Biochem. J. (1973) **131**, 97–105
Printed in Great Britain

The Significance of Abrupt Transitions in Lineweaver–Burk Plots with Particular Reference to Glutamate Dehydrogenase

NEGATIVE AND POSITIVE CO-OPERATIVITY IN CATALYTIC RATE CONSTANTS

By PAUL C. ENGEL and WILLIAM FERDINAND
Department of Biochemistry, University of Sheffield, Western Bank, Sheffield S10 2TN, U.K.

(*Received* 18 *July* 1972)

1. Lineweaver–Burk plots for glutamate dehydrogenase, glucose 6-phosphate dehydrogenase and several other enzymes show one or more abrupt transitions between apparently linear sections. These transitions correspond to abrupt increases in the apparent K_m and $V_{max.}$ with increasing concentration of the varied substrate. 2. The generalized reciprocal initial-rate equation for a multi-site enzyme requires several restrictions to be put on it in order to generate such plots. These mathematical conditions are explored. 3. It is shown that the effective omission of a term in the denominator of the reciprocal initial-rate equation represents a minimal requirement for generation of abrupt transitions. This corresponds in physical terms to negative co-operativity followed by positive co-operativity affecting the catalytic rate constant for the reaction. 4. Previous models for glutamate dehydrogenase cannot adequately account for the results. On the other hand, the model based on both negative and positive co-operativity gives a good fit to the experimental points. 5. The conclusions are discussed in relation to current knowledge of the structure and mechanism of glutamate dehydrogenase.

One of the original arguments advanced in favour of the Monod–Wyman–Changeux model for allosteric proteins was the fact that all the well-documented cases of homotropic interaction appeared to be of the positive type, binding at each site assisting binding at the next (Monod *et al.*, 1965). Koshland *et al.* (1966) pointed out that their model based on interacting sites allowed, in theory at least, the opposite type of interaction in which binding at each site hinders binding at the next. Since then 'negative co-operativity' has been invoked to explain results obtained with several enzymes, notably NAD^+-binding results for glyceraldehyde 3-phosphate dehydrogenase from rabbit muscle (de Vijlder & Slater, 1968; Conway & Koshland, 1968) and Lineweaver–Burk plots of e_0/v against $1/[NAD(P)^+]$ for ox liver glutamate dehydrogenase (Dalziel & Engel, 1968; Engel & Dalziel, 1969), where e_0 is the total enzyme site concentration and v is the initial rate.

Negative co-operativity leads to Lineweaver–Burk plots that are concave downward, i.e. apparent substrate activation. Dalziel & Engel (1968) therefore sought the condition for

$$\frac{d^2(e_0/v)}{d(1/s)^2}$$

to be negative and used this condition to discriminate between various models for a two-site enzyme. No attempt, however, was made to fit the kinetic results for glutamate dehydrogenase. Interpretation of these results suggested that, if there is a random pathway of substrate addition to the enzyme, the negative interactions should be found not in the binding of $NAD(P)^+$ to form a binary complex but in its binding to enzyme–glutamate to form the ternary complex (Engel & Dalziel, 1969). It has now been found (Dalziel & Egan, 1972) that binding of $NAD(P)^+$ to free enzyme is non-co-operative, whereas binding in the presence of saturating concentrations of the inhibitory substrate analogue, glutarate, shows strong negative co-operativity.

The Lineweaver–Burk plots of e_0/v against $1/[NAD(P)^+]$ for glutamate dehydrogenase are, however, not merely concave downwards. Distinct pseudo-linear sections can be discerned, separated by fairly abrupt transitions (Dalziel & Engel, 1968; Engel & Dalziel, 1969). With NAD^+ as coenzyme and at pH 7, three such transitions were noted within a 1000-fold range of coenzyme concentration.

Abrupt transitions of this kind are not a unique feature of ox liver glutamate dehydrogenase. Similar transitions have been reported for glutamate dehydrogenase from pig heart (Godinot & Gautheron, 1971) and yeast (Fourcade & Venard, 1971), and also for glucose 6-phosphate dehydrogenase from human erythrocytes (Pinto *et al.*, 1966), yeast (Anderson *et al.*, 1968) and sweet potato (Muto & Uritani,

1970). Single transitions only were seen in most of these cases, but Muto & Uritani (1970) found two abrupt transitions in the plot of e_0/v against the reciprocal of the glucose 6-phosphate concentration.

It was thought initially (Dalziel & Engel, 1968) that such observations could be explained solely on the basis of very strong negative binding interactions, a view questioned by Schwert (1970). The mathematical requirements for such behaviour are re-examined in the present paper and shown to be more restrictive than originally envisaged. On the basis of the criteria developed here an equation is presented which fits the experimental results. The possible physical implications of the mathematical restrictions are considered in the Discussion section.

Theory

The essential features of the Lineweaver–Burk plots in question as shown in Fig. 1 are as follows. (*a*) Several apparently linear sections are separated by clear abrupt transitions. (*b*) With increasing concentration of the varied substrate the successive linear sections show at each step an increase in slope and a decrease in intercept, both approximately two-fold. Thus at each transition both '$V_{max.}$' and 'K_m' appear to increase.

Any kinetic model for a multi-site enzyme will give rise to an equation of the form:

$$\frac{e_0}{v} = \frac{N_0 + N_1 s + N_2 s^2 \cdots N_q s^q}{D_1 s + D_2 s^2 \cdots D_q s^q} \qquad (1)$$

where N_0–N_q and D_1–D_q are numerical constants and s is the substrate concentration.

The physical significance of the coefficients N_0 to N_q and D_1 to D_q depends on the mechanism. The value of s determines which terms in the numerator and denominator of eqn. (1) are dominant. At very low values of s, for instance, the equation reduces to the linear form:

$$\frac{e_0}{v} = \frac{N_0 + N_1 s}{D_1 s} \qquad (2)$$

Other terms successively assume dominance over ranges of s determined by the relative values of the coefficients in eqn. (1). Thus, in theory, with suitable values of the coefficients eqn. (1) may generate further linear regions in addition to the low-concentration region effectively governed by eqn. (2) e.g.:

$$\frac{e_0}{v} = \frac{N_3 s^3 + N_4 s^4}{D_4 s^4} = \frac{N_3 + N_4 s}{D_4 s}$$

In general, if eqn. (1) is to give rise to pseudo-linear regions separated by abrupt transitions the following conditions must be satisfied.

Rule 1: For a given transition in the plot of e_0/v against $1/s$ the corresponding transitions in the denominator and numerator of eqn. (1) must coincide fairly closely.

Rule 2: Where there are multiple transitions these must not overlap.

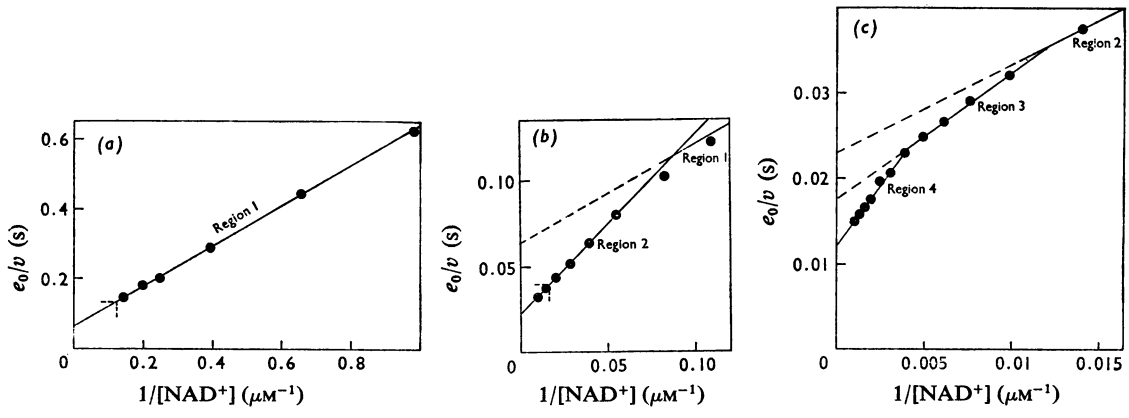

Fig. 1. *Lineweaver–Burk plot of initial-rate results obtained with glutamate dehydrogenase*

The data are taken from Engel & Dalziel (1969, 1970). The glutamate concentration was 50mM and the buffer sodium phosphate, pH7.0, $I = 0.25$. Fig. 1(*a*) shows measurements made with low NAD^+ concentrations (1–7μM). The concentration region (8–100μM) beyond the dashed bracket is shown on a larger scale in (*b*), with the extrapolated line from (*a*) also drawn in. Measurements made at high concentrations (60–1000μM), beyond the dashed bracket in (*b*), are shown on a still larger scale in (*c*), with the extrapolated Region 2 line from (*b*) also drawn in.

Rule 3: The narrow concentration range over which a transition occurs must include the value of s at which the extrapolated linear portions intersect.

Let us consider first the possibility (Engel, 1970) that each of the terms assumes overall dominance at some value of s and that, with appropriate coefficients, eqn. (1) can give rise to a series of pseudo-linear sections approximately fitting the equations:

$$\frac{e_0}{v} = \frac{N_0 + N_1 s}{D_1 s} \qquad \text{Region 1}$$

$$\frac{e_0}{v} = \frac{N_1 s + N_2 s^2}{D_2 s^2} = \frac{N_1 + N_2 s}{D_2 s} \qquad \text{Region 2}$$

$$\frac{e_0}{v} = \frac{N_2 s^2 + N_3 s^3}{D_3 s^3} = \frac{N_2 + N_3 s}{D_3 s} \qquad \text{Region 3}$$

$$\frac{e_0}{v} = \frac{N_{q-1} s^{q-1} + N_q s^q}{D_q s^q} = \frac{N_{q-1} + N_q s}{D_q s}$$

Region q

In this situation, designated Case 1, neighbouring pseudo-linear sections would always have a numerator term in common: for example $N_1 s$ is a significant term in both Region 1 and Region 2. The transition between these regions occurs as $N_2 s^2$ becomes large compared with N_0 and $D_2 s^2$ becomes large relative to $D_1 s$.

For the numerator transition:

$$N_0 = N_2 s^2 \quad \therefore s^2 = N_0/N_2 \tag{3}$$

For the denominator transition:

$$D_1 s = D_2 s^2 \quad \therefore s = D_1/D_2 \tag{4}$$

Therefore, applying Rule 1:

$$(D_1/D_2)^2 \doteq N_0/N_2 \tag{5}$$

Also, applying Rule 2:

$$D_1/D_2 \gg D_2/D_3 \quad \therefore D_1 D_3 \gg D_2^2 \tag{6}$$

and

$$N_0/N_2 \gg N_1 N_3 \tag{7}$$

The two lines obeying the equations:

$$y = m_1\left(\frac{1}{s}\right) + c_1$$

and

$$y = m_2\left(\frac{1}{s}\right) + c_2$$

intersect when:

$$\frac{1}{s} = \frac{c_1 - c_2}{m_2 - m_1} \tag{8}$$

where m_1, m_2, c_1, and c_2 are numerical constants.

Thus in the present case the lines intersect when:

$$\frac{1}{s} = \frac{\frac{N_1}{D_1} - \frac{N_2}{D_2}}{\frac{N_1}{D_2} - \frac{N_0}{D_1}} = \frac{N_1 D_2 - N_2 D_1}{N_1 D_1 - N_0 D_2} \tag{9}$$

If this point of intersection is to coincide approximately with the denominator transition, then, from eqn. (4):

$$\frac{D_1}{D_2} \doteq \frac{N_1 D_1 - N_0 D_2}{N_1 D_2 - N_2 D_1}$$

$$\therefore N_2 D_1^2 \doteq N_0 D_2^2$$

unless $N_1 D_1 \gg N_0 D_2$ and $N_1 D_2 \gg N_2 D_1$. This equation is equivalent to eqn. (5). Thus for Case 1, the condition for denominator and numerator transitions to coincide also ensures that these transitions occur at the intersection of the theoretical straight lines for the two regions.

In Case 1 the numerator transition occurs fairly rapidly as s is raised: $N_2 s^2$ increases from 0.1 N_0 to 10 N_0 over a tenfold range of s. The denominator transition, however, involves terms differing by only one power of s. For $D_2 s^2$ to increase from 0.1 $D_1 s$ to 10 $D_1 s$ requires a 100-fold increase in s, and this transition therefore occurs much more gradually. It is still possible for a Case 1 equation to generate pseudo-linear sections in a Lineweaver–Burk plot, but the obviously curved transitional portions must cover a considerable concentration range, and the breaks cannot be as sharp as those that have been experimentally observed. Moreover, successive transitions in Case 1 must be very widely separated to be distinct. Accordingly an attempt to fit the initial-rate results for glutamate dehydrogenase by a Case 1 equation failed, yielding a smooth curve.

Case 1 has also been analysed by the use of differential calculus. If the plot of e_0/v against $1/s$ shows an abrupt transition between two pseudo-linear regions, then the plot of

$$\frac{\mathrm{d}\frac{e_0}{v}}{\mathrm{d}\left(\frac{1}{s}\right)}$$

must show an abrupt fall from one plateau value to another. The second differential must pass through a corresponding minimum and the third differential must pass through zero at least once. It can be shown, however, that if, taking the simplest equation for a single Case 1 transition:

$$\frac{e_0}{v} = \frac{N_0 + N_1 s + N_2 s^2}{D_1 s + D_2 s^2}$$

then the third differential is given by:

$$\frac{d^3\left(\frac{e_0}{v}\right)}{d\left(\frac{1}{s}\right)^3} = \frac{6D_1(N_1 D_1 D_2 - N_0^2 D_2 - N_2 D_1^2)}{\left(\frac{D_1}{s}+N_2\right)^4}$$

This expression cannot pass through zero. No abrupt transition can ever be generated by a second-degree equation. For an equation of higher degree the numerator of the third differential is a complex function of s which may in principle pass through zero, since both positive and negative terms are present. The conditions, however, for this to occur are not readily obtainable.

Since Case 1 cannot produce abrupt transitions one has to consider what extra restrictions must be placed on the coefficients of eqn. (1) for it to do so. It is clear that the major shortcoming of Case 1 lies in the fact that the denominator transition occurs over too wide a range of s. The range can be decreased if the two terms involved differ by more than one power of s.

If we consider as a general case two adjacent pseudo-linear sections described approximately within the appropriate range of values of s by the equations:

$$\frac{e_0}{v} = m_1\left(\frac{1}{s}\right) + c_1$$

and

$$\frac{e_0}{v} = m_2\left(\frac{1}{s}\right) + c_2$$

then the overall equation must be of the form:

$$\frac{e_0}{v} = \frac{\cdots m_1 s^{p-1} + c_1 s^p + am_2 s^{q-1} + ac_2 s^q \cdots}{\cdots s^p + as^q \cdots} \quad (10)$$

where $q-p \geqslant 2$ for the reason just given, and a is a numerical constant. This equation does not include Case 1 in which adjacent regions share a common numerator term. At the denominator transition:

$$s^p = as^q$$

$$\therefore s^{q-p} = 1/a \quad (11)$$

At the numerator transition:

$$am_2 s^{q-1} + ac_2 s^q = m_1 s^{p-1} + c_1 s^p$$

$$\therefore s^{q-p} = \frac{m_1 + c_1 s}{m_2 + c_2 s}\cdot\frac{1}{a} \quad (12)$$

If Rule 1 is obeyed, eqns. (11) and (12) both apply and lead to:

$$\frac{m_1 + c_1 s}{m_2 + c_2 s} = 1$$

$$\therefore \frac{m_1 - m_2}{c_2 - c_1} = s$$

This is the same as eqn. (8). In other words, if Rule 1 is obeyed, so is Rule 3, just as was found for Case 1. Eqns. (11) and (8) lead to the conclusion that:

$$a = \left(\frac{c_1 - c_2}{m_2 - m_1}\right)^{q-p} \quad (13)$$

which is the condition for obeying Rules 1 and 3.

On the basis of the considerations outlined above, equations may be constructed to give Lineweaver–Burk plots with abrupt transitions, the degree of abruptness being governed by the value of $(q-p)$ in eqn. (10). The equation may be extended as follows, to give any desired number of linear sections:

$$\frac{e_0}{v} = \frac{\cdots m_1 s^{p-1} + c_1 s^p + am_2 s^{q-1} + ac_2 s^q + bm_3 s^{r-1} + bc_3 s^r + \cdots}{\cdots \quad s^p \quad + as^q \quad + bs^r + \cdots} \quad (14)$$

We have examined most thoroughly the cases in which:

$$2 = q-p = r-q = \cdots \quad \text{(Case 2)}$$

and

$$3 = q-p = r-q = \cdots \quad \text{(Case 3)}$$

In Case 2 eqn. (1) takes the form:

$$\frac{e_0}{v} = \frac{N_0 + N_1 s + N_2 s^2 + N_3 s^3 + N_4 s^4 + N_5 s^5 + \cdots}{D_1 s \quad + D_3 s^3 \quad + D_5 s^5 + \cdots}$$

Case 3, however, gives an equation of the form:

$$\frac{e_0}{v} = \frac{N_0 + N_1 s + N_3 s^3 + N_4 s^4 + N_6 s^6 + N_7 s^7 \cdots}{D_1 s \quad + D_4 s^4 \quad + D_7 s^7 \cdots}$$

The physical significance of omitting coefficients in this way will be considered in the Discussion section.

To explore the potentialities of the equations discussed above a program was written for the Wang 700 desk-top computer equipped with a graph-plotter. This allowed the plotting of the quotient of two polynomials of power twelve or less. When supplied with the appropriate coefficients in eqn. (1) the instrument produced the equivalent Lineweaver–Burk plot.

The simple case of a single transition involving twofold changes in slope and intercept in opposite senses was first examined by using Cases 2 and 3. This is illustrated in Fig. 2. A fairly abrupt transition

is seen in both cases, although it is clearly most abrupt in Case 3. On examination of the sections on either side of the transition/intersection point it is clear that they do not exactly follow the theoretical lines, although they are asymptotic to those lines. There is a point of inflexion on either side of the transition point resulting in a 'knee' in the curve. Nevertheless the two sections of the curve do approximate to straight lines, and would undoubtedly be regarded as such in an experimental situation.

Attempts were also made to fit the kinetic results for glutamate dehydrogenase by using both Cases 2 and 3. The procedure followed was to take the experimentally observed slopes and intercepts (Table 1) of plots of e_0/v against $1/[NAD^+]$ taken from the results of Engel & Dalziel (1969, 1970) and use these in the appropriate form of eqn. (14) as m_1, m_2, m_3, m_4 and c_1, c_2, c_3, c_4. Values of a, b etc. were calculated from eqn. (13). The computer plot of the resulting equation was then compared with the experimental points. With both Cases 2 and 3 this initial attempt gave relatively good fit in Regions 1 and 4 but poor fit in Regions 2 and 3. This is because, as shown in Fig. 2, the apparent slope or intercept for each region is modified by those terms in the equation that become dominant in adjoining regions. Slopes and intercepts were amended by a process of manual iteration until the best fit to the results was obtained, as shown in Fig. 3. The values of m and c for each region used in obtaining the best fit are shown in Table 1 for comparison with the slopes and intercepts actually observed. The coefficients used in the equations are given in Table 2.

Fig. 3 shows that Case 3 gives, as might be expected, rather more abrupt transitions. On the other hand, the fit achieved with Case 2 is surprisingly good. A definite decision as to whether Case 2 or

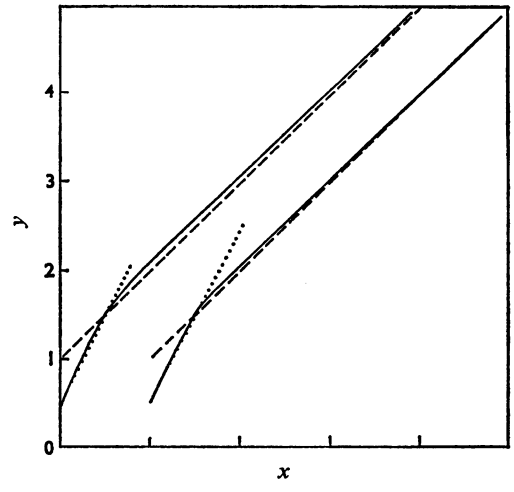

Fig. 2. *Comparison of single abrupt Case 2 and Case 3 transitions*

The Case 2 curve, drawn nearest the left-hand vertical axis, is given by the equation:

$$y = \frac{0.125 + 0.5x + x^2 + x^3}{0.25 \qquad + x^2}$$

The case 3 curve, displaced one division to the right, is given by the equation:

$$y = \frac{0.0625 + 0.25x + x^3 + x^4}{0.125 \qquad + x^3}$$

In both cases the theoretical lines (· · ·) to which the curve is asymptotic as x approaches 0 and infinity respectively are given by equations $y = 2x + 0.5$ and $y = x + 1$. Each division on the abscissa represents 1.

Table 1. *Observed values of slope (m) and intercept (c) compared with those used to obtain optimum fit of the results*

The observed slopes and intercepts are those obtained from the separate sections of the Lineweaver–Burk plot shown in Fig. 1. The numbers in the other columns are values of m_1, m_2, m_3, m_4 and c_1, c_2, c_3, c_4 inserted in eqn. (14) to give a good fit to the experimental results.

	m			*c*		
	Observed	Case 2 Fit	Case 3 Fit	Observed	Case 2 Fit	Case 3 Fit
Region 1	0.575	0.575	0.575	0.064	0.060	0.062
Region 2	1.04	0.93	0.98	0.023	0.023	0.0237
Region 3	1.49	1.49	1.475	0.0176	0.0167	0.0171
Region 4	2.94	2.59	2.65	0.0122	0.0122	0.0122

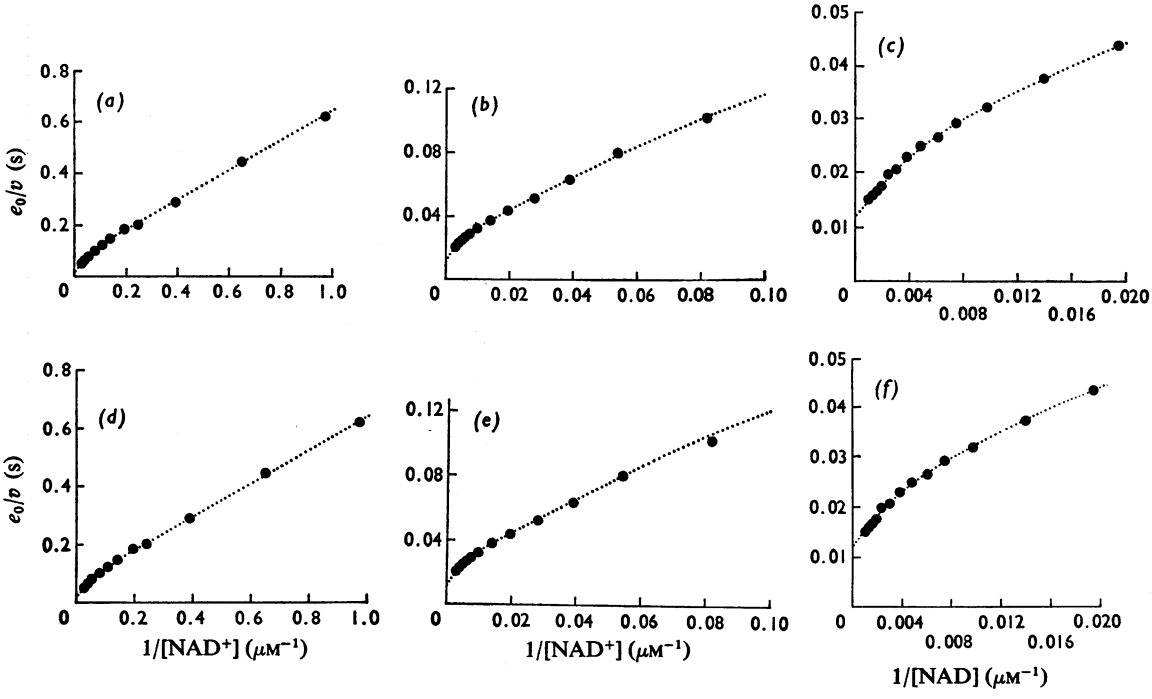

Fig. 3. *Comparison of Case 2 and Case 3 simulations of initial-rate results*

The experimental points (●) are those of Fig. 1. The dotted curves are computer-drawn lines corresponding to the optimum fit achieved. The coefficients used in eqn. (1) to achieve this fit in each case are given in Table 2. The Case 2 fit is shown in (*a*), (*b*) and (*c*) and the Case 3 fit in (*d*), (*e*) and (*f*). Low (*a*, *d*), intermediate (*b*, *e*) and high (*c*, *f*) NAD^+ concentrations are shown.

Table 2. *Values of coefficients in eqn.* (1) *used to obtain optimum fit of the experimental results for glutamate dehydrogenase (see the text)*

Case 2		Case 3	
$N_0 = 0.575$	$D_0 = 0$	$N_0 = 0.575$	$D_0 = 0$
$N_1 = 0.06$	$D_1 = 1$	$N_1 = 0.062$	$D_1 = 1$
$N_2 = 10^{-2}$	$D_2 = 0$	$N_2 = 0$	$D_2 = 0$
$N_3 = 2.48 \times 10^{-4}$	$D_3 = 1.08 \times 10^{-2}$	$N_3 = 8.29 \times 10^{-4}$	$D_3 = 0$
$N_4 = 2.04 \times 10^{-6}$	$D_4 = 0$	$N_4 = 2.01 \times 10^{-5}$	$D_4 = 8.46 \times 10^{-4}$
$N_5 = 2.29 \times 10^{-8}$	$D_5 = 1.37 \times 10^{-6}$	$N_5 = 0$	$D_5 = 0$
$N_6 = 5.90 \times 10^{-11}$	$D_6 = 0$	$N_6 = 2.96 \times 10^{-9}$	$D_6 = 0$
$N_7 = 2.78 \times 10^{-13}$	$D_7 = 2.28 \times 10^{-11}$	$N_7 = 3.43 \times 10^{-11}$	$D_7 = 2.01 \times 10^{-9}$
		$N_8 = 0$	$D_8 = 0$
		$N_9 = 3.87 \times 10^{-16}$	$D_9 = 0$
		$N_{10} = 1.78 \times 10^{-18}$	$D_{10} = 1.46 \times 10^{-16}$

Case 3 fits the experimental results more closely would require many more experimental measurements of greater precision than those employed in the present paper.

Discussion

It has been demonstrated in this paper that an abrupt transition between pseudo-linear sections in a Lineweaver–Burk plot can only be generated by the

omission of one or more terms in the reciprocal initial-rate expression (eqn. 1).

This mathematical exercise is an attempt to utilize the entire information content of a body of experimental results. Significant details of the shape of experimentally obtained curves are easily overlooked or disregarded on the grounds that verification of such detail places a heavy demand on the experimental method. Abrupt transitions in Lineweaver–Burk plots of kinetic results for glutamate dehydrogenase represent a definite and reproducible experimental observation. The present analysis shows that the results cannot be accounted for by any of the simple models previously advanced. Dalziel & Engel (1968) showed that the general downward concavity of the Lineweaver–Burk plot could be accounted for by negative homotropic co-operativity in an Adair model, and suggested that reasonably strong interactions in such a model would give rise to abrupt transitions. It is clear from the results shown here that the occurrence of several clearly separated transitions within a relatively narrow range of co-enzyme concentration requires a more complex model.

Barton & Fisher (1971) have re-examined the kinetics of glutamate dehydrogenase and obtained smooth curves in Lineweaver–Burk plots against $1/[NAD^+]$. They conclude that a random-order steady-state mechanism accounts adequately for the results without any need to postulate subunit interactions, and they present an empirical rate equation. Their results were, however, obtained with phosphate buffer at pH 8, and the abrupt transitions that are so striking in results obtained at pH 7 are not discernible at pH 8 (Engel & Dalziel, 1969). For a fixed glutamate concentration the equation of Barton & Fisher (1971), when expanded into a single expression, is of the fourth power in $[NAD^+]$ in the numerator and denominator. Such an equation could not therefore account for the results obtained at pH 7.

Since a Case 2 or Case 3 equation appears to be required to describe the results, it is necessary to consider the physical significance of such equations. When eqn. (1) describes either binding without catalysis or a 'rapid-equilibrium' catalytic reaction with a rate limited by the first-order reaction of the enzyme–ligand complex(es), each numerator term represents the contribution of a single species to the total enzyme concentration. Thus N_0 represents the fractional contribution of free enzyme, $N_1 s$ that of ES, $N_2 s^2$ that of $E(S)_2$ and so on. The denominator terms correspond to the contributions of each species to the overall degree of saturation in the case of binding, or to the overall rate in the case of a 'rapid-equilibrium' catalytic reaction, although in the more general case of a steady-state reaction individual enzyme-containing species contribute to more than one term in the numerator and therefore to more than one in the denominator. It seems less likely that a mechanism of the latter type could give rise to the equations under present consideration, and attention is confined to the simpler 'rapid-equilibrium' situation.

Taking first the equation for a single Case 2 transition:

$$\frac{e_0}{v} = \frac{N_0 + N_1 s + N_2 s^2 + N_3 s^3}{D_1 s \qquad + D_3 s^3}$$

the enzyme-containing species E, ES, $E(S)_2$ and $E(S)_3$ are all significant in the rate equation, since all the corresponding terms appear in the numerator, but one term, the term in s^2, is missing from the denominator. This means that the total catalytic reactivity of $E(S)_2$ is very low compared with the catalytic reactivity of both ES and $E(S)_3$. This implies a negative interaction affecting the catalytic rate constant followed by a positive interaction: the binding of the second molecule of S 'switches off' the enzyme, but the binding of the third switches it on again. The second site may indeed not be an active site, but mere inactivity is insufficient: occupation of this site must greatly decrease the catalytic activity at the first site, otherwise that activity would be manifest in a significant $D_2 s^2$ term.

Clearly a simple binding system without catalytic rate constants cannot give rise to an equation that has a term missing from the denominator but nevertheless contains the corresponding numerator term. The mathematical condition for the disappearance of the denominator term in s^x is:

$$D_{(x-1)} D_{(x+1)} \geqslant D_x^2$$

ensuring that the term in s^x is always much smaller than at least one of the adjacent terms. Now for a simple binding system each denominator term is given by multiplying the corresponding numerator term by the power to which s is raised in that term. Hence the inequality above becomes:

$$N_{(x-1)} N_{(x+1)} (x^2 - 1) \geqslant N_x^2 x^2$$

$$\therefore N_{(x-1)} N_{(x+1)} \geqslant N_x^2$$

This, however, is the condition for disappearance of the numerator term in s^x. A simple binding system, therefore, cannot give rise to a Case 2 equation.

For Case 3, the equation for a single transition is of the form:

$$\frac{e_0}{v} = \frac{N_0 + N_1 s + N_3 s^3 + N_4 s^4}{D_1 s \qquad + D_4 s^4}$$

The absence of the numerator term in s^2 means that $E(S)_2$ never constitutes a significant fraction of the total enzyme concentration. This again requires negative co-operativity followed by positive co-operativity, but now affecting binding rather than the catalytic rate: any $E(S)_2$ formed is almost entirely

converted into $E(S)_3$. This automatically deletes the s^2 term in the denominator also, unless $E(S)_2$ is abnormally catalytically reactive. In Case 3, however, the s^3 term also is missing from the denominator. Superimposed on the binding co-operativity, therefore, there is also a marked decrease in site reactivity in $E(S)_3$ which is reversed in $E(S)_4$. Thus although $E(S)_3$ contributes significantly to the total enzyme concentration at some stage during the overall saturation process it is nevertheless essentially inactive.

Case 2 clearly involves simpler assumptions than Case 3 and in the absence of compelling reasons for adopting Case 3 it is concluded that a Case 2 equation accounts adequately for the existing results for glutamate dehydrogenase.

For glutamate dehydrogenase the present analysis implies that a sequence of negative followed by positive co-operativity occurs, not merely once, but three times within the saturation range covered by the experimental results. The results within each linear region have been analysed to give a set of kinetic parameters (Engel & Dalziel, 1969). In using these parameters it must be borne in mind that:

(*a*) Notwithstanding the abruptness of the transitions the slope and intercept of each section is affected by contributions from the neighbouring regions. The experimentally obtained parameters will therefore only approximate to 'true' parameters.

(*b*) Successive pseudo-linear sections definitely do not correspond to successive saturation steps. If Case 2 applies, for instance, the four sections reflect the first, third, fifth and seventh saturation steps.

The idea of both negative and positive co-operativity occurring in the binding of a single ligand to a single protein has been clearly enunciated previously by Teipel & Koshland (1969). These authors attempted to explain the occurrence of intermediate plateau regions in plots of v against s for several enzymes, including glutamate dehydrogenase from *Blastocladiella* (Lé John & Jackson, 1968) and have shown by inflexion-point analysis of a four-site model that negative co-operativity alone cannot account for such behaviour. Appropriately 'bumpy' curves could be generated by postulating negative followed by positive co-operativity in either the binding constants or the catalytic rate constants. Although this situation is somewhat analogous to that analysed in the present paper, the plot of v against s for ox liver glutamate dehydrogenase shows no plateaux. Such plateaux correspond in the Lineweaver–Burk representation to a long smooth curved transition between the two pseudo-linear sections. The difference in slope between the two sections is also much greater than is found with ox liver glutamate dehydrogenase. It is precisely because there is no plateau, but instead an abrupt transition between two sections with slopes and intercepts differing only by factors of approximately two, that (i) the Lineweaver–Burk plot is the most sensitive method for detecting this aspect of the kinetics; and (ii) interactions involving binding alone cannot adequately account for the behaviour.

At the concentrations normally used in kinetic experiments glutamate dehydrogenase exists as a hexamer consisting of identical subunits (references are given in Goldin & Frieden, 1972). If eqn. (1) were based on a rapid-equilibrium mechanism involving six interacting active sites, the highest exponent of s in the numerator and denominator would be 6. Such an equation could not adequately fit the experimental results. The highest exponent of s in the numerator and denominator of the expression used to fit the result (Fig. 3) is 10 if Case 3 is used or 7 by Case 2, and the presence of such high powers of s clearly requires explanation.

One possibility is that the protein exists in two conformational states that equilibrate only very slowly (cf. Frieden, 1972). This would be kinetically equivalent to having a mixture of two enzymes. Each form would give an initial-rate expression containing powers of s up to s^6 and the summed rate combined into a single expression would contain terms up to s^{12} in denominator and numerator.

A second possibility is that there are two NAD^+-binding sites per subunit, making a total of twelve per hexamer. There is abundant evidence from many sources (e.g. Jallon & Iwatsubo, 1971; Koberstein & Sund, 1971; di Prisco, 1971; Melzi D'Eril & Dalziel, 1972; Goldin & Frieden, 1972) for more than one nucleotide site per subunit and it appears that NADH can bind to two sites per subunit, the active site and one inactive site. Engel (1972) has found evidence in the amino acid sequence for a partial gene duplication which may be a structural reflection of the presence of a secondary coenzyme site. Dalziel & Egan (1972) have, however, measured NAD^+ binding directly by equilibrium dialysis, simulating the active ternary complex by using the inhibitory substrate analogue glutarate. They found only one NAD^+ site per subunit under these conditions. The weight of this evidence depends on the extent to which glutarate mimics the substrate glutamate. Conformational changes involved in the unmasking of new NAD^+ sites might depend on the presence of the α-amino group on the substrate molecule. Shafer *et al.* (1972) have obtained evidence from rapid-reaction kinetics that there must be non-catalytic NAD^+ sites on the enzyme molecule.

A third possibility is that in the presence of substrates the hexamers associate to forms with higher molecular weight as they do at high protein concentrations even in the absence of substrates. The evidence bearing most directly on this point is that of Cohen & Mire (1971) who performed ultracentrifugation experiments on glutamate dehydrogenase layered

over a complete reaction mixture. Under these conditions the sedimentation coefficient was still that of the hexameric enzyme. The enzyme was, however, detected in these experiments by measuring the absorption at 340nm due to NADH, which is known to dissociate larger species of glutamate dehydrogenase to the hexamer (Frieden, 1959). It may therefore be argued that under true initial-rate conditions the sedimentation coefficient would have been different.

It is not possible at present to choose among these possibilities. The mathematical considerations developed in the present paper should prove useful, however, in assessing future models of the action of glutamate dehydrogenase and similar regulatory enzymes.

References

Anderson, W. B., Horne, R. N. & Nordlie, R. C. (1968) *Biochemistry* **7**, 3997–4004

Barton, J. S. & Fisher, J. R. (1971) *Biochemistry* **10**, 577–584

Cohen, R. & Mire, M. (1971) *Eur. J. Biochem.* **23**, 276–281

Conway, A. & Koshland, D. E., Jr. (1968) *Biochemistry* **7**, 4011–4022

Dalziel, K. & Egan, R. R. (1972) *Biochem. J.* **126**, 975–984

Dalziel, K. & Engel, P. C. (1968) *FEBS Lett.* **1**, 349–352

de Vijlder, J. J. M. & Slater, E. C. (1968) *Biochim. Biophys. Acta* **167**, 23–34

di Prisco, G. (1971) *Biochemistry* **10**, 585–589

Engel, P. C. (1970) in *Pyridine Nucleotide-Dependent Dehydrogenases* (Sund, H., ed.), p. 254, Springer-Verlag, Berlin

Engel, P. C. (1972) *Nature (London)* in the press

Engel, P. C. & Dalziel, K. (1969) *Biochem. J.* **115**, 621–631

Engel, P. C. & Dalziel, K. (1970) in *Pyridine Nucleotide-Dependent Dehydrogenases* (Sund, H., ed.), pp. 245–253, Springer-Verlag, Berlin

Fourcade, A. & Venard, R. (1971) *Biochim. Biophys. Acta* **242**, 331–344

Frieden, C. (1959) *J. Biol. Chem.* **234**, 809–814

Frieden, C. (1972) *Biochem. J.* **130**, 1P

Godinot, C. & Gautheron, D. (1971) *FEBS Lett.* **13**, 235–240

Goldin, B. R. & Frieden, C. (1972) *Curr. Top. Cell Regul.* **4**, 77–117

Jallon, J.-M. & Iwatsubo, M. (1971) *Biochem. Biophys. Res. Commun.* **45**, 964–971

Koberstein, R. & Sund, H. (1971) *FEBS Lett.* **19**, 149–151

Koshland, D. E. Jr., Némethy, G. & Filmer, D. (1966) *Biochemistry* **5**, 365–385

Lé John, H. B. & Jackson, A. (1968) *J. Biol. Chem.* **243**, 3447–3457

Melzi D'Eril, G. & Dalziel, K. (1972) *Biochem. J.* **130**, 3P

Monod, J., Wyman, J. & Changeux, J.-P. (1965) *J. Mol. Biol.* **12**, 88–118

Muto, S. & Uritani, I. (1970) *Plant Cell Physiol.* **11**, 767–776

Pinto, P. V. C., Newton, W. A., Jr. & Richardson, K. E. (1966) *J. Clin. Invest.* **45**, 823–831

Schwert, G. W. (1970) in *Pyridine Nucleotide-Dependent Dehydrogenases* (Sund, H., ed.), p. 254, Springer-Verlag, Berlin

Shafer, J. A., Chiancone, E., Yielding, K. L. & Antonini, E. (1972) *Eur. J. Biochem.*, **28**, 528–532

Teipel, J. & Koshland, D. E. Jr. (1969) *Biochemistry* **8**, 4656–4663