IMMOBILISED NUCLEOTIDES AND COENZYMES FOR AFFINITY CHROMATOGRAPHY

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Abstract - General methods for the preparation of coenzyme and nucleotide analogues substituted with spacer molecules at various positions on the purine, phosphate and ribose moieties and suitable for attachment to insoluble support matrices are given. The relative efficiencies of the resulting differently immobilised nucleotides are compared both in regard to the position of substitution and in terms of the relative merits of whole coenzyme immobilisation versus the "AMP-half". Both the biospecificity and the problem of non-specific adsorption to nucleotide adsorbents is discussed. Finally, examples are given to illustrate the range of potential applications of immobilised coenzymes.

INTRODUCTION

Affinity chromatography exploits the unique biospecificity displayed between a small ligand, typically a substrate or inhibitor, and its complementary biological macromolecule such as an enzyme. The ligand is immobilised by covalent attachment to a porous hydrophilic matrix often via an intermediate "spacer" molecule that is long enough to minimise steric interference between the two binding partners. The matrix-immobilised ligand is established as a chromatographic bed, the mixture of proteins applied and, in principle at least, only that enzyme that biospecifically recognises the immobilised ligand will be retained by the adsorbent. All other inert proteins will pass through unretracted. The biospecifically adsorbed enzyme may subsequently be eluted by changing the column irrigants to favour dissociation of the enzyme-immobilised ligand complex. These principles and practice of affinity chromatography are well documented in a number of recent review articles (Refs. 1 - 4).

Two different chemical approaches to the immobilisation of ligands can be distinguished. The first, or "solid phase modular assembly" approach couples the desired ligand to a matrix to which spacer molecules have already been attached. However, severe problems of multipoint attachment and consequent heterogeneity of the resulting adsorbent can arise with multifunctional ligands. The present author therefore adopts the more systematic or "preassembly" approach where the ligand is derivatised with a suitable spacer molecule bearing a terminal functional group prior to attachment to an activated matrix. This approach permits adequate characterisation of the ligand-spacer molecule assembly by conventional chemical and physical procedures prior to immobilisation and thus leads to a more homogenous adsorbent. For analytical applications of affinity chromatography such confidence is essential.

This paper is concerned with the preparation and applications of preassembled ligands which display broad specificity for groups of enzymes. These highly versatile ligands, "group specific" or "general" ligands reduce the elaborate organic syntheses required in the preparation of adsorbents since new synthetic routes need not be constructed for every putative biospecific purification. Enzymes requiring coenzymes, particularly the adenine and pyridine nucleotide coenzymes, are especially amenable to this "group specific" or "general ligand" approach (Refs. 2, 5-8). The advantages of this type of adsorbent are immediately apparent; the chemistry of ligand immobilisation is restricted to a single species and once the adsorbent is prepared it is generally applicable to the purification of a number of complementary enzymes. For example, of the 2000 or so recorded enzymes, approximately 8% bind NAD, 8% bind NADP, 11% bind ATP and 4% coenzyme A. Adenosine 5'-monophosphate (AMP) is a constituent of the above coenzymes and is therefore a likely candidate for immobilisation as as a potential general ligand.

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nucleotides with hexamethylene diisocyanate to generate 6-ureido purine derivatives (Ref. 18).

The carbon atom located at position 8 of the purine nucleus has the highest electron density and is thus susceptible to direct electrophilic substitution. For example, derivatives bearing terminal amino or carboxyl functions substituted at position 8 of the adenine nucleus may be prepared by bromination of the nucleotide followed by nucleophilic displacement of the halogen by 1,6-diaminohexane (Ref. 19) or 3-mercaptopropionic acid (Ref. 16) respectively. This leads to a facile synthesis of 8-(6-aminohexyl)-AMP (Ref. 19) and the method is equally applicable to the preparation of C8-linked analogues of NAD, NADP, cAMP, adenosine-2' (3'), 5'-bisphosphate plus many other nucleotides containing bases other than adenine. The 8-position is also susceptible to substitution with diazonium cations although in most cases these reactions form part of a solid phase modular assembly approach (Ref. 5) and therefore lead to adsorbents of ill-defined and heterogeneous character. Nevertheless, nucleotides such as NAD may be readily immobilised with diazonium reactions and yield adsorbents which display great potential for the purification of pyridine-nucleotide dependent dehydrogenases.

Phosphate-linked Nucleotides.
The synthetic methods available to immobilise nucleotides through the terminal phosphate moiety are generally applicable to the preparation of adsorbents comprising all ribosyl and deoxyribosyl nucleotides. For example, the carbodiimide promoted synthesis of p-aminophenyl esters of nucleotides is simple but often results in the formation of symmetrical pyrophosphates (Ref. 20). This problem may be circumvented by employing phosphate-displacement reactions such as the anion displacement method (Ref. 21) or the imidazolide procedure (Ref. 22). The latter procedure is a particularly convenient and widely applicable approach. Hexitolamine is phosphorylated to 6-aminohexan-1-ol phosphate, reacted with 1,1'-carbonyldimidazole and the resulting imidazolide of hexitolamine phosphate reacted with an appropriate nucleotide to generate the corresponding asymmetric pyrophosphate. For example, with AMP this is P'-{(6-aminohexyl)-P'-{S'-adenosyl}-pyrophosphate (ref. 23).
The synthesis of nucleotide analogues for affinity chromatography

A number of methods are available for the derivatisation and subsequent immobilisation of the adenine nucleotide coenzymes. Fig. 1 summarises several different ways of introducing a spacer molecule into an AMP (or NAD') moiety to permit attachment to a solid support. They may be broadly classified into base-linked, ribose-linked and phosphate-linked nucleotides.

**Fig. 1.** Procedures for the immobilisation of Adenosine 5'-monophosphate (AMP).

**Base-linked Nucleotides**
The base-linked nucleotides substituted at the exocyclic N\textsuperscript{6} amino group are most effectively synthesised by nucleophilic displacement of a 6-chloro (Ref. 9), 6-mercapto (Ref. 10) or 6-methylsulphone (Ref. 11) group by a large excess of a suitable diaminoalkane spacer molecule to prevent bis-nucleotide formation. For example, N\textsuperscript{6}-(6-aminohexyl)-AMP is readily synthesised by heating the sodium salt of 6-mercapto-purine riboside 5'-monophosphate and 1,6-diaminohexane in a sealed ampoule overnight at 85 - 95°C (Ref. 10). The course of the reaction is followed by a characteristic change in absorbance maximum from 309 nm to 267 nm. The synthetic route obviates the need for the seven-stage sequence of reactions starting from inosine to produce the same product via the 6-chloronucleotide (Ref. 9).

Introduction of a spacer molecule at the N\textsuperscript{6} position of the base may also be effected by prior alkylation at position N\textsuperscript{7} with iodoacetic acid (Ref. 12), aziridine (Ref. 13), propionolactone (Ref. 14) or epoxides (Refs. 15, 16) followed by a Dimroth rearrangement to the corresponding N\textsuperscript{6} derivatives by heating under alkaline conditions. A procedure involving alkylation with iodoacetic acid for 5 - 10 days at pH 6.5, followed by alkaline rearrangement to the corresponding N\textsuperscript{6}-carboxymethyl substituent and subsequent coupling of 1,6-diaminohexane to the terminal carboxyl group with a carbodiimide promoted reaction has been adapted to the synthesis of the N\textsuperscript{6}-(6-aminohexyl)-carbamoylmethyl-derivatives of AMP, ADP, ATP, NAD\textsuperscript{+} and NADP\textsuperscript{+}. Fig. 2 illustrates a typical synthesis of an NADP\textsuperscript{+} analogue for affinity chromatography by this relatively facile approach (Ref. 17). An alternative approach involves direct carbamoylation of the exocyclic N\textsuperscript{6} amino of adenine.
Ribosyl-linked Nucleotides.
The immobilisation of nucleotides through the vicinal diol of the ribose moiety is relatively straightforward. The ribose hydroxyls of the coenzyme or nucleotide are oxidised to aldehyde groups by the action of periodate and then allowed to react with an agarose-bound hydrazide, such as adipic acid dihydrazide coupled to CNBr-activated agarose (Ref. 8). Alternatively, acetalisation of the nucleotide with ethyl levulinate leads to levulinic acid (O'-O'-nucleoside) acetal which is readily converted to the corresponding nucleotide by phosphorylation with phosphoryl chloride. Saponification followed by condensation with 1,6-diaminohexane yields a ribose-substituted analogue suitable for attachment to a solid support (Ref. 23). However, it should be pointed out that ribosyl-linked nucleotides are relatively ineffectual in the purification of the NAD-linked dehydrogenases. Nevertheless, ribosyl-linked nucleotides are effective in the purification of deaminases and equivalently linked NADP adsorbents have proved invaluable in the purification of several NADP-dependent dehydrogenases.

Characterisation of Nucleotide Analogues
In most cases the spacer arm-conjugated coenzyme and nucleotide analogues were purified to homogeneity by ion exchange chromatography and characterised in terms of their behaviour on thin layer chromatography in several solvent systems, functional group and elemental analysis and their UV, IR, 1H and 13C-NMR spectroscopic properties. Furthermore, treatment of the NAD and NADP analogues with sodium dithionite, enzymes or 1M KCN generated the chemically and enzymically reduced forms (λmax 340nm) and cyanide adducts (λmax 328nm) respectively in almost quantitative yield. The purified and characterised analogues have subsequently been covalently attached to CNBr-activated agarose to yield adsorbents containing 1.5-5μmol nucleotide/g moist wt. gel. The immobilised ligand concentrations were determined by direct UV spectroscopy or phosphate analysis (Ref. 17). Fig. 3 illustrates the structures of several adsorbents comprising base, phosphate and ribosyl-linked adenine nucleotides.

Fig. 3. The structures of several immobilised nucleotide and coenzyme analogues: (a) N'-(6-aminohexyl)-AMP; (b) 8-(6-aminohexyl)-AMP; (c) P'=-(6-aminohexyl)-P(5'-adenosyl)-pyrophosphate; (d) ribosyl-linked AMP.
THE EFFICIENCY OF DIFFERENTLY IMMOBILISED NUCLEOTIDE ADSORBENTS

Preassembled NAD* and NADP* analogues and the complementary nucleotide "half molecules" 5'-AMP and 2',5'-ADP when covalently attached to CNBr-activated agarose have proved excellent adsorbents for the purification of NAD* and NADP* -dependent dehydrogenases. However, differently immobilised nucleotide adsorbents exhibit markedly different effectivities in the purification of complementary enzymes. For example, Table 1 summarises the binding of several AMP-dependent enzymes to agarose-bound N'- (6-aminohexyl)-AMP and 8-(6-aminohexyl)-AMP under equivalent experimental conditions i.e. 50mM potassium phosphate buffer pH 7.5 at 4°C. The affinity of the enzymes for the respective adsorbents is expressed in terms of the concentration of eluant (NADH or AMP) required to elute the enzyme with peak activity on a linear gradient of the eluant under standardised conditions. At the immobilised ligand concentration of 2.5mmol AMP analogue/g moist weight gel used in this study, most of the mammalian enzymes, including alcohol, lactate and malate dehydrogenase, bound more tightly to the N'-substituted AMP adsorbent. In marked contrast, however, the two B. subtilis enzymes, alanine dehydrogenase and malate dehydrogenase, and the E. coli enzyme, IMP dehydrogenase, display a preference for the 8-substituted AMP-analogue. At the ligand concentration used in this study, neither Pseudomonas fluorescens D-galactose dehydrogenase nor pig heart lipoamide dehydrogenase bound to either gel. These differences in binding patterns observed for the two immobilised-AMP adsorbents can be ascribed to differences in the affinity of the enzymes for the AMP analogues. Thus, Table 1 also lists the competitive inhibition constants (K_i) for each of the free 6- and 8-substituted AMP-derivatives derived from initial velocity measurements. In most cases the K_i values for N'- (6-aminohexyl)-AMP were up to 20-times lower than those for 8-(6-aminohexyl)-AMP measured under identical conditions. In contrast, in the case of alanine dehydrogenase from B. subtilis, the K_i value for the 8-substituted analogue was considerably lower than the N'-substituted AMP derivative. Furthermore, it is apparent from Table 1 that the K_i value for the prospective free AMP analogue must be < 2mM in order to effect adsorption of an enzyme under the experimental conditions chosen i.e. an immobilised ligand concentration of 2.5mmol/g moist weight gel. This conclusion is supported from evidence of the inhibition of mammalian hexokinase by various N-aminoacyl glucosamine derivatives where, again, a K_i < 2mM was necessary in order to construct an effective affinity adsorbent (Ref. 24).

In general terms, NAD* -dependent dehydrogenases bind more tightly to base-substituted nucleotide matrices than to either phosphate-immobilised or ribose-linked adsorbents. In fact, in the absence of prior knowledge of how a particular dehydrogenase binds to these adsorbents, the preference in binding to an immobilised-AMP adsorbent might be expected to lie in the order: N' > phosphate > C' > ribose. These data are entirely consistent with the results of X-ray crystallographic analysis of several NAD* -dependent enzymes. For example, X-ray studies on dogfish muscle lactate dehydrogenase have indicated that the coenzyme NAD* is bound in a

**Table 1.** The binding of several NAD* -dependent dehydrogenases to N'- (6-aminohexyl)-AMP-agarose and 8-(6-aminohexyl)-AMP-agarose.

<table>
<thead>
<tr>
<th>Enzyme: Dehydrogenase</th>
<th>Source</th>
<th>Eluant conc.</th>
<th>K_i</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C8</td>
<td>N'</td>
</tr>
<tr>
<td>Alanine</td>
<td>B. subtilis</td>
<td>80</td>
<td>8</td>
</tr>
<tr>
<td>Alcohol</td>
<td>Horse liver</td>
<td>30</td>
<td>75</td>
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<tr>
<td>Galactose</td>
<td>Ps. fluorescens</td>
<td>0</td>
<td>*</td>
</tr>
<tr>
<td>IMP</td>
<td>E. coli</td>
<td>4.6</td>
<td>3*8</td>
</tr>
<tr>
<td>Lactate</td>
<td>Rabbit muscle</td>
<td>30</td>
<td>190</td>
</tr>
<tr>
<td>Lactate</td>
<td>Pig heart</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>Lipoamide</td>
<td>Pig heart</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Malate</td>
<td>B. subtilis</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Malate</td>
<td>Pig heart</td>
<td>0</td>
<td>35</td>
</tr>
</tbody>
</table>

*Eluant was a linear gradient of AMP (0 - 10 mM).*
deep pocket with the adenine moiety outermost (Ref. 25). In particular, it appears that the exocyclic N^2-amino and to a lesser extent the carbon atom at position C8 of the purine nucleus are free of binding to the apoenzyme and protrude out of a shallow hydrophobic crevice. Not surprisingly, therefore, substitution at positions N^6 and C8 yield effective adsorbents whilst substitution at the ribose hydroxyls yields relatively ineffectual matrices.

A COMPARISON OF BINDING TO IMMOBILISED AMP AND NAD\(^+\) ADSORBENTS

The data presented in Table 2 compare the binding of several pyridine-nucleotide dependent dehydrogenases to adsorbents comprising agarose-bound NAD\(^+\) and AMP at identical ligand concentrations of 2\,μmol/g moist weight gel. With the exception of those enzymes which display no affinity for either adsorbent under these conditions, stronger binding is observed in most cases to the agarose-bound N^6-(6-aminohexyl)-AMP adsorbent than to agarose-bound N^6-(6-aminohexyl) carbamoylmethyl-NAD\(^+\) gel. These rather surprising data may be rationalised in terms of the nature of the spacer molecules employed since intuitively one would expect almost all the enzymes to bind more tightly to the NAD\(^+\) adsorbent in view of the known affinities of the enzymes for free underivatised NAD and AMP. However, the NAD\(^+\) analogue comprises a more hydrophilic arm enclosing a peptide linkage (NH\(_2\)(CH\(_2\))\(_6\)NHCO\(_2\)NH-N^-NAn) rather than the AMP-analogue with its hydrophobic hexanethylene chain (NH\(_2\)(CH\(_2\))\(_6\)NH-N^-AMP). It is known that hydrophilic arms weaken the binding of dehydrogenases to immobilised nucleotide adsorbents (Ref. 19). This conclusion is supported by comparisons between agarose-bound N^6-(6-aminohexyl)-NAD\(^+\) and N^6-(6-aminohexyl)-AMP where stronger binding is observed to immobilised NAD\(^+\) than AMP when both adsorbents comprise identical polymethylene spacer molecules (Ref. 26).

These latter data clearly emphasise that there is often no advantage in embarking on a complex synthesis of a complete coenzyme analogue when a more facile synthesis of an AMP-analogue is possible. Furthermore, adequate adsorption of dehydrogenases to AMP-adsorbents can often be achieved simply by alterations in the operating conditions such as immobilised ligand concentration and irrigant buffers.

SPECIFICITY OF IMMOBILISED NUCLEOTIDE ADSORBENTS

An important prerequisite for affinity chromatography on immobilised "group specific" ligands is that the complementary enzymes specifically recognise their respective immobilised nucleotides or coenzymes. For example, an NADP^+ dependent enzyme should be able to discriminate between immobilised-NAD\(^+\) and NADP\(^+\). Experience has shown that this is in fact so in most cases. For example, as Fig. 4 demonstrates, agarose-bound NADP^+ synthesised according to the procedure given in Fig. 2, proved to be an effective biospecific adsorbent for the resolution of a mixture of bovine serum

<table>
<thead>
<tr>
<th>Enzyme: Dehydrogenase</th>
<th>Source:</th>
<th>Eluant concn. (μM NADH)(^a)</th>
<th>N^6-AMP</th>
<th>N^6-NAD(^+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>B. subtilis</td>
<td>40</td>
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</tr>
<tr>
<td>Alcohol</td>
<td>Yeast</td>
<td>85</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Alcohol</td>
<td>Horse liver</td>
<td>60</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Galactose</td>
<td>Ps. fluorescens</td>
<td>0</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>Yeast</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Lactate</td>
<td>Pig heart</td>
<td>80</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>Lactate</td>
<td>Rabbit muscle</td>
<td>300</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Lipoamide</td>
<td>Pig heart</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Malate</td>
<td>Pig heart</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Columns (1g) containing NAD\(^+\)- or AMP-agarose (2μmol analogue/g agarose) were equilibrated with 20mM potassium phosphate buffer pH 7.5 at 4°C. Enzymes were eluted with a linear gradient of NADH (0 - 0.4mM).
Fig. 4. Resolution of an enzyme mixture (a) prior to and (b) after treatment of an immobilised-NADP⁺ adsorbent with alkaline phosphatase.

albumin, lactate dehydrogenase and glucose-6-phosphate dehydrogenase. Neither bovine serum albumin nor lactate dehydrogenase displayed any affinity for the NADP⁺-gel and appeared in the void volume of the column whereas glucose-6-phosphate dehydrogenase remained adsorbed even after exhaustive washing with equilibrating buffer. The adsorbed glucose-6-phosphate dehydrogenase could not be eluted by pulses of 0.5mM NAD⁺ or NADH but was quantitatively released with 0.5mM NADP⁺. Treatment of the gel-bound NADP⁺ with calf intestinal alkaline phosphatase generated matrix-bound NAD⁺ by removal of the 2'-phosphate and reversed this binding pattern. Thus, the modified gel adsorbed lactate dehydrogenase and subsequently released it with a pulse of 0.5mM NADH whilst most of the glucose-6-phosphate dehydrogenase activity appeared with the albumin in the void volume.

The binding specificity of immobilised nucleotide adsorbents is also demonstrated by the relative effectivities of immobilised AMP and IMP. Immobilised IMP was synthesised from 8-(8-aminoctyl)-AMP by protection of the terminal amino group of the spacer molecule with S-ethyltrifluoroacetyl thiol acetate (CF₃COSC₂H₅) followed by diazotisation at 0°C for 3h in the presence of nitrous acid (Ref. 27). The terminal amino group of the 8-substituted IMP derivative was finally deprotected by incubation at pH 12 for 3h. The purified analogue, 8-(8-aminoctyl)-IMP and the parent analogue, 8-(8-aminoctyl)-AMP were subsequently coupled to CNBr-activated agarose to yield adsorbents containing 2μmol nucleotide/g adsorbent. Fig. 5 illustrates the behaviour of B. subtilis alanine dehydrogenase on columns of the two adsorbents under identical chromatographic conditions. Adsorbents comprising agarose-bound
8-(8-aminooctyl)-IMP displayed no affinity for the enzyme which appeared in the void volume, whilst the enzyme was strongly bound to immobilised-AMP and required a gradient of NADH to effect release. Similar binding to the AMP adsorbent but not the IMP was observed for pig heart lactate dehydrogenase. In contrast, E. coli IMP dehydrogenase, which displays affinity for both IMP and NAD⁺, is strongly adsorbed to both matrices with preference being shown for the immobilised-IMP. These observations are entirely consistent with the known $K_m$ values of IMP dehydrogenase for IMP and AMP respectively.

**NON-SPECIFIC ADSORPTION TO NUCLEOTIDE ADSORBENTS**

The biospecificity of adsorption involved in the binding of enzymes to nucleotide and coenzyme affinity adsorbents is amply illustrated by the above two examples. The enzymes were able to discriminate between subtle differences in the immobilised ligand structure. However, it has become evident in recent years that non-specific

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**Fig. 5.** Chromatography of a crude extract of *B. subtilis* on (A) 8-(8-aminooctyl)-IMP-agarose and (B) 8-(8-aminooctyl)-AMP-agarose. The bacterial extract (100μl) was applied to a column (0.5g) of the immobilised nucleotide (2μmol/g adsorbent) and allowed to preincubate for 15min. The column was washed with 50mM potassium phosphate buffer pH 7.5 (10ml) and developed with a linear gradient of NADH (0 - 0.5 μM; 20 ml total volume) in the phosphate buffer. Alanine dehydrogenase activity (- - -), protein concentration (mg/ml) (- - -) and concentration of NADH (----) were assayed in the eluant fractions (1.5 ml).
adsorption phenomena can augment or even drastically distort true biospecific adsorption to a prospective biospecific ligand (Refs. 19, 28). It is now accepted that such effects are induced partly by the charged isourea linkages introduced during CNBr-activation of the matrix and partly by the hydrophobic nature of the spacer molecules interposed between the ligand and matrix backbone. These charged and hydrophobic effects interfere with the natural bioaffinity between the enzyme and the immobilised ligand by interacting non-specifically with charged and hydrophobic sites on the enzyme surface. Non-specific electrostatic interactions may be readily eliminated by using bishydrazides as spacer molecules in preference to bisamines (Ref. 8) or by using alternative activation procedures such as bisoxiranes or epichlorohydrin (Refs 2,4) which result in stable, neutral ether linkages between the matrix and ligand. However, the problem of hydrophobic interactions is not so readily solved. The absolute requirement for "spacer molecules" separating the ligand from the steric occlusion effects of the matrix backbone was established several years ago. The spacer arms currently employed comprise aliphatic poly-methylene chains bearing terminal functional groups to permit anchorage at one end to the activated matrix and at the other to the ligand itself. However, it has only been recently appreciated that the hydrocarbon spacer molecules can generate non-specific adsorption effects which supplement or overrule true biospecific effects. Two main approaches may be considered for the circumvention of these effects.

The Use of Organic Solvents

The chromatography may be performed in buffers supplemented with low concentrations of water-immiscible solvents (Ref. 29). For example, the effect of the weak polarity reducing agent, ethylene glycol, on the binding of pig heart lactate dehydrogenase to agarose-bound N'-(6-aminohexyl)-AMP has been investigated in detail (Ref. 29). In the absence of the reagent, recoveries of enzyme activity from the adsorbent as low as 60% were observed on elution with a linear gradient of NADH. However, on inclusion of low concentrations of ethylene glycol in the column irrigants drastically improved the recovery of enzyme activity with quantitative recoveries being observed in the presence of 20-30% ethylene glycol. Low concentrations of dioxane (1% v/v), N,N-dimethylformamide, urea or detergents also produced similar effects in discharging hydrophobic effects created by hydrophobic spacer molecules. However, it is important to realise that higher concentrations of organic solvents alter the native conformation of enzymes and lead to a decreased affinity for the immobilised ligand (Ref. 29).

The Use of Hydrophilic Spacer Arms

In principle at least, many of the problems associated with aliphatic spacer arms may be circumnavigated by the synthesis of ligands bearing hydrophilic spacer arms. Experience has shown, however, that this is not necessarily so. Thus, for example, a series of 8-substituted derivatives of AMP bearing spacer molecules of differing composition but similar length have been synthesised and characterised by conventional organic chemistry (Ref. 19). Fig. 6 shows the structures of four of these immobilised-AMP derivatives prepared by condensation of appropriate 8-(ω-aminoalkyl)-AMP derivatives with N-trifluoroacetyl-glycine or 8-alanine in the presence of a water-soluble carbodiimide and subsequent attachment to CNBr-activated agarose. The chromatographic behaviour of the resulting adsorbents was investigated in terms of their ability to bind rabbit muscle lactate dehydrogenase and B.subtilis alanine dehydrogenase. Both enzymes bound more tightly to the most hydrophobic adsorbent (Fig. 6) and progressively less and less tightly to the adsorbents comprising AMP linked via arms of increasing hydrophilicity. Indeed, under the conditions of the experiment, 10mM potassium phosphate buffer pH 7.5 and a ligand concentration of 2.5μM AMP analogue/g moist weight gel, little or no affinity was shown for the derivative bearing the most hydrophilic arm. These data concur with those of Trayer et al. (Ref. 24) where hydrophilic derivatives of glucosamine proved relatively ineffectual in the purification of rat hepatic glucokinase whilst hydrophobic derivatives proved eminently satisfactory. Interestingly, in the latter case (Ref. 24) and the present example (Ref. 19), little difference was found in the free solution Kᵣ values for the hydrophobic and hydrophilic analogues. For example, in free solution [AMP]-8-NH(CH₂)₇-NH₃ and [AMP]-8-NH₂(CH₂)₅-NHCOCH₃-NH₃ were strictly competitive with NADH for rabbit muscle lactate dehydrogenase with Kᵣ values of 2.0mM and 1.8mM respectively (Ref. 19). These data and those of Trayer (Ref. 24) suggest that the observed differences in chromatographic behaviour of these immobilised derivatives may reflect a difference in accessibility of the analogues when attached to agarose rather than a fundamental difference in affinity. It is conceivable that the more hydrophilic derivatives of AMP may hydrogen bond to the matrix backbone and therefore exhibit reduced accessibility to interaction with the complementary enzymes.
Fig. 6. The structures of four 8-substituted AMP-agarose adsorbents with spacer molecules of differing composition [Reproduced with permission from C.R. Lowe, Eur. J. Biochem. 73, 265-274 (1977)].

ELUTION TECHNIQUES

Although to some extent the problems of non-specific adsorption may be circumvented by careful choice of operating conditions and adsorbent design, biospecific elution often affords an additional tool to manipulate. This is particularly true for group specific adsorbents where in addition to these non-specific effects, the general applicability of these adsorbents often leads to problems involving the resolution of complex mixtures of related enzymes. A number of elution techniques are available for the resolution of enzymes bound to "group specific" or "general ligand" adsorbents. Thus any chemical agent or alteration in a physical parameter that alters the apparent affinity of an adsorbed enzyme for any affinity matrix is potentially capable of effecting its elution from an affinity gel. For example, changes in physical parameters such as the pH (Ref. 30), ionic strength (Ref. 5), temperature (Ref. 31) or dielectric constant (Ref. 29) are effective. Thus, for example, ethylene glycol concentrations above 40 -45% (v/v) alter the intrinsic protein fluorescence of pig heart lactate dehydrogenase and markedly increase the dissociation constant for NADH (Ref. 29). Fig 7 shows that lactate dehydrogenase is eluted from a column of agarose-bound N-(6-aminohexyl)-AMP by a 0 - 70% (v/v) linear gradient of ethylene glycol in the presence of 1M KCl. The enzyme is eluted with peak activity at 43% (v/v) ethylene glycol and is recovered in a yield of approximately 45%. Furthermore, the
enzyme is elutable at 1.2M urea with quantitative recovery on a 0 - 2.5M urea gradient and at 30% (v/v) dioxane with 80 - 90% recovery on a 0 - 50% dioxane gradient (Ref. 29).

However, in general terms, the demands on the specificity of the elution regime are greater with "group specific" affinity adsorbents than with "monospecific" affinity chromatography and has prompted the development of biospecific elution conditions. These approaches employ ligands or ligand combinations which display a higher affinity for the enzyme than the immobilised ligand. The competitive counterligands may be applied as steps, pulses or for more resolving power as gradients of oxidised or reduced coenzymes (Ref. 32), ternary complexes (Ref. 27) or preformed coenzyme-substrate adducts (Ref. 33).

APPLICATIONS OF GROUP SPECIFIC ADSORBENTS

The general usefulness of these adsorbents has prompted their application in a number of preparative and analytical situations, summarised in Table 3. Some examples will be given to illustrate their potential.

Enzyme Purification from Crude Extracts
Alanine dehydrogenase from B. subtilis displays remarkably high affinity for 8-substituted AMP-analogues. The competitive inhibition constant (K_i) for 8-(6-aminohexyl)-AMP was 0.08mM compared to 2.0mM for N^6-(6-aminohexyl)-AMP (Table 1). The enzyme is adsorbed to an immobilised 8-substituted AMP adsorbent and eluted quantitatively as a single protein homogenous by SDS electrophoresis. Furthermore, IMP dehydrogenase from a crude extract of E. coli may be purified to homogeneity in a single step on immobilised AMP adsorbents (Ref. 27).

Enzyme Mechanistic Studies
As yet the full potential of chemically defined nucleotide adsorbents for studies in mechanistic enzymology has not been fully appreciated. Nevertheless, there is evidence of the value of affinity chromatography in the assessment of the order of binding of ligands to enzymes, in the detection of non-productive or abortive complexes and in the demonstration of ternary complex formation (Ref. 34). Furthermore, the use of differently immobilised nucleotides to assess the nature and evolution of nucleotide binding sites and of immobilised coenzyme fragments to yield information
on binding site topographies has proved fruitful (Ref 34).

TABLE 3. Applications of Immobilised Coenzymes

<table>
<thead>
<tr>
<th>Application</th>
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<tr>
<td>Enzyme purification from crude extracts</td>
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<tr>
<td>Preparative scale enzyme isolation</td>
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<td>Isoenzyme resolution</td>
</tr>
<tr>
<td>Contaminant removal</td>
</tr>
<tr>
<td>Resolution of active/non-active or chemically modified species</td>
</tr>
<tr>
<td>Separation of mutant and wild type enzymes</td>
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Coenzymically Active Coenzymes

In addition to their undeniable biospecificity in affinity chromatography, immobilised coenzymes such as NAD and NADP, also retain a useful proportion of their biological activity. For example, an N'-substituted NADP-analogue, synthesised according to the route illustrated in Fig. 2, and subsequently attached to a water-soluble dextran polymer, retained up to 35% of its coenzymic activity relative to the underivatised NADP (Ref. 17). The agarose-bound coenzyme, however, displayed a relative activity of 1% under similar conditions.

CONCLUSIONS

This discussion emphasises the preparation and remarkable versatility of immobilised coenzymes. Their applications span enzyme purification from crude extracts through various analytical applications to their use as immobilised active coenzymes in enzyme technology. Nevertheless, it is important to realise that for many of these applications criteria should be applied to affirm the biospecificity of the interaction with the complementary enzymes. Where this is suspect, steps should be taken to reduce or eliminate non-specific adsorption effects which can seriously marr true biospecific interactions.

REFERENCES