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Some Parameters Relevant to Affinity Chromatography on Immobilized Nucleotides

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1. The suitability of cellulose and Sepharose as supports for affinity chromatography of two groups of cofactor-linked enzymes, dehydrogenases and kinases, was examined. Sepharose was found to be superior. 2. The selective capacities of the columns were measured by frontal analysis and are discussed in relation to the nucleotide contents. 3. The effect of various concentrations of enzyme and of non-specific protein on the performance of the affinity columns, and the effects of equilibration time, flow rate, sample volume and dilution of the nucleotide were examined. 4. The effect of interposing polymethylene and polyglycine extension arms between the matrix backbone and the nucleotide was investigated for several cofactor-dependent enzymes. Maximum binding was observed with an extension arm 0.8–1 nm long.

Much interest has been shown in the development and application of chromatographic adsorbents based on biological specificity (Cuatrecasas & Anfinsen, 1971*a*). Rapid advances have led to new approaches to the selective purification of enzymes and to the exploration of complex biological interactions. Cuatrecasas & Anfinsen (1971*b*) have described some of the more important experimental conditions necessary for satisfactory binding of proteins to immobilized ligands.

More recently the effectiveness of immobilized cofactors in resolving mixtures of proteins by affinity chromatography has been demonstrated (Lowe & Dean, 1971; Lowe *et al.*, 1972). To date, however, no systematic study has been presented to verify the basic postulates of the technique or to delineate any of its attributes of limitations. The interaction of the cofactor-dependent dehydrogenases and kinases with immobilized nucleotides allows many of the theories to be tested under similar experimental conditions.

In this paper we have attempted to show the need to rationalize the principles of affinity chromatography in terms of the known free-solution kinetics of the system. It is hoped that some information on the mode of binding of pyridine and adenine nucleotides to dehydrogenases and kinase can thus be obtained.

Materials and Methods

Materials

Enzymes and their substrates were purchased from C. F. Boehringer, Mannheim, Germany except for malate dehydrogenase (EC 1.1.1.37) which was obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. CNBr, 3-aminopropane-1,2-diol, β-alanine, 4-aminobutyric acid, 5-aminovaleric acid, 6-aminohexanoic acid and 11-aminoundecanoic acid were obtained from R.N. Emanuel Ltd., Wembley, Middx., U.K. 7-Aminoheptanoic acid and 12-aminododecanoic acid were purchased from Research Organic/Inorganic Chemical Corp., Sun Valley, Calif., U.S.A. and 8-amino-octanoic acid was from Fluka A.G., Buchs, Switzerland. Sepharose 4B was obtained from Pharmacia (G.B.) Ltd., London W.5, U.K. Nicotinamide and adenine nucleotides were supplied by Whatman Biochemicals, Maidstone, Kent, U.K. All other chemicals were of the highest purity available from BDH Chemicals Ltd., Poole, Dorset, U.K.

Methods

Enzymes were assayed by the methods cited by Barman (1969) and protein was measured by the u.v.-absorption method of Warburg & Christian (1931).

The various ω-aminoalkyl-carboxylic acids, H_2N–$[CH_2]_x$–CO_2H, where $x = 2$ to 10, were coupled to Sepharose 4B activated with CNBr (Axen *et al.*, 1967). These products are designated as, e.g. ε-aminohexanoyl-NAD^+–Sepharose. Nicotinamide and adenine nucleotides were linked to these spacer molecules by the dicyclohexyl carbodi-imide-promoted reaction in aq. 80% (v/v) pyridine, as described by Larsson & Mosbach (1971).

Except where stated, all experiments were performed on columns (5mm × 20mm) of NAD^+ and ATP linked to ε-aminohexanoyl-Sepharose. The equilibration and elution buffers employed in this study were 10mM-KH_2PO_4–KOH (pH 7.5) for the

dehydrogenases and 5mM-$MgCl_2$–10mM-Tricine [*N*-tris-(hydroxymethyl)methylglycine]–NaOH (pH 7.5) for the kinases. For glycerokinase the latter buffer was supplemented with 10mM-glycerol and 1mM-EDTA.

For determination of the E_{260} of the bound nucleotide the moist gel was hydrolysed for 15min in 0.1M-HCl at 100°C. For the phosphate determinations 1.0ml of moist gel was washed with 10ml of methanol followed by 10ml of diethyl ether and dried under reduced pressure before analysis by the method of Meun & Smith (1968).

Results and Discussion

Cellulose as a matrix for affinity chromatography

Early studies of affinity chromatography making use of cofactors immobilized on cellulose (Lowe & Dean, 1971) have shown that these polymers possess interesting properties. The results, however, were difficult to interpret in terms of the known properties of the enzymes.

It has been suggested that the CNBr activation technique of Axen *et al.* (1967) introduces an ion-exchange capacity into the polysaccharide matrix (Axen & Ernbäck, 1971). Table 1 demonstrates how the binding of glucose 6-phosphate dehydrogenase (EC 1.1.1.49) was dependent on the amount of CNBr used to activate the cellulose rather than the amount of NAD^+ coupled. Further, activated cellulose to which no ligand had been coupled also bound lactate dehydrogenase (EC 1.1.1.27) (Table 2). These effects probably arise from activated species on the matrix, which are not coupled to nucleotide (Dean & Lowe, 1972). The ion-exchange capacity can be eliminated by reaction of the residual activated species with 3-aminopropane-1,2-diol or D-glucosamine (Table 2).

Sepharose as an affinity matrix

Sepharose activated with CNBr (Lowe & Dean, 1971), to which no ligand was coupled, did not bind lactate dehydrogenase or bovine serum albumin; however, NAD^+–Sepharose retained lactate dehydrogenase and showed no affinity for bovine serum albumin. With polymers prepared by the dicyclohexyl carbodi-imide-promoted reaction of Larsson & Mosbach (1971) significant binding of lactate dehydrogenase occurred only when NAD^+ was included in the dicyclohexyl carbodi-imide reaction mixture (Table 3) and consequently was covalently attached to the matrix. ε-Aminohexanoyl-ATP–Sepharose was prepared by the same procedure. Both immobilized nucleotides showed reasonable stability (6 months at 0°C) and could be used repeatedly. Some irreversible adsorption of proteins was observed when the columns were used repeatedly over a period of weeks, especially with crude extracts.

The amount of bound nucleotide determined by u.v.-absorption and phosphate analysis was 2μmol of NAD^+ or ATP/g wet wt. of gel, corresponding to 38.5μmol/g dry wt. This is in good agreement with the value quoted by Larsson & Mosbach (1971) for an NAD^+-containing polymer.

Frontal analysis chromatography

When a constant concentration of bovine serum albumin and lactate dehydrogenase or glycerokinase (EC 2.7.1.30) was applied to a column of immobilized NAD^+ or ATP about 12.5 units of added lactate dehydrogenase and 7 units of added glycerokinase were adsorbed before either enzyme appeared in the eluate (Figs. 1 and 2). When this capacity was surpassed a further 23 units of lactate dehydrogenase and 12 units of glycerokinase were adsorbed before the concentration of these enzymes in the eluate approached that

Table 1. *Effect of CNBr on the binding properties of NAD^+–cellulose*

Columns were prepared from 10g of cellulose activated under the conditions below. The binding, β (mM-KCl), was determined by applying a linear salt gradient (usually 0–500mM) to the column and determining the salt concentrations subsequently by measuring the conductivity of the fractions collected. At the concentrations used, protein does not interfere. Other experimental details are given in the text.

Activation and coupling conditions		Binding (mM-KCl)	
CNBr (g)	NAD^+ (mg)	Glucose 6-phosphate dehydrogenase	Bovine serum albumin
0.5	5	115	95
0.5	25	106	90
6.5	5	315	240
6.5	25	300	195

Table 2. *Ion-exchange properties of CNBr-activated cellulose*

For details see Table 1.

Conditions	Bovine serum albumin		Lactate dehydrogenase	
	% Bound	β	% Bound	β
CNBr-activated cellulose	33	20	100	40
CNBr-activated cellulose plus aminopropanediol	0	0	0	0
NAD^+–cellulose	23	200	100	210
NAD^+–cellulose plus aminopropanediol	29	100	30	130

Table 3. *Effect of dicyclohexylcarbodi-imide-reaction components on bovine serum albumin and lactate dehydrogenase*

Experimental details are given in the text.

Conditions	Binding (mM-KCl)	
	Bovine serum albumin	Lactate dehydrogenase
NAD^+ and dicyclohexylcarbodi-imide plus ε-aminohexanoyl-Sepharose	0	280
Dicyclohexylcarbodi-imide plus ε-aminohexanoyl-Sepharose	50	95
NAD^+ plus ε-aminohexanoyl-Sepharose	0	0 (85%); 50 (15%)
ε-Aminohexanoyl-Sepharose	0	0 (80%); 50 (20%)

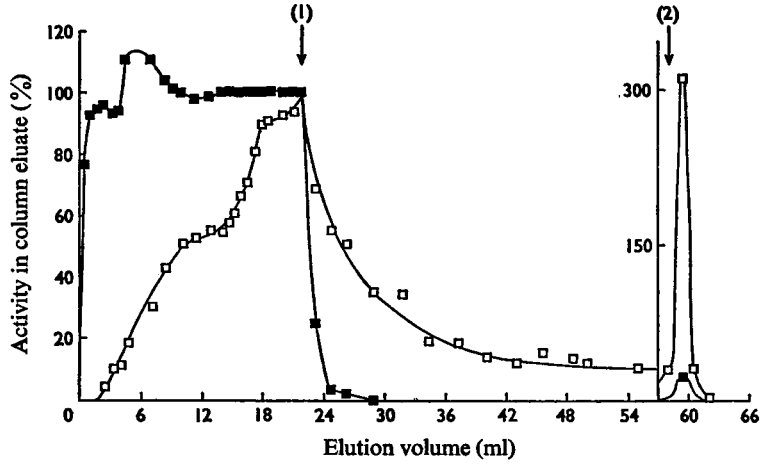

Fig. 1. *Frontal analysis chromatography of lactate dehydrogenase on ε-aminohexanoyl-NAD⁺–Sepharose*

A column (2mm×20mm) containing ε-aminohexanoyl-NAD^+–Sepharose (125mg moist wt.) was equilibrated with 10mM-KH_2PO_4–KOH, pH7.5. A mixture containing bovine serum albumin (E_{280}, 0.340) and lactate dehydrogenase (3 units/ml) in the same buffer was applied until the concentration of lactate dehydrogenase in the eluate reached that of the applied solution. Subsequent elution was carried out with (1) 10mM-KH_2PO_4–KOH, pH7.5, and (2) 1M-KCl–10mM-KH_2PO_4–KOH, pH7.5. Bovine serum albumin and lactate dehydrogenase were measured as described in the Materials and Methods section. ■, Bovine serum albumin; □, lactate dehydrogenase.

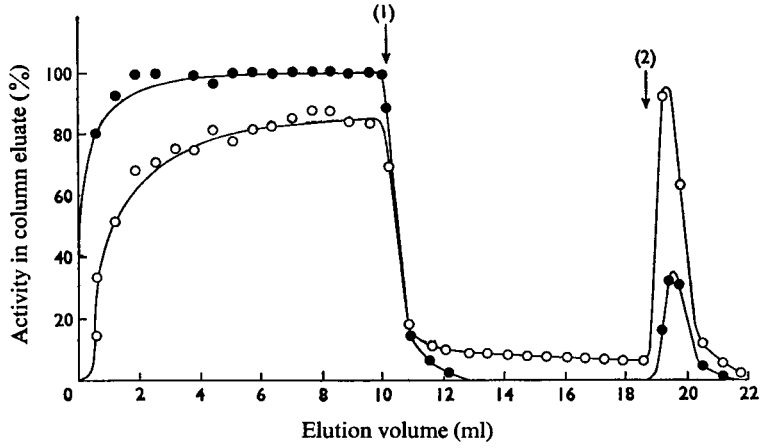

Fig. 2. *Frontal analysis chromatography of glycerokinase on ε-aminohexanoyl-ATP–Sepharose*

A column (2mm×20mm) containing ε-aminohexanoyl-ATP–Sepharose (41mg moist wt.) was equilibrated with 5mM-$MgCl_2$–10mM-glycerol–1mM-EDTA in 10mM-Tricine–NaOH buffer, pH 7.5. A mixture containing bovine serum albumin (E_{280}, 0.485) and glycerokinase (6.2 units/ml) in the same buffer was applied until the concentration of glycerokinase in the eluate reached that of the applied solution. Subsequent elution was carried out with (1) equilibration buffer and (2) 1M-KCl in equilibration buffer. Bovine serum albumin and glycerokinase were measured as described in the Materials and Methods section. ●, Bovine serum albumin; ○, glycerokinase.

of the applied solution. Before saturation a shoulder was apparent in the lactate dehydrogenase profile, which could indicate the presence of a second species of NAD^+ bound to the matrix; this shoulder was not present in the glycerokinase profile. The application of bovine serum albumin and enzyme-free buffer to these columns resulted in an initial rapid decrease in the concentration of enzyme in the eluate followed by a gradual decline, with some 25 units of lactate dehydrogenase and 9.3 units of glycerokinase being eluted from the columns. Subsequent addition of 1M-KCl eluted a further 12.6 units of lactate dehydrogenase and 7.1 units of glycerokinase. The elution profiles for bovine serum albumin showed a rapid rise to the concentration of the applied solution. Apart from a slight deviation, before the plateau in the lactate dehydrogenase profile, the bovine serum albumin concentration remained constant until application ceased, when there was a rapid fall to zero, indicating no interaction with the polymer.

These studies have indicated that there are at least two binding sites on both the NAD^+- and ATP-linked polymers. Only one of these sites was associated with the tight binding observed under normal experimental conditions, requiring high salt concentrations for enzyme elution. The other sites, having capacities of 23 units of lactate dehydrogenase and 12 units of glycerokinase, exhibited very weak binding properties in that all the enzyme activity was eluted with the equilibration buffer. It can be calculated from a knowledge of the capacities of the tight binding sites (12.6 units of lactate dehydrogenase and 7.1 units of glycerokinase) together with the known nucleotide content of the respective columns, that of the immobilized cofactor only 0.1% of the NAD^+ and 0.4% of the ATP was utilized in the tightest binding site of lactate dehydrogenase and glycerokinase respectively.

The immobilization technique using dicyclohexyl carbodi-imide to attach nucleotides to Sepharose (Larsson & Mosbach, 1971) is not without complications, since frontal analyses of these gels indicate that probably more than one immobilized species is present. The capacity of these columns is less than that expected from the nucleotide content determined by phosphate and E_{260} analyses, in that less than 1% of the total immobilized nucleotide is available for binding. These findings parallel the observations of Larsson & Mosbach (1971) who showed that immobilized NAD^+ displayed about 0.2% coenzymic function when compared with an equivalent amount of NAD^+ in free solution. These results could reflect the stringent requirements for interaction between the enzyme and nucleotide, and suggest that most of the immobilized nucleotide is bound in a form un-

acceptable to the active site of the enzyme. It is suspected that the active nucleotide species is linked by N^6 substitution of the adenine moiety.

Effect of protein and enzyme concentration

The binding of constant amounts of lactate dehydrogenase and glycerokinase to their respective nucleotide columns was independent of bovine serum albumin concentration up to an equivalent of 80mg/ml in the applied sample. At low protein concentrations the recovery of lactate dehydrogenase was approx. 75%; quantitative recoveries were obtained at protein concentrations greater than 20mg/ml. This suggests that irreversible adsorption sites in the NAD^+-containing polymer were being saturated by bovine serum albumin. The recovery of glycerokinase from the ATP-containing polymer was independent of the bovine serum albumin concentration, although some bovine serum albumin did bind to this polymer and was eluted at 40mM-KCl. This binding was independent of glycerokinase and did not affect the binding characteristics of the enzyme. The ability for a polymer to bind bovine serum albumin seems to depend not so much on the nucleotide attached as on the individual preparations.

Increasing the enzyme concentration (for lactate dehydrogenase from 1.9 to 9.6 units; for glycerokinase from 1.7 to 11.9 units) in a constant sample volume (bovine serum albumin concn. 20mg/ml), had no effect on the binding of the enzymes to the columns containing the respective nucleotide. No significant effect on the enzyme-binding characteristics of the columns was observed when the bovine serum albumin/enzyme ratio in the applied sample was varied over the range 15–300. Since the binding constants are independent of enzyme concentration it would appear that the tight binding sites observed in frontal analysis chromatography are probably homogeneous. It is true, of course, that the range of concentration covered is small compared with that of the immobilized NAD^+ and ATP. The lack of effect of inert protein is consistent with these binding sites being specific for nucleotide-dependent enzymes.

Dilution studies

The effect of diluting Sepharose-bound nucleotide with unmodified Sepharose or ε-aminohexanoyl-Sepharose is shown in Fig. 3. The binding of lactate dehydrogenase and glycerokinase to their respective affinity columns decreased exponentially over a 21-fold dilution range. Increasing the concentration of NAD^+ available for the dicyclohexylcarbodi-imide-promoted coupling to ε-aminohexanoyl-Sepharose led to an increase in the amount of NAD^+ immobilized. Table 4 shows how the strength of binding of lactate dehydrogenase increased with the concentration of immobilized NAD^+, reaching a maximum at a concentration equivalent to 40μmol of NAD^+/g dry wt. of gel.

Clearly, the concentration of bound ligand is a dominant feature in determining the strength of binding. The equilibrium that exists between the bound ligand and the enzyme is presumably being altered in favour of the free enzyme when the ligand concentration is decreased. Similar results have been observed with other affinity adsorbents, for example, in the purification of oestradiol-17β dehydrogenase (Nicolas *et al.*, 1972).

Dilution of the enzyme sample by 2-, 6- and 21-fold had no effect on the subsequent binding characteristics of either nucleotide column, nor on the enzyme recovery or elution volume. Hence these small columns can effectively be applied to concentrating dilute enzyme samples.

Thus dilution of the enzyme sample does not have the same effect on binding as does dilution of the bound nucleotide. This difference can be rationalized in terms of the dissociation of the enzyme–nucleotide complex in the solid phase. Application of an ionic gradient alters the dissociation constant in favour of the free enzyme, and subsequent partition through the column will be dependent on the concentration of the immobilized nucleotide. Further observations on the effect of temperature on these parameters support the above conclusions (Lowe & Dean, 1973).

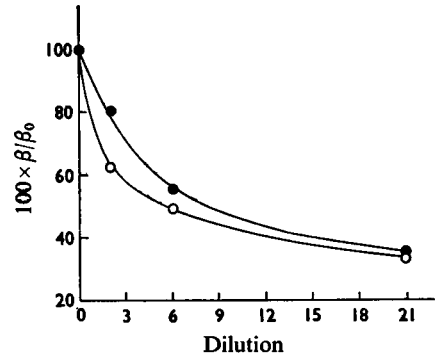

Fig. 3. *Binding of lactate dehydrogenase and glycerokinase to diluted NAD- and ATP-linked polymers*

Experimental procedures are given in the Materials and Methods section. β and β_0 represent the concentration of KCl required to elute the enzyme from the diluted and undiluted polymers respectively. ●, Glycerokinase on ε-aminohexanoyl-ATP–Sepharose diluted with Sepharose 4B; ○, lactate dehydrogenase on ε-aminohexanoyl-NAD^+–Sepharose diluted with ε-aminohexanoyl-Sepharose.

Table 4. *Effect of the concentration of immobilized nucleotide on the binding of lactate dehydrogenase to ε-aminohexanoyl-NAD⁺–Sepharose*

Experimental details are given in the text.

Concn. of NAD⁺ in dicyclohexylcarbodi-imide-coupling reaction (μmol/g wet wt. of gel)	Concn. of immobilized NAD⁺ (μmol/g wet wt. of gel)	Concn. of immobilized NAD⁺ (μmol/g dry wt. of gel)	Lactate dehydrogenase binding (mM-KCl)
0.65	0.33	6.5	55
2.26	0.40	7.9	190
6.80	1.83	36.7	245
13.60	2.13	42.6	295
27.20	3.31	66.1	285
81.60	4.11	82.2	300

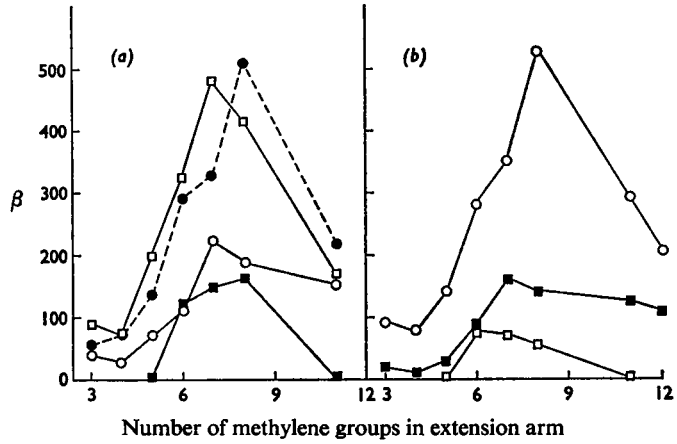

Fig. 4. *Effect of extension-arm length on the binding of dehydrogenases and kinases*

Experimental procedures are given in the Materials and Methods section. β represents the concentration of KCl (mM) required to elute the enzyme. (*a*) NAD⁺–Sepharose column: □, lactate dehydrogenase from rabbit skeletal muscle, M_4; ●, lactate dehydrogenase from pig heart, H_4; ○, malate dehydrogenase; ■, glucose 6-phosphate dehydrogenase. (*b*) ATP–Sepharose column: □, hexokinase; ■, phosphoglycerate kinase; ○, glycerokinase.

Equilibration time and flow rate

No effect of equilibration time could be found on the binding of bovine serum albumin or lactate dehydrogenase and glycerokinase when they were left for 1.5 and 20h in contact with their respective nucleotide columns before elution. This could prove particularly useful in the storage of enzymes that depend on the presence of substrate or cofactor for stability.

Increasing the flow rate of an ε-aminohexanoyl-NAD⁺–Sepharose column from 6.7 to 41ml/h had no effect on the binding of either lactate dehydrogenase or bovine serum albumin. The latter flow rate was equivalent to 40 void volumes/h; columns were normally run at 8ml/h.

Ligand extension studies

The effect of increasing the hydrocarbon chain length between the matrix and the ligand on the binding of dehydrogenases and kinases is shown in Figs. 4(*a*) and 4(*b*).

Three distinct phases were observed. Initially, with the nucleotide in close proximity to the matrix backbone, the enzyme binding was relatively independent of the number of methylene bridges interposed. This situation prevailed with extensions up

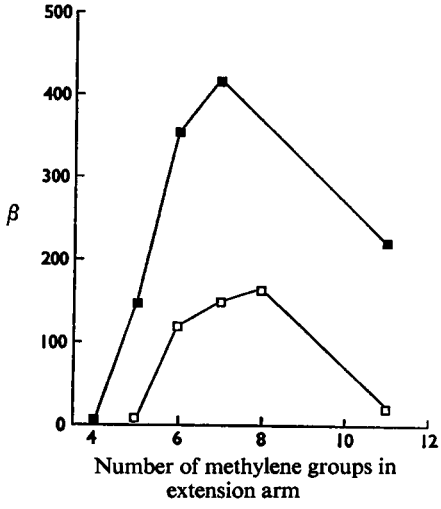

Fig. 5. *Effect of extension-arm length on the binding of glucose 6-phosphate dehydrogenase to polymers containing NAD⁺ and NADP⁺*

Experimental procedures are given in the Materials and Methods section. β represents the concentration of KCl (mM) required to elute the enzyme. □, ε-Aminohexanoyl-NAD⁺–Sepharose; ■, ε-aminohexanoyl-NADP⁺–Sepharose.

to four carbon atoms in length, a distance of approx. 0.5nm. The second phase, corresponding to four to eight carbon atoms interposed between the matrix and the nucleotide and a distance of 0.5–1nm, was associated with a steady increase in the strength of binding up to a maximum before decreasing again in the final phase. Fig. 5 shows the effect of various extension arm lengths on the binding of glucose 6-phosphate dehydrogenase to immobilized NAD^+ and $NADP^+$. The binding of glucose 6-phosphate dehydrogenase to immobilized NAD^+ is weaker than to the analogous $NADP^+$-containing polymer, although maximum binding in both cases was observed with a chain extension containing six methylene groups. This indicates that physical (i.e. length of extension arm) as well as chemical (i.e. nucleotide species) factors, determine the strength of binding.

Cuatrecasas (1970) has proposed that steric interference by the matrix on the ligand–macromolecule interaction can be decreased by introduction of extension arms between the matrix and ligand. It has been shown in the present study that an extension arm of at least four methylene groups was required before significant affinity chromatography was achieved. It is suggested that the use of an extension arm at least 0.5nm long enables the nucleotide to traverse a barrier imposed by the micro-environment associated with the polymer. This effect could be due to a diffusion barrier of ordered water molecules surrounding the matrix backbone, or vibrational motion of the lattice. For enzymes with weak affinities for the cofactor this barrier would prohibit any interaction with the specific ligand. This was apparent with glucose 6-phosphate dehydrogenase, hexokinase (EC 2.7.1.1) and 3-phosphoglycerate kinase (EC 2.7.2.3), in which at least five methylene groups were necessary before any interaction was observed. With these enzymes, further increases in the length of the extension arm resulted in only slightly enhanced binding. On the other hand, enzymes with high affinities for the ligand (lactate dehydrogenase and glycerokinase) showed progressive increases in binding when the extension arm was lengthened. There was also a correlation between molecular weight and the binding in relation to the extension arm length. For low-molecular-weight proteins, e.g. 3-phosphoglycerate kinase and malate dehydrogenase, the length of the extension arm was not as critical as for proteins with molecular weights greater than 70000, although this might reflect the conformation of the nucleotide-binding site of the enzyme. In all cases examined, maximum binding occurred when the length of the extension arm was in the region of 0.8–1nm. The decreased binding observed with longer extension arms might reflect the folding of the hydrocarbon chain back towards the matrix backbone, so that 11 methylene groups become equivalent to a distance of 0.7nm. Steric interference related to the pore size of the Sepharose gel might also account for these observations. It is perhaps significant that the binding of malate dehydrogenase and 3-phosphoglycerate kinase was virtually unaffected when the extension arm was increased beyond eight methylene groups, suggesting that the extended ligand was unavailable to those enzymes having molecular weights greater than 70000.

We have also examined the use of other types of extension arm, such as sesquipeptides of glycine, in an attempt to obtain further insight into this problem. There is little effect (190–290mM-KCl) of interposing polyglycine extension arms between the matrix and NAD^+ on the binding of the two isoenzymes of lactate dehydrogenase. This may be a reflection of either the increased rigidity or the more hydrophilic nature of the glycine analogues compared with the polymethylene series.

General discussion

The interaction of dehydrogenases and kinases with immobilized nucleotides has presented a unique opportunity to provide evidence for some of the basic postulates of affinity chromatography (Cuatrecasas & Anfinsen, 1971*a*,*b*).

Evidence is presented here which suggests that the use of cellulose as a matrix for affinity chromatography is hampered by the introduction of ion-exchange groups during, or as a result of, CNBr treatment. This effect was not observed when agarose (Sepharose) was used as the inert matrix, and consequently this is the preferred insoluble support for nucleotide affinity chromatography.

The important feature of affinity chromatography on immobilized nucleotides is that the enzyme interacts specifically with its complementary nucleotide. In the present study the selectivity of the interaction is shown by the following observations. First, lactate dehydrogenase is only bound tightly to the polymer prepared by coupling NAD^+ to ϵ-aminohexanoyl-Sepharose in the presence of dicyclohexylcarbodi-imide. Insignificant binding is observed when any of the dicyclohexylcarbodi-imide reaction components are omitted (Table 3). Secondly, the strength of binding increases with the amount of NAD^+ coupled to the gel (Table 4). Thirdly, lactate dehydrogenase binding is not influenced by increased concentrations of inert protein. Finally, the specificity of the interaction has been shown by frontal analysis chromatography.

The mode of binding of either nucleotide has not been established. However, Lowe *et al.* (1972) have shown that NAD^+-linked dehydrogenases bind strongly to N^6-aminohexyl-AMP–Sepharose, and that the binding resembles that observed with ϵ-aminohexanoyl-NAD^+–Sepharose. Therefore we surmise that the tight binding site observed for polymers containing either NAD^+ or ATP corresponds to attachment through an amide linkage between the carboxyl group of the spacer molecule and the 6-amino group of the adenine moiety of the nucleotide (Dean & Lowe, 1972). Coupling through the ribose hydroxyl groups probably accounts for the weak binding sites observed in the enzyme profiles. This could explain the failure of some investigators to observe a change in u.v. absorption associated with N^6 substitution (Larsson & Mosbach, 1971).

In summary, the binding of the free enzyme is independent of equilibration time and flow rate, indicating a rapid adsorption phase. Further, the concentration of immobilized ligand is a more important parameter than the concentration of freely diffusible macromolecule.

Work with chemically defined nucleotide adsorbents should resolve some of the difficulties experienced with the dicyclohexylcarbodi-imide-linked nucleotide matrices. Nevertheless, the general utility of these adsorbents for the purification of groups of cofactor-dependent enzymes has been demonstrated (Lowe *et al.*, 1972).

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