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The Binding of Oxidized Coenzymes by Glutamate Dehydrogenase and the Effects of Glutarate and Purine Nucleotides

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1. The binding of NAD^+ and $NADP^+$ to glutamate dehydrogenase has been studied in sodium phosphate buffer, pH 7.0, by equilibrium dialysis. Approximate values for the dissociation constants are 0.47 and 2.5 mM respectively. For NAD^+ the value agrees with that estimated from initial-rate results. 2. In the presence of the substrate analogue glutarate both coenzymes are bound more firmly, and there is one active centre per enzyme subunit. The binding results cannot be described in terms of independent and identical active centres, and binding is stronger at low coenzyme concentrations than at high concentrations. Either the six subunits of the oligomer are not identical or there are negative interactions between them in the binding of coenzymes in ternary complexes with glutarate. The latter explanation is favoured. 3. The binding studies support the conclusions drawn from earlier kinetic studies of the glutamate reaction. 4. ADP and GTP respectively decrease and increase the affinity of the enzyme for NAD^+ and $NADP^+$, in both the presence and absence of glutarate. The negative binding interactions in the presence of glutarate are abolished by ADP, which decreases the affinity for the coenzymes at low concentrations of the latter. 5. In the presence of glutarate, GTP and NAD^+ or $NADP^+$, the association of enzyme oligomers is prevented, and the solubility of the enzyme is decreased; the complex of enzyme and ligands readily crystallizes. 6. The results are discussed in relation to earlier kinetic studies.

The active oligomer of ox liver glutamate dehydrogenase (EC 1.4.1.3) has a molecular weight of $3.2\times10^5\pm0.2\times10^5$ and appears to be composed of six polypeptide chains of molecular weight $5.7\times10^4\pm0.3\times10^4$ (Cassman & Schachman, 1971; Smith *et al.*, 1970). Fluorescence-enhancement and polarization measurements indicate one binding site for NADH on each subunit (Bayley & Radda, 1966; Krause & Sund, 1970). The enzyme forms an abortive ternary complex with NADH or NADPH and glutamate in which the nicotinamide chromophore is perturbed to a much greater extent than in the binary enzyme–coenzyme compounds (Egan & Dalziel, 1971). This large hypochromic effect allowed spectrophotometric titration of the enzyme with NADH and NADPH, in the presence of excess of glutamate, from which an active-centre equivalent weight of 5.7×10^4 was obtained (Egan & Dalziel, 1971).

Detailed initial-rate studies of the oxidative deamination of glutamate showed that, contrary to earlier conclusions (Olson & Anfinsen, 1953; Frieden, 1959*a*), coenzyme 'activation' is exhibited with $NADP^+$ as well as with NAD^+ (Dalziel & Engel, 1968; Engel & Dalziel, 1969). In phosphate buffer at pH 7.0, the 'activation' is of a peculiar type; with a constant large glutamate concentration Lineweaver–Burk plots for wide ranges of $NADP^+$ or NAD^+ concentration showed three or four linear regions of different slope with relatively sharp discontinuities. On the assumption that the enzyme subunits are identical, it was suggested that negative interactions between the active centres of an oligomer are a more likely explanation of these findings than complexities of the reaction mechanism affecting identical and independent active centres, such as a non-equilibrium, random-order mechanism, abortive-complex formation, or an 'activating' site for the oxidized coenzymes in addition to the active centre (Dalziel & Engel, 1968; Engel & Dalziel, 1969).

It is established that the catalytic reaction sequence involves a ternary complex of enzyme, oxidized coenzyme and glutamate, but not whether the complex is formed solely by ordered combination of enzyme and coenzyme followed by glutamate, or by a random mechanism in which glutamate can also combine with the free enzyme (Engel & Dalziel, 1969). The rate-limiting steps with large glutamate and non-saturating coenzyme concentrations, and therefore the steps at which the proposed negative interactions occur, would be different in the two cases. For an ordered mechanism in which coenzyme combines first it would be the rate of combination of the latter with the enzyme. For a random mechanism, it would be the dissociation of coenzyme from the active ternary complex with glutamate (Engel & Dalziel, 1969).

Co-operative interactions between enzyme subunits, whether positive or negative, cannot be conclusively demonstrated by kinetic studies alone. Thermodynamic studies of ligand binding will serve to characterize positive interactions, but will not distinguish negative interactions between identical sites from independent binding at sites that have different intrinsic affinities (Wyman, 1948). Binding studies with NADH have not as yet revealed any such effects with glutamate dehydrogenase, and no detailed studies of the binding of oxidized coenzymes have been reported.

In the present paper, studies by equilibrium dialysis of the binding of NAD^+ and $NADP^+$ to the enzyme, and the effects of the substrate analogue glutarate and the allosteric effectors ADP and GTP on coenzyme binding, are reported. The objectives were to obtain further evidence of the mechanism of the overall reaction and to test the hypothesis that negative interactions in the ternary complex are responsible for the complex pattern of coenzyme 'activation'.

Materials and Methods

Ox liver glutamate dehydrogenase was obtained from Boehringer Corp. (London) Ltd. (London W.5, U.K.), as a crystalline suspension in $(NH_4)_2SO_4$ solution. Several batches of enzyme were used in the course of the work, and no significant differences of specific activity or variations in the results of binding experiments were observed. In earlier work, no evidence of inhomogeneity of enzyme preparations from this source was obtained (Engel & Dalziel, 1969; Engel, 1968). Recrystallization with Na_2SO_4 gave crystals and mother liquid with the same specific activity of 1.00 ± 0.05 μmol of NAD^+/min per mg, and chromatography on DEAE-cellulose also showed no heterogeneity. Recently dry-weight and absorption measurements (Egan & Dalziel, 1971) gave $E^{1\%}_{1cm} = 9.3$ at 280nm, which is 4% smaller than the earlier value (Olson & Anfinsen, 1952). For each experiment a sample of the crystalline suspension was thoroughly dialysed against 0.11M-sodium phosphate buffer, pH7.0, containing 10μM-EDTA, and clarified by centrifugation.

NAD^+, $NADP^+$, GTP and ADP were from Boehringer Corp. (London) Ltd. Monosodium L-glutamate was obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.). Glutaric acid, from BDH Chemicals Ltd. (Poole, Dorset, U.K.), was recrystallized from benzene–ether.

[^{14}C]NAD^+ was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The specific radioactivity was 55mCi/mmol of nucleotide. Spectrophotometric and enzymic assay showed that 74% of the total nucleotide was coenzymically active NAD^+. The scintillation fluid consisted of 8g of 5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole in 700ml of toluene and 300ml of Triton X-100, and was prepared at least 2 days before use. Phosphate buffers were prepared from AnalaR $NaH_2PO_4,2H_2O$ and Na_2HPO_4.

Equilibrium dialysis

Equilibrium dialyses were carried out at 23°C in the medium used in the kinetic studies of Engel & Dalziel (1969), namely 0.11M-sodium phosphate buffer, pH7.0, containing 10μM-EDTA, in which the enzyme is very stable. Enzyme, nucleotide and glutarate solutions were made up in this buffer. Special dialysis cells were not used; 0.5–0.8ml samples of enzyme solution were placed in sacs of narrow Visking tubing, knotted and tied with linen thread to leave a small air space, and dialysed against 5.2ml portions of coenzyme solutions in tubes with ground-glass stoppers secured by adhesive tape. The tubes were clipped to a revolving wheel for 24h. Control experiments with buffer solution in the sac showed that equilibrium was reached within 8h, and occasional duplicates in dialysis experiments gave the same results after 18h as after 24h.

The Visking tubing was prepared by boiling in 0.05M-NaOH containing 10mM-EDTA, washing in water, soaking in the phosphate buffer and drying by squeezing with filter paper just before filling. The enzyme concentration was 10.4mg/ml in all experiments. Since glutarate was used at relatively large concentrations (39–154mM) it was added initially to the enzyme solution as well as to the diffusates. After dialysis, the sac contents were centrifuged for 5min at 40000g before analysis. The protein concentration after dialysis was within 5% of the initial value, and in several experiments initial-rate measurements showed no loss of activity. The recovery of the total coenzyme was about 95%.

Spectrophotometric methods of analysis

Spectrophotometric measurements were made with a Unicam SP.500 series 2 spectrophotometer.

Protein concentrations were determined either by measurements of E_{280}, by using $E^{1\%}_{1cm} = 9.3$ (Egan & Dalziel, 1971), or by the method of Lowry *et al.* (1951) calibrated with glutamate dehydrogenase. Blanks were measured on samples of diffusates.

The equilibrium concentrations of NAD^+ and $NADP^+$ in diffusates and sac contents were estimated by measurements of E_{340} in cells of 1cm path-length, after reduction by excess of glutamate and glutamate dehydrogenase at pH7.9. Calibrated pipettes were used, and duplicate or triplicate analyses were made. For diffusates, the assay mixture consisted of 0.05–1.5ml of diffusate, according to the coenzyme concentration, together with sufficient 0.1M-NaOH

to adjust the pH to 7.9, 0.05–0.2ml of glutamate dehydrogenase (10.4mg/ml) and 0.4M-sodium phosphate buffer, pH7.9, to give a total volume of 2.6ml. After measurement of the blank, 0.5ml of 1.0M-glutamate in pH7.9 buffer was added. Reduction was complete within 3min or less. Sac contents containing more than 180μM-NAD(P)$^+$ were assayed in the same way, 0.05–0.2ml samples without additional enzyme being used. The coenzyme concentration was calculated, after appropriate blank corrections, by using $\epsilon_{mM} = 6.22$ for NAD(P)H at 340nm (Horecker & Kornberg, 1948). Controls with dilutions of NAD(P)$^+$ solutions assayed by a standard technique (Engel & Dalziel, 1967) showed that these methods were valid with the range of enzyme concentrations used, and in the presence of glutarate, ADP and GTP.

For smaller coenzyme concentrations in sac contents, semi-micro cells were used, with 0.2–0.3ml of sac contents and the requisite 0.1M-NaOH and pH7.9 buffer to give a total volume of 1.1ml. After measurement of the blank, 0.2ml of 1.0M-glutamate was added. However, controls for this method, in which the enzyme concentration is large and the active-centre concentration greater than that of the coenzyme, showed that the coenzyme concentrations calculated from the extinction coefficient of 6.22 were 19% smaller than the theoretical values. Further investigation showed that a ternary complex of enzyme, NAD(P)H and glutamate is formed under these conditions, and that the extinction coefficient at 340nm is 19% smaller than that of free NAD(P)H (Egan & Dalziel, 1971). A value of $\epsilon_{mM} = 5.0$ was therefore used to calculate the coenzyme concentration from these measurements.

Analyses by scintillation counting

In some equilibrium-dialysis experiments, [^{14}C]-NAD$^+$ was used and the ligand concentrations were determined with a Beckman CPM200 liquid-scintillation counter. The same concentration of [^{14}C]-NAD$^+$ (0.2ml = 0.2μCi) was added initially to all the diffusates, and standards were included in which the sac contents consisted of buffer solution in place of enzyme solution. The NAD$^+$ concentrations in sac contents and diffusates were then calculated from [c.p.m. in diffusate or sac contents/c.p.m. in standard (10000)] $\times$ [NAD$^+$]$_0$, where [NAD$^+$]$_0$ is the theoretical NAD$^+$ concentration corresponding to equal distribution in diffusate and sac contents, calculated from the total volume of diffusate and sac contents and the amount of NAD$^+$ added initially to each diffusate. For radioactivity counting, duplicate 0.2ml samples, 0.8ml of water and 10ml of scintillation fluid were mixed by hard shaking. Clear solutions were obtained, and after standing in the dark for 1h were counted for radioactivity for 20min. Background counts (100c.p.m.) were obtained for diffusates and sac contents without [^{14}C]NAD$^+$ and control experiments showed that glutarate, ADP, GTP and variation of total NAD$^+$ concentration did not affect the counts. A correction of 2% was, however, required for quenching by protein. Duplicates agreed to within 1%.

Although spectrophotometric and radioisotopic analyses gave qualitatively similar binding curves, the differences between the two methods of analysis, and between experiments with different samples of [^{14}C]NAD$^+$, were larger than the expected analytical errors. Because of the relatively low purity of the [^{14}C]NAD$^+$ samples, and the possible effects of labelled or unlabelled nucleotide impurities, it was considered that the spectrophotometric analyses were more reliable, and the results reported were obtained by the latter method except where otherwise stated.

Ultracentrifuge measurements

Sedimentation-velocity measurements were made at 20°C in a Spinco model E analytical ultracentrifuge with schlieren optics and rotor AN-D2. The rotor speed was 59780rev./min, and photographs were taken every 4min for 30min.

Results

Effect of glutarate on NAD(P)$^+$ binding

The results of several equilibrium-dialysis experiments on the binding of NAD$^+$ in the presence and absence of glutarate are shown in Fig. 1 as a plot of the reciprocal of the concentration of bound NAD$^+$ against that of free NAD$^+$. In the absence of glutarate the binding is weak, and the experimental error of estimates of bound NAD$^+$ is large. The maximum binding capacity of the enzyme, as well as the dissociation constant, cannot be estimated accurately, since at the highest concentration of NAD$^+$ used (2mM) the bound NAD$^+$ is less than 10% of the total NAD$^+$ in the protein solution. The results suggest that the maximum binding capacity may be about half that in the presence of glutarate, but this is not clearly outside the experimental error. From the line drawn, an approximate estimate of 0.47mM for the dissociation constant of the enzyme–NAD$^+$ compound is obtained as the ratio of the slope to the intercept (Klotz *et al.*, 1946).

The experiments show unequivocally that glutarate greatly increases the affinity of the enzyme for NAD$^+$. The more reliable extrapolation in this case indicates a maximum binding capacity of about 0.18mM-NAD$^+$ for the enzyme concentration of 10.4mg/ml, consistent with a combining weight of 57000 (Egan & Dalziel, 1971; Cassman & Schachman, 1971).

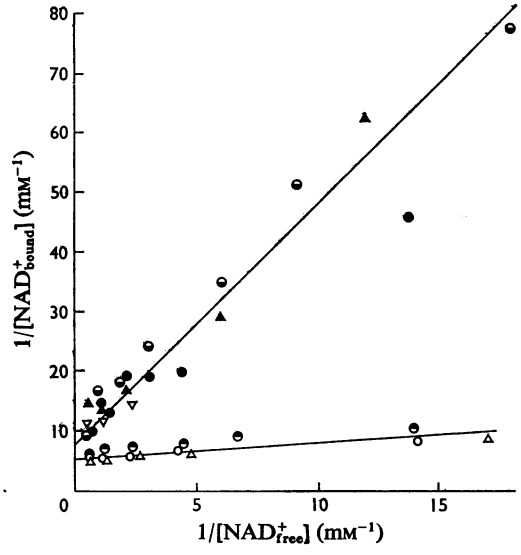


Fig. 1. *Effect of glutarate on the binding of NAD$^+$ to glutamate dehydrogenase*

Equilibrium-dialysis experiments at 23°C in 0.11M-sodium phosphate buffer, pH 7.0, were carried out as described in the Materials and Methods section. The concentrations of NAD^+ in diffusates and protein solution were measured by scintillation counting of [^{14}C]NAD^+ in two experiments (⊖, ⊖) and by spectrophotometric analysis in the other experiments. The results of several experiments in the absence of glutarate (⊖, ●, ▲, ▽) and in the presence of 39mM-glutarate (○, ⊖) and 154mM-glutarate (△) are shown. The protein concentration was 10.4mg/ml in all experiments.

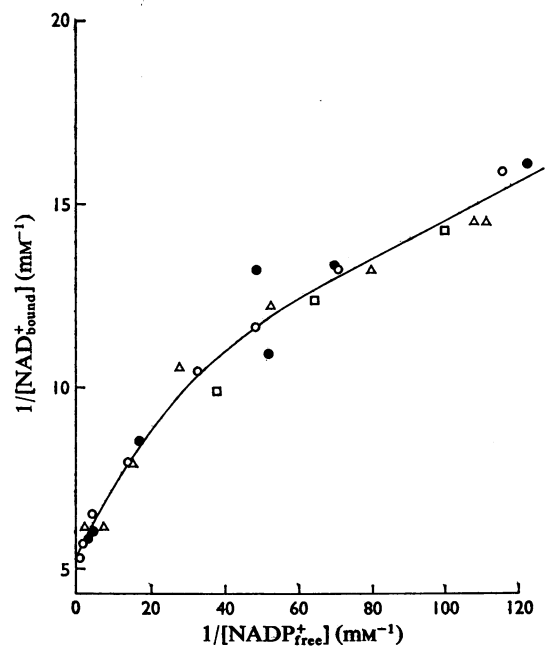


Fig. 2. *Effect of glutarate on the binding of NAD$^+$ to glutamate dehydrogenase*

The glutarate concentrations were 39mM (○, △, □) and 154mM (●). Other experimental details were as given for Fig. 1.

Results from these experiments in the presence of two concentrations of glutarate down to smaller free NAD^+ concentrations are shown in Fig. 2. The plots are clearly not linear, and the binding cannot be described by a single dissociation constant. The slope at low free NAD^+ concentrations corresponds to a dissociation constant of 6μM, whereas a tangent at high free NAD^+ concentrations suggests a dissociation constant of at least 40μM (cf. Fig. 1).

The results of analogous experiments with $NADP^+$ were similar in all respects, and the slope at low free $NADP^+$ concentrations in the presence of glutarate (Fig. 3) indicates a dissociation constant of about 8μM.

Effects of ADP and GTP

In the absence of glutarate, no significant binding of NAD^+ to the enzyme could be detected in the presence of 1mM-ADP with NAD^+ concentrations up to 2mM. However, 0.8mM-GTP did not prevent NAD^+ binding, and indeed appeared to increase the affinity of the enzyme for the coenzyme slightly, the dissociation constant from the results shown in Fig. 4 being 0.3mM.

ADP and GTP also significantly affected the binding of the oxidized coenzymes when glutarate was present. As shown in Fig. 5, ADP does not then prevent the binding of NAD^+, but does profoundly affect the form of the saturation curve. The Klotz plot is linear within the experimental error and gives a dissociation constant of 60μM. Thus the firm binding at low concentrations of NAD^+ in the presence of glutarate is eliminated by ADP and the binding can be described by a single dissociation constant, which is of the same order of magnitude as that indicated with high concentrations of glutarate, but considerably smaller than that for NAD^+ binding to the enzyme alone, in the absence of glutarate or ADP (Fig. 1). Similar results were obtained for the binding of $NADP^+$ in the presence of glutarate and ADP (Fig. 6), the dissociation constant being 93μM.

The effect of GTP on NAD^+ binding in the presence of glutarate is different from that of ADP. The fractional saturation of the enzyme with NAD^+ at

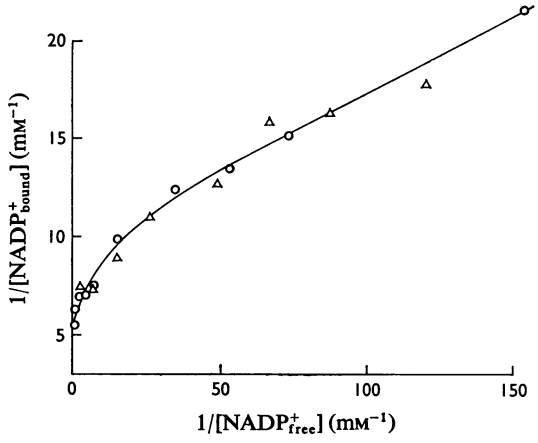


Fig. 3. *Binding of NADP+ to glutamate dehydrogenase in the presence of* 39 mM-*glutarate*

Experimental conditions were as given for Fig. 1.

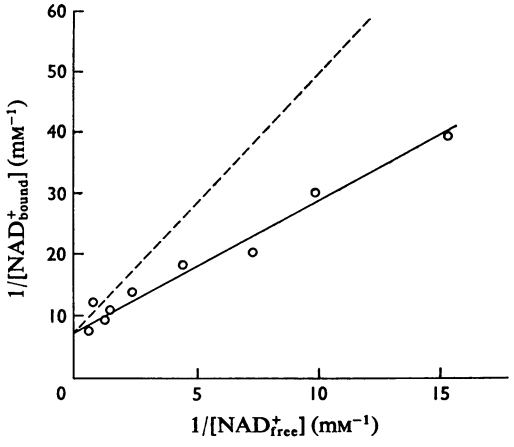


Fig. 4. *Binding of NAD+ to glutamate dehydrogenase in the presence of* 0.8 mM-*GTP*

The broken line represents the binding in the absence of GTP, taken from the results of Fig. 1. Other experimental details were as given for Fig. 1.

low free-coenzyme concentrations is increased, but there is still evidence of deviations from linearity in the Klotz plot (Fig. 7). The dissociation constant for NAD^+ at low concentration is about 3 μM.

Effects of ligands on the sedimentation coefficient of glutamate dehydrogenase

The active oligomer of glutamate dehydrogenase exhibits a concentration-dependent association,

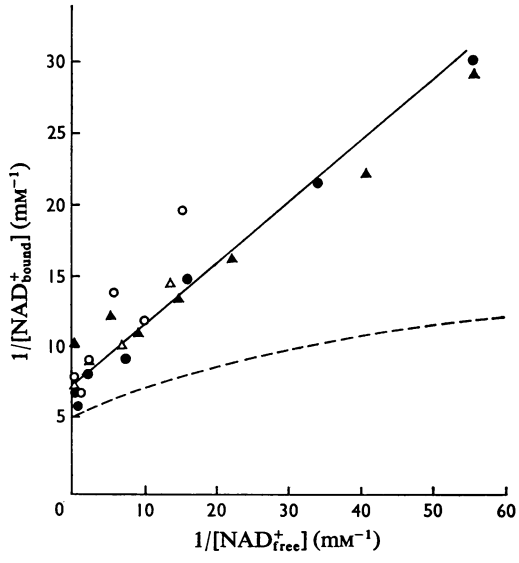


Fig. 5. *Binding of NAD+ to glutamate dehydrogenase in the presence of glutarate and ADP*

The results of five separate experiments are shown. The ligand concentrations were 39 mM-glutarate and 1 mM-ADP. In one experiment, 0.3 mM-ADP was also used with three different NAD^+ concentrations and gave the same results as 1 mM-ADP. Other details were as given for Fig. 1. The broken line shows the binding curve in the presence of glutarate and absence of ADP, taken from Fig. 2.

which is affected by coenzymes and other nucleotides (Frieden, 1959*a*,*b*). Since association is extensive at the enzyme concentrations used in the present binding studies, the effects of glutarate, ADP and GTP on the binding of NAD^+ might be related to their effects on the degree of association of the enzyme. Sedimentation-velocity measurements were therefore made in the presence of the various ligands, and the results are shown in Table 1. There is a slight increase in sedimentation coefficient in the presence of NAD^+, previously noted (Frieden, 1959*a*), but glutarate and ADP have no further significant effect. In the presence of glutarate and NAD^+, however, GTP decreases the sedimentation coefficient to the value for the unassociated oligomer of the enzyme. It has been shown previously that GTP has the same effect in the presence of NADH and NADPH, but not in the absence of the coenzyme (Frieden, 1963).

Discussion

In the absence of substrate analogue, the binding of NAD^+ and $NADP^+$ to the enzyme is weak, and the

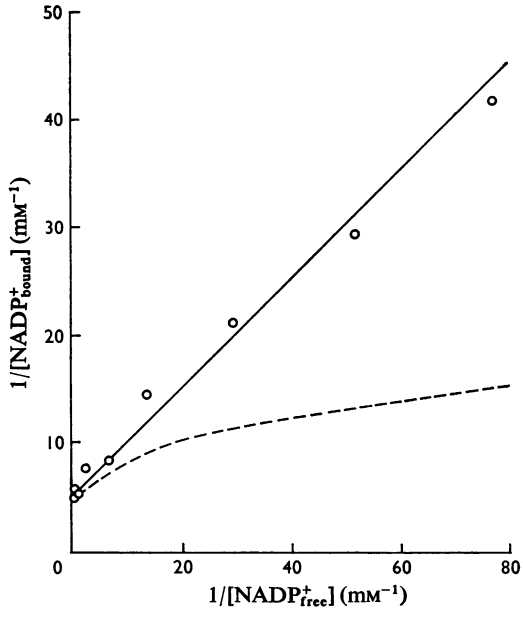


Fig. 6. *Binding of NADP+ to glutamate dehydrogenase in the presence of* 39 mM-*glutarate and* 1 mM-*ADP*

The broken line is the binding curve in the presence of glutarate and the absence of ADP, taken from Fig. 3.

estimates of bound coenzyme concentration are therefore subject to a large experimental uncertainty. It appears that the binding of each coenzyme can be described by a single dissociation constant. For NAD^+, the approximate estimate of 0.47 mM agrees well with the value of 0.43 mM calculated from the ratio of initial-rate parameters, ϕ'_{12}/ϕ'_2, for the oxidative deamination of glutamate in the same buffer; this ratio (Engel & Dalziel, 1969) should be equal to the dissociation constant of the binary enzyme–NAD^+ complex for a ternary-complex mechanism, whether random- or compulsory-order. The only other direct estimate of this constant, by the ultracentrifuge method, is 0.3–0.4 mM in 0.1 M-phosphate buffer, pH 6.8 (Kubo *et al.*, 1957). For $NADP^+$, a single equilibrium-dialysis experiment gave a value of about 2.5 mM, compared with the kinetic estimate of 0.75 mM (Engel & Dalziel, 1969); the difference is not outside the combined experimental uncertainties.

With such weak binding it is not possible to obtain a reliable estimate of the maximum coenzyme-binding capacity in the absence of substrate analogue. Extrapolation of the results from several experiments with NAD^+ suggests that it may be less than six

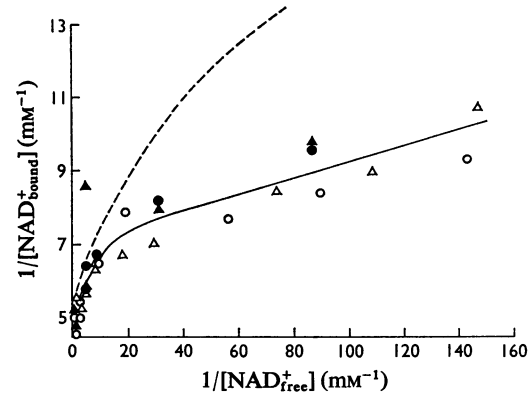


Fig. 7. *Binding of NAD+ to glutamate dehydrogenase in the presence of glutarate and GTP*

The glutarate concentration was 39 mM. The GTP concentrations were 0.8 mM (○, △), 0.7 mM (▲) and 1.3 mM (●). The broken line is the binding curve without GTP present, taken from Fig. 2.

Table 1. *Sedimentation coefficients for glutamate dehydrogenase in the presence of ligands*

The measurements were made in 0.11 M-sodium phosphate buffer, pH 7.0, at 20°C in a double-sector cell. The protein concentration was 10.4 mg/ml.

Ligands present	*S*
None	22.8
1.6 mM-NAD^+	24.3
32 mM-Glutarate	22.9
1.6 mM-NAD^+ + 32 mM-glutarate	25.1
0.2 mM-NAD^+ + 40 mM-glutarate + 1 mM-ADP	24.8
2.0 mM-NAD^+ + 40 mM-glutarate + 1 mM-ADP	25.7
0.2 mM-NAD^+ + 40 mM-glutarate + 0.7 mM-GTP	12.5
2.0 mM-NAD^+ + 40 mM-glutarate + 0.7 mM-GTP	12.9

molecules per oligomer (Fig. 1). This could indicate that two or three subunits do not bind NAD^+, or that their affinity for NAD^+ is extremely small, either because the six subunits are not identical or because there are negative interactions between them; but it is obviously impossible to establish the point by binding studies.

The experiments in the presence of glutarate show clearly that the substrate analogue greatly strengthens the binding of both NAD^+ and $NADP^+$, and that the

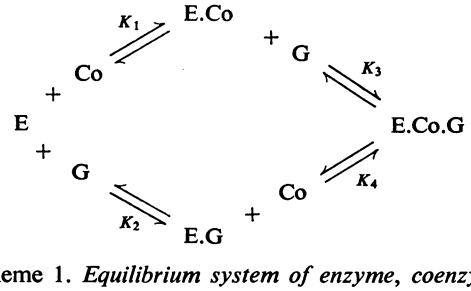
maximum binding capacity is one molecule per enzyme subunit (Figs. 1–3). This confirms the conclusion, from spectrophotometric titrations of the enzyme with NAD(P)H in the presence of excess of glutamate, that there is one active centre per subunit (Egan & Dalziel, 1971). However, the binding of the oxidized coenzymes in the presence of glutarate cannot be described by a single dissociation constant. For both NAD^+ and $NADP^+$, the Klotz plots show reproducible deviations from linearity which are outside the experimental error.

At the concentration of 10mg/ml used in these experiments, the enzyme exists as high-molecular-weight aggregates of the oligomer (Olson & Anfinsen, 1952). It is conceivable that the affinity of the oligomer for coenzyme may be altered in the polymers, and that glutarate may exert its effect on coenzyme binding by changing the degree of association. Sedimentation-velocity experiments (Table 1) showed that this does not occur.

Glutarate and other dicarboxylic acids in which the dissociable protons are 7.45Å apart, as in glutamate, are strong competitive inhibitors of the oxidative deamination reaction (Caughey *et al.*, 1957). Glutarate also forms a ternary complex with the enzyme and NADH or NADPH, and its absorption spectrum is very similar to that of the enzyme–NAD(P)H–glutamate complex (Egan & Dalziel, 1971). The formation of stable ternary complexes of enzyme, oxidized coenzymes and glutarate is therefore to be expected and it may be taken as a model for the active ternary complex with glutamate.

The most general representation of the equilibrium system of enzyme, oxidized coenzyme and glutarate is shown in Scheme 1, where E represents a single active centre. If it is assumed that the active centres are identical and independent of one another, it can be shown that, at equilibrium, eqn. (1) or eqn. (2) holds.

$$[B] = \frac{[E][F]}{K_{app.} + [F]} \quad (1)$$



Scheme 1. *Equilibrium system of enzyme, coenzyme and glutarate*

or

$$\frac{1}{[B]} = \frac{1}{[E]} + \frac{K_{app.}}{[E][F]} \quad (2)$$

The apparent dissociation constant for oxidized coenzyme, $K_{app.}$, is

$$K_{app.} = \frac{K_1(1 + [G]/K_2)}{1 + [G]/K_3} \quad (3)$$

In these equations, K_1, K_2 and K_3 are dissociation constants of the complexes E.Co, E.G and E.Co.G in Scheme 1; for example, $K_3 = [E.Co][G]/[E.Co.G]$. The concentration of bound coenzyme is $[B] = [E.Co] + [E.Co.G]$. [F] is the concentration of free coenzyme, [G] that of free glutarate and [E] the total concentration of active centres.

Scheme 1 is analogous to a random-order mechanism for the overall enzymic reaction. If glutarate cannot combine with the free enzyme, i.e. $K_2 = \infty$ and $K_4 = 0$, analogous to a compulsory-order mechanism, a saturation function of the same form as eqn. (1) is obtained with

$$K_{app.} = \frac{K_1}{1 + [G]/K_3} \quad (4)$$

In either case, if the total glutarate concentration is much greater than that of the active centres [G] will be constant and effectively equal to the total glutarate concentration. In the present experiments, $[E] = 0.18$mM and $[G] = 39$mM or 154mM. Therefore the observed deviations from linearity are not consistent with either representation of the system of two ligands in terms of identical and independent active centres.

For glutarate as a competitive inhibitor of glutamate, with a large concentration of NAD^+, $K_I = 0.58$mM (Caughey *et al.*, 1957). This constant should approximate to K_3 in Scheme 1, and is much smaller than [G]. For the case analogous to a compulsory-order mechanism therefore, eqn. (4) simplifies to $K_{app.} = K_1K_3/[G]$. The apparent dissociation constant for coenzyme should be inversely proportional to [G] and approach zero at very large glutarate concentrations. In fact, an increase in glutarate concentration from 39 to 154mM had no significant effect on NAD^+ binding (Fig. 2), which indicates that dissociation of coenzyme from the ternary complex can occur without prior dissociation of glutarate, i.e. $K_4 \neq 0$. If this were not the case, with a sufficiently large glutarate concentration the enzyme would be titrated stoicheiometrically with coenzyme; it may be noted that the experiment is analogous to the method of isotope exchange at equilibrium for distinguishing between a random- and a compulsory-order mechanism (Silverstein & Boyer, 1964).

For the general case of Scheme 1, with $[G] \gg K_3$, eqn. (3) simplifies to

$$K_{app.} = \frac{K_1 K_3(1 + [G]/K_2)}{[G]} \quad (5)$$

If also $[G] \gg K_2$, then $K_{app.} = K_1 K_3/K_2 = K_4$. The value of K_2 is unknown, but again the absence of any significant effect of a fourfold increase in glutarate concentration on coenzyme binding suggests that this condition is approached, and that the experimental saturation functions represent binding of coenzyme in a ternary complex with glutarate.

The non-linear Klotz plots, as well as the complex Lineweaver–Burk plots reported by Engel & Dalziel (1969), might arise from the presence of more than one protein species or of competing impurities in the coenzyme preparations. It is unlikely, however, that such pronounced deviations from linearity could be caused in these ways. No evidence of heterogeneity of similar enzyme preparations, used in the kinetic studies of Engel & Dalziel (1969), was obtained by recrystallization and chromatography, and coenzyme preparations purified by chromatography gave kinetic results similar to those obtained with commercial preparations. The latter, used in the present work, may contain up to 3% of nucleotide impurities, and similar small amounts of degradation products of $NAD(P)^+$ might be formed during the equilibrium dialysis. The effects of even such small proportions of nucleotide impurities may be considerable in kinetic measurements with small concentrations of enzyme (Dalziel, 1962), but are unlikely to be significant in binding studies, with a concentration of active centres greater than the possible concentration of impurities or coenzyme degradation products.

The deviations from linearity in the Klotz plots (Figs. 2 and 3) show that, in the ternary complex, either the six coenzyme-binding sites of an oligomer are not identical, i.e. do not have the same intrinsic affinities for coenzyme, or there are negative interactions between them. The plots are qualitatively similar to Lineweaver–Burk plots of initial-rate data obtained with large and constant glutamate concentrations and variable concentrations of NAD^+ and $NADP^+$ (Dalziel & Engel, 1968; Engel & Dalziel, 1969). The binding studies with glutarate therefore support the earlier conclusion that, if the enzyme subunits are identical, the coenzyme 'activation' is due to negative interactions, and suggest that dissociation of coenzyme from the active ternary complex with enzyme and glutamate is the rate-limiting step at which the negative interactions occur. In this case, K_m for the coenzyme is the dissociation constant for coenzyme from the ternary complex. The initial-rate data were described in terms of several values for K_m corresponding to different coenzyme concentration ranges. For NAD^+, the values were 4.1 μM for the concentration range 1–7 μM, 26 μM (12–60 μM), 76 μM (60–200 μM) and 220 μM (300–1000 μM). For $NADP^+$, the values were 5.3 μM (2–20 μM), 20 μM (20–200 μM) and 73 μM (200–1000 μM). The values are smaller than the dissociation constants of the binary enzyme–coenzyme compounds. Thus glutamate, like glutarate, increases the affinity of the enzyme for the oxidized coenzymes, especially at low concentrations of the latter.

It is noteworthy that the apparent dissociation constants for the ternary complexes with glutarate at low coenzyme concentrations, about 6 μM for NAD^+ and 8 μM for $NADP^+$ (Figs. 2 and 3), are similar to the K_m values obtained with low coenzyme concentrations.

However, the binding results are intrinsically much less precise and detailed than the earlier initial-rate data, especially in the higher coenzyme concentration ranges, and it is not possible to discern discontinuities in the Klotz plots, nor to derive meaningful constants by fitting the experimental data by a theoretical equation for a particular model. Negative interactions cannot be accounted for by the allosteric model of Monod *et al.* (1965), even if the two enzyme conformations have different affinities for substrate and maximum turnover rates (Dalziel & Engel, 1968). Some form of sequential model is indicated in which ligand binding at one or more sites decreases the affinity of the remaining sites for coenzyme (cf. Koshland *et al.*, 1966; Conway & Koshland, 1968).

There remains, however, the possibility that the six active centres are not identical, i.e. they differ in their intrinsic affinities for coenzyme, either because the subunits do not have identical amino acid sequences or because they are arranged asymmetrically in the oligomer. Sequence studies have revealed no difference between the six polypeptide chains (Smith *et al.*, 1970) but a single amino acid replacement at the active centre could escape detection. There is evidence that in each oligomer there are only three tyrosyl residues involved in binding purine nucleotides (di Prisco, 1971), and that there are nine binding sites for anilinonaphthalenesulphonate (Dodd & Radda, 1969). A model for the oligomer has been proposed by Eisenberg & Reisler (1970), based on light-scattering and electron-microscope investigations, in which two layers, each consisting of three subunits arranged in triangular fashion, are stacked on top of one another. Thus a particular possibility is that the oligomer consists of two groups of subunits, which differ in their binding properties, as a result either of differences of primary structure or of geometrical arrangement. The saturation curves for the coenzymes in the presence of glutarate (Figs. 2 and 3) could indeed be described, within the large experimental error, by only two dissociation constants. On the other hand, the kinetic data are more difficult to

account for in terms of this hypothesis, in particular the occurrence of more than one discontinuity in Lineweaver–Burk plots, and the fact that there is no such evidence of non-identical active centres in the kinetics of the oxidative deamination of norvaline or the reductive amination of 2-oxoglutarate (Engel & Dalziel, 1969). Nevertheless, this interpretation cannot be discounted at present.

At pH 8.0, ADP activates and GTP inhibits the glutamate dehydrogenase reaction (Frieden, 1963). At pH 7.0, both nucleotides are partial inhibitors, the degree of inhibition increasing with nucleotide concentration to a constant value; maximal inhibition is reached with 0.2mM-ADP and about 1mM-GTP (Engel, 1968). This and much other evidence shows that there are separate binding sites for ADP, GTP and coenzyme, although the sites may overlap (cf. Cross & Fisher, 1970; di Prisco, 1971). The present work shows that 1mM-ADP and 0.8mM-GTP have opposite effects on NAD^+ binding. With and without glutarate present, ADP weakens the binding of NAD^+ by the enzyme whereas GTP strengthens it. This effect of ADP is consistent with its behaviour as an inhibitor of the partially competitive type with respect to NAD^+ (Engel, 1968), but the inhibition of the NAD^+–glutamate reaction by GTP must be due to effects on the rate of reaction within the ternary complex or the release of products. From stopped-flow experiments on the $NADP^+$–glutamate reaction at pH 7.5, Iwatsubo & Pantaloni (1967) concluded that GTP increases and ADP decreases the affinity of the enzyme for oxidized and reduced coenzyme and substrate.

The most striking effect of ADP is that, in the presence of excess of glutarate, the binding of NAD^+ and $NADP^+$ no longer shows evidence of negative interactions, and can be described by a single dissociation constant, which is, however, smaller than that for coenzyme binding to the enzyme alone. The main effect of the nucleotide is to decrease the affinity of coenzyme binding at low coenzyme concentrations (Figs. 5 and 6). It is significant that the Klotz plots for coenzyme binding in the ternary complex are affected by ADP in the same way as Lineweaver–Burk plots for the overall reaction with a large, constant glutamate concentration and variable NAD^+ concentration; the latter show the inhibition to be 'partial competitive', and in the presence of ADP the Lineweaver–Burk plots are effectively linear over the whole range of coenzyme concentration, and no longer show the discontinuities observed in the absence of ADP (Engel, 1968). In terms of the hypothesis of negative interactions, it appears that ADP induces a conformational change of all the subunits similar to that induced by combination of all or most of the subunits of an oligomer with NAD^+ or $NADP^+$, resulting in a decreased affinity for the oxidized coenzymes in the ternary complex with glutarate or glutamate. The effect of GTP, on the other hand, is to maintain the subunits in a conformation with high affinity for coenzyme up to a higher degree of saturation with coenzyme (Fig. 7). The behaviour of GTP as an inhibitor is also broadly consistent with this interpretation (Engel, 1968), but more detailed studies of the kinetics of inhibition by ADP and GTP are needed. Thus, like the effectors of other allosteric enzymes, these nucleotides alter the homotropic interactions of the substrates, albeit negative interactions in this case.

Sedimentation-velocity experiments showed that in the presence of glutarate, NAD^+ and GTP, disaggregation of the enzyme to free oligomers occurs. It is not known whether this has an effect on coenzyme binding. Such dissociation is also brought about by NADH and GTP, and biphasic conformational changes of the oligomer, independent of the disaggregation, are brought about by these two ligands, as shown by fluorescence probes (Dodd & Radda, 1969). Similar studies of the combined effects of glutarate, $NAD(P)^+$ and the nucleotide effectors on probe fluorescence would be of value. In the equilibrium-dialysis experiments with glutarate, coenzyme and GTP, it was also found that the enzyme crystallized spontaneously from the sac contents on storage in ice for a few minutes. All three ligands were needed, and the extent of crystallization increased with the coenzyme concentration, indicating that the crystals are a complex of enzyme oligomer and all three ligands.

Finally, the similarity of behaviour of NAD^+ and $NADP^+$ in these experiments should be emphasized. Early kinetic studies (Olson & Anfinsen, 1953; Frieden, 1959*a*) seemed to show that coenzyme activation occurred with NAD^+ but not with $NADP^+$. When it was found that the binding of NADH to the enzyme was accompanied by perturbation of the adenine chromophore, whereas NADPH binding was not, it was suggested that binding at an adenine site, not accessible to $NADP^+$, was responsible for coenzyme activation by NAD^+ (Pantaloni & Iwatsubo, 1967; Pantaloni & Dessen, 1969). This conclusion is no longer tenable in the light of more detailed kinetic studies, which show 'activation' with both coenzymes (Dalziel & Engel, 1968; Engel & Dalziel, 1969), and the similar deviations from simple binding now observed with both coenzymes in the presence of glutarate. There is no evidence in the present work for coenzyme-binding sites in addition to the active centres, and negative interactions between active centres in the ternary complex is the most likely explanation of the results for both coenzymes. The difference spectra no doubt indicate real differences of binding for the two reduced coenzymes in their binary complexes with the enzymes, but they may not be significant in the ternary complexes of the oxidized coenzymes. Indeed, Pantaloni & Iwatsubo

(1967) found that glutamate weakened the binding of NADH to the enzyme at the adenine site.

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