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 insol-
uble support matrices are given. The relative efficiencies of the result-
ing differently immobilised nucleotides are compared both in regard to the
position of substitution and in terms of the relative merits of whole co-
enzyme immobilisation versus the "AMP-half". Both the biospecificity and
and the problem of non-specific adsorption to nucleotide adsorbents is dis-
cussed. 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 macro-
molecule 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 unretarded. 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 chromato-
graphy 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 char-
acterisation 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 preassem-
bled ligands which display broad specifity 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 especial-
ly 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
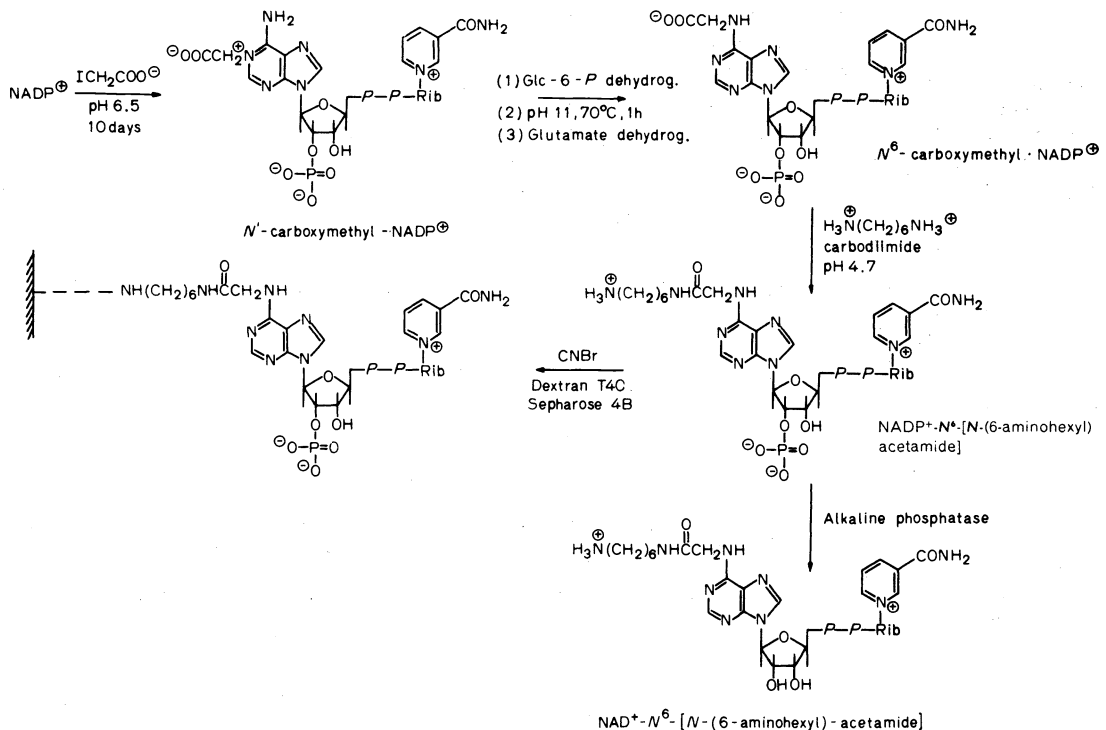for immobilisation as as a potential general ligand.

Fig. 2.   The synthesis of a matrix-immobilised NADP⁺ analogue for affinity
chromatography. P-P, Pyrophosphate; Rib, ribose. [Reproduced with permission from C.R. Lowe and K. Mosbach, Eur. J. Biochem. 49, 511-520 (1974)].

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'-bis-phosphate 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 heterogenous 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-amino-hexan-1-ol phosphate, reacted with 1,1'-carbonyldiimidazole and the resulting imidazolide of hexitolamine phosphate reacted with an appropriate nucleotide to generate the corresponding asymmetric pyrophosphate.   For example, with AMP this is P¹-(6-aminohexyl)-P²-(5'-adenosyl)-pyrophosphate (ref. 23).

THE SYNTHESIS OF NUCLEOTIDE ANALOGUES FOR AFFINITY CHROMATOGRAPHY

A number of methods are available for the derivatisation and subsequent immobilisat-
ion 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
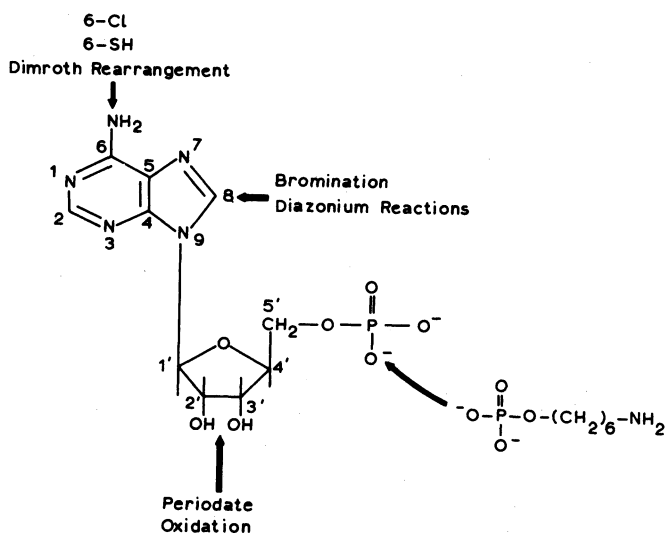and phosphate-linked nucleotides.

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

Base-linked Nucleotides

The base-linked nucleotides substituted at the exocyclic N^6 amino group are most
effectively synthesised by nucleophilic displacement of a 6-chloro (Ref.9), 6-mer-
capto (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^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^6 position of the base may also
be effected by prior alkylation at position N^1 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^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^6-
carboxymethyl substituent and subsequent coupling of 1,6-diaminohexane to the term-
inal carboxyl group with a carbodiimide promoted reaction has been adapted to the
synthesis of the N^6- (6-aminohexyl)-carbamoylmethyl-derivatives of AMP, ADP, ATP,
NAD^+ and $NADP^+$. Fig. 2 illustrates a typical synthesis of an $NADP^+$ analogue for
affinity chromatography by this relatively facile approach (Ref. 17). An alternat-
ive approach involves direct carbamoylation of the exocyclic N^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, acetalation of the nucleotide with
ethyl levulinate leads to levulinic acid $(0^2, {}^3$-nucleoside) acetal which is readily
converted to the corresponding nucleotide by phosphorylation with phosphoryl chlor-
ide.   Saponification followed by condensation with 1,6-diaminohexane yields a rib-
ose-substituted analogue suitable for attachment to a solid support (Ref. 23). How-
ever, it should be pointed out that ribosyl-linked nucleotides are relatively in-
effectual in the pruification of the NAD^+-linked dehydrogenases.   Nevertheless,
ribosyl-linked nucleotides are effective in the purification of deaminases and equi-
valently linked $NADP^+$ adsorbents have proved invaluable in the purification of sev-
eral $NADP^+$-dependent dehydrogenases.

Characterisation of Nucleotide Analogues
In most cases the spacer arm-conjugated coenzyme and nucleotide analogues were pur-
ified 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 ^{13}C-NMR spectroscopic propert-
ies.   Furthermore, treatment of the NAD^+ and $NADP^+$ analogues with sodium dithionite,
enzymes or 1M KCN generated the chemically and enzymically reduced forms (λ max 340nm)
and cyanide adducts (λ max 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 spectro-
scopy or phosphate analysis (Ref. 17).   Fig. 3 illustrates the structures of sever-
al adsorbents comprising base, phosphate and ribosyl-linked adenine nucleotides.
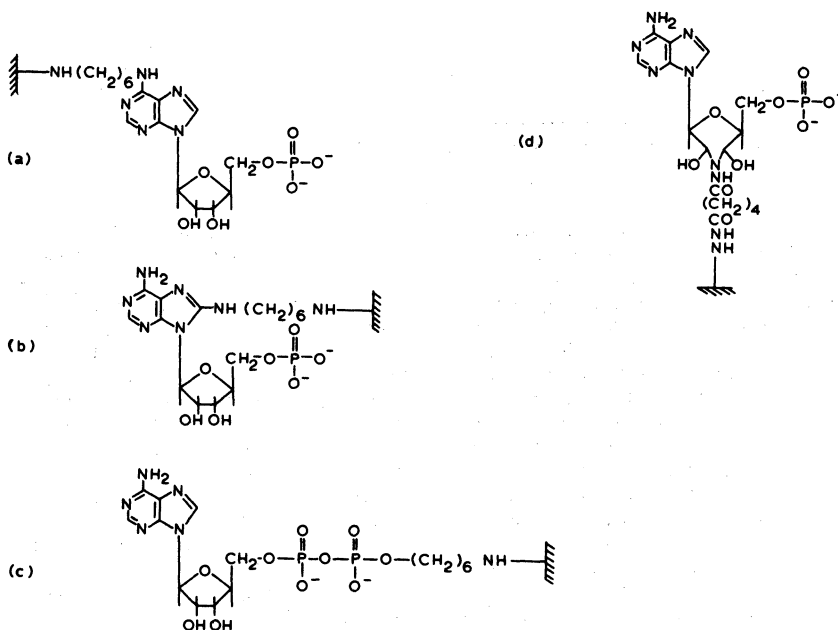

Fig. 3.   The structures of several immobilised nucleotide and coenzyme
analogues: (a) N^6-(6-aminohexyl)-AMP;   (b) 8-(6-aminohexyl)-AMP;   (c)
P^1-(6-aminohexyl)-P^2-(5'-adenosyl)-pyrophosphate;   (d) ribosyl-linked AMP.

THE   EFFICIENCY   OF   DIFFERENTLY   IMMOBILISED   NUCLEOTIDE   ADSORBENTS

Preassembled NAD^+ and $NADP^+$ analogues and the complementary nucleotide "half mole-
cules" 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 dehy-
drogenases. However, differently immobilised nucleotide adsorbents exhibit marked-
ly different effectivities in the purification of complementary enzymes. For exam-
ple, Table 1 summarises the binding of several AMP-dependent enzymes to agarose-
bound N^6-(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 2.5μmol 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^6-substituted AMP adsorbent. In marked contrast, however, the

TABLE 1.   The binding of several NAD^+-dependent dehydrogenases to N^6-(6-
aminohexyl)-AMP-agarose and 8-(6-aminohexyl)-AMP-agarose.

Enzyme: Dehydrogenase	Source	Eluant conc. (μM NADH)		K_i (mM)	
		C8	N^6	C8	N^6
Alanine	B.subtilis	80	8	0.08	2.0
Alcohol	Horse liver	30	75	1.5	0.4
Galactose	Ps. fluorescens	0*	0*	11.0	2.5
IMP	E. coli	4.6	3·8	-	-
Lactate	Rabbit muscle	30	190	2.0	0.1
Lactate	Pig heart	10	30	2.4	0.1
Lipoamide	Pig heart	0	0	-	-
Malate	B. subtilis	10	0	-	-
Malate	Pig heart	0	35	4.4	0.2

*Eluant was a linear gradient of AMP (0 - 10 mM).

two B. subtilis enzymes, alanine dehydrogenase and malate dehydrogenase, and the E.
coli enzyme, IMP dehydrogenase, display a preference for the 8-substituted AMP-ana-
logue.   At the ligand concentration used in this study, neither Pseudomonas fluor -
escens 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 vel-
ocity measurements.   In most cases the K_i values for N^6-(6-aminohexyl)-AMP were up
to 20-times lower than those for 8-(6-aminohexyl)-AMP measured under identical con-
ditions.   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^6-substit-
uted 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 con-
centration of 2.5μmol/g moist weight gel.   This conclusion is supported from evi-
dence 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 effect-
ive 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-link-
ed adsorbents.   In fact, in the absence of prior knowledge of how a particular
dehydrogenase binds to these adsorbents, the preference in binding to an immobilis-
ed-AMP adsorbent might be expected to lie in the order : N^6 > phosphate ⩾ C^8 ⩾ ribose.
These data are entirely consistent with the results of X-ray crystallographic ana-
lysis 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

deep pocket with the adenine moiety outermost (Ref. 25). In particular, it appears that the exocyclic N^6-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 positons 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 $2\mu mol/g$ 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

TABLE 2. The binding of several dehydrogenases to agarose-bound N^6-(6-aminohexyl)-AMP and N^6-(6-aminohexyl)-carbamoylmethyl -NAD^+.

Enzyme: Dehydrogenase	Source:	Eluant concn. $(\mu M \text{ NADH})^\alpha$	
		N^6-AMP	N^6-NAD^+
Alanine	B. subtilis	40	0
Alcohol	Yeast	85	0
Alcohol	Horse liver	60	30
Galactose	Ps. fluorescens	0	40
Glucose-6-phosphate	Yeast	0	0
Lactate	Pig heart	80	70
Lactate	Rabbit muscle	300	100
Lipoamide	Pig heart	0	0
Malate	Pig heart	0	0

α 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).

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 ($NH_3^+(CH_2)_6NHCOCH_2NH-N^6-NAD^+$) than the AMP-analogue with its hydrophobic hexamethylene chain ($NH_3^+(CH_2)_6NH-N^6$-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
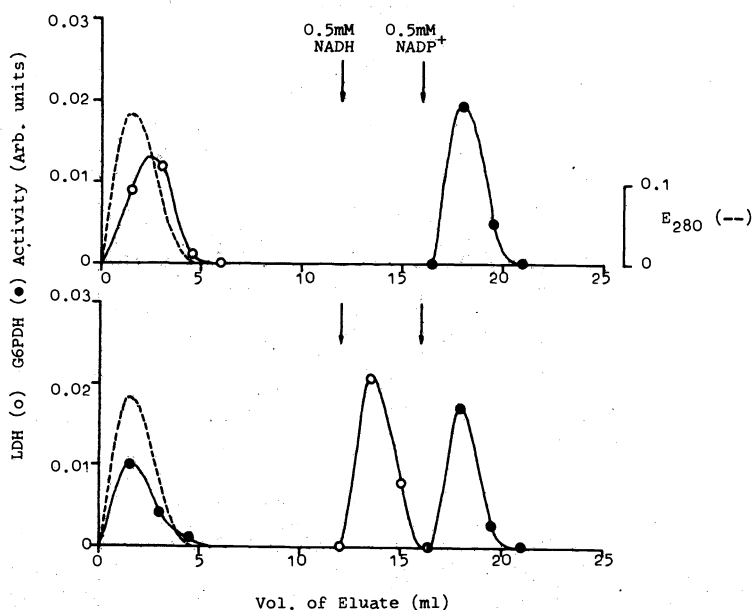
Fig. 4.   Resolution of an enzyme mixture (a) prior to and (b) after
           treatment of an immobilised-NADP$^+$ adsorbent with alkaline phosphatase.

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 ad-
sorbed 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 re-
moval 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
serum albumin in the void volume.
        The binding specificity of immobilised nucleotide adsorbents is also demon-
strated by the relative effectivities of immobilised AMP and IMP.   Immobilised IMP
was synthesised from 8-(8-aminooctyl)-AMP by protection of the terminal amino group
of the spacer molecule with S-ethyltrifluoroacetyl thiol acetate ($CF_3COSC_2H_5$) followed
by diazotisation at 0°C for 3h in the presence of nitrous acid (Ref. 27).   The ter-
minal amino group of the 8-substituted IMP derivative was finally deprotected by in-
cubation at pH 12 for 3h.   The purified analogue, 8-(8-aminooctyl)-IMP and the par-
ent analogue, 8-(8-aminooctyl)-AMP were subsequently coupled to CNBr-activated aga-
rose 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 grad-
ient 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 obser-
vations 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
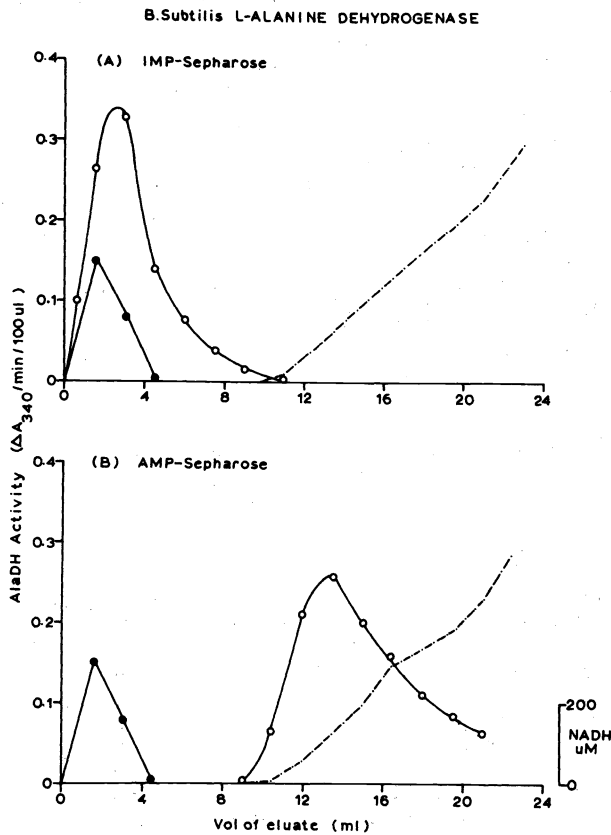ligand structure.   However, it has become evident in recent years that non-specific

Fig. 5.   Chromatography of a crude extract of B. subtilis on (A) 8-(8-amino-
octyl)-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 mM; 20 ml total volume) in the phosphate
buffer.   Alanine dehydrogenase activity (-0-), 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-(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^1-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 β-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 2.5μmol 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 K_i values for the hydrophobic and hydrophilic analogues. For example, in free solution [AMP]-8-NH(CH$_2$)$_6$NH$_3^+$ and [AMP]-8-NHCH$_2$CHOHCH$_2$NHCOCH$_2$NH$_3^+$ were strictly competitive with NADH for rabbit muscle lactate dehydrogenase with K_i 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.

NH₂ structure 1: —NH–CH₂–CH₂–CH₂–CH₂–CH₂–CH₂–NH————

NH₂ structure 2: —NH–CH₂–CH₂–CH₂–NH–CO–CH₂–NH————

NH₂ structure 3: —NH–CH₂–CH₂–NH–CO–CH₂–CH₂–NH————

NH₂ structure 4: —NH–CH₂–CH(OH)–CH₂–NH–CO–CH₂–NH————

(Each with adenine ring, ribose with OH OH, and CH₂–O–P(=O)(O⁻)–O⁻ phosphate group)
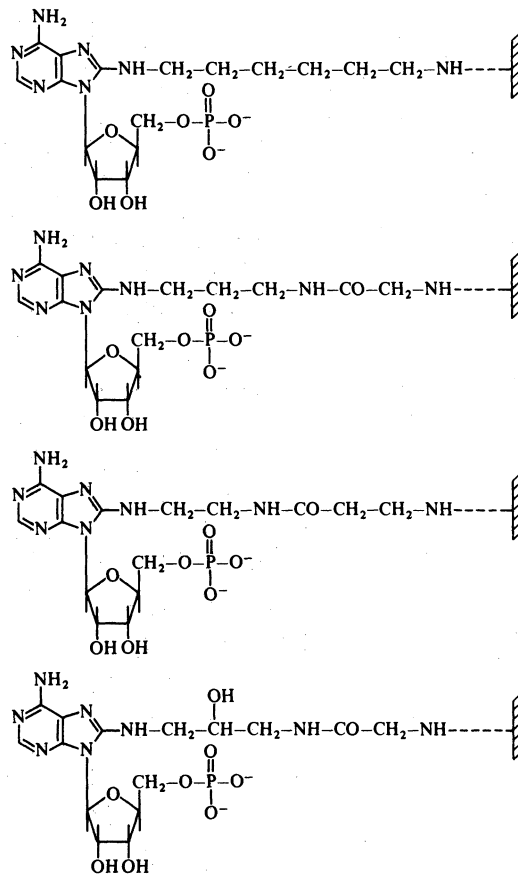
Fig. 6. The structures of four 8-substituted AMP-agarose adsorbents with spacer molecules of differing composition [Reproduced with permission from C.R. Lowe, Eur. J. Biochem. 73, 265-274 (1977)].

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]-(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
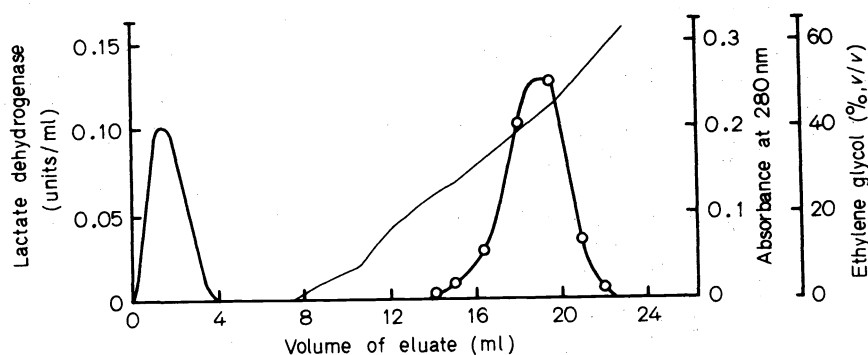
Fig. 7.   Elution of pig heart lactate dehydrogenase from N^6-(6-aminohexyl)-AMP-agarose by a linear gradient of ethylene glycol (0 - 70%; 20ml total volume). [Reproduced with permission from C.R. Lowe and K. Mosbach, Eur. J. Biochem. 52, 99-105 (1975)].

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 homogenaus 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

Enzyme purification from crude extracts
Preparative scale enzyme isolation
Isoenzyme resolution
Contaminant removal
Resolution of active/non-active or chemically modified species
Separation of mutant and wild type enzymes
Enzyme mechanistic studies
Binding site topographies
Determination of dissociation and equilibrium constants
Coenzymically active immobilised coenzyme preparations
Co-immobilised enzyme/coenzyme systems

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^6-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.

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