Kinetic Studies of Glutamate Dehydrogenase with Glutamate and Norvaline as Substrates

COENZYME ACTIVATION AND NEGATIVE HOMOTROPIC INTERACTIONS IN ALLOSTERIC ENZYMES

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1. Kinetic studies of glutamate dehydrogenase were made with wide concentration ranges of the coenzymes NAD^+ and $NADP^+$ and the substrates glutamate and norvaline. Initial-rate parameters were evaluated. 2. Deviations from Michaelis–Menten behaviour towards higher activity were observed with increasing concentrations of either coenzyme with glutamate as substrate, but not with norvaline as substrate. 3. In phosphate buffer, pH 7·0, Lineweaver–Burk plots with either coenzyme as variable and a constant, large glutamate concentration showed three or four linear regions of different slope with relatively sharp discontinuities. Maximum rates obtained by extrapolation and Michaelis constants for the coenzymes increased in steps with increase of coenzyme concentration. 4. In the absence of evidence of heterogeneity of the enzyme and coenzyme preparations, the results are interpreted in terms of negative homotropic interactions between the enzyme subunits. It is suggested that sharp discontinuities in Lineweaver–Burk plots or reciprocal binding plots may be characteristic of this new type of interaction, which can be explained in terms of an Adair–Koshland model, but not by the model of Monod, Wyman & Changeux.

Glutamate dehydrogenase (EC 1.4.1.3) displays wide specificity towards amino acid substrates (Struck & Sizer, 1960; Tomkins, Yielding, Curran, Summers & Bittensky, 1965) and nicotinamide nucleotide coenzymes (Olson & Anfinsen, 1953; Strecker, 1953; Kaplan, Ciotti & Stolzenbach, 1956). These properties offer unusual opportunities for investigations of mechanism by comparative kinetic studies. Because deviations from Michaelis–Menten behaviour towards higher rates were observed with high concentrations of NAD^+ (Olson & Anfinsen, 1953; Frieden, 1959a), detailed kinetic studies have hitherto been confined to the apparently simpler reaction with $NADP^+$ as coenzyme (Frieden, 1959b). In the present work, advantage has been taken of the great sensitivity of fluorimetry to make initial-rate studies with wide concentration ranges of both NAD^+ and $NADP^+$, and to compare the kinetics with glutamate and the next most active substrate, norvaline, under identical conditions, despite their different pH optima. Initial-rate parameters have been determined for the reaction with both substrates. Complex patterns of 'activation' by both coenzymes are interpreted in terms of

antagonistic homotropic interactions (Dalziel & Engel, 1968).

MATERIALS AND METHODS

Glutamate dehydrogenase. Ox liver glutamate dehydrogenase was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, as a crystalline suspension in $(NH_4)_2SO_4$ solution and stored at 2°. For each experiment a sample of the suspension was freed from $(NH_4)_2SO_4$ by dialysis at 2° against 0·1 M-sodium phosphate buffer, pH 7·0, containing 10 μM-EDTA, and clarified by centrifugation. The protein concentrations of these stock solutions (about 2 mg./ml.) were calculated from E_{280} measurements and the extinction coefficient of 0·97 cm.^{2}/mg. (Olson & Anfinsen, 1952). The E_{280}/E_{260} ratio was 1·94±0·02 in phosphate buffer, pH 7·0, higher than values previously reported (Olson & Anfinsen, 1953; Frieden, 1959a).

Dilute enzyme solutions for kinetic experiments were assayed before and after use by initial-rate measurements at 25° with a standard reaction mixture containing 0·111 M-sodium phosphate buffer, pH 7·0, 10 μM-EDTA, 20 mM-sodium glutamate and either 50 μM-NAD^+ or 230 μM-$NADP^+$. The specific activities of the enzyme preparation in this assay were 1·02 μmoles of NAD^+/min./mg. and 1·62 μmoles of $NADP^+$/min./mg., independent of the enzyme concentration used in the assay (0·03–7·6 μg./ml.). The proportionality between initial rate and enzyme concentration was also repeatedly confirmed in kinetic experiments.

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The specific activity of the crystalline suspension of the enzyme remained unchanged for several months. When kept at 2°, stock solutions containing about 2 mg./ml. in 0·1 M-phosphate buffer, pH 7·0, were stable for at least a week, and dilute solutions containing 0·1 mg./ml. in the same buffer for at least 24 hr. The enzyme was also remarkably stable at much lower concentrations in reaction mixtures in phosphate buffers, pH 7·0 and pH 8·0, as shown by the linearity of progress curves for 1–2 min. By contrast, progress curves for the reaction in 0·1 M-tris–acetate buffers showed a rapid decrease of rate. Stock solutions of the enzyme in this buffer at pH 7·0 lost half their activity in 3 days, and in dilute solution (0·1 mg./ml.) the activity fell by 60% in 20 hr. For studies of the kinetics in tris–acetate buffer, the stock solution of the enzyme was dialysed against this buffer and fresh dilutions were made every hour and assayed.

No evidence for heterogeneity of the enzyme preparations was obtained by recrystallization, chromatography or ultracentrifugation. These experiments are described in detail by Engel (1968). After recrystallization with Na₂SO₄ (Olson & Anfinsen, 1953) the specific activity was unchanged in both the product and the mother liquor. When the enzyme was adsorbed on to DEAE-cellulose from 0·04 M-sodium phosphate buffer, pH 7·0, and eluted with a concentration gradient of the same buffer, all the enzyme activity was recovered in a single elution peak across which the specific activity was uniform.

Sucrose-density-gradient centrifugation with nine enzyme concentrations in the range 12·6 μg./ml. to 12·7 mg./ml. was carried out at 20° essentially as described by Martin & Ames (1961), except that 0·111 M-sodium phosphate buffer, pH 7·0, 10 μM-EDTA and, in two experiments, 1·3 mM-NAD⁺, were added to the sucrose solutions. The sedimentation coefficient increased from 13–14 s with 12·6–63·5 μg. of enzyme/ml. to 17 s with 635 μg./ml. and 23 s with 12·7 mg./ml. These findings agree, within the experimental error, with the results of the more detailed studies of Rogers, Hellerman & Thompson (1965) and Sund & Burchard (1968). The presence of NAD⁺, with 48·5 μg. or 3·23 mg. of enzyme/ml., did not significantly affect the sedimentation coefficient. In conventional ultracentrifugation studies at 20°, the well-established dependence of sedimentation coefficient on protein concentration was also confirmed. Values for $S_{20,w}$ increased from 17 s with 0·6 mg. of enzyme/ml. to 28 s with 9·0 mg./ml., and at the lower and higher protein concentrations were not significantly affected by the presence of NAD⁺ (1·3–16·2 mM).

Substrates and coenzymes. Monosodium L-glutamate was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., and found to be pure within the error of enzymic assay as described by Engel & Dalziel (1967). L-Norvaline was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. NAD⁺ and NADP⁺ were purchased from C. F. Boehringer und Soehne G.m.b.H., and assayed as described by Engel & Dalziel (1967). For some experiments, NADP⁺ was purified by chromatography on DEAE-cellulose as described by Dalziel & Dickinson (1966a), except that the coenzyme was eluted with a linear gradient of NaCl, and was freeze-dried after removal of salt with Sephadex G-10 (Pharmacia, Uppsala, Sweden).

Initial-rate measurements. An improved version of the recording fluorimeter described by Dalziel (1961, 1962) was used, and is described in detail by Engel (1968). The principal modifications were a greater variable backing-off potential, more stable high-voltage supply (E.H.T. unit type 532/D; Isotope Developments Ltd., Aldermaston, Berks.) for the photomultiplier, and shorter light-paths from sample to light-source and detector. These modifications increased the sensitivity 20-fold, and rates down to 1 nM-NADH/min. could be measured accurately. The calibration of the fluorimeter and the technique of initial-rate measurements at 25° were essentially as described previously (Dalziel, 1961, 1962). Reactant solutions other than enzyme solutions were filtered three times through sintered glass to remove particulate debris and fluorimeter tubes were cleaned by boiling in 0·1 M-NaOH to avoid the retention of small air bubbles in reaction mixtures. To start the reaction, 5–20 μl. of enzyme solution was pipetted on to a piece of Parafilm with which the fluorimeter tube containing the reaction mixture was covered; the tube was inverted and quickly replaced in the fluorimeter, and the record started with a time-lag of less than 5 sec. All measurements were made at least in duplicate. The reproducibility of initial rates was generally ± 1–2% in phosphate buffer and ± 3–4% in tris buffer.

Kinetic coefficients in the inital-rate equation:

$$\frac{e}{v_0} = \phi_0' + \frac{\phi_1'}{[S_1]} + \frac{\phi_2'}{[S_2]} + \frac{\phi_{12}'}{[S_1][S_2]} \tag{1}$$

were evaluated from linear regions of primary and secondary plots of initial-rate data (Dalziel, 1957). In eqn. (1), S_1 and S_2 are coenzyme and substrate respectively and e is the concentration of the enzyme. Because of the known dissociation of the enzyme molecule in solution and uncertainty regarding the number of active centres per molecule, the earlier value of 10^6 for the molecular weight has been arbitrarily assumed in order to express e in molar units. This simplifies the dimensions of the kinetic coefficients, which can easily be corrected when the molecular weight and number of active sites are established.

RESULTS

Reaction of glutamate and NADP⁺

Kinetics in phosphate buffer. Initial-rate measurements were made in 0·111 M-sodium phosphate buffer, pH 7·0 (I 0·25), containing 10 μM-EDTA. The reactant concentrations were 0·1–500 mM-glutamate, 2–1000 μM-NADP⁺ and 0·38–3·8 μg. of enzyme/ml. The variation of the initial rate with the NADP⁺ concentration, with a constant glutamate concentration of 50 mM, is shown in Figs. 1(a) and 1(b). The reciprocal plots exhibit three distinct linear regions with fairly sharp discontinuities at about 20 μM- and 140 μM-NADP⁺. With smaller fixed glutamate concentrations, similar plots showed discontinuities at smaller values of the NADP⁺ concentration and the changes of slope were also smaller; with a constant glutamate concentration of 0·3 mM the plots were linear within the experimental error over the whole range of NADP⁺ concentration (Fig. 2). The alternative primary plots of the initial-rate data with the glutamate
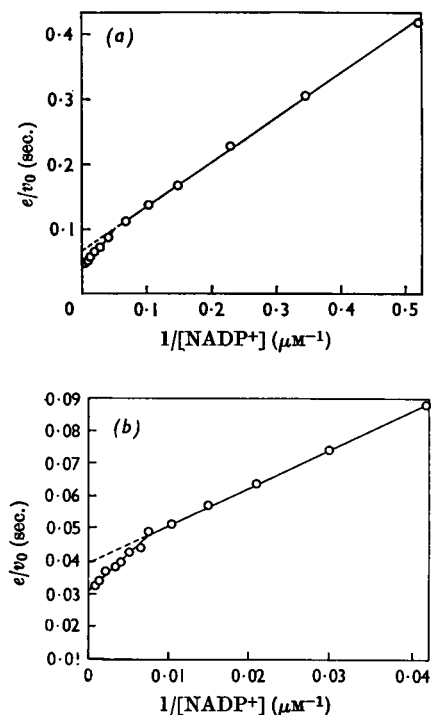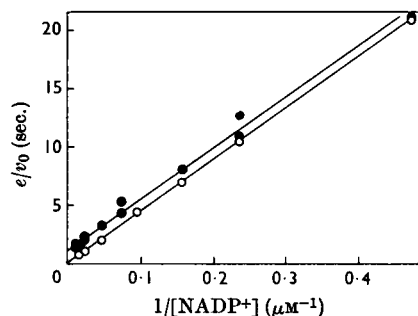
Fig. 1. Variation of the reciprocal of the specific initial rate with the reciprocal of the NADP+ concentration, with sodium phosphate buffer, pH7·0 and I 0·25, at 25°. The glutamate concentration was 50 mM. (a) 1·9–100 μM-NADP+; (b) 24–1000 μM-NADP+.

Fig. 2. Variation of the reciprocal of the specific initial rate with the reciprocal of the NADP+ concentration, with sodium phosphate buffer, pH7·0 and I 0·25, at 25°. The glutamate concentration was 0·3mM. Results with two concentration ranges of NADP+ are shown: ○, 2·1–100 μM-NADP+; ●, 21–1000 μM-NADP+, the specific rates and the NADP+ concentrations having been divided by 10.

concentration as variable and several fixed concentrations of NADP+ were linear from 0·1mM- to 50mM-glutamate (Fig. 3). With larger glutamate
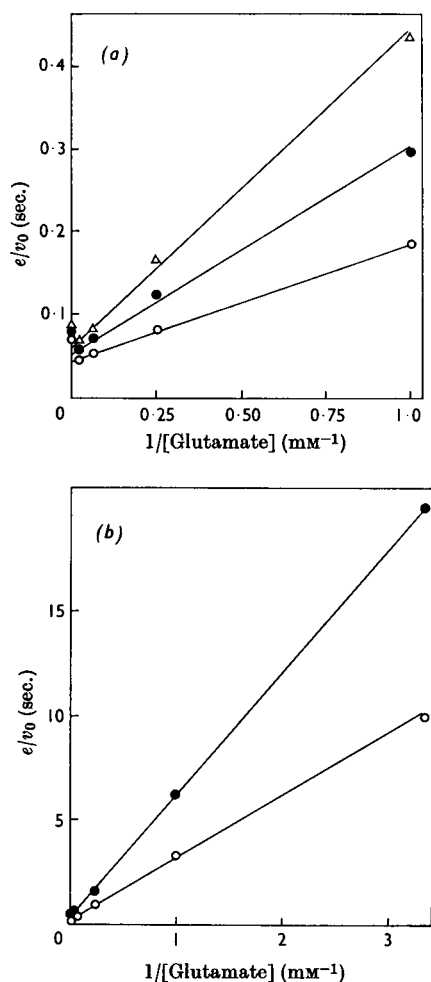

Fig. 3. Variation of the reciprocal of the specific initial rate with the reciprocal of the glutamate concentration, with sodium phosphate buffer, pH7·0 and I 0·25, at 25°, for several constant NADP+ concentrations: (a) ○, 143 μM; ●, 66 μM; △, 44 μM. (b) ○, 4·4 μM; ●, 2·2 μM.

concentrations, substrate inhibition was observed, but only when the fixed NADP+ concentration was greater than 10 μM, as illustrated in Fig. 3(a).

For glutamate concentrations less than 50mM, the initial-rate data for each of the three ranges of NADP+ concentration indicated in Fig. 1 may be empirically described by eqn. (1). The fact that the changes in slope and intercept in Fig. 1 are most marked with high glutamate concentrations indicates that they may be ascribed to alterations of ϕ_0' and ϕ_1' rather than ϕ_2' and ϕ_{12}'. The slopes of the primary plots with glutamate concentration as variable, including those of Fig. 3, are plotted

against the reciprocal of the fixed NADP$^+$ concentration in Fig. 4. This plot is linear over the entire coenzyme concentration range: the slope is ϕ'_{12} in eqn. (1), which is clearly the same for all three concentration ranges of NADP$^+$. The intercept of this plot is equal to ϕ'_2. From eqn. (1), with these
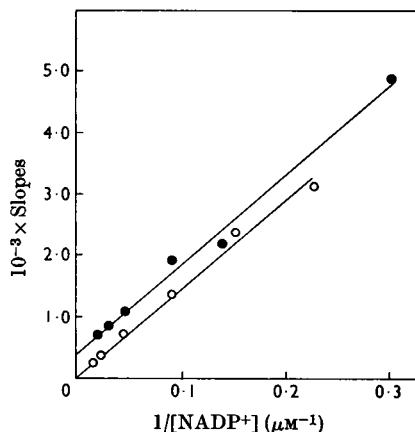

Fig. 4. Variation of the slopes of Lineweaver–Burk plots such as those in Fig. 3 with the reciprocal of the NADP$^+$ concentration, for two ranges of the latter: O, 2·1–100 μM-NADP$^+$; ●, 66–1000 μM-NADP$^+$, the slopes having been multiplied, and the NADP$^+$ concentrations divided, by 20.

estimates of ϕ'_2 and ϕ'_{12} and [S$_2$] = 50mM, values for ϕ'_0 and ϕ'_1 were calculated from the slopes and intercepts, obtained by extrapolation of the linear regions of Fig. 1. The initial-rate parameters are given in Table 1.

The variation of initial rate with NADP$^+$ concentration, at a constant glutamate concentration of 50mM, was also studied with larger and smaller enzyme concentrations of 12 and 0·12 μg./ml., with similar results. Since commercial NADP$^+$ preparations contain small amounts of NAD$^+$ and other nucleotide impurities (Dalziel & Dickinson, 1966a), the experiment was also repeated with chromatographically purified NADP$^+$, and with NADP$^+$ deliberately contaminated with 1% and 5% NAD$^+$. A similar activation pattern was obtained in each case. Increase of the EDTA concentration of the buffer to 800 μM did not affect the rate obtained with 20mM-glutamate and 200 μM-NADP$^+$.

The variation of the initial rate with NADP$^+$ concentration (2–1090 μM) was also studied in detail at pH 8·0 in sodium phosphate buffer, I 0·25. The results with 3mM- and 50mM-glutamate are shown in Fig. 5. The plots are linear up to about 10 μM-NADP$^+$ (Fig. 5a); above this value deviations towards higher activity are again apparent (Fig. 5b), but the pattern is more complex than at pH 7·0. Although only two glutamate concentrations were used, it was assumed that Lineweaver–Burk plots with the glutamate concentration as variable would

Table 1. *Kinetic coefficients for the oxidative deamination of glutamate and norvaline in phosphate buffers, I 0·25, at 25°*

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

$$\frac{e}{v_0} = \phi'_0 + \frac{\phi'_1}{[S_1]} + \frac{\phi'_2}{[S_2]} + \frac{\phi'_{12}}{[S_1][S_2]}$$

where S$_1$ is the coenzyme and S$_2$ is the substrate. The kinetic coefficients were calculated from initial-rate measurements with coenzyme concentrations in the ranges indicated, as described in the text.

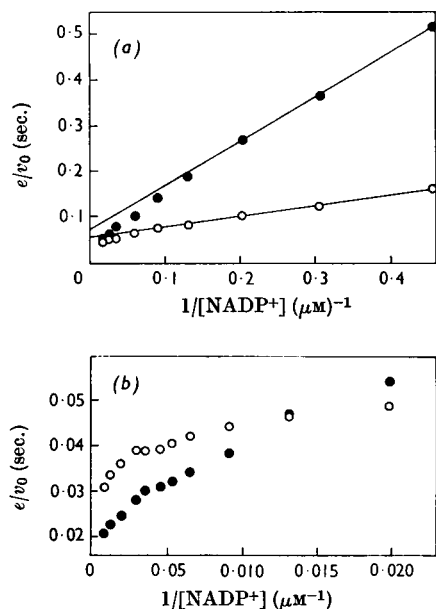| pH	Coenzyme and concn. (μM)		ϕ'_0 (sec.)	ϕ'_1 (μM sec.)	ϕ'_2 (μM sec.)	ϕ'_{12} (mM^2 sec.)	ϕ'_1/ϕ'_0 (μM)	ϕ'_2/ϕ'_0 (μM)	ϕ'_{12}/ϕ'_2 (μM)
Glutamate									
7·0	NADP$^+$	2–20	0·071 (0·05*)	0·38 (0·07*)			5·3	0·28	
		20–200	0·043	0·88	20	0·015	20	0·47	750
		200–1000	0·031	2·27	(50*)	(0·055*)	73	0·65	
7·0	NAD$^+$	1–7	0·068	0·28			4·1	0·59	
		12–60	0·025	0·66	40	0·017	26	1·6	425
		60–200	0·017	1·25			76	2·3	
		300–1000	0·012	2·55			220	3·3	
8·0	NADP$^+$	2–8	0·053	0·19	50	0·0024	3·6	0·95	480
8·0	NAD$^+$	0·2–14	0·015	0·1			6·7	0	
		35–140	0·010	0·44	4	0·002	44	0·4	500
		200–500	0·006	1·1			180	0·7	
Norvaline									
8·0	NAD$^+$	1·3–1300	0·2	54	2500	2·0	270	12	800

* 0·1 M-tris buffer, pH 7·2.

Fig. 5. Variation of the reciprocal of the specific initial rate with the reciprocal of the NADP+ concentration, with sodium phosphate buffer, pH 8·0 and I 0·25, at 25°. The glutamate concentrations were: O, 50mM; ●, 3mM. (a) 2·0–50 μM-NADP+; (b) 50–1090 μM-NADP+.

Fig. 6. Variation of the reciprocal of the specific initial rate with the reciprocal of the NADP+ concentration for gluta-mate concentrations of 50mM (O) and 3mM (●), with 0·1 M-tris–acetate buffer, pH 7·2, at 25°. The lines are extrapolations from measurements with smaller NADP+ concentrations (see the text).

be linear, as at pH 7·0; secondary plots of their slopes and intercepts against the reciprocals of the four NADP+ concentrations used in the range 2·5–10 μM were linear, and allowed estimates of the initial-rate parameters in eqn. (1) for this range of coenzyme concentration (Table 1). It may also be noted that the plots in Fig. 5 for the two glutamate concentrations used cross at about 70 μM-NADP+, indicating that with high coenzyme concentrations there is inhibition with the higher glutamate concentration.

Kinetics in tris–acetate buffer. In 0·1 M-tris–acetate buffer, pH 7·2, initial-rate measurements were made under the same conditions as in the preceding experiment. Lineweaver–Burk plots were linear in the range 2–20 μM-NADP+ (six concentrations), and deviated towards higher activities with higher coenzyme concentrations, as shown in Fig. 6, in which the lines are extrapolations of the linear plots obtained with smaller NADP+ concentrations. The activation is apparently complex, as in phos-phate buffer, pH 8·0. Initial-rate parameters in eqn. (1), evaluated from measurements with 2–20 μM-NADP+ as in the preceding experiment, are given in Table 1.

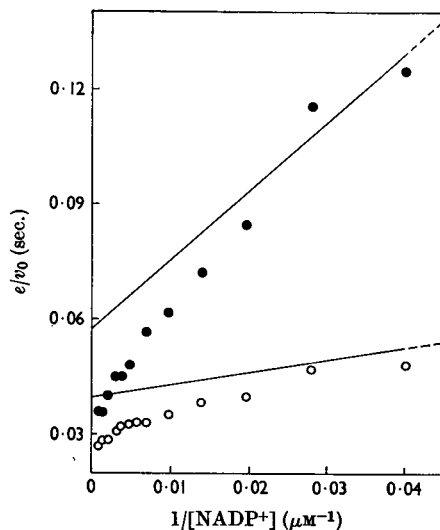A rather more detailed experiment at pH 8·0, in which four glutamate concentrations (3–50mM)

were used, showed a similar activation pattern in Lineweaver–Burk plots against NADP+, but no clear linear regions could be discerned even in the concentration range 2–10 μM-NADP and with the smallest glutamate concentration used; a smooth curve, concave downwards, fitted the points within the experimental error, which was greater than in the preceding experiments. Because of the instability of the enzyme in this buffer, there was marked curvature of progress curves, especially with small glutamate concentrations. No meaningful values for ϕ_0' and ϕ_1' in eqn. (1) could therefore be evaluated. Lineweaver–Burk plots against glutamate, with six NADP+ concentrations in the range 3–750 μM, were linear, as were secondary plots of their slopes against the reciprocal of the NADP+ concentration.

Reaction of glutamate and NAD+

Kinetic measurements with NAD+ as coenzyme were made only in sodium phosphate buffers, I 0·25. At pH 7·0, glutamate concentrations of 3·5, 12 and 50mM were used with 24 different concentrations of NAD+ in the range 1–1000 μM. The enzyme concentration was 1·3 μg./ml. Primary plots with glutamate concentration as variable, some of which are shown in Fig. 7, were all linear. Primary plots with the NAD+ concentration as variable consisted of four linear regions of systematically changing
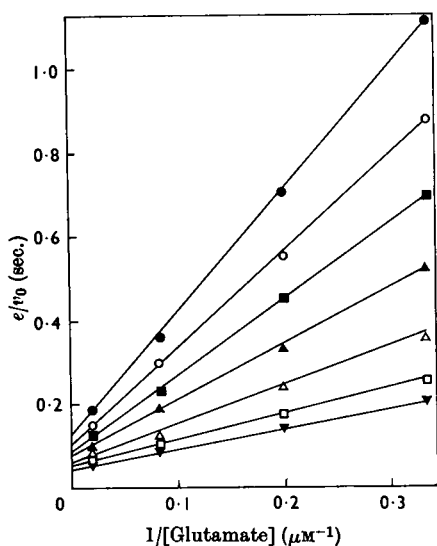
Fig. 7. Variation of the reciprocal of the specific initial rate with the reciprocal of the glutamate concentration, with sodium phosphate buffer, pH 7·0 and I 0·25, at 25°, for several constant NAD⁺ concentrations: ▼, 35·7 μM; □, 25·5 μM; △, 18·4 μM; ▲, 12·2 μM; ■, 9·18 μM; ○, 7·14 μM; ●, 5·1 μM.

slope, with fairly sharp discontinuities at about 10 μM-, 50 μM- and 300 μM-NAD⁺. The results for three glutamate concentrations are shown in Fig. 8 in separate plots for different ranges of NAD⁺ concentration, in order to make clear the closeness of fit to a linear relation in each range; the results for 50 mM-glutamate were shown elsewhere (Dalziel & Engel, 1968). With the smallest glutamate concentration of 3 mM, the slope is 5·23 in Fig. 8(a) and 9·4 in Fig. 8(d). With 50 mM-glutamate, the corresponding slopes were 0·575 and 2·75 respectively.

All the initial-rate parameters in eqn. (1) were evaluated for each of the four regions of NAD⁺ concentration in the usual way, from secondary plots of the slopes and intercepts of Fig. 8 against the reciprocals of the glutamate concentrations; the secondary plots were linear within the experimental error. The parameters were also evaluated by the usual alternative secondary plots of the slopes and intercepts from Fig. 7 against the reciprocals of the NAD⁺ concentrations. The slopes gave linear secondary plots, from which values for ϕ_2' and ϕ_{12}' applicable over the whole range of coenzyme concentration were obtained. The intercepts, which correspond to reciprocal rates with infinitely large glutamate concentrations, gave secondary plots consisting of four linear regions from each of

which ϕ_0' and ϕ_1' were evaluated. The mean values for the parameters are given in Table 1.

At pH 8·0, a very detailed study of the variation of rate with NAD⁺ concentration was made with four glutamate concentrations. With 8 mM-glutamate, 57 NAD⁺ concentrations were used, from 0·2 μM to 2000 μM. Over the 70-fold concentration range of 0·2–15 μM, the Lineweaver–Burk plot against NAD⁺ was accurately linear; at higher concentrations there were marked deviations towards higher activity, but without the sharp discontinuities seen at pH 7·0. Within the experimental error, the plots were linear in the ranges 35–140 μM-NAD⁺ and 200–500 μM-NAD⁺, and for descriptive and comparative purposes the initial-rate parameters in eqn. (1) were evaluated from data for each of these concentrations range as well as the lower range (Table 1). With 8 mM-glutamate, there appeared to be a fourth linear region from 500 μM- to 2000 μM-NAD⁺, but measurements were not made in this range with other glutamate concentrations.

Reaction of norvaline and NAD⁺

Initial rates were measured in sodium phosphate buffer, pH 8·0, I 0·25, with five concentrations of norvaline (5–25 mM) and five concentrations of NAD⁺ (19·9–119 μM). Primary Lineweaver–Burk plots against reciprocal substrate and coenzyme concentrations were all linear, as were secondary plots of the slopes and intercepts, from which the parameters in eqn. (1) were evaluated (Table 1). With a constant norvaline concentration of 15 mM, 12 further concentrations of NAD⁺ were used to cover the range 1·33–1325 μM in detail. As shown in Fig. 9, the Lineweaver–Burk plot is linear over this 1000-fold range of NAD⁺ concentration.

DISCUSSION

Because of the unusual stability of glutamate dehydrogenase in phosphate buffer, it proved possible to make exceptionally precise measurements of initial rates at pH 7–8 by using small concentrations of enzyme and recording the progress of a negligible fraction of reaction to equilibrium with a very sensitive fluorimeter. Progress curves were linear for at least 2 min., even with small concentrations of coenzyme and substrate, and true initial rates in the steady state could be estimated with a precision of ± 1–2%.

For the oxidative deamination of norvaline by NAD⁺ in phosphate buffer, pH 8·0, primary Lineweaver–Burk plots with coenzyme or substrate concentration as variable, and secondary plots of the slopes and intercepts, were linear over the wide ranges of concentrations used. For the oxidative
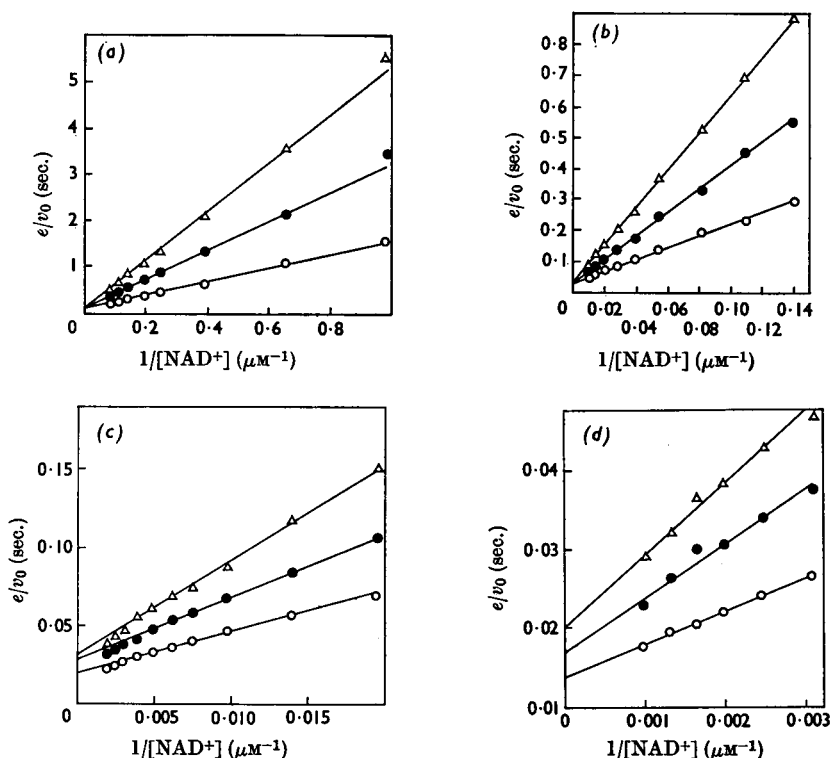
Fig. 8. Variation of the reciprocal of the specific initial rate with the reciprocal of the NAD concentration, with sodium phosphate buffer, pH7·0 and I, 0·25 at 25°, for constant glutamate concentrations: O, 12mM; ●, 5mM; △, 3mM. The concentration ranges of NAD⁺ are: (a)1–12·2μM; (b) 7·1–102μM; (c) 50–500μM; (d) 326–1020μM.
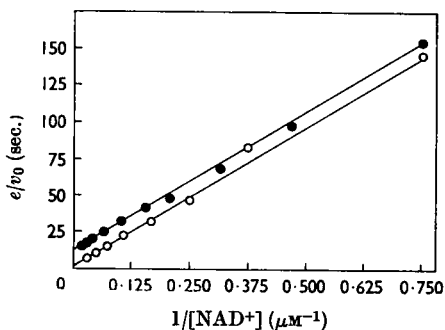

Fig. 9. Variation of the reciprocal of the specific initial rate with the reciprocal of the NAD⁺ concentration for a constant norvaline concentration of 15mM. Two ranges of NAD⁺ concentration are shown: O, 1·33–33μM; ●, 33–1325μM, the specific rates and the NAD concentrations having been divided by 25.

deamination of glutamate, the reciprocal of the initial rate was also linearly related to the reciprocal of the glutamate concentration, except that with

very large glutamate concentrations (> 20mM) substrate inhibition occurred. However, with the NAD⁺ concentration as variable, Lineweaver–Burk plots showed progressive deviations towards higher activity with increasing coenzyme concentration. Such 'activation' by NAD⁺ has been observed previously in phosphate buffer by Olson & Anfinsen (1953) and in tris buffer by Frieden (1959b). Contrary to the findings of these workers, similar activation was observed here with NADP⁺ as coenzyme, in both phosphate and tris buffers, pH7 and pH8.

The measurements in phosphate buffers were sufficiently detailed and precise to establish that with both coenzymes the activation assumes a remarkable pattern: at pH7·0, Lineweaver–Burk plots consisted of three or four linear regions separated by fairly sharp discontinuities, the slopes of the plots increasing in steps as the coenzyme concentration increased. At pH8·0, the plots were clearly linear only up to 8μM-NADP⁺ and 14μM-NAD⁺; above these values, the plots were generally concave downwards, although with NADP⁺ there were significant irregularities and with NAD⁺ there

appeared to be further linear regions. In tris buffer, the results with NADP$^+$ as coenzyme were similar to those in phosphate buffer, pH 8·0, but instability of the enzyme made the measurements less reliable, especially at pH 8·0.

Single discontinuities in Lineweaver–Burk plots for glutamate dehydrogenase have occasionally been recorded previously (di Prisco, Arfin & Strecker, 1965; Anderson & Reynolds, 1966; di Prisco, 1967; H. Sund, personal communication). Such findings might be due to heterogeneity of the enzyme or coenzyme. In the present work, no evidence was found for the presence of more than one protein by recrystallization, chromatography or ultracentrifuge studies of the enzyme, and NADP$^+$ from which nucleotide impurities had been removed by chromatography gave the same results as commercial preparations. In sucrose-gradient-centrifugation experiments with enzyme concentrations similar to those used in kinetic measurements, there was no evidence for more than one molecular species ($S_{20,w}$ 13–14s), and the presence of NAD$^+$ did not affect the sedimentation coefficient. Moreover, the rate of the reaction was proportional to the enzyme concentration over a wide range, and the enzyme activity was not altered by preincubation with NAD$^+$, nor with NAD$^+$ and the substrate analogue glutaric acid. It must be concluded that the peculiar pattern of activation is a real kinetic property of the enzyme in the oxidative deamination of glutamate under the conditions of these experiments, and cannot be explained by an association-dissociation equilibrium of the enzyme molecule.

Initial-rate data for the norvaline reaction, and for the glutamate reaction over each range of coenzyme concentration within which Lineweaver–Burk plots are linear, may be described empirically by eqn. (1) with the experimental values for the kinetic coefficients in Table 1. In the reaction with glutamate the coenzyme activation became more pronounced with increase of the glutamate concentration, and with a sufficiently small constant glutamate concentration, Lineweaver–Burk plots were linear over the whole range of coenzyme concentration. The activation can therefore be described in terms of systematic changes in the values of ϕ_0' and ϕ_1' in eqn. (1) as the coenzyme concentration changes. It appears that in each experiment ϕ_2' and ϕ_{12}' are independent of the coenzyme concentration; with small glutamate concentrations, the rate is largely determined by these kinetic coefficients, and variations of ϕ_0' and ϕ_1' have no significant effect on the rate.

The values for the kinetic coefficients in phosphate buffer in Table 1 show that with glutamate as substrate the enzyme utilizes NAD$^+$ and NADP$^+$ with similar efficiency under all the conditions studied, and it seems likely that the same mechanism

is involved with the two coenzymes. In general, the rate is smaller with NADP$^+$ than with NAD$^+$, especially at pH 8·0, and also at pH 7·0 with large glutamate and coenzyme concentrations. With NAD$^+$, all the kinetic coefficients are smaller at pH 8·0 than at pH 7·0, i.e. the rate is greater at the higher pH value with all substrate and coenzyme concentrations. In 0·1 M-tris buffer, especially at pH 8·0, the data were less reliable because of enzyme instability but indicated that at pH 7·2, ϕ_1' is smaller than in phosphate buffer, whereas ϕ_0' is about the same. The kinetics of the glutamate–NADP$^+$ reaction in 0·1 M-tris buffer, pH 8·0, were previously studied by Frieden (1959b) with limited ranges of coenzyme and substrate concentration, by a spectrophotometric method. The coenzyme activation was not detected. Approximate Michaelis constants estimated from the present data in 0·1 M-tris buffer for the same range of NADP$^+$ concentration (50–500 μM) are of the same order as those reported by Frieden (1959b).

With NAD$^+$ as coenzyme at pH 8·0, norvaline is a poorer substrate than glutamate, and not only because its Michaelis constant (ϕ_2'/ϕ_0') is much greater; the Michaelis constant for NAD$^+$ (ϕ_1'/ϕ_0') is also much bigger, and the maximum rate ($1/\phi_0'$) smaller, with norvaline as substrate. The kinetic differences between the two substrates resemble those between primary and secondary alcohols, rather than those between different primary alcohols as substrates of liver alcohol dehydrogenase (Dalziel & Dickinson, 1966b), and indicate that with norvaline the ternary complex of enzyme, coenzyme and substrate is formed more slowly, and also undergoes reaction to products more slowly, than with glutamate. That the reaction with both substrates involves a ternary complex, as with other dehydrogenases, rather than a double-displacement (enzyme substitution or 'ping-pong') mechanism, is shown by the clear-cut values obtained for ϕ_{12}' under all conditions. For a double-displacement mechanism the last term of eqn. (1) is lacking in the initial-rate equation, i.e. $\phi_{12}' = 0$ (Alberty, 1953; Dalziel, 1957).

The ternary complex may be formed by either ordered or random combination of coenzyme and substrate with the enzyme. In both cases ϕ_{12}' and the corresponding initial-rate parameter, ϕ_{123}, for the reverse reaction, which involves three substrates, should be related to the overall equilibrium constant K by the Haldane relation $\phi_{123}/\phi_{12}' = K/[\text{H}^+]$ (Dalziel, 1969). This relationship is tested in Table 2 with values for ϕ_{123} determined in detailed kinetic studies of the reverse reaction (P. C. Engel & K. Dalziel, unpublished work) and direct estimates of K under the same conditions (Engel & Dalziel, 1967). The agreement with both coenzymes at two pH values is very close. Also, for both an ordered mechanism in which the coenzyme combines

Table 2. *Haldane relationships for the glutamate dehydrogenase reaction in phosphate buffer, I 0·25, at 25°*

Values for the kinetic constant ϕ'_{12} for the oxidative deamination reactions were obtained as described in the text, and those for ϕ_{123} from kinetic studies of the reverse reaction (P. C. Engel & K. Dalziel, unpublished work). The overall equilibrium constants, K, are from Engel & Dalziel (1967).

pH	Coenzyme	ϕ_{123}/ϕ'_{12} (μM)	$K/[H^+]$ (μM)
7·0	NAD$^+$	1·5	1·28
	NADP$^+$	1·0	0·77
8·0	NAD$^+$	12	12·8
	NADP$^+$	7·3	7·7

first, and a rapid-equilibrium random mechanism, ϕ'_{12}/ϕ'_2 should be equal to the dissociation constant of the binary enzyme–coenzyme compound (Dalziel, 1957). Within the experimental errors, this ratio is constant for all the experiments in phosphate buffers (Table 1). It may be noted that ϕ'_2 is particularly difficult to determine accurately in the experiments with glutamate because of the coenzyme activation. A value of 300–400 μM was reported by Kubo *et al.* (1957) for the dissociation constant of the enzyme–NAD$^+$ compound in phosphate buffer, pH 6·8, estimated by the ultracentrifuge method of Hayes & Velick (1954). This is in satisfactory agreement with the value of ϕ'_{12}/ϕ'_2 from the experiment with NAD$^+$ at pH 7·0 in Table 1. Further, this ratio should be independent of the substrate used. The values obtained in comparable experiments with glutamate and norvaline agree within the combined experimental errors in spite of the enormous differences between the individual initial-rate parameters for the two substrates.

A compulsory-order mechanism in which the substrate must combine first with the enzyme is unlikely, since the coenzymes are known to combine with the enzyme in absence of substrates. In the initial-rate equation for this mechanism ϕ'_2 is the reciprocal of the velocity constant for the combination of enzyme and substrate (Dalziel, 1957), and should therefore be independent of the nature of the coenzyme. This is not so in the experiments with glutamate, especially at pH 8·0. Similarly, if the coenzyme combines before the substrate in a simple compulsory-order mechanism, ϕ'_1 will be the reciprocal of the velocity constant for this initial reaction, and the same values should be obtained in comparable experiments with glutamate and norvaline. Although coenzyme activation with glutamate as substrate makes this simple interpretation equivocal, the fact that the value for ϕ'_1 from the experiment with norvaline is 50 times the highest estimate

from the experiment with glutamate is strong evidence against a simple ordered sequence with coenzyme leading in the reaction with both substrates. Such a mechanism could apply, however, with one of the two substrates.

A similar situation exists for liver alcohol dehydrogenase with primary and secondary alcohols as substrates. There is very strong evidence for a compulsory-order mechanism with primary alcohols but with secondary alcohols, which are poorer substrates, much larger values for ϕ'_0 and ϕ'_1 were obtained than with primary alcohols (Dalziel & Dickinson, 1966b). It was nevertheless concluded that a modified compulsory-order mechanism also operates with secondary alcohols; the slower intramolecular reaction of the ternary complex results in larger values for ϕ'_0 and also a larger steady-state concentration of this complex, which permits dissociation of NAD$^+$ and the formation of a 'dead-end' enzyme–substrate complex. The value of ϕ'_1 may then be determined by the dissociation constant for NAD$^+$ from this complex rather than the rate of combination of NAD$^+$ with free enzyme. A similar interpretation is possible for the glutamate dehydrogenase reactions, and receives some support from recent pre-steady-state studies by Pantaloni & Iwatsubo (1967), who concluded that glutamate oxidation is limited by the rate of liberation of the reduced coenzyme, whereas with norvaline and alanine the formation of the 'Michaelis complex' is rate-limiting.

It is concluded that the available data indicate a ternary-complex mechanism, and are consistent with either random combination of coenzyme and substrate, or a compulsory-order mechanism in which the coenzyme combines first with enzyme. Further evidence of the mechanism might be obtained by studies of isotope exchange at equilibrium (Silverstein & Boyer, 1964), of binding of substrates by the enzyme, and of the kinetics of the reactions of the enzyme with its coenzymes by rapid-reaction techniques.

Substrate inhibition with sufficiently large concentrations of glutamate was observed under all the experimental conditions, and was most marked with large coenzyme concentrations. This is consistent with the formation of an abortive complex, E–NAD(P)H–glutamate, from which NAD(P)H dissociates more slowly than from the normal product complex E–NAD(P)H. There is evidence for the formation of such a complex from the effect of glutamate on the fluorescence of the enzyme–NADH compound (F. M. Dickinson, unpublished work).

Coenzyme activation was observed previously only with NAD$^+$, and was attributed to binding at an allosteric site, distinct from the active site, at which purine nucleotide effectors also bind (Frieden,

1959a, 1963). Other possible explanations, which do not require the postulate of a binding site for the coenzyme other than the active site, are formation of an abortive complex (Dalziel & Dickinson, 1966b), and a random-order mechanism (Dalziel, 1958). For an enzyme with more than one active centre per molecule, interactions between the active centres provide yet another possible explanation of substrate or coenzyme activation (Dalziel & Engel, 1968; Conway & Koshland, 1968). At the concentrations used in kinetic experiments glutamate dehydrogenase has a molecular weight of about 300000 and consists of four to six identical subunits (cf. Jirgensons, 1961; Marler & Tanford, 1964; Rogers, Hellerman & Thompson, 1965; Appella & Tomkins, 1966; Eisenberg & Tomkins, 1968; Sund & Burchard, 1968). Although it does not exhibit the characteristic sigmoid relations between rate and substrate or coenzyme concentration, indicative of positive homotropic interactions between active centres, the enzyme has long been considered to be an allosteric protein. Lineweaver–Burk plots that are concave downwards correspond to plots of rate against substrate concentration that do not obviously differ from a rectangular hyperbola, but are in fact steeper below the half-saturation point and less steep above it. This is the opposite type of deviation from Michaelis–Menten behaviour to a sigmoid rate plot, which corresponds to a Lineweaver–Burk plot that is concave upwards, and both types of plot can be described by a rate equation of the same form as that of Adair (1925) for ligand binding by a multivalent protein, with appropriate relations between the constants (Dalziel & Engel, 1968). The existence of negative interactions between the active centres of glutamate dehydrogenase therefore provides a feasible explanation of the coenzyme activation. Moreover, Lineweaver–Burk plots that are not simply concave downwards, but show several sharp changes of slope, are to be expected if the negative interactions are strong enough, as has been shown theoretically (Dalziel & Engel, 1968), and are not easily explained by the other mechanisms considered. The results of this kind obtained with both coenzymes in phosphate buffer at pH 7·0 therefore greatly strengthen the hypothesis.

Monod, Wyman & Changeux (1965) pointed out that allosteric systems appeared to be always positive or co-operative, and their model for such systems cannot account for variations of rate (or substrate binding) with substrate concentration of the type described here. On the other hand, the model for allosteric proteins put forward by Koshland, Nemethy & Filmer (1966), based on the site-interaction hypothesis of Adair (1925), is symmetrical in that interactions may be positive or negative, i.e. ligand binding at one site may increase or decrease the ligand affinity of the remaining sites.

The present results therefore favour the latter model for glutamate dehydrogenase (Dalziel, 1968; Dalziel & Engel, 1968). Similar conclusions have been reached by Conway & Koshland (1968) for glyceraldehyde 3-phosphate dehydrogenase, but their kinetic data, presented as plots of v_0 against NAD+ concentration, are not sufficiently detailed to show whether the characteristic discontinuities in Lineweaver–Burk plots occur. It is worth emphasizing that negative interactions are more clearly revealed in the latter type of plot, and in analogous reciprocal plots of binding data, than in the direct plots of rate or fractional saturation usually employed for allosteric enzymes.

The complete absence of coenzyme activation in the norvaline reaction, even with large norvaline concentrations and rate-limiting coenzyme concentrations, is striking. This suggests either that the negative interactions involve conformational changes of a ternary complex of enzyme, coenzyme and glutamate that do not occur with the poorer substrate, or that the negative interactions involve steps that are rate-limiting in the reaction with glutamate but not in the much slower reaction with norvaline. The first explanation could apply if an equilibrium random-order mechanism is followed with both substrates; ϕ_0' would then be the reciprocal of the velocity constant for the rate-limiting formation of products in the ternary complex, and ϕ_1'/ϕ_0' would be the dissociation constant for NAD(P)+ from the ternary complex (Dalziel, 1957). The second explanation would be applicable to the compulsory-order mechanisms for the two substrates discussed above. With glutamate as substrate, ϕ_1' would be the reciprocal of the velocity constant, k_1', for the combination of oxidized coenzyme with the free enzyme, and ϕ_0 the reciprocal of the velocity constant, k_{-1}, for the dissociation of the reduced coenzyme product. The variation of these parameters with coenzyme concentration would then indicate stepwise decreases of k_1' for the combination of successive NAD(P)+ molecules and possibly decreases of k_{-1} for the dissociations of successive NAD(P)H molecules. This was illustrated previously by steady-state analysis of a simple model with two active centres (Dalziel & Engel, 1968). With norvaline, on the other hand, these parameters may be determined by the same steps as in a random-order mechanism (Dalziel & Dickinson, 1966b) and conformational changes of binary enzyme–coenzyme complexes would then not affect the overall rate.

The detailed experimental data obtained with glutamate could of course be fitted by a theoretical equation, and values for the constants derived, but this would be premature until sufficiently detailed equilibrium and kinetic studies of the enzyme–coenzyme reactions are available to establish the

number of active centres per molecule and whether negative interactions are involved in these steps. The number of discontinuities in Lineweaver–Burk plots does not of course necessarily indicate the number of active centres. The fact that the results with NAD+ show four such discontinuities and those with NADP+ only three may indicate that the negative interactions are weaker in the latter case, but there may also be other discontinuities outside the concentration range of these experiments.

Whereas the effect of positive interactions is to make an enzyme very sensitive to small changes of substrate or coenzyme concentration over a narrow range, the effect of negative interactions is to make the reaction rate continuously responsive to changes in concentration over a very wide range. For example, in phosphate buffer, pH 7·0, the Michaelis constant for NAD+ (ϕ_1'/ϕ_0') is 220 μM for the concentration range 300–1000 μM, 76 μM for the range 60–200 μM, 26 μM for the range 12–60 μM and 4·1 μM for the range 1–7 μM (Table 1). The maximum rate ($1/\phi_0'$) decreases sixfold from the highest to the lowest concentration range. The result is that a reasonable rate of reaction is maintained with small coenzyme concentrations because of the rapid combination of the first coenzyme molecule, and yet the rate increases substantially with greater coenzyme concentrations. The effect is as if the number of binding sites increases with the coenzyme concentration, the successive sites having larger Michaelis constants for the coenzyme and perhaps greater maximum turnover rates (Dalziel & Engel, 1968). Similar considerations were put forward by Conway & Koshland (1968) in terms of affinities, and the possible advantages in metabolic control were discussed. As has been pointed out previously (Dalziel & Engel, 1968), sufficiently detailed and precise initial-rate or binding studies may reveal evidence of negative interactions with other enzymes, now that the characteristic double-reciprocal plots associated with the phenomenon have been recognized.

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