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Evaluation of Equilibrium Constants for the Interaction of Lactate Dehydrogenase Isoenzymes with Reduced Nicotinamide–Adenine Dinucleotide by Affinity Chromatography

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Rabbit muscle lactate dehydrogenase was subjected to frontal affinity chromatography on Sepharose–oxamate in the presence of various concentrations of NADH and sodium phosphate buffer (0.05 M, pH 6.8) containing 0.5 M-NaCl. Quantitative interpretation of the results yields an intrinsic association constant of $9.0\times10^4\,M^{-1}$ for the interaction of enzyme with NADH at 5°C, a value that is confirmed by equilibrium-binding measurements. In a second series of experiments, zonal affinity chromatography of a mouse tissue extract under the same conditions was used to evaluate association constants of the order $2\times10^5\,M^{-1}$, $3\times10^5\,M^{-1}$, $4\times10^5\,M^{-1}$, $7\times10^5\,M^{-1}$ and $2\times10^6\,M^{-1}$ for the interaction of NADH with the M_4, M_3H, M_2H_2, MH_3 and H_4 isoenzymes respectively of lactate dehydrogenase.

Previous investigations (Andrews *et al.*, 1973; Dunn & Chaiken, 1974; Nichol *et al.*, 1974) have shown the potential of affinity chromatography for obtaining equilibrium constants for interactions affecting ligand-dependent elution of a macromolecule. The studies of Andrews *et al.* (1973) and Dunn & Chaiken (1974) represent analogous treatments of zonal data for affinity-chromatography systems exhibiting ligand-retarded and ligand-facilitated elution respectively. A general quantitative treatment of affinity chromatography (Nichol *et al.*, 1974) has yielded more rigorous expressions for the ligand-dependence of elution volume, and has provided a possible means of determining both of the pertinent equilibrium constants that characterize the simplest affinity-chromatography systems. Such characterization requires a series of frontal-chromatographic experiments (Winzor & Scheraga, 1963) on mixtures with fixed concentration of acceptor (enzyme) and various amounts of specific ligand, the free concentration of which also needs to be known.

In the present study of the effect of NADH on the binding of lactate dehydrogenase to a column of Sepharose–oxamate (O'Carra & Barry, 1972) we show how the theoretical expressions of Nichol *et al.* (1974) may be used to salvage the magnitude of one of the two equilibrium constants from experiments with insufficient information for complete characterization of the system. First we determine the equilibrium constant for the interaction of NADH with rabbit muscle lactate dehydrogenase from frontal experiments on mixtures in which only the total concentrations of enzyme and NADH are known. In the second series of experiments the interaction of NADH with the five isoenzymes (M_4, M_3H, M_2H_2, MH_3 and H_4) of lactate dehydrogenase is studied by zonal affinity chromatography of a mouse tissue extract. The partial separation of the isoenzymes described here should be distinguished from the separations described previously by O'Carra *et al.* (1974), which depended on differential binding of the isoenzymes to immobilized oxamate in the presence of NAD^+, rather than on differential affinity for NADH.

Experimental

Materials

Rabbit muscle lactate dehydrogenase was obtained as a freeze-dried salt-free powder (type XI) from Sigma Chemical Co., St. Louis, Mo., U.S.A., who also supplied the NADH. Molar enzyme concentrations were determined spectrophotometrically at 280 nm on the basis of an extinction coefficient ($E^{1\%}_{1cm}$) of 14.4 and a molecular weight of 140000 (Jaenicke & Knof, 1968). A molar extinction coefficient at 6220 litre·mol^{-1}·cm^{-1} at 340 nm was used to define the concentrations of NADH solutions; on the basis of absorbance measurements at 340 and 260 nm the NADH was judged to be essentially free from NAD^+ contamination ($E_{260}/E_{340} = 2.33$).

A mouse tissue extract was obtained by homogenizing the heart, liver and kidneys of a female Quackenbush mouse in 2 vol. of cold (5°C) sodium phosphate buffer (0.05 M, pH 6.8) containing 0.5 M-NaCl. The homogenate was then centrifuged for 60 min at 14000*g* and 5°C, and the supernatant

dialysed for 16h at 5°C against more (500ml) of the same buffer.

Sephadex G-25 and Sepharose 4B were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. The Sepharose 4B was converted into an oxamate derivative by the method of O'Carra & Barry (1972).

Methods

Frontal affinity chromatography on Sepharose–oxamate. Mixtures of rabbit muscle lactate dehydrogenase (0.90μM) and NADH (0–4.5μM) were applied to a column (1cm×40cm) of Sepharose–oxamate equilibrated at 5°C with 0.05M-sodium phosphate (pH6.8) containing 0.5M-NaCl, the buffer used to prepare the enzyme–NADH solutions. Fractions (1–4ml) of the eluate were collected and analysed spectrophotometrically at 280nm. $\bar{V}_A$, the weight-average elution volume of protein, was obtained from the centroid (Longsworth, 1943) of the ascending elution profile. Results were interpreted in terms of eqn. (1), in which A represents enzyme and B the nucleotide.

$$\frac{1}{\bar{V}_A - V_A^*} = \frac{1}{V_s[\bar{X}]K_3 K_{AB}[B]} + \frac{1+K_3[\bar{A}]}{V_s[\bar{X}]K_3} \qquad (1)$$

V_A^* denotes the value of $\bar{V}_A$ for [NADH]=0, V_s is the volume of stationary phase, [$\bar{A}$] the total enzyme concentration and [$\bar{X}$] the effective total concentration of immobilized oxamate. K_{AB} is the intrinsic association constant (Klotz, 1946) for the binding of NADH to the four equivalent and independent binding sites on lactate dehydrogenase, and K_3 the binding constant for the interaction of enzyme–NADH complexes with immobilized oxamate (Nichol *et al.*, 1974).

Zonal affinity chromatography of mouse tissue extract. Before affinity chromatography, dialysed tissue extract (1ml) was equilibrated with NADH by passage through a column (1.0cm×26cm) of Sephadex G-25 pre-equilibrated with 0.05M-sodium phosphate buffer (pH6.8) containing 0.5M-NaCl and the required concentration of NADH (2.0–3.3μM). The material eluted at the void volume was diluted with the equilibrating solution such that the lactate dehydrogenase activity was 60units/ml by the Wroblewski & La Due (1955) assay. The resulting extract–NADH mixture (2ml) was then applied to a column (1.0cm×36cm) of Sepharose–oxamate pre-equilibrated at 5°C with the same phosphate–chloride–NADH buffer. Fractions (2ml) of the eluate were collected and monitored for lactate dehydrogenase activity by the method of Wroblewski & La Due (1955). Elution volumes for the five peaks of enzymic activity were again interpreted in terms of eqn. (1).

Frontal gel chromatography on Sephadex G-25. The binding of NADH to rabbit muscle lactate dehydrogenase was also determined by frontal gel chromatography (Nichol & Winzor, 1964) on a column (1.0cm×26cm) of Sephadex G-25 equilibrated at 5°C with 0.05M-sodium phosphate buffer (pH6.8) containing 0.5M-NaCl. A mixture (20ml) of lactate dehydrogenase (2.8μM) and NADH (15.0μM) in the phosphate–chloride buffer was applied to the column, after which elution with buffer was recommenced and the eluate collected in 1ml fractions. Absorbance measurements at 340nm were made on each fraction to determine the distribution of NADH in the trailing elution profile. Since enzyme and enzyme–NADH complexes are excluded from the stationary phase of Sephadex G-25 and thus co-migrate, the concentration of NADH in the slower-moving boundary corresponds to the free concentration of nicotinamide nucleotide in the original mixture (Gilbert & Jenkins, 1959; Nichol & Winzor, 1964).

Results and Discussion

Frontal affinity chromatography of lactate dehydrogenase

As noted in the Experimental section, results are to be interpreted in terms of eqn. (1), which refers to a system in which interaction of the enzyme–NADH complexes with immobilized oxamate is restricted to a single linkage because of steric requirements (Nichol *et al.*, 1974). Although this situation cannot be presumed to hold generally for multi-subunit enzymes, results obtained with the phosphocellulose–aldolase–fructose diphosphate system (Masters *et al.*, 1969) show the assumption to be reasonable in that case at least: non-fulfilment of the single-linkage concept would be detected (theoretically) by sigmoidality of the plot of $(\bar{V}_A - V_A^*)$ against [B] (Masters *et al.*, 1969).

From eqn. (1) a double-reciprocal plot of retardation $(\bar{V}_A - V_A^*)$ as a function of free NADH concentration is linear, with intercept $(1+K_3[\bar{A}])/V_s[\bar{X}]K_3$ and slope $1/V_s[\bar{X}]K_3K_{AB}$. As noted previously (Nichol *et al.*, 1974), combination of the statistically determined slope and intercept is insufficient to define the system in terms of the enzyme–NADH equilibrium (K_{AB}) and the enzyme–NADH–oxamate equilibrium (K_3), because [$\bar{X}$], the effective total concentration of accessible oxamate, is a parameter that also requires evaluation from the analysis of results. Complete characterization thus requires another series of frontal affinity-chromatography experiments with different enzyme concentration [$\bar{A}$]. However, for the present system the intercept is effectively $1/V_s[\bar{X}]K_3$, since [$\bar{A}$] is only 0.9μM (3.6μM in terms of subunit concentration) and K_3 for oxamate (not immobilized) is 7×10^3 M^{-1} (Winer & Schwert,

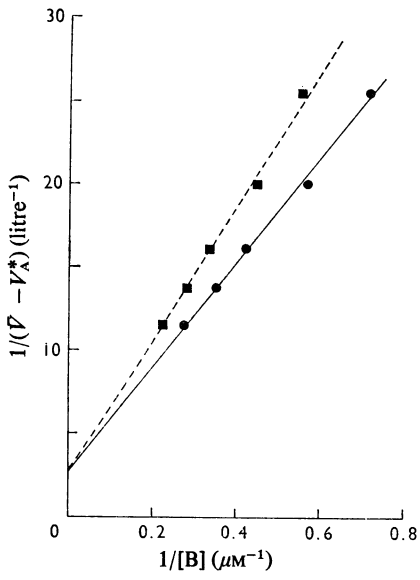

Fig. 1. *Evaluation of the association constant for the interaction of NADH with lactate dehydrogenase by affinity chromatography*

Mixtures of rabbit muscle lactate dehydrogenase (0.9 μM) and NADH (0–4.5 μM) in sodium phosphate buffer (0.05 M, pH 6.8) containing 0.5 M-NaCl were subjected to frontal affinity chromatography on a column (1 cm × 40 cm) of Sepharose–oxamate. ■, Initial plot of data with total NADH concentration [$\bar{B}$] used as an estimate of [B], its free concentration; ●, final plot of data with [B] obtained by an iterative procedure (see the text).

1959); thus consideration of $(1+K_3[\bar{A}])$ as unity is likely to introduce an error of less than 2.5%. The ratio intercept/slope may therefore be taken as a reasonable value of the intrinsic association constant K_{AB} for the binding of NADH to rabbit muscle lactate dehydrogenase.

Fig. 1 summarizes the results of frontal affinity-chromatography experiments on rabbit muscle lactate dehydrogenase. Although eqn. (1) requires the abscissa of the double-reciprocal plot to be in terms of the free concentration of NADH, the only quantity directly available from the experiment is [$\bar{B}$], the total NADH concentration; the squares in Fig. 1 denote the plot with [$\bar{B}$] used as a first approximation to [B]. The resulting estimate of K_{AB} (5.6×10^4 M^{-1}) was then used to obtain improved estimates of [B] from eqn. (2), which expresses mass conservation of ligand:

$$[\bar{B}] = [B]+\{4K_{AB}[\bar{A}][B]/(1+K_{AB}[B])\} \quad (2)$$

From the ensuing double-reciprocal plot with the improved values of [B] a revised estimate of K_{AB} was calculated, and the process of refining K_{AB} continued until iteration produced no change in the estimate of K_{AB}. The circles in Fig. 1 represent the outcome of the iterative procedure, and from the intercept and slope of the line an association constant of $9.0\times10^4\pm2.0\times10^4$ M^{-1} is deduced. The broken line in Fig. 1, which is the simulated relationship with 1/[$\bar{B}$] as abscissa, confirms the earlier assertion (Nichol *et al.*, 1974) that such plots should be curvilinear, and that the slope and intercept of the limiting tangent as 1/[$\bar{B}$]$\rightarrow$0 are the quantities required for substitution in eqn. (1). With the Sephadex–lysozyme–glucose system studied previously (Nichol *et al.*, 1974) construction of the limiting tangent posed no difficulty because [$\bar{B}$]$\gg$[$\bar{A}$] and hence [B]$\simeq$[$\bar{B}$] in their experiments, whereupon the slope and intercept of a plot of $1/(\bar{V}_A-V^*_A)$ versus 1/[$\bar{B}$] sufficed. The present study demonstrates a means by which the limiting tangent may be defined more precisely in instances where the difference between [B] and [$\bar{B}$] is significant experimentally.

Although the magnitude of K_{AB} is smaller than the values of 2×10^5–3×10^5 M^{-1} that are usually quoted (e.g. Brodelius & Mosbach, 1974) for the interaction of NADH with mammalian muscle lactate dehydrogenases, the experimental conditions differ considerably with respect to temperature (5°C instead of 20–30°C) and ionic strength (0.6 M instead of 0.1–0.2 M). These two factors have been considered in detail by Stinson & Holbrook (1973) for the pig muscle enzyme at pH 7.2. From their Fig. 9 the value of K_{AB} at 20°C is 4.4×10^4 M^{-1} in buffer of ionic strength 0.6 M. On the basis of Fig. 7 of Stinson & Holbrook (1973), which gives the temperature-dependence of K_{AB} in 0.067 M-sodium phosphate buffer (pH 7.2), the value of 4.4×10^4 M^{-1} at 20°C would be equivalent to 8.2×10^4 M^{-1} at 5°C; such interpretation presumes, of course, the applicability of the same ΔH^0 to both sets of conditions.

The value of 9.0×10^4 M^{-1} for the binding of NADH to rabbit muscle lactate dehydrogenase is thus seemingly consistent with the findings of Stinson & Holbrook (1973) for the interaction with pig muscle lactate dehydrogenase under conditions similar to, but not identical with, those used in the present study. Confirmation of the present result was obtained by frontal gel chromatography on Sephadex G-25 (Nichol & Winzor, 1964), which provides a direct measure of the concentration of free NADH in an equilibrium mixture for which the total NADH concentration is known. The elution profile obtained with a mixture comprising 2.8 μM-lactate dehydrogenase and 15.0 μM-NADH is shown in Fig. 2, from which the equilibrium concentration of nicotinamide nucleotide is 9.5 μM. Substitution

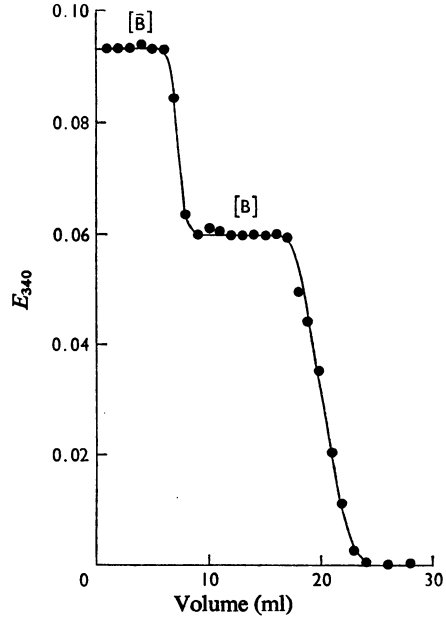

Fig. 2. *Evaluation of the association constant for the interaction of NADH with lactate dehydrogenase by gel chromatography*

A mixture of rabbit muscle lactate dehydrogenase (2.8μM) and NADH (15μM) in sodium phosphate buffer (0.05M, pH6.8) containing 0.5M-NaCl was subjected to frontal gel chromatography on a column (1.0cm×26cm) of Sephadex G-25; only the trailing elution profile is shown.

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of these values in eqn. (3) gives $1.0\times10^5\,M^{-1}$ for K_{AB}, a value in excellent agreement with the $9.0\times10^4\,M^{-1}$ obtained by affinity chromatography.

$$K_{AB}=\frac{[\bar{B}]-[B]}{(4[\bar{A}]-[\bar{B}]+[B])[B]} \qquad (3)$$

Zonal affinity chromatography of lactate dehydrogenase isoenzymes

We now consider the use of zonal affinity chromatography for quantitative study of the binding of NADH to lactate dehydrogenase. One disadvantage of zonal chromatographic procedures is the lack of information about the solute (enzyme) concentration [Ā] to which a measured elution volume $\bar{V}_A$ refers (Winzor, 1966). However, for the present system we have demonstrated above that by use of sufficiently low lactate dehydrogenase concentrations the $K_3[\bar{A}]$ term in eqn. (1) can be disregarded; since eqn. (1) then becomes independent of enzyme concentration, zonal experiments should suffice to evaluate K_{AB} provided that [B], the equilibrium concentration of NADH, is known in each experiment. Indeed, such zonal experiments formed the basis of the quantititive affinity-chromatography studies reported by Andrews *et al.* (1973) and Dunn & Chaiken (1974), although in neither case was the existence of the $K_3[\bar{A}]$ term recognized; in retrospect its disregard was justified, as with the present system.

In instances where either zonal or frontal affinity chromatography is feasible, the frontal technique is to be preferred, since [Ā] is then clearly defined. Although it is true that $\bar{V}_A$ is seemingly independent of $[\bar{V}]$ provided that $K_3[\bar{A}]\ll1$ (see eqn. 1), the enzyme concentration can influence $\bar{V}_A$ indirectly through its effect on free ligand concentration [B]. This indirect dependence on [Ā] is likely to be encountered whenever the total enzyme and total ligand concentrations are of comparable magnitudes, a factor clearly evident in the above frontal study (see Fig. 1). Because frontal experiments had been used, allowance could be made for the binding of NADH to the lactate dehydrogenase, and hence the data were still amenable to quantitative interpretation. In zonal studies (Andrews *et al.*, 1973; Dunn & Chaiken, 1974) the enzyme is applied to a column pre-equilibrated with ligand, and the pre-equilibrating ligand concentration must be presumed to apply throughout the migrating zone of enzyme. This assumption is certainly reasonable in experiments with $[B]\gg[\bar{A}]$, but becomes more tenuous as $[B]\rightarrow[\bar{A}]$ (see Bethune & Kegeles, 1961; Cann & Goad, 1965).

However, there are systems for which zonal affinity chromatography seems to offer advantages over the frontal technique, and as an example we consider the determination of equilibrium constants for the interaction of NADH with the five isoenzymes of lactate dehydrogenase in a crude mouse tissue extract. First, the retardation of the lactate dehydrogenase allows the remainder of the tissue-extract components to be eluted from the column ahead of the enzyme, and hence the interaction of the isoenzymes with NADH is studied essentially in the absence of the contaminants that were present initially. In this respect Sepharose–oxamate is presumably a more selective affinity-chromatographic medium than Sepharose–AMP (Brodelius & Mosbach, 1973), which would interact with other dehydrogenases as well as with some kinases. Secondly, the preferential binding of hybrid tetramers richer in the heart-type subunit of lactate dehydrogenase to Sepharose–oxamate is to be expected because of their stronger interaction with NADH [see Brodelius & Mosbach (1974) for a comparison of equilibrium constants for the parent M_4 and H_4 forms]; indeed, evidence of the preferential retardation of H-rich isoenzymes has been reported (Spielman *et al.*, 1973). Although some

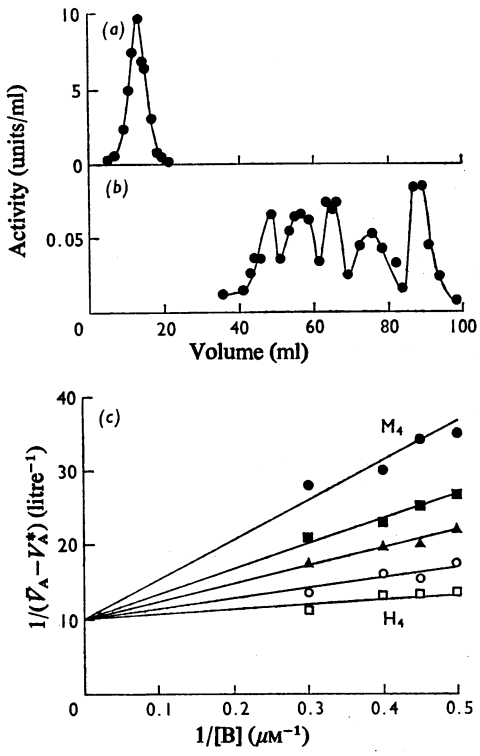

Fig. 3. *Zonal affinity chromatography of a mouse tissue extract on Sepharose–oxamate*

Samples of a mouse tissue extract in dialysis equilibrium with various concentrations of NADH (0–3.3 μM) were subjected to zonal affinity chromatography on a column (1.0cm×36cm) of Sepharose–oxamate pre-equilibrated with the same concentration of nucleotide; conditions were as in Fig. 1. (*a*) Elution profile obtained in the absence of NADH; (*b*) profile for [NADH] = 2.5 μM; (*c*) double-reciprocal plot of the data in accordance with eqn. (1). ●, M_4; ■, M_3H; ▲, M_2H_2; ○, MH_3; □, H_4 isoenzyme.

separation of isoenzymes is to be expected, the resolution is likely to be incomplete, and in such circumstances the positions of zones are much more clearly defined than those of boundaries; with the degree of resolution that is likely to be achieved, recognition of individual isoenzyme boundaries would almost certainly be precluded. Thus, provided that (i) the concentration of lactate dehydrogenase in the applied zone is low enough for K_3[Ā] to be ignored, and (ii) precautions are taken to ensure that the concentration of free NADH in the applied zone matches that used for pre-equilibration of the affinity column, zonal studies offer the potential of yielding approximate values of all five isoenzyme–NADH binding constants.

In order to meet the second proviso, the mouse tissue extract was equilibrated with the appropriate NADH concentration by zonal gel filtration on a Sephadex G-25 column equilibrated with the same phosphate–chloride–NADH solution that was used to pre-equilibrate the Sepharose–oxamate column. This procedure has the advantage of ensuring that all NADH-binding components of the tissue extract are in dialysis equilibrium with the NADH concentration used for the affinity-chromatography run: of the various dehydrogenase–NADH complexes present, however, only those of lactate dehydrogenase should interact with the immobilized oxamate. Figs. 3(*a*) and 3(*b*) show the results of typical experiments in which extracts with a lactate dehydrogenase concentration of 0.5–1.0 μM were subjected to affinity chromatography in the presence of 0 and 2.5 μM-NADH respectively. Partial resolution of five peaks is clearly evident in Fig. 3(*b*), their identification with the five isoenzymes being on the basis of starch-gel electrophoresis (Georgiev *et al.*, 1970) of samples from an experiment with higher enzyme loading. Double-reciprocal plots of results for four NADH concentrations are presented in Fig. 3(*c*); from the slopes and intercepts association constants were found of the order of 2×10^5 M^{-1}, 3×10^5 M^{-1}, 4×10^5 M^{-1}, 7×10^5 M^{-1} and 2×10^6 M^{-1} for the five peaks of enzymic activity, identified as M_4, M_3H, M_2H_2, MH_3 and H_4 respectively, of lactate dehydrogenase. Of interest is the observation of a common intercept ($1/V_s[\bar{X}]K_3$), which implies identity of K_3 for the interaction of immobilized oxamate with both types of lactate dehydrogenase subunit. On this basis the preferential retardation of the H_4 isoenzyme and H-rich hybrids relative to their muscle-type counterparts simply reflects the stronger binding of NADH to heart-type subunits of lactate dehydrogenase.

The value of 2×10^6 M^{-1} for the H_4 isoenzyme is approximately two-thirds that of 3.1×10^6 M^{-1} inferred from Figs. 7 and 9 of Stinson & Holbrook (1973), which present the temperature- and ionic-strength-dependence of K_{AB} for pig heart enzyme at pH 7.2. However, the accuracy of the present data is insufficient to attach significance to this difference. Nor can the difference between the K_{AB} of 2×10^5 M^{-1} obtained from Fig. 3(*c*) for M_4 isoenzyme and the earlier value (Fig. 1) of 0.90×10^5 M^{-1} be considered significant in the light of the approximations inherent in the zonal study. First, the identity of peak positions with centres of overlapping zones has been assumed. Secondly, although the free concentration of NADH in the tissue-extract zone matched the pre-equilibrating concentration of nicotinamide nucleotide at the time of application to the column, the migrating zone is a reaction zone within which the concentrations of NADH, enzyme and enzyme–NADH complexes can all be expected to vary to some

extent (Bethune & Kegeles, 1961; Cann & Goad, 1965); thus in systems with [Ā] not so very much smaller than the pre-equilibrating ligand concentration, [B] cannot be regarded as defined unequivocally despite the pre-equilibration step. However, irrespective of any shortcomings of this nature in the data, reasonable estimates for at least two and presumably all five equilibrium constants have been obtained, whereupon it must be concluded that the re-adjustment of NADH within the migrating protein zone must have been relatively small.

Whereas frontal experiments on the tissue extract would almost certainly have yielded a single value of K_{AB} that represented a weighted mean of the five separate constants, the zonal study has provided estimates of all five binding constants that are of the right order of magnitude. From the very nature of the experiment, namely zonal chromatography of a crude tissue extract, it would be over-optimistic to expect to characterize an equilibrium reaction more quantitatively than has been achieved. Should greater accuracy be required, the various isoenzymes would need to be separated and studied individually by frontal techniques. However, as a means of obtaining approximate equilibrium constants with impure systems the zonal procedure has proved successful. In contrast with the gradient affinity-chromatography procedure suggested by Brodelius & Mosbach (1974) the present method is free from empiricism, and yields equilibrium constants that are pertinent to the environment (pH, temperature, ionic strength) in which the interaction is being studied.

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