Biochem. J. (1973) **135**, 595–607
Printed in Great Britain

Affinity Chromatography of Nicotinamide–Adenine Dinucleotide-Linked Dehydrogenases on Immobilized Derivatives of the Dinucleotide

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(*Received* 2 *April* 1973)

1. Three established methods for immobilization of ligands through primary amino groups promoted little or no attachment of NAD^+ through the 6-amino group of the adenine residue. Two of these methods (coupling to CNBr-activated agarose and to carbodi-imide-activated carboxylated agarose derivatives) resulted instead in attachment predominantly through the ribosyl residues. Other immobilized derivatives were prepared by azolinkage of NAD^+ (probably through the 8 position of the adenine residue) to a number of different spacer-arm–agarose derivatives. 2. The effectiveness of these derivatives in the affinity chromatography of a variety of NAD-linked dehydrogenases was investigated, applying rigorous criteria to distinguish general or non-specific adsorption effects from truly NAD-specific affinity (bio-affinity). The ribosyl-attached NAD^+ derivatives displayed negligible bio-affinity for any of the NAD-linked dehydrogenases tested. The most effective azo-linked derivative displayed strong bio-affinity for glyceraldehyde 3-phosphate dehydrogenase, weaker bio-affinity for lactate dehydrogenase and none at all for malate dehydrogenase, although these three enzymes have very similar affinities for soluble NAD^+. Alcohol dehydrogenase and xanthine dehydrogenase were subject to such strong non-specific interactions with the hydrocarbon spacer-arm assembly that any specific affinity was completely eclipsed. 3. It is concluded that, in practice, the general effectiveness of a general ligand may be considerably distorted and attenuated by the nature of the immobilization linkage. However, this attenuation can result in an increase in specific effectiveness, allowing dehydrogenases to be separated from one another in a manner unlikely to be feasible if the general effectiveness of the ligand remained intact. 4. The bio-affinity of the various derivatives for lactate dehydrogenase is correlated with the known structure of the NAD^+-binding site of this enzyme. Problems associated with the use of immobilized derivatives for enzyme binding and mechanistic studies are briefly discussed.

Affinity chromatography, initially applied almost exclusively to mono-substrate enzymes, has recently been applied increasingly to kinetically complex, multi-substrate enzymes, in particular the NAD-dependent dehydrogenases. Two approaches to affinity chromatography of these NAD-dependent enzymes have been advocated: (*a*) The 'general-ligand' approach, advocated by Mosbach *et al.* (1972) and Lowe & Dean (1971*a*), utilizing immobilized derivatives of the 'cofactor', i.e. the common or general substrate, NAD^+; (*b*) the 'specific-ligand' approach, involving the development of effective immobilized analogues of the specific substrates, e.g. of pyruvate in the case of lactate dehydrogenase (O'Carra & Barry, 1972).

Since ultra-specificity is generally the goal of affinity chromatography as a purification method, the latter 'specific-ligand' approach seemed generally preferable to us in view of its much greater inherent specificity (O'Carra & Barry, 1972). However, the 'general-ligand' approach seemed to have the advantage that a single immobilized derivative might be useful in the purification of a wide range of enzymes (Mosbach *et al.*, 1972; Lowe & Dean, 1971*a*). Moreover, an effective immobilized NAD^+ derivative would be useful for studies of enzyme-binding mechanisms (see O'Carra & Barry, 1972; Ohlsson *et al.*, 1972).

Consequently we investigated immobilized NAD^+ derivatives as well as immobilized specific substrates. The present paper describes our investigations into a number of potential methods for the immobilization of NAD^+ and model affinity chromatographic studies designed to test the effectiveness of the resulting immobilized derivatives. The conclusions we draw differ in important respects from those of other authors who have studied immobilized NAD^+ derivatives. A preliminary account of some of our findings has been presented elsewhere (O'Carra & Barry, 1973).

Experimental

Terminology

In its original usage, the term 'affinity chromatography' denoted a type of adsorption chromatography dependent on functionally or biologically specific adsorption effects, such as enzyme–substrate binding (Cuatrecasas *et al.*, 1968). However, the term does not explicitly convey such a specific meaning since the word 'affinity' could be validly applied to any type of adsorption. Indeed it has been increasingly used in this more general sense in connexion with adsorption effects not considered to have any direct dependence on biological specificity (e.g. Hofstee, 1973; Röschlau & Hess, 1972). Moreover, some of the affinity chromatographic procedures originally considered to depend on biologically specific adsorption have recently been shown to depend largely on generalized adsorption effects related to gross physico-chemical properties rather than to active-site specificities (O'Carra *et al.*, 1973; O'Carra, 1973; and evidence presented in the present paper).

To circumvent this confusion and to convey more explicitly the original concept of enzymically specific adsorption chromatography, the modified terms bio-affinity and bio-specific adsorption have been used (O'Carra, 1973). These modified terms will be used here to facilitate discussion, which otherwise becomes laden with clumsy qualifying phrases. The practical, as well as the theoretical, importance of maintaining a rigorous distinction between bio-affinity and more general or 'non-specific' adsorption effects is discussed in detail elsewhere (O'Carra, 1973) and is touched on in the present paper.

Materials

Chemicals and biochemicals. These were obtained as follows. Sepharose 4B from Pharmacia (G.B.) Ltd., London W.5, U.K.; 1,6-diaminohexane and 6-aminohexanoic acid from Koch–Light Laboratories, Colnbrook, Bucks., U.K.; 1,3-diaminopropane from Ralph Emanuel, Wembley, Middx., U.K.; inosine, 5'-AMP, DL-glyceraldehyde-3-phosphoric acid, 2,4,6-trinitrobenzenesulphonic acid and 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide from Sigma (London) Chemical Co., London S.W.6, U.K.; β-NAD^+ and β-NADH from Boehringer Corp. (London) Ltd., London W.5, U.K.; CNBr and *p*-nitrobenzoyl azide from Eastman–Kodak, Rochester, N.Y., U.S.A.

Enzymes. Xanthine dehydrogenase (EC 1.2.3.2), from turkey liver, was a gift from Dr. M. P. Coughlan and Mr. W. Cleere, Galway. Sigma (London) Chemical Co., supplied all the following: alcohol dehydrogenase (EC 1.1.1.1) from yeast and from horse liver; lactate dehydrogenase (EC 1.1.1.27) isoenzyme (LDH_1) from pig heart, type VII; glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) from rabbit muscle; malate dehydrogenase (EC 1.1.1.37) from ox heart; NADP-specific isocitrate dehydrogenase (EC 1.1.1.42) from pig heart, type IV; glucose 6-phosphate dehydrogenase (EC 1.1.1.49) from *Torula* yeast.

General methods

Enzyme assays. The various dehydrogenases were assayed at 30°C by spectrophotometrically monitoring the change in E_{340}. In all cases the final volume of the assay mixtures was 3ml. The individual assay procedures were as follows. Lactate dehydrogenase was assayed by the method of Fritz *et al.* (1970) but with 0.02M-potassium phosphate, pH7.4, instead of Tris buffer. Yeast alcohol dehydrogenase was assayed in 0.01M-sodium pyrophosphate, pH8.8, containing 0.8mM-NAD^+ and 1.2M-ethanol; the reaction was started with 0.1ml of enzyme sample. Horse liver alcohol dehydrogenase was assayed in 0.05M-potassium phosphate buffer, pH7.5, containing 0.2mM-NADH and 3mM-acetaldehyde; the reaction was started with 0.1ml of enzyme sample. D-Glyceraldehyde 3-phosphate dehydrogenase was assayed at pH8.0, in 66mM-sodium pyrophosphate, containing 10mM-EDTA, 10mM-sodium arsenate, 6mM-L-cysteine and 0.1ml of enzyme sample; the reaction was started with 35μl of DL-glyceraldehyde 3-phosphoric acid solution prepared from the diethyl acetal (barium salt) following Sigma technical bulletin no. 10. Malate dehydrogenase was assayed in 0.1M-potassium phosphate buffer, pH7.4, containing 0.2mM-NADH and 0.1ml of enzyme solution; the reaction was started with 0.1ml of 6mM-oxaloacetate solution. Xanthine dehydrogenase was assayed by the method of Rajagopalan & Handler (1967). Isocitrate dehydrogenase was assayed by the method of Rose (1960), and glucose 6-phosphate dehydrogenase was assayed by the method of Kuby & Noltmann (1966) except that 2mM-glucose 6-phosphate was used.

Spectral and chemical monitoring of substitution. The substituted gels, after thorough washing, were scanned in a Unicam SP.1800 spectrophotometer with the sample and blank cuvettes mounted near the detector to decrease the effect of light-scattering. The blank consisted of a suspension containing a balanced quantity of the appropriate Sepharose–spacer-arm derivative unsubstituted with ligand.

Some substitutions and derivatizations could be monitored positively, and others negatively, by testing for free amino groups before and after derivatization by the trinitrobenzenesulphonate colour test described by Cuatrecasas (1970). NAD^+ does not react positively to this test, the amino group of the adenine residue being comparatively inert, as indicated below (see the Discussion section).

The degree of substitution of the gels with immobi-

lized NAD^+ was roughly calculated on a comparative basis from a combination of the spectral data and indications of full substitution of the spacer-arm assemblies obtained by chemical monitoring, which allowed an assumption of equivalence between the extent of substitution of the spacer-arm and ligand. The extent of substitution varied from 2 to 8 μmol/ml of packed gel. The extent of substitution of all the gels used in the affinity chromatographic studies described here was about 2 μmol/ml.

Preparation of immobilized derivatives

Coupling of spacer-arms and NAD^+ to activated Sepharose. Sepharose 4B was activated with CNBr by the general procedure of Cuatrecasas (1970). CNBr (150mg/ml of packed Sepharose gel) in a strong and tightly closed plastic bag was ground to a fine powder before the addition to the Sepharose suspension. Activation was allowed to proceed for 15–20min, or until the CNBr had all dissolved and the pH had stabilized.

The activated Sepharose was washed with 15–20 vol. of ice-cold 0.1M-$NaHCO_3$ buffer, pH10.0, and was immediately added to an equal volume of an ice-cold 2M aqueous solution of spacer-arm precursor (i.e. 1,3-diaminopropane, 1,6-diaminohexane or 6-aminohexanoate) titrated to pH10 with HCl. The mixtures were stirred gently at 4°C for 16h and the gels were then washed with large volumes of 0.1M-NaCl and stored at 4°C, after addition of 0.02% sodium azide as a preservative. The gels were washed free of sodium azide before use.

For direct coupling of NAD^+ or its analogues, the 'spacer-arm solution' was replaced by an equal volume of a solution (40mg/ml) of the nucleotide, titrated to pH10.

Further derivatization of spacer-arms. The full matrix–spacer-arm precursors of derivatives (III), (IV), (V) and (VI) (Fig. 2) were prepared by further derivatization of derivatives described in the previous section following methods described by Cuatrecasas (1970), as also were bromoacetylated derivatives.

Attachment of NAD^+ and NAD^+ analogues to derivatized Sepharose preparations. NAD^+ was coupled to hexanoyl–Sepharose and succinylamidohexyl–Sepharose by the water-soluble carbodi-imide method outlined by Cuatrecasas (1970). Derivatized Sepharose (5ml packed vol.) was added to NAD^+ (0.43mmol in 5ml of water) and the mixture was adjusted to pH4.8 with 0.5M-HCl. 1-Ethyl-3-(3-dimethylpropylamino)carbodi-imide (1.14mmol dissolved in 1ml of water immediately before use) was then added to the stirred mixture over a 5min period while the pH was maintained at 4.8 with 0.5M-HCl. The mixture was stirred gently at 28°C for a further 20h, the pH being readjusted occasionally to pH4.8 during the first hour. The gel was then washed with 1 litre of 0.2M-NaCl. Analogues of NAD^+ were similarly treated.

p-Aminobenzyl derivatives of Sepharose were diazotized, broadly as described by Cuatrecasas (1970), and coupled to NAD^+. In a typical experiment sodium nitrite (138mg) in 10ml of ice-cold distilled water was added to an ice-cold mixture of 5ml of *p*-aminobenzamidohexyl–Sepharose (washed with 0.5M-HCl) and 5ml of 0.5M-HCl. After 8min at 4°C, NAD^+ (200mg) in 10ml of ice-cold saturated sodium borate was added to the diazotized mixture and the pH was immediately adjusted to pH8 with KOH, whereon the gel turned yellow–orange. The mixture was stirred slowly at 4°C for 16h and the gel was then washed with 1 litre of 0.1M-NaCl and stored in a dark-coloured container at 4°C. $NADP^+$ was attached in identical fashion. NAD^+ was coupled in the same way to *p*-aminobenzamidopropyl–Sepharose (the matrix–spacer-arm assembly of derivative IV, Fig. 2) and to the matrix–spacer-arm assembly of derivative (VI), Fig. 2.

Chromatographic procedures

Chromatography was carried out at 15°C in miniature columns (7mm internal diam.; 4–5ml bed vol.) with a flow rate of approx. 1 column-volume/11min. Enzyme samples were applied in 0.4ml of the irrigating buffer. Effluent was collected as fractions (0.4ml) in a Gilson micro-fractionator. Column volumes (corresponding to break-through or straight-through elution volumes) were measured by dissolving a little glucose (3–4mg) as internal marker in the applied samples and monitoring its elution as described by O'Carra & Barry (1972).

Results

Methods of immobilization and their chemistry

In our initial approach to the preparation of an immobilized NAD^+ derivative for affinity chromatography we concentrated on attempted attachment of the NAD^+ via the 6-amino group of the adenine residue. This also seems to have been the initial approach of other workers. Three methods commonly used for attachment of ligands through amino groups (see Cuatrecasas, 1970) were applied. (1) Direct coupling to CNBr-activated Sepharose; (2) carbodi-imide-promoted coupling to carboxylated Sepharose derivatives; (3) coupling to bromoacetylated Sepharose derivatives. None of these methods promoted a significant degree of attachment through the 6-amino group. Method (3) resulted in no detectable attachment at all, and the other two methods resulted in anomalous attachment which, on the basis of evidence presented below, seems to be predominantly through the ribosyl hydroxyl groups (although the

possibility of a small proportion of NAD^+ residues being linked via the amino group cannot be entirely ruled out).

These immobilized NAD^+ derivatives are represented in Fig. 2; derivative (I) being produced by the CNBr method (method 1) and derivatives (II) and (III) by the carbodi-imide-promoted attachment method (method 2) applied to two different Sepharose–spacer-arm derivatives.

Derivatives (I), (II) and (III) displayed well-defined u.v. absorbance spectra similar to that of soluble NAD^+ (maxima at 259nm); derivatives (I) and (II) had an absorbance maximum at 255–260nm and derivative (III) at 260–265nm, the precise location of these maxima being rendered somewhat uncertain by difficulties in balancing the turbidity of the gel samples exactly. In the case of derivatives (I) and (II) the unshifted position of the absorbance maximum is evidence against substitution through the 6-amino group, since such substitution (by the anticipated amide-bond formation) causes a bathochromic shift of about 15nm (see Mosbach *et al.*, 1972).

The approximately 5nm spectral shift displayed by derivative (III) relative to derivative (II) is unlikely to be attributable to any difference in covalent attachment, since the chemistry of the attachment procedure was the same for these two derivatives, only the length of the spacer arm being different. In any case, the shift seems too small to be attributable to a covalent modification of the adenine residue, and may be due to non-covalent factors, possibly hydrophobic interactions. Molecular models of derivatives (II) and (III) show that hydrophobic interactions between the adenine residue and the hydrocarbon spacer arm are possible in derivative (III), with its longer, flexible arm which permits a 'curled-back' conformation of the type postulated by O'Carra *et al.* (1973) to explain apparent masking of certain immobilized ligand-arm assemblies.

Mosbach *et al.* (1972) have also suggested that carbodi-imide-promoted coupling of NAD^+ to hexanoate-derivatized Sepharose does not take place through the amino group, but through either the ribosyl hydroxyl groups or through the pyrophosphate grouping.

To ascertain the groups involved in covalent linkage of NAD^+ by methods 1 and 2, we performed control experiments in which fragments and analogues of NAD^+ were subjected to the relevant coupling procedures. Inosine coupled as readily as NAD^+ both to CNBr-activated Sepharose (method 1) and to carbodi-imide-activated hexanoyl–Sepharose (method 2). The resulting gels retained the absorption spectrum characteristic of inosine ($\lambda_{max.}$ 248nm) after extensive washing with 0.2M-NaCl (which completely removes uncoupled inosine). Since inosine (Fig. 1) contains neither the 6-amino nor the phosphate grouping, this seems to leave only the ribosyl hydroxyl groups as possible points of attachment. Preliminary experiments with simple sugars indicate that these undergo similar coupling.

In the case of the carbodi-imide-promoted coupling, ester formation between a ribosyl hydroxyl and the carboxyl function of the spacer-arm is clearly indicated (attempts to attach NAD^+ directly to underivatized Sepharose with carbodi-imide were unsuccessful, indicating that the sugar hydroxyl groups of the Sepharose itself are not involved in immobilization by method 2).

The type of linkage involved in the coupling to CNBr-activated Sepharose is more obscure but it may be similar to that involved in the 'stabilization' of Sepharose itself which takes place during CNBr activation, and which seems to involve the formation of cross-linkages of unknown type between the dextran chains There seems to be no reason to suppose that the reactions involved would distinguish between the four free ribosyl hydroxyl groups of NAD^+, so

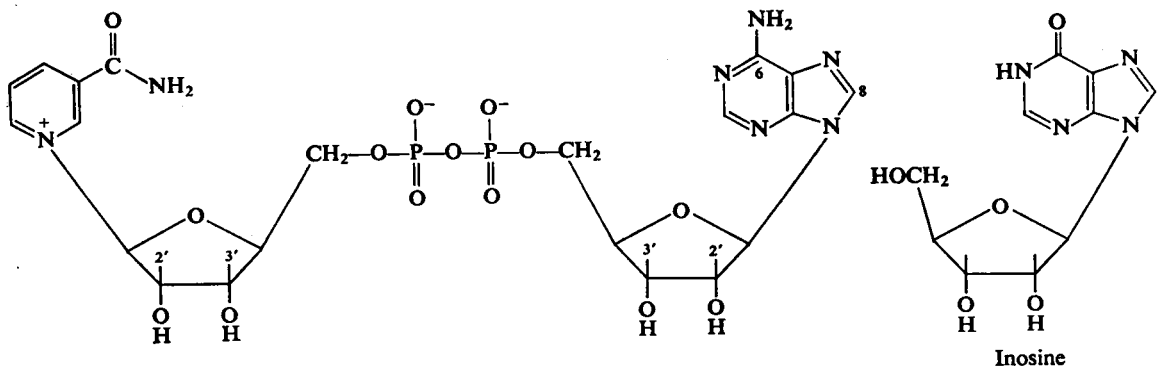

Fig. 1. *Structures of NAD^+ and inosine*

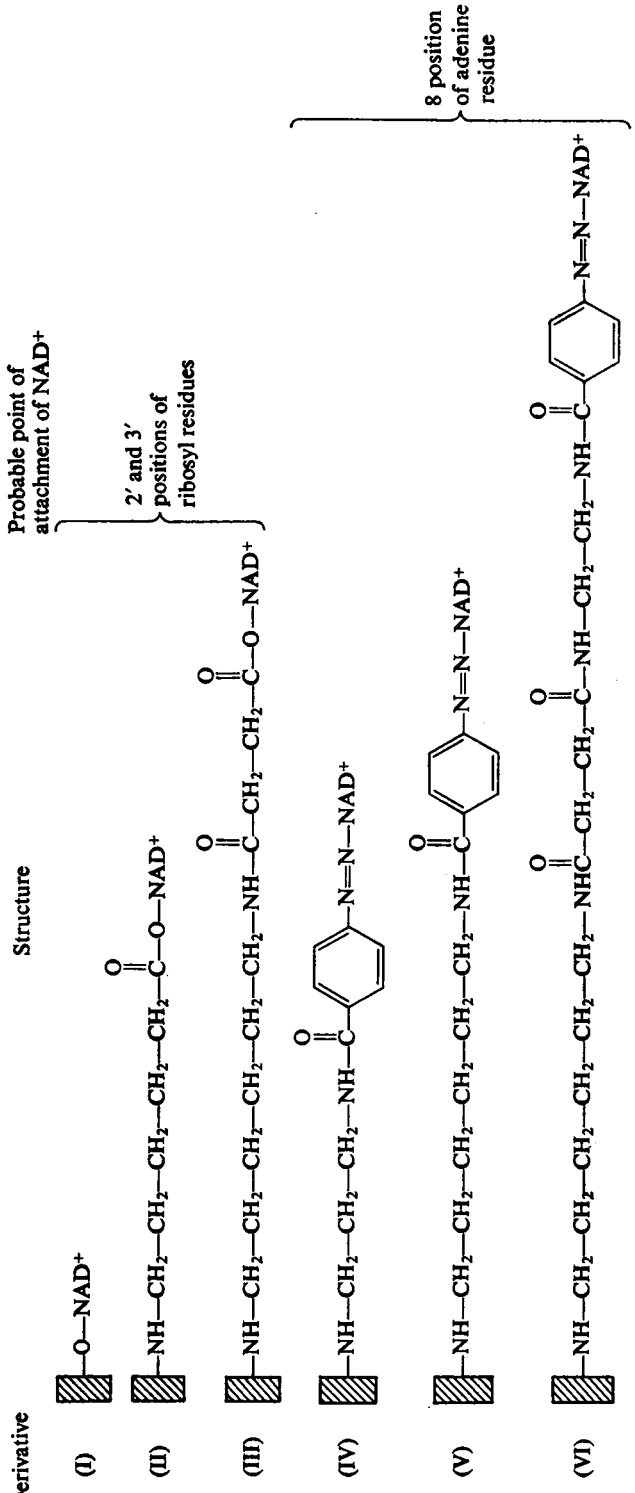

Fig. 2. *Structures of immobilized NAD⁺ derivatives*

The hatched areas to the left represent the agarose matrix.

that the immobilized derivatives (I), (II) and (III) are presumably heterogeneous in containing a proportion of NAD^+ molecules linked through the ribosyl residue of the nicotinamide end and a proportion linked through the ribosyl residue of the adenine end.

The facility with which adenine and its derivatives form azo derivatives suggested an alternative method for the immobilization of NAD^+, i.e. by treating NAD^+ with diazotized *p*-aminobenzoylated derivatives of Sepharose, to yield the azo-linked NAD^+ derivatives represented by structures (IV), (V) and (VI) of Fig. 2. The resulting gels were yellow–orange and displayed an absorbance maximum at about 350nm with a lesser peak at 240nm. Other adenine-containing molecules (e.g. AMP, $NADP^+$) could be similarly immobilized and displayed similar spectral properties. Weibel *et al.* (1971) described an immobilization of NAD^+ based on the same chemical principle but with a complex glass–silane derivative as a matrix. The point of attachment of the NAD^+ in these azo-linked derivatives has been discussed by Weibel *et al.* (1971) and is probably through the 8 position of the adenine residue (see Fig. 1). This is the position in purines generally that reacts with diazo compounds.

Model affinity chromatographic studies

In our model affinity chromatographic studies on the immobilized NAD^+ derivatives we were initially influenced by the general-ligand concept to the extent that we concentrated on a representative dehydrogenase to test the effectiveness of the various derivatives. Lactate dehydrogenase was chosen as the representative NAD-linked enzyme since it is often so regarded by other workers and is by far the most extensively studied enzyme in this group. Further, our previous experience had shown that this enzyme was less subject to non-specific binding effects than many other dehydrogenases. Glyceraldehyde 3-phosphate dehydrogenase was later included as a second 'representative enzyme' because of the evidence that it binds NAD^+ in the folded conformation rather than the open conformation that binds to lactate dehydrogenase (see, e.g., Velick, 1961; Eby & Kirtley, 1971). When we came to doubt the validity of extrapolating from results obtained with these supposedly representative dehydrogenases (see below) we included other NAD-linked dehydrogenases at random, the main criteria for their choice being availability, stability and ease of assay.

Non-specific adsorption effects and their control. Our experience has shown that affinity chromatography may be grossly distorted or completely obscured by non-specific interactions between enzymes and features of the gel other than the bio-specific ligand. In particular, the hydrocarbon spacer-arm assemblies promote much non-specific binding, which appears to be attributable to complex interactions involving hydrophobic, Van der Waals and electrostatic components (O'Carra, 1973; O'Carra *et al.*, 1973; P. O'Carra, S. Barry & T. Griffin, unpublished work). In some cases this non-specific binding may be minimized or abolished by adjusting the ionic strength of the irrigating buffer to a high enough value. Thus for our 'model enzyme' lactate dehydrogenase (Fig. 3) salt-sensitive adsorption of the dehydrogenase on derivative (V) (an effective affinity system for lactate dehydrogenase, see below) is duplicated on a 'control gel' representing the matrix–spacer-arm assembly of derivative (V) minus the bio-specific ligand.

These results clearly show that lactate dehydrogenase binds non-specifically to the spacer-arm

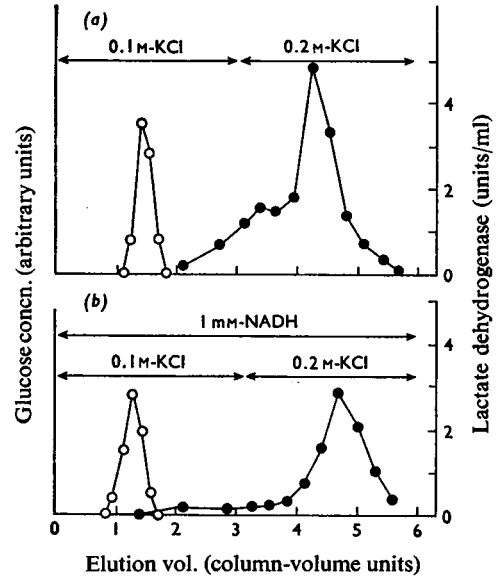

Fig. 3. *Salt-sensitive, non-specific adsorption of lactate dehydrogenase* (●) *on derivative* (*V*)

See Fig. 2 for structure of derivative (V). (*a*) Chromatographic behaviour on an analogue of derivative (V) substituted with the spacer-arm assembly but bearing no immobilized NAD^+ ligand. (*b*) Behaviour on the complete derivative (V) but with soluble NADH added to competitively eliminate bio-specific adsorption (see Fig. 4*a*). Glucose (○) was added to the applied samples to mark the break-through position. The irrigating buffer was 0.05 M-potassium phosphate, pH 7.4, to which KCl and NADH were added as indicated by the horizontal arrows. For further particulars, see description of chromatographic procedures in the Experimental section.

Table 1. *Influence of KCl concentrations on kinetic constants for lactate dehydrogenase (LDH_1) from pig heart*

The constants were calculated from double-reciprocal plots of initial velocity data determined at 30°C in 0.05M-potassium phosphate buffer, pH 7.0, containing 0.5mM-sodium pyruvate, and the appropriate KCl concentration.

KCl concn. (M) (in 0.05M-phosphate buffer, pH 7.0)	NADH K_m (M)	NAD^+ K_i (M)	5'-AMP K_i (M)
0	1×10^{-5}	2.1×10^{-4}	0.94×10^{-3}
0.1	1×10^{-5}	2.1×10^{-4}	0.94×10^{-3}
0.2	1.2×10^{-5}	1.9×10^{-4}	
0.5	2.1×10^{-5}	1.1×10^{-4}	1.6×10^{-3}

assembly and that this binding is abolished by 0.2M-KCl. In the complete affinity gel (derivative V) the enzyme continues to participate in this salt-sensitive non-specific binding while binding specifically to the immobilized NAD^+ residue. The specific binding is abolished competitively by including NAD^+ or NADH in the irrigant (Fig. 4*a*), this being a rigorous criterion of true bio-affinity chromatography (see below) but, as can be seen in Fig. 3(*b*), this effect is not apparent unless the non-specific binding is abolished at the same time by inclusion of 0.2M-KCl in the irrigant.

Kinetic studies, summarized in Table 1, indicate that KCl concentrations in this range have little effect on the binding of NAD^+, NADH or 5'-AMP to lactate dehydrogenase and there is no evidence that such salt concentrations significantly decrease the NAD^+-specific affinity of any of the other dehydrogenases employed in this study. Therefore 0.2M-KCl was added to the irrigant to minimize non-specific adsorption. This is not sufficient to abolish such non-specific binding of some dehydrogenases.

Ribosyl-linked immobilized NAD^+ derivatives. Chromatography of lactate dehydrogenase in the standard irrigating buffer (0.05M-potassium phosphate, pH 7.4, containing 0.2M-KCl) revealed no detectable affinity for any of the ribosyl-linked NAD^+ derivatives (I), (II) or (III) (Fig. 2). Similar results were observed for glyceraldehyde 3-phosphate dehydrogenase, yeast and horse liver alcohol dehydrogenase and malate dehydrogenase. When KCl was omitted from the irrigating buffer, some slight retardation of lactate dehydrogenase on these three immobilized derivatives was observed and this retardation was decreased by addition of soluble NAD^+ to the irrigating buffer. However, these effects seemed too slight to be of any practical value. Because of non-specific interference at low salt concentrations, it was not clear whether the slight observed retardation was attributable to very weak bio-specific affinity, to complex non-specific interactions, or to a combination of both (see the Discussion section) and we did not study derivatives (I), (II) and (III) further.

Azo-linked immobilized NAD^+ derivatives. By contrast with the ribosyl-linked derivatives, columns of the azo-linked derivative (V) strongly retarded lactate dehydrogenase in the standard irrigating buffer (containing 0.2M-KCl). The enzyme was specifically eluted on addition of soluble NADH to the irrigant (Fig. 4*a*), providing clear-cut evidence of true bio-affinity. Recovery of the enzyme was almost quantitative (over 95%).

The spacer-arm assembly of derivative (V) (including the phenylazo grouping) is approx. 1.6nm long when fully opened out. The longer spacer arm of derivative (VI) (approx. 2.4nm) did not noticeably improve the affinity chromatographic effectiveness. However, the shorter arm of derivative (IV) (approx. 1.3nm including the phenyl azo group) decreased the affinity (Fig. 4*b*). On columns of this latter derivative lactate dehydrogenase was retarded only by about 1 column volume.

Because the spacer-arm of derivative (V) seemed to give near-optimal results, it was employed for further detailed studies.

The lactate dehydrogenase begins to leak very slightly off the column soon after the breakthrough volume and before the addition of NADH to the irrigant (Fig. 4*a*). This spontaneous 'bleeding' of the enzyme off the column occurred with a number of preparations of derivative (V). It was very slight with freshly synthesized gels but became progressively worse as the gels aged. Some gel preparations eventually lost all their ability to retard lactate dehydrogenase after they had been re-utilized several times during 4–5 months and stored at 2–4°C between experiments. This loss of chromatographic effectiveness is presumably caused by instability of the immobilized NAD^+.

Glyceraldehyde 3-phosphate dehydrogenase was also effectively adsorbed in a bio-specific fashion on derivative (V) and was specifically eluted with NAD^+. No 'bleeding' of this enzyme off the columns was observed. When the glyceraldehyde 3-phosphate dehydrogenase was eluted immediately after adsorp-

tion, recovery was almost quantitative, but when the enzyme was left adsorbed on the columns, progressively lower recovery was obtained on elution. This time-dependent decrease in recovery was far in excess of the slight loss of activity of a control unadsorbed sample of enzyme. Raising the KCl concentration in the irrigating buffer to 0.5M produced no noticeable change in the chromatographic behaviour of glyceraldehyde 3-phosphate dehydrogenase, but it weakened the binding of lactate dehydrogenase (compare Figs. 4*a* and 4*c*).

To further check the bio-specificity of the affinity of lactate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase for derivative (V), an exactly equivalent derivative was prepared but replacing NAD^+ by $NADP^+$ in the final synthetic step. This $NADP^+$ analogue of derivative (V) had no affinity for either of these dehydrogenases. Further, the NADP-linked isocitrate dehydrogenase and glucose 6-phosphate dehydrogenase had no affinity for derivative (V).

Of the other NAD-linked dehydrogenases studied, malate dehydrogenase was not retarded at all by derivative (V), but both yeast alcohol dehydrogenase and liver xanthine dehydrogenase were strongly adsorbed by the gel. This strong adsorption was largely non-specific as these dehydrogenases could not be eluted by soluble NAD^+ (5mM) even with 0.5M-KCl. (The presence of the adsorbed enzymes on the columns was demonstrated by passing the appropriate assay mixtures through the columns and checking the effluent for enzymic conversion.) The behaviour of these last two enzymes is consistent with their strong adsorption on gels derivatized with almost any hydrocarbon group (P. O'Carra, S. Barry & T. Griffin, unpublished work). Horse liver alcohol dehydrogenase was also strongly adsorbed by derivative (V) but in contrast with the yeast enzyme a proportion of the enzyme (27%) could be eluted with NAD^+. The remainder of the enzyme remained firmly and non-specifically bound to the column.

In addition to being elutable with NAD^+ and NADH, lactate dehydrogenase was also readily elutable from columns of derivative (V) by inclusion of 5'-AMP (10mM) in the irrigant. By contrast, glyceraldehyde 3-phosphate dehydrogenase was not elutable with 5'-AMP, and this provided a means of differentially eluting these two enzymes from derivative (V). Fig. 5 illustrates the separation of three NAD-linked dehydrogenases on a column of derivative (V).

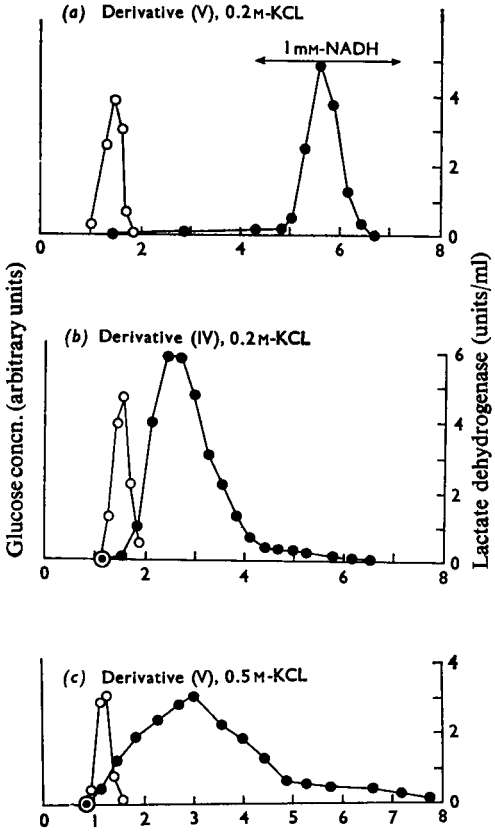

Fig. 4. *Affinity chromatography of lactate dehydrogenase (●) on derivatives (IV) and (V)*

The irrigant was 0.05M-potassium phosphate, pH 7.4, containing the indicated KCl concentrations. A little glucose (○) was added to mark the break-through position. Other details as for Fig. 3.

Discussion

Our initial approach to the immobilization of NAD^+ (through the 6-amino group of the adenine residue) was prompted first by the specificity studies available for NAD-linked dehydrogenases, which suggest that the adenine residue, and its amino group in particular, is the least critical feature of the NAD^+ molecule as regards binding specificity. For example, many dehydrogenases can effectively utilize the deamino analogue of NAD^+ (see, e.g., Sund, 1968). Further, the 6-amino group seemed, *a priori*, to be the easiest point for the chemical attachment of NAD^+ since at least three well-established procedures existed for immobilization of ligands through amino groups (see Cuatrecasas, 1970). However, of these three procedures, one yielded no immobilization of NAD^+ at all, whereas the other two yielded derivatives immobilized largely through the ribosyl hydroxyl groups rather than the 6-amino group. The

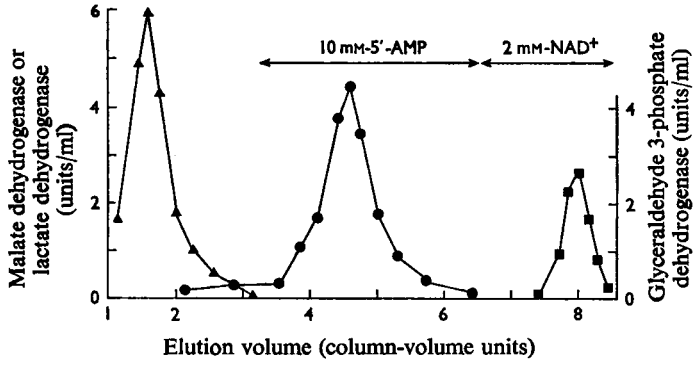

Fig. 5. *Separation of three NAD-linked dehydrogenases on a column of derivative* (V) (*see Fig.* 2)

The column was irrigated with 66mM-sodium pyrophosphate buffer, pH8.0, containing 10mM-EDTA, 6mM-L-cysteine and 0.2M-KCl, and was run at a flow rate of one column volume/11min. A sample (0.4ml) was applied containing malate dehydrogenase, lactate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase, in the same buffer system. Other additions to the irrigating buffer, i.e. 10mM-5'-AMP and 2mM-NAD^+, are indicated by the horizontal arrows (which are correlated with the volume of effluent collected when such changes were made). ▲, Malate dehydrogenase; ●, lactate dehydrogenase; ■, glyceraldehyde 3-phosphate dehydrogenase.

atypical inertness of the 6-amino group seems to be caused by interaction with the heterocyclic ring, resulting in a net positive charge on the 6-amino group (see, e.g., Bock, 1960; Pullman & Pullman, 1963; Kochetkov & Budovskii, 1971).

The ribosyl-linked immobilized derivatives (I, II and III, Fig. 2) displayed only slight affinity for the representative test enzyme, lactate dehydrogenase, or for glyceraldehyde 3-phosphate dehydrogenase, and this affinity was abolished by 0.2M-KCl. As 0.2M-KCl has little effect on the affinity of lactate dehydrogenase for NAD^+ (Table 1), it seems logical to conclude that even the slight affinity observed was at least partly attributable to non-specific adsorption.

Lowe & Dean (1971*a*,*b*) described immobilized NAD^+ derivatives closely analgous to derivatives (I) and (II), and Mosbach *et al.* (1972) used a more drastic version of the carbodi-imide method to prepare a derivative that is presumably analogous to derivative (II). These authors have claimed encouraging affinity chromatographic results for these derivatives. However, the complex treatment to which Lowe & Dean (1971*a*) subjected their experimental data obscures a lack of real correlation between their chromatographic results and the $NAD^+/NADP^+$ specificity of the dehydrogenases studied. The generally puzzling results of Lowe and co-workers (Lowe & Dean, 1971*a*,*b*; Lowe *et al.*, 1972) can be ascribed to the criterion of affinity-chromatographic effectiveness adopted, this being 'the salt concentration required to elute a particular enzyme' from an immobilized NAD^+ derivative. The kinetic and other data available and in particular that in Table 1 for lactate dehydrogenase (one of the principal test enzymes used by Lowe and co-workers) shows that such elution is not a valid index of specific affinity for NAD^+ and is likely to reflect non-specific binding rather than bio-affinity. Closer inspection of the results of Lowe and co-workers shows that, by their criteria, even totally non-specific proteins such as serum albumin should be given quite a high 'affinity rating' for the immobilized NAD^+ derivatives.

The results of Mosbach *et al.* (1972) are much more valid, since these authors, although using an irrigating buffer of comparatively low ionic strength, were able to demonstrate apparently specific elution with soluble NAD^+.

As mentioned above, we have observed some retardation of lactate dehydrogenase by derivatives (I), (II) and (III) at low ionic strength, and this retardation is abolished by soluble NAD^+ as well as by raising the salt concentration. It is difficult to decide whether this indicates slight bio-affinity or mild non-specific adsorption, or a combination of both. The latter possibility now seems the more likely, and some studies on other affinity chromatographic systems (P. O'Carra & S. Barry, unpublished work) provide compelling evidence for such a reinforcement of weak bio-affinity by non-specific interactions, in such a way that abolition of either component of the binding leaves the other alone incapable of retaining the enzyme which, as a result, is eluted.

The higher degree of adsorption reported for the carbodi-imide-prepared derivative of Mosbach *et al.* (1972), compared with that observed by us for derivative (II), possibly reflects the more drastic coupling

conditions employed by Mosbach *et al.* (1972) [17 days treatment in 80% (v/v) pyridine, as against overnight treatment under purely aqueous conditions]. This procedure of Mosbach *et al.* (1972) appears to allow a higher degree of substitution than the milder aqueous conditions involved in the preparation of our derivatives, and this would be expected to increase the strength of any slight bio-affinity. A high degree of substitution was avoided by us since there is evidence that this leads to a disproportionate increase in non-specific interference (M. A. Raftery, personal communication; S. Barry & P. O'Carra, unpublished work). It is also possible that the comparatively severe coupling conditions of Mosbach *et al.* (1972) may have resulted in a more complex and heterogeneous population of immobilized ligand molecules.

The weakness of the bio-affinity of the ribosyl-linked derivatives for lactate dehydrogenase is unlikely to be attributable to insufficient length of spacer-arm, since the bio-affinity does not noticeably improve through the series of derivatives (I) to (III). This weakness of affinity is fully consistent with the detailed molecular model of the binary lactate dehydrogenase–NAD^+ complex which has recently emerged from the X-ray diffraction studies of Rossmann's group (Rossmann *et al.*, 1972). These models show the hydroxyl groups of both ribosyl residues buried in the binding site in such a way that any substituent on these hydroxyl groups would be expected to greatly inhibit or abolish affinity. The slight and problematical bio-affinity that we observe may originate from the proportion of NAD^+ molecules attached via the ribosyl residue of the nicotinamide part, since such attachment would leave the AMP part of the molecule unsubstituted except through the phosphate group. Lactate dehydrogenase has a weak affinity for 5′-AMP and its phosphate-substituted derivatives (such as ADP and ATP) but not for NMN (representing the nicotinamide part on its own; see McPherson, 1970). A ribosyl-immobilized 5′-AMP analogue of derivative (II) showed no affinity for lactate dehydrogenase which could be construed as bio-affinity.

The above-mentioned molecular models of the lactate dehydrogenase–NAD^+ binary complex show further that the C-8 position of the adenine residue is readily accessible from the exterior of the binding site. Immobilization through this position would therefore be expected to introduce little direct steric hindrance and this is consistent with the clear-cut bio-affinity of azo-linked NAD^+ derivatives (IV), (V) and (VI) for lactate dehydrogenase. The lower affinity of derivative (IV) compared with derivatives (V) and (VI) appears at first sight to be indicative of a need for a long spacer group. However, the molecular models reveal no compelling necessity for a spacer group longer than that provided by derivative (IV) from the point of view of steric hindrance, and a more complex explanation is possible, involving a more positive role for the longer spacer-arms, as outlined below.

The evidence in favour of true bio-affinity for derivatives (IV)–(VI) seems conclusive, in particular the specific elution of lactate dehydrogenase with NAD^+ and 5′-AMP, and the lack of affinity of this enzyme for the $NADP^+$ analogue of derivative (V). However, as can be seen by comparison of Figs. 4(*a*) and 4(*c*), the affinity is sensitive to salt concentrations higher than those of our 'standard irrigant'. This salt-sensitivity can hardly be attributed to an effect on the bio-affinity, since the kinetic results presented in Table 1 show that KCl concentrations up to 0.5M have little effect on the binding of NAD^+ to lactate dehydrogenase. We suggest that under our standard irrigating conditions the bio-affinity is aided or reinforced by residual non-specific interactions with the spacer-arm that are not sufficient on their own to cause any detectable retardation. This is much the same explanation as suggested above for the much weaker effects displayed by the ribosyl-linked NAD^+ derivatives. Further evidence in favour of such 'compound affinity' will be presented elsewhere.

If it is accepted that such compound affinity is operative in the adsorption of lactate dehydrogenase by derivative (V), it is clearly possible that the long spacer-arm of this derivative *vis-à-vis* that of derivative (IV) produces its effect by providing stronger non-specific interactions to re-inforce the bio-affinity rather than by passively eliminating steric interference by the matrix backbone.

The fact that the even longer spacer-arm of derivative (VI) does not further improve the effectiveness in affinity chromatography is not necessarily in conflict with this explanation. In the first place, the positive effect of the spacer-arm in such compound affinity must remain marginal so as not to dominate or eclipse the bio-affinity. Any further increase in the amount of non-specific adsorption beyond a critical value merely becomes a nuisance as evidenced by the intractable non-specific adsorption of some other dehydrogenases on derivative (V) (see below) and by our re-investigation of the role of the spacer-arms in the affinity chromatographic systems of Steers *et al.* (1971) for β-galactosidase (O'Carra *et al.*, 1973; O'Carra, 1973). In any case, lengthening of the arm *per se* need not necessarily increase the amount of non-specific adsorption, since such adsorption depends as much on the chemical nature of the arm as on its length (see O'Carra, 1973). Undue lengthening of the arm may even cause a decrease in the affinity by permitting the entire ligand-arm assembly to adopt a folded conformation in which the ligand may 'curl back' on the spacer-arm and become masked or occluded (O'Carra, 1973). The possibility of such 'curl-back' has also been mentioned above in connexion with derivative (III) (see the Results section).

In view of these considerations, it is probable that the true (i.e. un-reinforced) bio-affinity of the azo-linked NAD^+ for lactate dehydrogenase is best reflected by the chromatographic results obtained with derivative (IV) or with derivative (V) in the presence of 0.5M-KCl (Figs. 4*b* and 4*c*).

Although the contribution of non-specific adsorption in the case of lactate dehydrogenase may be considered a help rather than a hindrance from a practical point of view, the reverse is the case with most of the other dehydrogenases tested.

Glyceraldehyde 3-phosphate dehydrogenase displays strong and unambiguous bio-affinity for derivative (V), but non-specific interactions cause low recoveries of the enzyme from the columns. The recovery is time-dependent. If the enzyme is eluted immediately from the column with NAD^+, recovery is almost quantitative, but if it is left adsorbed for any length of time it appears to become progressively more enmeshed and non-specifically bound so that the proportion elutable with NAD^+ decreases progressively with time. Extensive investigation of this phenomenon confirms that non-specific adsorption rather than simple inactivation of the enzyme is responsible (P. O'Carra, S. Barry & T. Griffin, unpublished work).

With xanthine dehydrogenase and yeast alcohol dehydrogenase, very strong and apparently instantaneous non-specific adsorption on the spacer-arm of derivative (V) prevented any significant recovery of the enzymes from the columns, even when high salt concentrations together with high NAD^+ concentrations were added to the irrigant. Yeast alcohol dehydrogenase was not adsorbed at all on derivative (IV), and some other independent evidence (P. O'Carra, S. Barry & T. Griffin, unpublished work) indicates that this enzyme displays little true bio-affinity for the azo-linked NAD^+, the strong adsorption on derivative (V) being almost totally non-specific.

The differences among the dehydrogenases as regards these positive sources of interference are clearly likely to complicate general-ligand affinity chromatography. However, a more fundamental complication of the general-ligand concept is introduced by the differential operation of steric hindrance caused by the immobilization linkage itself.

This is well illustrated by the differing affinities of derivative (V) for lactate, malate and glyceraldehyde 3-phosphate dehydrogenases. The optimum retardation, due solely to bio-affinity, should be in the same general range for these three enzymes since their K_i values with NAD^+ are all within the same range: lactate dehydrogenase, $K_i = 1.9 \times 10^{-4}$M (the present study); malate dehydrogenase, $K_i = 1 \times 10^{-4}$ (Grimm & Doherty, 1961); glyceraldehyde 3-phosphate dehydrogenase (last binding site), $K_i = 1 \times 10^{-4}$M (Furfine & Velick, 1965). (It is presumed that the last binding site of the latter enzyme is operative in affinity-binding, the other sites being already occupied by NAD^+.) At the substitution value of immobilized NAD^+ on the columns of derivative (V) used in this work (about 2×10^{-3}M) these K_i values would suggest that the bio-specific retardations, in the absence of interference, should range upwards from about 10 column volumes (O'Carra *et al.*, 1973; O'Carra, 1973).

Bio-affinity of this order is indeed observed for glyceraldehyde 3-phosphate dehydrogenase, but the true (i.e. un-reinforced) bio-affinity for lactate dehydrogenase is considerably sub-optimal, and malate dehydrogenase shows no detectable bio-affinity at all for derivative (V).

These chromatographic differences presumably reflect differences in the NAD-binding sites of these three enzymes. Since the immobilization linkage in derivative (V) is probably through the 8 position of the adenine residue, differences in the importance of this portion of NAD^+ in the binding process may be involved. Preliminary affinity chromatographic studies with immobilized analogues representing fragments of the NAD^+ molecule seem to confirm that the AMP portion of the molecule does not play the same primary role in the binding of NAD^+ to glyceraldehyde 3-phosphate dehydrogenase as it does in the binding to the other two dehydrogenases (S. Barry & P. O'Carra, unpublished work). This also presumably explains the fact that, by contrast with lactate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase is not susceptible to elution from derivative (V) with 5′-AMP even at very high AMP concentrations (e.g. 20 times the concentration of the immobilized NAD^+). NAD^+ itself must be added to the irrigant as counter-ligand to elute this enzyme.

These results broadly support the view, originally put forward by Velick (1961), that the mode of binding of NAD^+ to glyceraldehyde 3-phosphate dehydrogenase differs considerably from its mode of binding to lactate and malate dehydrogenases (see, e.g., the recent n.m.r. studies by Lee *et al.*, 1973).

In any event it is clear that although a group of enzymes may bind a general ligand in the free form with similar affinities, subtle differences in the mode of the ligand-binding to the individual enzymes may cause the immobilization linkage to introduce considerable alterations in the general specificity of the immobilized ligand. Presumably some loss of generality can be expected no matter what point of attachment or method of immobilization is employed and it is clearly invalid to expect the generality to be affected in an all-or-none fashion. Therefore it is also clearly inadvisable to extrapolate from results with one or two supposedly representative enzymes. For example, the ribosyl-linked derivatives, shown above to have little effective bio-affinity for lactate dehydrogenases or glyceraldehyde 3-phosphate dehydrogenase, might

possibly be quite effective for some other NAD-linked dehydrogenases (although to date none of the other dehydrogenases we have tested on these derivatives has shown any greater bio-affinity than that observed with lactate dehydrogenase).

Weibel *et al.* (1971) have described the preparation of an immobilized NAD^+ derivative by an azo-linkage procedure similar to that adopted here for the preparation of derivatives (IV)–(VI), but by using a complex silanized glass preparation as matrix–spacer assembly, as opposed to the agarose–hydrocarbon assemblies described here. Weibel *et al.* (1972) tested this derivative as an affinity chromatographic system, although with only one enzyme, yeast alcohol dehydrogenase. They reported indications of effectiveness but extremely poor recoveries of the enzyme (in the region of 10%). In view of our observation of negligible bio-affinity of yeast alcohol dehydrogenase for azo-NAD^+ derivatives, but nearly total non-specific adsorption on derivative (V) and also on a variety of 'spacer-arm' assemblies carrying no immobilized NAD^+ (P. O'Carra, S. Barry & T. Griffin, unpublished work), the 90% loss of activity observed by Weibel *et al.* (1972) is presumably attributable to non-specific adsorption on the glass–silane complex. The misleading impression created by results obtained with this one enzyme in isolation illustrates the danger of extrapolating from experiments with supposedly representative enzymes.

Unimpaired general effectiveness, as envisaged in the original concept of general-ligand affinity chromatography, would presumably result in strong bio-specific adsorption of all NAD-linked dehydrogenase as a group, and intra-group separations (i.e. separations of individual dehydrogenases) would, in general, have required the development of complex elution procedures. Ironically, in practice, the impairment of general or group effectiveness results in a considerable facilitation of intra-group separations.

This is illustrated by the experiment presented in Fig. 5, involving fractionation of a mixture of NAD-linked dehydrogenases on a column of derivative (V). Malate dehydrogenase, showing no bio-affinity for this derivative, is washed straight through; lactate dehydrogenase, being adsorbed apparently by a combination of weakened bio-affinity and reinforcing non-specific interactions, can be eluted with the weak competitive inhibitor 5'-AMP; glyceraldehyde 3-phosphate dehydrogenase remains strongly bound and is eluted by NAD^+. (The low yield of the latter enzyme reflects losses through the time-dependent non-specific adsorption mentioned above.) Very 'sticky' enzymes such as yeast alcohol dehydrogenase or xanthine dehydrogenase, if present in the applied dehydrogenase mixture, are removed completely and can be eluted only by irrigating the column with strongly dissociating buffers (e.g. containing 8M-urea) or occasionally by 'deforming buffers', at relatively extreme pH values (P. O'Carra, S. Barry & T. Griffin, unpublished work). It is clearly advisable to wash affinity columns periodically with dissociating solutions to remove accumulated non-specifically adsorbed proteins.

The use of immobilized derivatives in enzyme mechanistic and binding studies has been advocated by O'Carra & Barry (1972) and by Ohlsson *et al.* (1972). Impairment of binding is clearly of interest in regard to binding-site topography, as illustrated by some of the results discussed above. However, in mechanistic studies (e.g. in attempting to determine the order or randomness of binding of NAD^+ and the specific substrates) it may obviously be difficult to decide whether negative results are attributable to intrinsic mechanistic factors or merely to interference introduced by the immobilization chemistry. Where the results are positive, it is clearly necessary to exercise great care in distinguishing true bio-affinity from non-specific adsorption.

We thank Mr. W. Cleere and Dr. M. P. Coughlan, Galway, for testing the affinity of xanthine dehydrogenase for the azo-linked NAD^+ derivatives. This work was supported by a grant-in-aid from the Medical Research Council of Ireland and, in part, from a National Science Council grant. S. B. is the recipient of a Research Maintenance Grant from the Irish Department of Education.

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