with both substrates or compulsory-ordered with one substrate but not both. The data in the present paper indicate that to a first approximation the kinetic mechanism with norvaline may be adequately described as rapid-equilibrium random-order.

Steady-state random-order model with norvaline

The non-linear double-reciprocal velocity-[substrate] profile (Fig. 1) with norvaline as the substrate with varied concentration suggests that higher-order norvaline concentration terms should be included in the initial-rate kinetic equation. The steady-state solution for a bisubstrate random-order reaction yields a velocity equation containing terms that are second-order in both substrate concentrations (Dalziel, 1958; King, 1956). The velocity equation with one substrate concentration fixed has the following form:

$$\frac{v}{[E]} = \frac{\alpha_1[S] + \alpha_2[S]^2}{\beta_0 + \beta_1[S] + \beta_2[S]^2} \quad (1)$$

Fig. 4. Velocity data for the oxidative deamination of norvaline by glutamate dehydrogenase with norvaline concentration varied fitted to first-order and second-order velocity models

Conditions were as indicated in Fig. 1 legend. Curve A (—), data fitted to:

$$\frac{v}{[E]} = \frac{\alpha'_1[N] + \alpha'_2[N]^2}{1 + \beta'_1[N] + \beta'_2[N]^2}$$

Curve B (.....), data fitted to:

$$\frac{v}{[E]} = \frac{\alpha''}{\beta'' + [N]}$$

where [N] represents norvaline concentration.

The symbols have their usual meanings and the coefficients α_i and β_i are complex functions of the rate constants and fixed substrate. In the most general case these coefficients include the rate constants for the reverse reaction (Dalziel, 1958). The experimental results shown in Fig. 1 were actually fitted to eqn. (2), where all the coefficients of eqn. (1) have been divided by β_0 :

$$\frac{v}{[E]} = \frac{\alpha'_1[Nva] + \alpha'_2[Nva]^2}{1 + \beta'_1[Nva] + \beta'_2[Nva]^2} \quad (2)$$

The data were also fitted to the following equation:

$$\frac{v}{[E]} = \frac{\alpha''[Nva]}{\beta'' + [Nva]} \quad (3)$$

which is expected to be followed if a hyperbolic velocity-[substrate] profile is obtained. The fits are

Table 5. Coefficients defined for an irreversible steady-state random-order model for the oxidative deamination of norvaline by glutamate dehydrogenase with norvaline as the substrate with varied concentration

See the text for details.

$$\begin{aligned} \alpha_1''' &= k_{+5}(k_{-3}k_{+1}k_{+2} + k_{-1}k_{+3}k_{+4})[N] + (k_{+5}k_{+1}k_{+2}k_{+4})[N]^2 \\ \alpha_2''' &= (k_{+5}k_{+2}k_{+3}k_{+4})[N] \\ \beta_0''' &= (k_{-2}k_{-1}k_{-3} + k_{-1}k_{-3}k_{-4} + k_{-1}k_{-3}k_{+5}) + \\ &\quad (k_{-2}k_{+1}k_{+4} + k_{+1}k_{+4}k_{+5})[N]^2 + \\ &\quad (k_{-1}k_{-2}k_{+4} + k_{-1}k_{+4}k_{+5} + k_{-2}k_{-3}k_{+1} + \\ &\quad k_{-3}k_{-4}k_{+1} + k_{-3}k_{+1}k_{+5})[N] \\ \beta_1''' &= (k_{+1}k_{+2}k_{+4})[N]^2 + (k_{-3}k_{-4}k_{+2} + \\ &\quad k_{-3}k_{+2}k_{+5} + k_{-1}k_{-2}k_{+3} + \\ &\quad k_{-1}k_{-4}k_{+3} + k_{-1}k_{+3}k_{+5}) + \\ &\quad (k_{+2}k_{+4}k_{+5} + k_{-2}k_{+3}k_{+4} + k_{-4}k_{+1}k_{+2} + \\ &\quad k_{-3}k_{+1}k_{+2} + k_{-1}k_{+3}k_{+4})[N] \\ \beta_2''' &= (k_{-4}k_{+2}k_{+3} + k_{+2}k_{+3}k_{+5}) + (k_{+2}k_{+3}k_{+4})[N] \end{aligned}$$

shown in Fig. 4, where clearly eqn. (2) more adequately describes the data points.

It has been shown that a steady-state random-order mechanism can give rise to linear double-reciprocal velocity-[substrate] plots or double-reciprocal velocity-[substrate] plots that exhibit apparent positive or negative co-operativity or substrate inhibition (Dalziel, 1958; Pettersson, 1969).

The simplest steady-state random-order model in the absence of added products that gives rise to non-linear double-reciprocal velocity-[substrate] plots assumes that the concentrations of enzyme-product complexes are negligible and that the reaction can be treated as essentially irreversible.

These assumptions lead to the following kinetic model for norvaline (N) and NAD⁺ (O):

At a fixed concentration of norvaline the velocity equation as a function of varied coenzyme is:

$$\frac{v}{[E]} = \frac{\alpha_1'''[O] + \alpha_2'''[O]^2}{\beta_0''' + \beta_1'''[O] + \beta_2'''[O]^2} \quad (4)$$

The coefficients are defined in Table 5. The reciprocal form of eqn. (4) is of course non-linear in [O]. However, as shown in the present work over a limited coenzyme concentration range, and as shown previously over a much wider coenzyme concentration range (Engel & Dalziel, 1969), the reciprocal velocity-[substrate] plots with varied coenzyme concentration are linear. From the definitions of the coefficients in Table 5, it may be seen

that α_2''' and β_2''' contain a first-order [norvaline] term whereas the coefficients α_1''' , β_0''' and β_1''' have a squared [norvaline] term in addition to a first-order term. In addition, α_2''' and β_2''' are multiplied by a squared [coenzyme] term.

In the kinetic experiments described in the present paper the concentration of norvaline was always at least 10-fold greater than the coenzyme concentration. These considerations suggest that $\alpha_1'''[O] > \alpha_2'''[O]^2$ and $\beta_0''' + \beta_1'''[O] > \beta_2'''[O]^2$. This reduces eqn. (4) to:

$$\frac{v}{[E]} = \frac{\alpha_1'''[O]}{\beta_0''' + \beta_1'''[O]} \quad (5)$$

The reciprocal form of eqn. (5) is of course linear in coenzyme concentration.

If the norvaline concentration becomes sufficiently high, the squared [norvaline] terms are dominant and the double-reciprocal velocity-[substrate] equation approximates to:

$$\frac{[E]}{v} \sim \frac{k_{-2}}{k_{+2}k_{+5}} + \frac{1}{k_{+2}} + \frac{1}{[O]} + \frac{1}{k_{+5}} \quad (6)$$

The terms on the right-hand side of eqn. (6) may be approximated from the ϕ -parameter experiment with varied coenzyme concentration, where:

$$\phi_0 = \frac{1}{k_{+5}} \text{ and } \phi_{\text{NAD}^+} = \frac{k_{-2}}{k_{+2}k_{+5}} + \frac{1}{k_{+2}}$$

The value for the catalytic rate constant $k_{+5} = 0.68 \text{ s}^{-1}$ is of course the same as that obtained assuming rapid equilibrium. The estimate for k_{+5} and the value for ϕ_{NAD^+} permits the following relationship to be obtained:

$$k_{-2} = (0.57 k_{+2} - 0.68) \text{ s}^{-1}$$

If the assumption is made that the second-order rate constant k_{+2} is approx. $10^3 \text{ mm}^{-1} \cdot \text{s}^{-1}$, then k_{-2} is approx. 570 s^{-1} .

This would suggest that coenzyme dissociates from the ternary complex about 800 times faster than it is converted into product, supporting the rapid-equilibrium assumption.

An analysis by Pettersson (1969) showed that if a steady-state bisubstrate random-order model is in fact obeyed and reciprocal plots are concave down, then k_{+2} must be greater than k_{+3} . This suggests that binding of the monocarboxylic substrate increases the rate of coenzyme binding to the enzyme, in apparent contradiction to the conclusion drawn when the rapid-equilibrium assumption is made. However, firstly the rapid-equilibrium assumption is an approximation and secondly the magnitude of the difference between k_{+2} and k_{+3} has to be large enough to generate a negative second derivative in the velocity equation but does not have to be

dramatically large. Also, an increased 'off' velocity constant for the coenzyme from the enzyme-norvaline-coenzyme complex could be compensated for by an increased 'on' velocity constant, so that synergism in binding is not detected. In general, when a double-reciprocal velocity-[substrate] plot is shown to be non-linear over a given range of a substrate concentration, then ϕ parameters calculated over apparently linear regions cannot be validly interpreted mechanistically. The steady-state treatment of the data in the present paper suggests that the step or steps involved in reduction of the coenzyme are primarily rate-limiting with norvaline as substrate. It can be shown that at very low substrate concentrations, where second-order concentration terms may be regarded as insignificant if $k_{+5} \ll k_{-3}$ and k_{-2} , then the rapid-equilibrium analysis is an adequate description of the kinetics, and experimentally determined ϕ parameters would provide useful estimates of equilibrium constants. Considering the concentration ranges used in these experiments, the use of ϕ parameters to determine equilibrium constants can only be regarded as first approximations to estimates that would be valid at lower substrate concentrations. These approximations may be considered as reasonable, considering the agreement obtained in two cases with equilibrium constants calculated from ϕ parameters and values obtained via direct binding studies (Table 4).

The apparent lack of convergence of the reciprocal velocity-[substrate] plots with varied norvaline concentration in the presence of inhibitors may in fact be mechanistically significant.

The most apparent lack of convergence in the double-reciprocal inhibition plots with varied norvaline concentration occurs with 2-oxovalerate as inhibitor (Fig. 3*d*). However, within the same inhibition ranges intercept differences appear to be similar for all inhibitors.

The addition of the product and pseudo-product 2-oxovalerate and 2-oxoglutarate would be expected to compete directly with norvaline for the active site. If enzyme-product complexes are negligible, convergence of the double-reciprocal plots is expected as the concentration of norvaline becomes saturating. If in fact enzyme-product complexes are not negligible, the presence of a product in the reaction could result in the accumulation of an enzyme-reduced coenzyme-keto acid product complex, introducing terms into the rate equation such that convergence is not obtained. This would require a product-release step(s) following chemical conversion of the substrates to be in part rate-limiting. This does not appear to be a likely possibility with norvaline as substrate. Another possible model involves binding of norvaline and inhibitor simultaneously to the active site. Presumably an addi-

tional binding site for the δ -carboxy group exists that may bind the inhibitor, thereby preventing catalysis. A more likely model suggests that norvaline has a much higher dissociation constant than has 2-oxovalerate (or the other inhibitors) and that sufficient concentrations of norvaline were not obtained. This means that in the presence of 15 mM 2-oxovalerate, the highest concentration used in the inhibition experiment, the norvaline concentration would have to exceed considerably the maximum (100 mM) used in these experiments. Internally consistent with this possibility is estimation of the dissociation constants obtained, namely 52 mM for norvaline and 7 and 11 mM for 2-oxovalerate binding to the free enzyme and enzyme-coenzyme complex respectively.

Despite the numerous kinetic investigations, the kinetic mechanism with glutamate as substrate has not been unequivocally established. Below pH 8.8 the published evidence supports a random-order addition of glutamate and coenzyme to form kinetically competent intermediates. Although, as described in the present paper, norvaline and NAD⁺ appear to obey to a first approximation a rapid-equilibrium random-order mechanism, it is not clear that a similar argument can be made when glutamate is the substrate. Transient-state kinetic studies suggest that product release is rate-limiting in the reaction with glutamate as substrate (Iwatsubo & Pantaloni, 1977; di Franco & Iwatsubo, 1972; di Franco, 1974). A deuterium isotope effect was observed on the burst but not on the steady-state portion of a kinetic study by Fisher *et al.* (1970). These data do not support a simple rapid-equilibrium model with glutamate as substrate, where it is assumed that catalysis is the rate-limiting step in the reaction. Clearly further steady-state and transient-state kinetic studies are necessary to elucidate further the mechanism with glutamate.

In summary, the data presented above support to a first approximation a rapid-equilibrium random-order model for the oxidative deamination of norvaline. However, a steady-state random-order model appears to reflect the kinetic data more accurately.

A major difference between norvaline and glutamate is the inability of the monocarboxylic substrate

to bind synergistically with co-enzyme to the enzyme.

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