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Affinity Chromatography of Nicotinamide Nucleotide-Dependent Dehydrogenases on Immobilized Nucleotide Derivatives

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A series of chemically-defined adenosine phosphate ligands attached to Sepharose 4B were used as active-site probes in studying the interaction of enzymes with their coenzymes and substrates and to test the suitability of these matrices for 'general ligand' affinity chromatography. Nicotinamide nucleotide-dependent dehydrogenases were used as models to test this methodology. Elution from these columns by NAD^+ and/or AMP gradients (in the presence or the absence of substrates and/or nicotinamide mononucleotide) was consistent with: (1) the compulsory ordered addition of substrates to lactate and malate dehydrogenase; (2) the necessity for the NMN moiety of NAD^+ to bind to these enzymes before the substrate; and illustrated: (3) that the binding of these two hydrogenases to these columns compared very well with the published three-dimensional models for these enzymes and (4) that separation of mixtures of dehydrogenases depended on the choice of matrix and displacing ion and whether any additions (e.g. substrates) were made to the gradients used. These techniques were used to purify UDP-glucose dehydrogenase from a crude starting material on a phosphate-linked UDP (or ADP) matrix. The binding of this enzyme to these two columns was not consistent with either an ordered or random addition of substrates and suggested a more complex mechanism.

The general potential of affinity chromatography for the isolation and purification of enzymes has been exploited extensively in recent years (Cuatrecasas & Anfinsen, 1971*a*,*b*; Cuatrecasas, 1972). Although initially this technique was applied almost exclusively to working with single enzymes, the idea of using an immobilized 'general ligand' which could interact simultaneously with a range of similar enzymes has received much attention (Mosbach *et al.*, 1971; Lowe & Dean, 1971; Collier & Kohlhaw, 1971). This 'general ligand' approach has been especially useful when insolubilized AMP and/or NAD^+ derivatives have been applied to the binding of NAD^+-dependent dehydrogenases. Different methods can then be used for increasing the selectivity of elution of the chosen dehydrogenase from the mixture (Lowe *et al.*, 1972; Mosbach *et al.*, 1972; Ohlsson *et al.*, 1972; Lowe *et al.*, 1973). The advantages of using these group-specific matrices are obvious on both economic and practical grounds. The effectiveness of the 'general ligand' approach has been questioned by some workers (O'Carra & Barry, 1972; Barry & O'Carra, 1973*a*) who claimed that some loss of specificity would be experienced. A later paper (Barry & O'Carra, 1973*b*) in this series, however, suggests that when a 6-linked NAD^+ analogue is attached to agarose it may be possible to overcome this problem.

The key to much of this controversy appears to be the chemistry of the immobilization process. When efforts are made to use chemically-defined ligands then 'general ligand' affinity chromatography appears to be a much more viable proposition [e.g., see Brodelius & Mosbach (1973) who separated all five isoenzymes of lactate dehydrogenase on one pass down a Sepharose-bound *N*-(6-aminohexyl)-AMP column]. Thus both the design of experiments and the interpretation of results to include enzyme-mechanism studies are made much simpler and more precise if the exact nature of the immobilized ligand is known.

Trayer *et al.* (1974) described the synthesis of AMP, ADP and ATP derivatives that may be attached to agarose through either the C-8 or the 6-amino group on the adenine ring (leaving the phosphate moieties unsubstituted) or through the phosphate group of ADP (leaving the adenine ring unsubstituted). These derivatives, together with a ribosyl-linked AMP derivative (Lamed *et al.*, 1973), have been used in this work, the aim of which was to demonstrate how affinity chromatography can be used to probe the active site of enzymes and reveal useful information concerning the binding of coenzymes and substrates. Several NAD^+-dependent dehydrogenases, some with known three-dimensional crystal structures, were chosen to show the sensitivity of this technique.

Materials and Methods

Chemicals

Coenzymes, nucleotides, all substrates and dithiothreitol were purchased from either Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K., or P–L Biochemicals Inc., Milwaukee, Wis., U.S.A. CNBr was from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K., and Sepharose-4B from Pharmacia (G.B.) Ltd., London W.5, U.K. Coomassie Brilliant Blue R was obtained from George T. Gurr Ltd., High Wycombe, Bucks., U.K. All other chemicals were A.R. grade and used as supplied.

Proteins

The following proteins were purchased from Sigma Chemical Co. Ltd.: bovine serum albumin; lactate dehydrogenase (L-lactate–NAD^+ oxidoreductase, EC 1.1.1.27, from rabbit muscle, crude preparation type 1) containing pyruvate kinase (ATP–pyruvate phosphotransferase, EC 2.7.1.40) activity; glyceraldehyde 3-phosphate dehydrogenase [D-glyceraldehyde 3-phosphate–NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12, from rabbit muscle] and uridine 5′-diphosphoglucose dehydrogenase (UDP-glucose–NAD^+ oxidoreductase, EC 1.1.1.22, from bovine liver). Ox heart lactate dehydrogenase was bought from Worthington Biochemical Corp., Freehold, N.J., U.S.A., and glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate–$NADP^+$ oxidoreductase, EC 1.1.1.49, from yeast, freeze-dried, A grade) from Calbiochem Ltd., San Diego, Calif., U.S.A. Cytoplasmic malate dehydrogenase (L-malate–NAD^+ oxidoreductase, EC 1.1.1.37, from pig heart) was obtained from Fluka A.G., Buchs, Switzerland and human serum albumin from Pentex Biochemicals Ltd., Kankakee, Ill., U.S.A. Rat hepatic glucokinase (ATP–D-glucose 6-phosphotransferase, EC 2.7.1.2) was purified 500-fold direct from liver extracts by affinity chromatography as described by Chesher *et al.* (1973).

Methods

Enzyme assays. Glucokinase assays were performed spectrophotometrically by the procedure of Parry & Walker (1966). Lactate dehydrogenase and pyruvate kinase activities were determined as described by Trayer *et al.* (1974). Malate dehydrogenase activity was measured from the decrease in E_{340} as NADH was oxidized in an incubation medium consisting of NADH (0.66μmol), oxaloacetate (1.5μmol), test solution (up to 0.15ml) and 0.1M-sodium phosphate buffer, pH7.0, to make a total volume of 3.0ml. Glyceraldehyde 3-phosphate dehydrogenase was assayed by the increase in E_{340} as NAD^+ was reduced in a medium containing NAD^+ (0.66μmol), DL-glyceraldehyde 3-phosphate (1.5μmol), dithiothreitol (100μmol), test solution (up to 0.15ml) and 0.015M-sodium pyrophosphate buffer, pH8.5, containing 0.03M-sodium arsenate to make a total volume of 3.0ml. In this instance, the incubation medium (minus the substrate but including the enzyme) was maintained at 25°C for 10min before commencing the reaction by addition of substrate. Glucose 6-phosphate dehydrogenase was assayed by the increase in E_{340} from the reduction of $NADP^+$ in an incubation medium containing $NADP^+$ (0.66μmol), $MgCl_2$ (20μmol), D-glucose 6-phosphate (3.0μmol), test solution (up to 0.1ml) and 0.1M-Tris–HCl buffer, pH7.6, to make up to 3.0ml. The oxidation of UDP-glucose by UDP-glucose dehydrogenase was also observed by the increase in E_{340} as NAD^+ was reduced to NADH in an incubation medium containing UDP-glucose (1.5μmol), NAD^+ (5.5μmol) and test solution (up to 0.2ml) made up to 3.0ml with 0.05M-glycine buffer, pH8.7.

All enzymes were assayed on a Gilford recording spectrophotometer, model 2000, with the cuvette chamber maintained at 25°C by an external water bath.

Preparation of the adenosine phosphate derivatives bearing single primary amino groups. 8-(6-Aminohexyl)amino-AMP (C8-AMP) (Fig. 1*a*), N^6-(6-aminohexyl)-AMP (N^6-AMP) [and 5′-diphosphate (N^6-ADP)] (Fig. 1*b*) and P^1-(6-aminohex-1-yl) P^2-(5′-adenosyl) pyrophosphate (P-ADP) (Fig. 1*c*) were prepared by the methods of Trayer *et al.* (1974). P^1-(6-Aminohex-1-yl) P^2-(5′-uridyl) pyrophosphate (P-UDP) (Fig. 1*c*) was synthesized as described by Barker *et al.* (1972, 1974). The reaction of periodate-oxidized AMP with the Sepharose–adipic dihydrazide was performed as described by Lamed *et al.* (1973).

Preparation of Sepharose adsorbents. The ligands were coupled to CNBr-activated Sepharose 4B essentially as described by Axen *et al.* (1967). The coupling conditions used were those given by Trayer *et al.* (1974). The extent of coupling was determined on the exhaustively washed gel by performing phosphate analyses after oxidation of the Sepharose–ligand conjugate by the method of Bartlett (1959). The concentration of ligand coupled to the gel was adjusted to 3.0 ($\pm$0.2)μmol/g of wet packed Sepharose 4B by addition of unsubstituted Sepharose 4B if necessary. (N.B. 1g of wet packed Sepharose 4B was the weight of Sepharose 4B taken after packing it down in a Buchner funnel connected to a water suction pump until the Sepharose 4B began to 'crack'. This is not entirely satisfactory but does give reproducible results in our experience.)

Chromatographic procedures. All affinity columns were operated at 4°C and at a flow rate of between

10 and 30ml/h. In the early experiments, the enzymes were dialysed to the appropriate starting buffer, but this was found not to be necessary except in the case of UDP-glucose dehydrogenase, which had to be equilibrated to 0.05M-sodium phosphate buffer, pH7.5, containing 0.01M-2-mercaptoethanol before chromatography. Unless specifically stated otherwise, quantitative recoveries of the enzymes from the columns were always obtained.

Between runs the columns were washed with three bed-volumes of 6M-urea–2M-KCl followed by the appropriate starting buffer. This was not always necessary but was performed as a routine.

The progress of the various gradient elutions used in this study was monitored spectrophotometrically on a Cary model 15 spectrophotometer by using an $\varepsilon_{259}^{\text{litre}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}}$ for AMP at pH7 of 15400 (Bock *et al.*, 1956) and an $\varepsilon_{260}^{\text{litre}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}}$ for NAD^+ at pH7 of 18000 (Siegel *et al.*, 1959).

Polyacrylamide-gel electrophoresis. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was carried out by the method of Weber & Osborn (1969). The stained gels were scanned on a Gilford recording spectrophotometer, model 2000, fitted with a Gilford gel-scanning attachment, model 2410.

Results and Discussion

The various nucleotide derivatives used in the present study are shown in Fig. 1. These were synthesized and purified (except Fig. 1*d*) as described above before attachment to CNBr-activated Sepharose 4B. The Sepharose-bound N^6-AMP derivative (Fig. 1*b*) is well established as a truly bio-specific affinity ligand for NAD^+-dependent dehydrogenases (e.g. see Mosbach *et al.*, 1971, 1972). Although the immobilized forms of the other derivatives are not so well documented, these too have been shown to act as affinity columns for this group of enzymes [Trayer *et al.* (1974) (C8-AMP and P-ADP); Barker *et al.* (1974) (R-AMP)]; for details of abbreviations see the legend to Fig. 1.

Comparison of the effectiveness of the various Sepharose-bound adenosine phosphate ligands in binding ox heart lactate dehydrogenase

The elution of ox heart lactate dehydrogenase (unless specifically stated otherwise, the lactate dehydrogenase used in this study was always the ox heart type), from a Sepharose–P-ADP derivative by an NAD^+ gradient, in the absence and presence of pyruvate, is shown in Fig. 2. The effect of pyruvate on elution of the lactate dehydrogenase by NAD^+ can be reconciled by assuming the formation of an abortive ternary complex between the enzyme, NAD^+ and pyruvate. Pyruvate alone does not effect elution. This experiment was repeated by using the Sepharose-bound conjugates of the other adenosine phosphate derivatives and the results are expressed in Table 1 (the results with the Sepharose–P-ADP column are included for comparison).

Before drawing any conclusions from these results it is worth making two comments. First, there was an exact reproducibility of the elution profiles in these studies (and in those described below) when the column dimensions and concentration gradients used were kept constant. Flow rate (up to 30ml/h at least) did not seem important. Given that the synthetic chemistry in preparing these ligands was precise (Trayer *et al.*, 1974) and the same concentration of ligand was used in each column [3.0 (±0.2) μmol of ligand/g wet wt. of Sepharose, see the Materials and Methods section], and assuming the same degree of availability of the Sepharose-bound ligand for the enzyme, then the results obtained with these different columns can be compared directly. Secondly, the formation of an abortive ternary complex between lactate dehydrogenase, NAD^+ and pyruvate was used by Ohlsson *et al.* (1972) to facilitate the elution of this enzyme from their Sepharose-bound N^6-AMP conjugate. These workers found it necessary to allow a 10–16h equilibration time for the formation of this complex on the column (see Everse *et al.*, 1971*a*) for efficient elution and preferred to use the oxidized form of the adduct synthesized by base-catalysed condensation of NAD^+ and pyruvate (Everse *et al.*, 1971*b*). In our hands and with the conditions described in the legend to Fig. 2, this was not found to be necessary and reproducible elution profiles were obtained with all of the adenosine phosphate columns. Lowe & Dean (1973) reported that they were unable to find any effect of either pyruvate or lactate on the elution of lactate dehydrogenase from their ε-aminohexanoyl-NAD^+–Sepharose conjugate by either a KCl or NAD^+ gradient. They attributed this anomaly to the fact that their experiments were completed within 2h whereas Ohlsson *et al.* (1972) required longer equilibration times for ternary-complex-formation. This is possible especially as a lower concentration of pyruvate (1mM) was used by Lowe & Dean (1973) than that used in the present study, and Everse *et al.* (1971*a*) do indicate that this variable affects the time-dependence of the ternary complex-formation. Another possible explanation may be that Lowe & Dean (1973) were using a chemically ill-defined polymer, which makes interpretation of their results more difficult.

The results shown in Fig. 2 and Table 1 do nevertheless describe the formation of both binary (enzyme–NAD^+) and ternary (enzyme–NAD^+–pyruvate) complexes and are consistent with a mechanism in which the substrate (or its analogue) cannot be

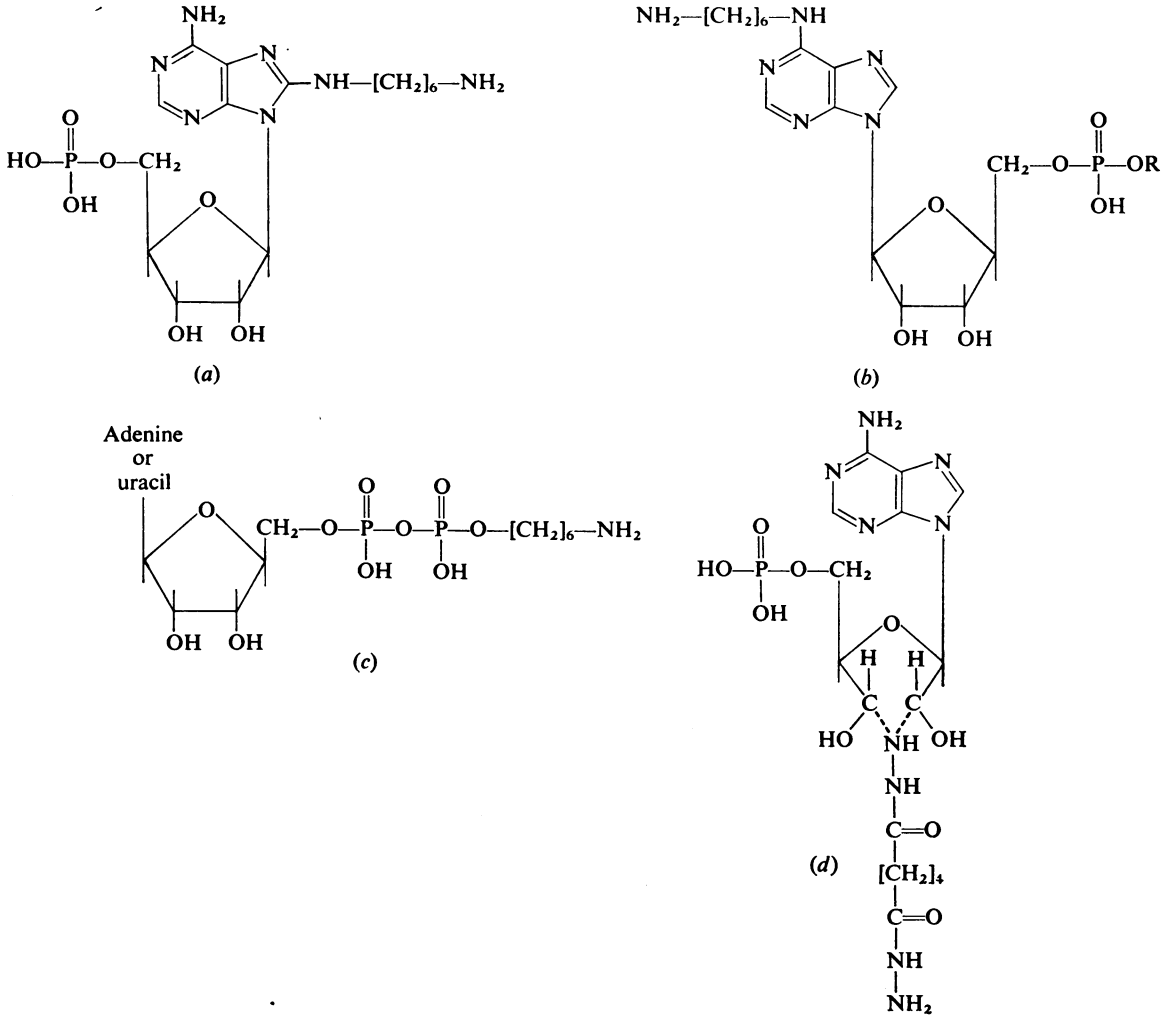

Fig. 1. *Structural formulae of nucleotide derivatives before attachment to an agarose matrix*

(*a*) 8-(6-Aminohexyl)amino-AMP (C8-AMP); (*b*) N^6-(6-aminohexyl)-AMP (R = H; N^6-AMP) or -ADP (R = $\begin{matrix}O\\\|\\P\text{—}OH\\|\\OH\end{matrix}$; N^6-ADP); (*c*), P^1-(6-aminohex-1-yl) P^2-(5′-adenosyl) pyrophosphate (P-ADP) or P^1-(6-aminohex-1-yl) P^2-(5′-uridyl) pyrophosphate (P-UDP); (*d*) ribosyl-linked AMP (R-AMP) prepared by reaction of periodate-treated AMP with an acyl hydrazide derivative of agarose (see the Materials and Methods section). The exact nature of the linkage between the periodate-oxidized ribose moiety and the acid hydrazide has not been established. The above abbreviations are used in the text to describe the various nucleotide derivatives shown here.

bound to lactate dehydrogenase unless the coenzyme is bound first. The results of these experiments, however, do not on their own exclude the formation of an enzyme–pyruvate complex. Such a mandatory order of binding has been established by kinetic observations (Zewe & Fromm, 1962; Gutfreund *et al.*, 1968) and by affinity chromatography (O'Carra & Barry, 1972) for lactate dehydrogenase and by kinetic studies for a number of other NAD^+-dependent dehydrogenases.

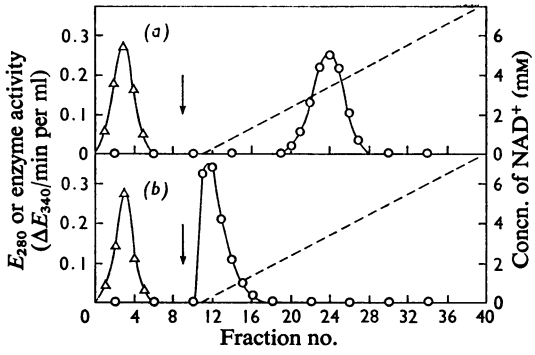

Fig. 2. *Effect of pyruvate on the elution by an NAD$^+$ gradient of lactate dehydrogenase from P-ADP–Sepharose*

Both columns (10cm×0.8cm) were equilibrated and eluted throughout with 50mM-sodium phosphate buffer, pH7.5, at a flow rate of 13ml/h and 1.3ml fractions were collected. The same protein mixture [human serum albumin (1ml, E_{280} 0.52) and 0.25ml of lactate dehydrogenase ($\Delta E_{340} \cdot min^{-1} = 2.5$)] was applied to each column. The columns were washed through with this buffer and developed with a linear gradient (at arrow) formed by 25ml of 50mM-sodium phosphate buffer, pH7.5, and 25ml of 10mM-NAD$^+$ dissolved in the phosphate buffer. In (*b*) conditions were as in (*a*) except that 5mM-sodium pyruvate was included in both of the gradient buffers. The fractions were monitored for enzymic activity and the progress of the gradient was determined as described in the Materials and Methods section. ○, Lactate dehydrogenase activity; △, E_{280} (human serum albumin); ----, gradient concentration.

Table 1. *Effect of pyruvate on the elution of ox heart lactate dehydrogenase by NAD$^+$ from immobilized adenosine phosphate derivatives*

The experiments were conducted exactly as described in the legend to Fig. 2.

Sepharose-bound adenosine phosphate derivative	Concentration of NAD$^+$ required for elution (mM)	
	NAD$^+$ alone	NAD$^+$+5mM-pyruvate
N^6-AMP*	9–9.5	0.5–0.7
P-ADP	3.50	0.20
C8-AMP	1.40	0.08
R-AMP	No binding	—

* The enzyme was eluted from this column in a broader peak than was observed with the other ligands.

The results were extended by repeating these experiments by using the reduced substrate, L-lactate, to effect the elution of lactate dehydrogenase by the oxidized coenzyme from these columns. Ternary-complex-formation was again demonstrated by the fact that NAD$^+$ and lactate together facilitated the desorption of the enzyme from the Sepharose–P-ADP columns over that observed with NAD$^+$ alone. This mixture was a less effective eluent than when the oxidized substrate was used. It may be that elution by NAD$^+$ plus lactate is caused by the formation of NADH *in situ* and, since NADH binds so much more tightly to this enzyme than NAD$^+$, only a small amount would be required. The effect was still observed, however, when 0.5mM-lactate was included in the gradient. These experiments were also shown to be applicable to other enzymes of this group since the elution of malate dehydrogenase from a Sepharose–P-ADP column by NAD$^+$ was similarly effected by the inclusion of either L-malate or oxaloacetate in the eluting gradient, again demonstrating ternary complex-formation. This effect is utilized to separate the two enzymes below.

The observations noted above are compatible with those of other workers for yeast alcohol dehydrogenase (Ohlsson *et al.*, 1972) and threonine dehydrogenase (Lowe *et al.*, 1973) where ternary-complex-formation between the enzyme, NAD$^+$ and substrate (or its analogue) has been used as a method of gaining specificity in elution from a general ligand. This model does depend on the enzyme under study displaying at least a preferred ordered addition of substrates.

As indicated above, the concentrations of NAD$^+$ required to elute lactate dehydrogenase from the various Sepharose-bound adenosine phosphate conjugates (Table 1) in the absence of any additions to the gradients can be compared directly and used to gain some insight into the topography of the nucleotide-binding site of this enzyme. Lactate dehydrogenase was chosen for this study so that the validity of the method could be tested against the known three-dimensional structure of this enzyme (Rossmann *et al.*, 1972; Chandrasekhar *et al.*, 1973; Adams *et al.*, 1973). The elution profiles show that the tightness of binding of lactate dehydrogenase to the adenosine phosphate ligands was in the order N^6-AMP > P-ADP > C8-AMP > R-AMP. This order is entirely consistent with the results of the X-ray crystallographic analysis. The 6-amino group of the adenosine is pointing away from the enzyme and attachment of the alkyl 'spacer' arm at this point on the AMP molecule (Fig. 1*b*) might be expected to allow maximum interaction of the AMP with the enzyme. The observation is also consistent with the fact that analogues of NAD$^+$ substituted in the 6-position of the adenine nucleus can serve as efficient coenzymes for lactate dehydrogenase (e.g. see McPherson, 1970; Windmueller & Kaplan, 1961). Although a comparison of the free energies of binding of various adenine nucleosides and nucleotides (McPherson, 1970) indicates the importance of the 5′-phosphate of AMP in binding this nucleotide to

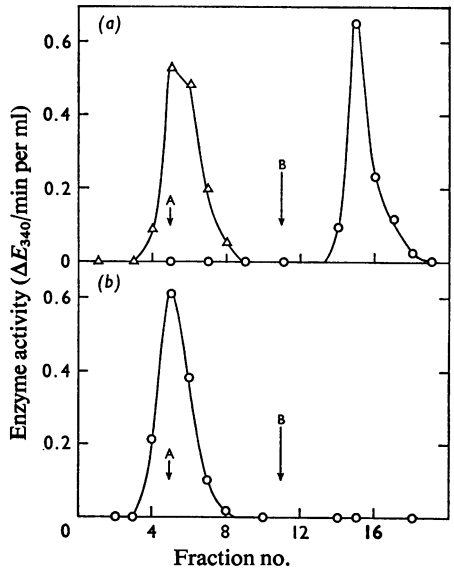

Fig. 3. *Comparison of the behaviour of lactate dehydrogenase from rabbit muscle (M_4) and ox heart (H_4) on R-AMP–Sepharose*

Both columns (10cm×0.8cm) were equilibrated and eluted with 50mM-sodium phosphate buffer, pH7.5, at a flow rate of 13ml/h and 1.3ml fractions collected. Samples were applied as follows: (*a*) 0.25ml of rabbit muscle lactate dehydrogenase (M_4) ($\Delta E_{340}\cdot min^{-1} = 1.53$) containing pyruvate kinase activity ($\Delta E_{340}\cdot min^{-1} = 1.81$); (*b*) 0.25ml of ox heart lactate dehydrogenase (H_4) ($\Delta E_{340}\cdot min^{-1} = 1.45$). The void volume of the column was determined by including human serum albumin in the sample and its elution position is shown at A (for the sake of clarity the E_{280} profile has been omitted). At B, 0.2mM-NADH was included in the developing buffer. The fractions were monitored for enzymic activity as described in the Materials and Methods section. ○, Lactate dehydrogenase activity; △, pyruvate kinase activity.

lactate dehydrogenase, the data also suggest that the second phosphate does not play a significant role in binding ADP. In fact, the ADP difference electron-density map shows two partially occupied sites for the second phosphate (Chandrasekhar *et al.*, 1973). Attachment of the 'spacer' arm through this second phosphate therefore provides another ligand capable of interacting strongly with the enzyme. This mode of attachment may, however, weaken the interactions between the 5′-phosphate(s) and the enzyme and thus it does not bind lactate dehydrogenase as well as the N^6-substituted AMP. The C-8 position on the adenine nucleus is orientated towards the surface of the hydrophobic crevice in which the adenosine portion of NAD^+ binds (Chandrasekhar *et al.*, 1973; Adams *et al.*, 1973), but it does not protrude from it as the 6-amino group of the adenine moiety. Thus attachment through this position might be expected to considerably limit the interactions with the enzyme. The 2′,3′-hydroxyl groups of the adenosine moiety are buried within the crevice and it is not surprising that the R-AMP derivative (Fig. 1*d*) did not interact with the enzyme.

Rabbit muscle lactate dehydrogenase (M_4) did, however, bind to the R-AMP column [and to the other three AMP–Sepharose derivatives (Trayer *et al.*, 1974)] under the same conditions that the ox heart (H_4) enzyme passed unretarded through this column (Fig. 3). It is noteworthy that low concentrations of KCl (<0.3M) would elute the M_4 enzyme from an R-AMP–Sepharose conjugate but not from columns made with the other three AMP analogues and so non-specific ion-exchange effects cannot be entirely ruled out. The bio-specificity of the Sepharose–R-AMP column is indicated, however, by the fact that pyruvate kinase activity appeared in the void column (Fig. 3) under conditions which caused interaction between the nucleotide and the M_4 dehydrogenase and this enzyme could be eluted by low concentrations of the reduced cofactor. This observation of the different behaviour of the two isoenzymes on the Sepharose–R-AMP column does suggest a difference in the NAD^+-binding site between them. Unfortunately, this is the least chemically defined of our ligands and the effect of the periodate oxidation on the three-dimensional integrity of the AMP molecule is difficult to assess. Thus only a small difference between the two isoenzyme NAD^+-binding sites could account for this observation. Small differences in the binding of NAD^+/NADH to either muscle (M_4) or heart (H_4) lactate dehydrogenases have been noticed by both equilibrium binding (Stinson & Holbrook, 1973) and affinity chromatography (Lowe & Dean, 1973) studies. Whether the differences observed in the present study are tissue- or species-specific remains to be answered.

The use of affinity chromatography in obtaining further insight into the binding of NAD^+ to lactate dehydrogenase can be seen in Fig. 4. Again, to compare the elution profiles directly the column dimensions and gradient concentrations used were kept constant. The concentration of AMP required to elute the enzyme was unaffected by the inclusion of either pyruvate or lactate in the gradient (Figs. 4*a* and 4*b*). This observation, when compared with those expressed in Fig. 2, is best explained by suggesting that substrate cannot bind to the enzyme–AMP binary complex, i.e. the NMN^+ half of the NAD^+ molecule is required for ternary-complex-formation. These results are entirely consistent with the conclusions reached by McPherson (1970) from kinetic data and by O'Carra & Barry (1972) from their affinity chromatography studies; i.e. the AMP portion of the coenzyme must

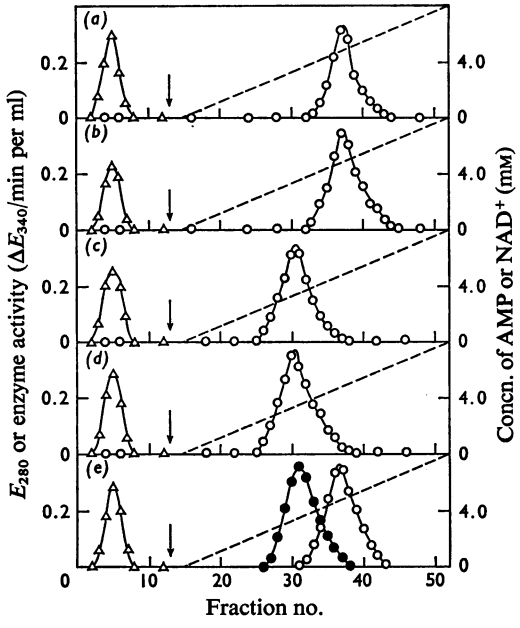

Fig. 4. *Effect of NMN(H) and substrates on the elution by AMP and NAD+ gradients of ox heart lactate dehydrogenase from P-ADP–Sepharose*

In each experiment, the columns (10cm×0.8cm) were operated in 50mM-sodium phosphate buffer, pH7.5, at a flow rate of 13ml/h and 1.1ml fractions were collected. To each column was applied 1ml of human serum albumin ($E_{280} = 0.52$) and 0.25ml of lactate dehydrogenase ($\Delta E_{340} \cdot min^{-1} = 2.1$). The arrow indicated where the linear gradient was applied. These gradients were formed from 25ml each of the phosphate buffer and either 10mM-AMP or 10mM-NAD^+ dissolved in this buffer. Enzyme activity and the progress of the gradient were determined as described in the Materials and Methods section. Each experiment was conducted as follows: (*a*) AMP gradient alone; (*b*) AMP gradient containing 5mM-sodium pyruvate (or 20mM-lactate) throughout; (*c*) AMP gradient containing 1.5mM-NMN^+ (or NMNH) throughout; (*d*) AMP gradient containing 1.5mM-NMN^+ plus 5mM-sodium pyruvate throughout; (*e*) NAD^+ gradient, either alone (●) or containing 1.5mM-NMN^+ (○) throughout. △, E_{280} (human serum albumin); ○ or ●, lactate dehydrogenase activity; ----, gradient concentration.

generate a site for the nicotinamide moiety before reaction with substrate. A comparison of profiles of Figs. 4(*a*) and 4(*c*) also supports the idea that NMN^+ can complex with the enzyme in the presence of AMP. Here, the inclusion of NMN^+ in the AMP gradient facilitates the dissociation of the enzyme–P-ADP–Sepharose complex, presumably through the formation of a tighter enzyme–AMP–NMN^+ complex. NMN^+ should be able to enhance the binding of lactate dehydrogenase to the Sepharose–P-ADP conjugates by a similar mechanism. This is indicated by the results described in Fig. 4(*e*), where a higher concentration of NAD^+ is required to elute the enzyme when NMN^+ is included in the gradient. The results shown in Figs. 4(*c*) and 4(*e*) are entirely consistent with one another if one assumes that AMP in free solution has less restrictions in binding to the enzyme than the P-ADP–Sepharose conjugate. In Fig. 4(*d*) an attempt was made to demonstrate that substrate could bind once AMP and NMN^+ have bound to the enzyme. No effect of including either pyruvate or lactate in the AMP gradient plus NMN^+ was noticed. Although several explanations are possible, it is noteworthy that the correct orientation of the pyrophosphate moiety of NAD^+ on this dehydrogenase may be a prerequisite for the conformational changes which allow substrate binding and thus ternary-complex-formation to occur (Adams *et al.*, 1973; Chandrasekhar *et al.*, 1973). It would be interesting to repeat the experiments shown in Figs. 4(*c*) and 4(*d*) by using an ADP-ribose gradient including nicotinamide with and without the substrate.

NMN(H) alone neither caused elution of lactate dehydrogenase from the P-ADP–Sepharose nor caused any inhibition of enzyme activity when the assays were performed in the presence of this nucleotide (up to 10mM). Similar results were also observed when a C8-AMP–Sepharose conjugate was used in the experiments described in Fig. 4.

The detailed manner in which NAD^+ and NADH interact with lactate dehydrogenase still remains to be resolved. It is not possible, at present, for example, to explain why NADH binds to this enzyme so much more tightly than NAD^+. Although our results cannot entirely rule out the existence of an enzyme–NMN(H) complex they are consistent with a model in which the AMP moiety of the coenzyme binds to the enzyme first, followed by the NMN^+ portion and so generates a site for the substrate.

Lactate dehydrogenase would also bind to an N^6-ADP–Sepharose conjugate and this column could be used to effect a separation between this enzyme and a kinase (rat liver glucokinase) by using their differing affinities for AMP and ATP (Fig. 5). Both enzymes bound to this column and development with 10mM-$MgATP^{2-}$ caused elution of the glucokinase but not the lactate dehydrogenase. The latter enzyme was dissociated from the column by including 30mM-AMP in the buffer (Fig. 5*a*). If the AMP was used before the ATP as an eluent then both enzymes appeared in the subsequent fractions (Fig. 5*b*). The effect of AMP on the glucokinase may, however, be a specific effect for this particular kinase (M. J. Holroyde & I. P. Trayer, unpublished work). If 1mM-NADH was used instead of the AMP then this caused specific elution of the dehydrogenase

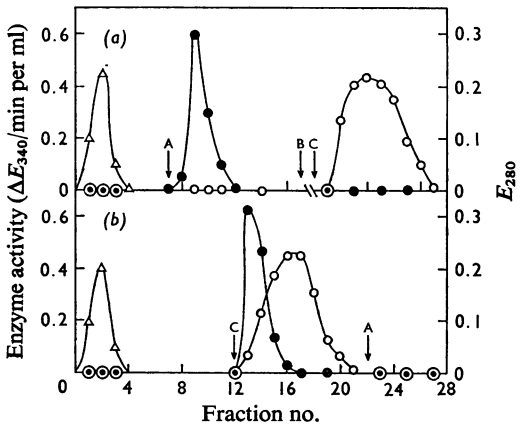

Fig. 5. *Separation of ox heart lactate dehydrogenase and rat liver glucokinase on N^6-ADP–Sepharose*

Each column (7cm×0.8cm) was equilibrated to and operated in 25mM-Tris–HCl, pH7.5, containing 10mM-KCl, 5mM-$MgCl_2$ and 0.5mM-dithiothreitol. The sample applied to each column contained 0.5ml of bovine serum albumin ($E_{280} = 1.1$), 0.7ml of glucokinase ($\Delta E_{340} \cdot min^{-1} = 4.2$), and 0.2ml of lactate dehydrogenase ($\Delta E_{340} \cdot min^{-1} = 8.0$). The columns were run at a flow rate of 20ml/h and 3.6–4.0ml fractions collected. The following inclusions were made to buffer at the points indicated: A, 10mM-ATP, 5mM-$MgCl_2$; B, nothing; C, 30mM-AMP. Enzyme activities were measured as described in the Materials and Methods section. In (*a*) ATP was used as an eluent before AMP and in (*b*) the order was reversed. △, E_{280} (bovine serum albumin); ○, lactate dehydrogenase activity; ●, glucokinase activity.

and no effect was observed on the kinase–N^6-ADP–Sepharose interaction. Thus specific elution of either enzyme in any order could be effected on this column.

Separation of mixtures of dehydrogenases on the Sepharose-bound conjugates of the various adenosine phosphate ligands

A mixture of three NAD^+-dependent dehydrogenases (lactate dehydrogenase, malate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase) and one $NADP^+$-dependent dehydrogenase (glucose 6-phosphate dehydrogenase) was applied in turn to three of the Sepharose–adenosine phosphate derivatives (Fig. 6). In each case the void volume of the column was estimated by including human serum albumin in the application mixture. The $NADP^+$-dependent glucose 6-phosphate dehydrogenase appeared in the first bed-volume of eluate in each case before the application of a linear NAD^+ gradient. No significant interaction could be noticed between this enzyme and the P-ADP and N^6-AMP ligands (Figs. 6*a* and 6*c*). Slight interaction was observed with the C8-AMP–Sepharose derivative and the passage of the enzyme through the column was retarded, and it appeared after the albumin (V_0) peak (Fig. 6*b*). These observations do suggest that the coenzyme, $NADP^+$, is orientated differently in the active site of this enzyme than is NAD^+ in the other enzymes tested. All of these NAD^+-dependent dehydrogenases have considerable tertiary structure homology in the coenzyme-binding site region [Adams *et al.*, 1973 (lactate dehydrogenase); Hill *et al.*, 1972; Webb *et al.*, 1973 (malate dehydrogenase); Buehner *et al.*, 1973 (glyceraldehyde 3-phosphate dehydrogenase)], and thus all would be expected to interact with the adenosine phosphate ligands. Clearly glucose 6-phosphate dehydrogenase does not 'recognize' AMP when it is attached through the ribose hydroxyl groups (Fig. 6*d*) after periodate oxidation. This is not really surprising since even though the exact configuration and integrity of the AMP molecule after this treatment is difficult to predict, one would expect the 3′-phosphate of $NADP^+$ to play a positive role in helping the enzyme to 'recognize' this cofactor. In this respect it is noteworthy that periodate oxidation of $NADP^+$, which would be restricted to the nicotinamide ribose, followed by coupling to a Sepharose–adipic dihydrazide derivative, does provide an immobilized ligand for this enzyme to interact with (Lamed *et al.*, 1973). Although glucose 6-phosphate dehydrogenase cannot be taken as wholly representative of $NADP^+$-dependent dehydrogenases, in this enzyme at least it is possible to speculate that the adenine and ribose phosphate moieties of AMP may play either a more important and/or different role in the enzyme–coenzyme interactions than is found in the NAD^+-dependent dehydrogenases tested. In view of the tertiary structure homology of the NAD^+-binding sites of the other dehydrogenases used and the apparent conservation of this nucleotide binding site in phosphoglycerate kinase (Bryant *et al.*, 1974; H. C. Watson, personal communication) it is quite possible that $NADP^+$-dependent dehydrogenases also possess this structural feature. The results obtained in the present study cannot be interpreted to either support or deny this conjecture. There appears to be considerable latitude by which an enzyme can bind nucleotides, e.g. in adenylate kinase the 6-amino group on the adenine ring of one of the nucleotide sites is buried (Schirmer & Schulz, 1973) whereas in lactate dehydrogenase it protrudes from the enzyme surface (Adams *et al.*, 1973; Chandrasekhar *et al.*, 1973).

When the elution profiles obtained by linear NAD^+ gradients of the three NAD^+-dependent dehydrogenases from the three Sepharose–adenosine phosphate conjugates are compared, however, the sensitivity of the method for separating these enzymes

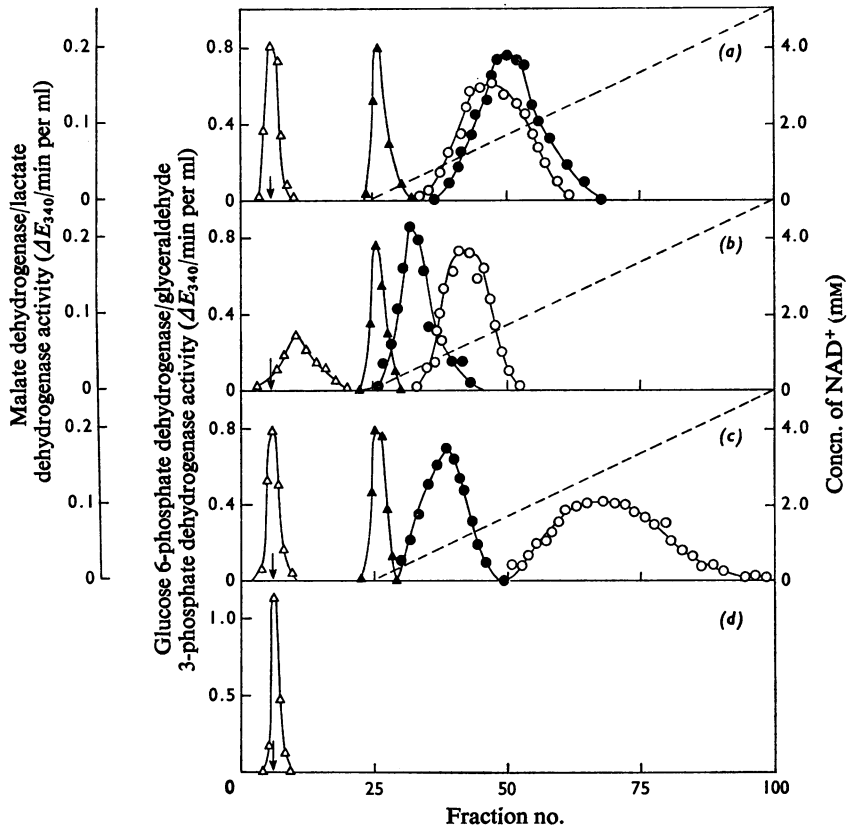

Fig. 6. *Separation of mixtures of dehydrogenases on sepharose-bound adenosine phosphate conjugates*

Each column (10cm×0.8cm) was equilibrated to and operated in 50mM-sodium phosphate buffer, pH7.5, at a flow rate of 13ml/h and 1.3ml fractions were collected. The different Sepharose-bound adenosine phosphate conjugates used were (*a*) P-ADP, (*b*) C8-AMP, (*c*) N^6-AMP and (*d*) R-AMP. The same sample containing 1.0ml of human serum albumin ($E_{280} = 0.52$), 0.2ml of lactate dehydrogenase ($\Delta E_{340} \cdot min^{-1} = 3.0$), 0.1ml of malate dehydrogenase ($\Delta E_{340} \cdot min^{-1} = 3.2$), 0.25ml of glyceraldehyde 3-phosphate dehydrogenase ($\Delta E_{340} \cdot min^{-1} = 3.3$), 0.2ml of glucose 6-phosphate dehydrogenase ($\Delta E_{340} \cdot min^{-1} = 3.4$), was applied to each column in turn except in (*d*) where only the human serum albumin and the glucose 6-phosphate dehydrogenase were applied. A linear gradient formed from 50ml of phosphate buffer and 50ml of 5mM-NAD^+ dissolved in this buffer was applied to each column at fraction number 21. The E_{280} profile of the human serum albumin has been omitted for the sake of clarity, but the position of the void volume so determined is indicated by the arrow. Enzymic activities and the progress of the gradients were monitored as described in the Materials and Methods section. △, Glucose 6-phosphate dehydrogenase activity; ▲, glyceraldehyde 3-phosphate dehydrogenase activity; ○, lactate dehydrogenase activity; ●, malate dehydrogenase activity; ----, gradient concentration.

is well illustrated. The N^6-AMP–Sepharose column clearly separates these enzymes in one operation (Fig. 6*c*), whereas on the C8-AMP–Sepharose derivative slight overlapping of the malate and lactate dehydrogenase activities occurred (Fig. 6*b*). The P-ADP–Sepharose conjugate gave a good separation of the glyceraldehyde 3-phosphate dehydrogenase from the malate and lactate dehydrogenases, but there was considerable contamination of these latter two enzymes one with the other. It should be noted that by using more shallow and/or different shape gradients the separation of the enzymes could be noticeably improved. Identical gradients are shown in Fig. 6, however, for comparative purposes.

In the case of malate and lactate dehydrogenases the crystallographic similarities have been extended by kinetic analyses where similar K_m values for the oxidized coenzyme have been reported: lactate dehydrogenase, $K_m = 1.8 \times 10^{-4}$M (McPherson, 1970); cytoplasmic malate dehydrogenase, $K_m = 2.0 \times 10^{-4}$M (Grimm & Doherty, 1961). Despite these similarities, however, it is still possible to achieve good separations

of these enzymes from one another by exploiting minor differences in their abilities to bind to the immobilized AMP ligand or to the displacing ion, NAD^+. This is seen in any one profile in Fig. 6. A comparison of the elution profiles of malate and lactate dehydrogenase shows that it is also possible to exploit minor differences in the orientation of AMP in the NAD^+-binding site by offering to the enzymes AMP immobilized in different but defined ways. Malate dehydrogenase was eluted slightly after lactate dehydrogenase on the Sepharose–P-ADP conjugate (Fig. 6*a*), but before it on the Sepharose-bound C8-AMP and N^6-AMP derivatives. The different elution positions of the two enzymes suggest that the adenine moiety of AMP may be more involved in binding this nucleotide to malate dehydrogenase than to lactate dehydrogenase. It is not possible at present to decide whether the X-ray crystallographic evidence supports this or not. The report by Webb *et al.* (1973) on the conformation of NAD^+ bound to cytoplasmic malate dehydrogenase does indicate that the orientation of the adenine moieties of enzyme-bound NAD^+ molecules may be slightly different between these two dehydrogenases.

These experiments were not repeated with either AMP or NADH as the displacing ions, but these might also be expected to influence the elution position of the enzymes. Thus extensive purifications of dehydrogenases from a crude mixture might be expected by varying either the choice of immobilized AMP derivative or the displacing ion or both. If these techniques alone are insufficient to achieve the required results then extra selectivity could be introduced by including substrate (or an analogue) into the eluting gradient as described above. To illustrate this a good separation of malate and lactate dehydrogenases was achieved on a Sepharose–P-ADP column by including either pyruvate or lactate or malate in the NAD^+ gradient. In the former case lactate dehydrogenase was eluted first and in the latter, malate dehydrogenase.

Glyceraldehyde 3-phosphate dehydrogenase was always eluted by very low NAD^+ concentrations in these experiments. It was retained on the columns when these were irrigated with salt solutions (0.2M-NaCl), suggesting this effect was specific. The most likely explanation is that this enzyme has a much greater affinity for NAD^+ than AMP and/or that this nucleotide may not play the same role in binding NAD^+ to this enzyme as it does to lactate dehydrogenase. (N.B. No attempt was made to determine how many of the coenzyme-binding sites were already occupied by NAD^+ in the commercial preparation of this enzyme used in this study.) It is noteworthy, however, that O'Carra & Barry (1973) were unable to elute this enzyme from a matrix-bound NAD^+ polymer by high concentrations of AMP although NAD^+ itself was an effective counter ligand.

Studies with uridine diphosphate glucose dehydrogenase

UDP-glucose dehydrogenase catalyses the oxidation of UDP-glucose to UDP-glucuronic acid by using 2mol of NAD^+ in the overall four-electron oxidation. It was chosen to test some of the methods described above for 'model' systems because (*a*) although a certain amount of knowledge is available on this enzyme its mechanism of action remains largely unelucidated and (*b*) a comparison of its behaviour on Sepharose-bound P-ADP and P-UDP conjugates (Fig. 1*c*) might be expected to yield some information on this subject. Preliminary experiments showed that it was necessary to include a thiol-protecting agent (10mM-2-mercaptoethanol) in the phosphate buffer in order to obtain 100% binding to the affinity columns and quantitative elution (Gainey *et al.*, 1972). This enzyme binds readily to both the Sepharose–P-ADP and Sepharose–P-UDP derivatives (Figs. 7*a* and 7*b*) and can be eluted quantitatively by including either NAD^+ or UDP-glucose respectively in the developing buffers. These two ligands differ from each other only by the base moiety, the synthesis and mode of attachment to the Sepharose matrix being identical (Barker *et al.*, 1974; Trayer *et al.*, 1974). Thus it was important to demonstrate that the P-UDP ligand was specific for the UDP-glucose-binding site and the P-ADP ligand for the NAD^+ site(s). The profiles shown in Figs. 7(*a*) and 7(*b*) where the appropriate counter ligand caused quantitative elution of the enzyme from the columns strongly suggests this. Similarly none of the dehydrogenases used before in this study showed any affinity whatsoever for the Sepharose–P-UDP conjugate. Fairly high concentrations of the displacing ions (10mM-UDP-glucose, 20mM-NAD^+) were required to obtain efficient elution of the enzyme from these columns. If lower concentrations were used then, although enzyme activity did appear in the eluate, it was considerably diluted and large volumes of eluate had to be collected (at considerable expense) before quantitative recovery of the enzyme was obtained. Elution could be effected by either AMP or ADP (from the Sepharose–P-ADP column) or UMP or UDP (from the Sepharose–P-UDP column), but since the enzyme is very sensitive to inhibition by these nucleotides (Zalitis & Feingold, 1968, 1969) it was necessary to dialyse the fractions before the assay and so this method of elution was abandoned. All of these experiments are consistent with the enzyme binding strongly yet specifically at either its UDP-glucose or NAD^+-binding sites.

NAD^+ (20mM) did not cause any significant elution of the enzyme from the P-UDP–Sepharose conjugate (Fig. 7*c*) as expected from the above observations. Subsequent application of 10mM-UDP-glucose (after a buffer wash) did elute enzymic activity, but there

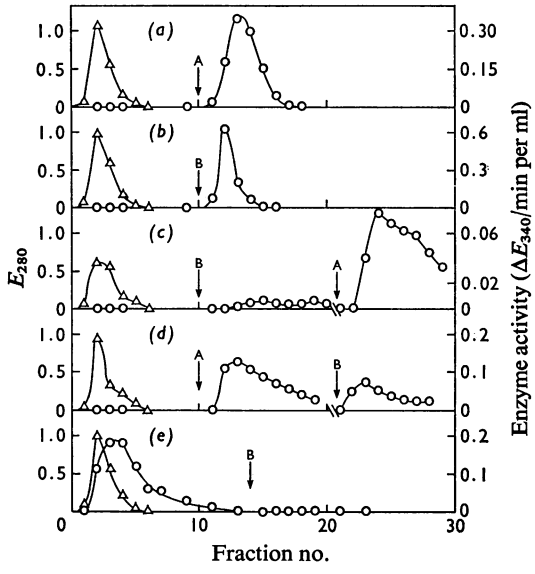

Fig. 7. *Affinity chromatography of UDP-glucose dehydrogenase on either P-ADP–Sepharose or P-UDP–Sepharose*

All columns (10cm×0.8cm) were operated in 50mM-sodium phosphate buffer, pH7.5, containing 10mM-2-mercaptoethanol at a flow rate of 20ml/h and 2–4ml fractions were collected. UDP-glucose dehydrogenase, 0.5ml ($\Delta E_{340} \cdot min^{-1} = 2.5$ to 4.0), previously equilibrated to this buffer, was applied to the following Sepharose-bound nucleotides: (*a*) P-UDP, (*b*) P-ADP, (*c*) P-UDP, (*d*) P-ADP, (*e*) P-ADP. At A, 10mM-UDP-glucose was included in the buffer and at B, 20mM-NAD^+. The breaks in the abscissa in profiles (*c*) and (*d*) indicate where 10ml of the phosphate buffer without any additions was run through the columns. In (*e*) the enzyme was applied to and eluted from the column with the phosphate buffer containing 1mM-UDP-glucose. Enzyme activity was determined as described in the Materials and Methods section. △, E_{280}; ○, UDP-glucose dehydrogenase activity.

was significant trailing and quantitative elution did not occur over the same volume of eluate as in Fig. 7(*a*). The most likely explanation is that complex-formation between the immobilized ligand, NAD^+ and the enzyme had occurred. When 10mM-UDP-glucose was applied to a P-ADP–Sepharose column (Fig. 7*d*), to which the dehydrogenase had been bound, a significant amount of activity appeared in the eluate [about 50–60% of that applied in the same volume as 100% appeared when 20mM-NAD^+ was the counter ligand (Fig. 7*b*)]. A further elution with 20mM-NAD^+ caused additional appearance of enzymic activity in the eluate and again trailing occurred indicating possible ternary (quaternary) complex-formation. These results were consistently obtained and require some explanation. It is unlikely that the P-ADP ligand is binding to the UDP-glucose site even though ADP-glucose does act as a poor substrate for this enzyme ($K_m = 20$mM, Zalitis & Feingold, 1968). If this were so, then efficient elution by UDP-glucose should have been observed and NAD^+ should not have caused dissociation of the enzyme–P-ADP complex. When the enzyme was applied to the Sepharose–P-ADP derivative in the presence of 1mM-UDP-glucose (Fig. 7*e*) then all of the applied activity appeared in the void volume with slight trailing, and no further enzyme was seen after applying 20mM-NAD^+. UDP-glucose dehydrogenase did bind to a P-UDP–Sepharose column, however, when various concentrations of NAD^+ (up to 20mM) were included in the application buffer.

It is difficult to reconcile these observations into a simple scheme and further affinity chromatographic studies in conjunction with a detailed kinetic analysis is necessary before any firm conclusions about the mechanism of action of this enzyme can be drawn. Certain points, however, can be made. These results are not consistent with a mechanism of compulsory order of addition in which the coenzyme binds before the substrate, a scheme into which lactate and malate dehydrogenase readily fit. A reversal in the order of binding in a compulsory order of addition scheme is equally unlikely by a similar reasoning. The fact that UDP-glucose will cause some elution from a P-ADP–Sepharose derivative (Fig. 7*d*) also argues against a completely random addition of substrates. It is perhaps wrong to think in terms of 'all or nothing' pathways, but rather one should consider a series of alternative pathways and the possible flux through each pathway. In this case, the binding of UDP-glucose dehydrogenase to these affinity columns can only be seen as indicating that there is not a virtual 100% flux through either a simple ordered or random pathway.

Some support for these experiments can be obtained from the work of Gainey *et al.* (1972) on the thiol-group reactivity of this enzyme. Although these workers do report that unpublished work indicated a mechanism with compulsory order of addition, the NAD^+ binding before UDP-glucose, they found that UDP-glucose alone protected their 'fast-reacting' thiol groups, suggesting that this molecule has a finite affinity for the enzyme even when NAD^+ is absent. Their results were also consistent with the assumption that the binding of either NAD^+ or UDP-glucose to the enzyme each results in different conformational effects.

The present work was performed on a crude commercial preparation of the enzyme and it was noted that when either column was used in the above experiments a large amount of inactive protein passed through unretarded. Fig. 8 shows the results of applying the enzyme eluted from a P-UDP–Sepharose

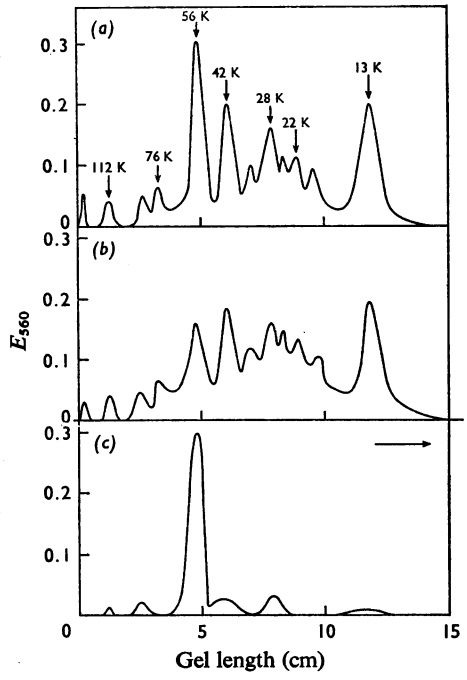

Fig. 8. *Sodium dodecyl–polyacrylamide-gel electrophoresis of UDP-glucose dehydrogenase fractions passed down a P-UDP–Sepharose column*

(*a*) Sample applied to column; (*b*) inactive material appearing in the void volume; (*c*) material eluted with UDP-glucose. The column was operated as described in Fig. 7(*a*). The ordinate is the intensity of Coomassie Brilliant Blue-stained material, as measured by its E_{560}. The abscissa indicates the distance that the proteins have migrated (in cm) from the origin. Migration is from left to right as indicated by the arrow. The molecular weights of the main peaks are indicated in (*a*) (K = $\times 10^3$) and were determined by comparison with known standards.

column to polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. The sample applied to the column contains a large number of proteins (Fig. 8*a*), most of which are inactive and appear in the void volume (Fig. 8*b*). The fraction eluted by UDP-glucose (Fig. 8*c*) contained all of the applied activity and was essentially a single component (greater than 90% homogeneous as judged by gel scanning) of subunit molecular weight 56000. This agrees very well with published values for the subunit molecular weight of this enzyme (Zalitis & Feingold, 1969; C. Phelps, personal communication). A further pass down either the P-UDP–Sepharose or the P-ADP–Sepharose column might be expected to yield an entirely homogeneous product. The breakthrough peak (Fig. 8*b*) still contained some material migrating in this position, but as no enzymic activity was found in this fraction it could indicate the presence of a considerable amount of denatured enzyme in the starting material, which was resolvable from active protein by these methods. Similar purifications could also be obtained on the P-ADP–Sepharose conjugate. No attempt was made to purify the enzyme direct from liver extracts, but this seems feasible particularly if both columns were used in series and the liver extract was first subjected to some initial fractionation procedure, e.g. $(NH_4)_2SO_4$ fractionation (Zalitis & Feingold, 1969; Wilson, 1965). Such a procedure could be expected to result in a homogeneous product in high yield.

General considerations

The results found in the present study support the concept of 'general ligand' affinity chromatography. Resolution of one enzyme from a mixture can be obtained by exercising careful choice over the ligand used and the elution conditions employed. Fine resolution of enzyme mixtures was obtained by allowing NAD^+ and AMP in free solution to compete with the variously immobilized AMP derivatives for the enzymes. Extra control was exerted by making various additions to the gradients. Lowe *et al.* (1974) and Harvey *et al.* (1974*a*, *b*) have shown that control of pH, ligand concentration on the matrix and temperature respectively can also be used to influence enzyme elution from the columns. The report by Lindberg *et al.* (1973) of the relatively simple synthesis of a chemically defined NAD^+ analogue should allow even further exploitation of this technique. The use of chemically defined ligands appears to remove many of the objections to the 'general ligand' affinity chromatography raised by Barry & O'Carra (1973*a*).

The enzymes chosen for part of this study were selected because their three-dimensional crystal structures and solution kinetics were well-documented. This enabled one to test affinity chromatography methodology as a means of gaining information into the mechanism of action and topography of the active site of these enzymes. The results obtained agreed very well with published observations and some of the methods were used to offer a simple means of purifying UDP-glucose dehydrogenase and to give some idea of its mode of action.

Clearly, affinity chromatography does not offer to be a panacea to all the problems of the enzymologist but it should be entered into his arsenal of weapons. It is readily acknowledged that the method has given a great boost to separation technology. By using different chemically defined ligands the results of this study indicate that it is also possible to use this procedure to probe the active site of an enzyme in such a way that valuable

information about the mechanism of action of the enzyme and the manner in which substrates or coenzymes are bound to it can be obtained.

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