SOME APPLICATIONS OF INSOLUBILISED COFACTORS TO THE PURIFICATION OF PYRIDINE NUCLEOTIDE-DEPENDENT DEHYDROGENASES.

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A study of the binding of several commercially available dehydrogenases to insolubilised NAD, NADP and AMP has revealed some interesting differences. The relative binding data provided can be used as a guide to the separation or purification of dehydrogenases from complex mixtures. The application of immobilised cofactors to the study of active sites is suggested.

The considerable potential of affinity chromatography for the purification of enzymes is now well established (1,2).Whilst many workers have concentrated on adsorbents specific for only one macromolecule (3), work in our laboratories (4 - 6) and those of others (7,8) has been directed at the development and analysis of insolubilised cofactors as affinity adsorbents for the purification of groups of enzymes. Good resolution of complex mixtures of enzymes, which are otherwise difficult to separate by conventional procedures, have been achieved.

This paper reports an analysis of four polymers containing covalently attached nucleotides. The data presented can be used as a guideline for the purification and resolution of complex mixtures of pyridine nucleotide dependent dehydrogenases. The versatility of this approach is demonstrated by the separation and purification of alcohol dehydrogenase and D-glucose 6-phosphate dehydrogenase from a crude yeast extract.

Affinity chromatography on immobilised cofactors and cofactor analogues can also provide a useful approach to mechanistic and binding studies on a wide range of enzymes. The binding of several pyridine nucleotide-dependent dehydrogenases to these matrices is interpreted tentatively in terms of the nature of the interaction with their respective cofactors.

MATERIALS AND METHODS: NAD and NADP were supplied by Whatman Biochemicals Ltd. All enzymes and 5'-AMP were purchased from Boehringer, Mannheim. N^6-Aminohexyl-AMP-sepharose was prepared by the method of Guilford et al (9) and the insolubilized derivatives of 5'-AMP, NAD and NADP prepared by coupling the appropriate nucleotide to ε-Amino-caproyl-sepharose by the carbodiimide reaction described by Larsson & Mosbach (10). Enzymatic and chromatographic analyses were performed as described in a previous publication (5). The yeast extract was prepared by the method of Darrow and Colowick (11).

RESULTS AND DISCUSSION: The binding of several pyridine nucleotide-dependent dehydrogenases to NAD or NADP covalently attached to sepharose by a hexacarbon spacer molecule is shown in Table 1. In general, NAD-dependent enzymes bind strongly to the polymer containing covalently bound NAD and weakly, if at all, to the corresponding NADP polymer. Rabbit muscle glyceraldehyde 3-phosphate dehydrogenase binds so tightly to ε-amino-caproyl-NAD-sepharose that it resists elution by gradients of KCl up to 1M. The enzyme is readily recovered by elution with a pulse of 5mM NADH. NADP-dependent enzymes, on the other hand, either do not discriminate between the two polymers or bind preferentially to the NADP-polymer.

Table I. The binding of NAD(P)-linked dehydrogenases to ε-Amino-Caproyl-NAD(P)-sepharose.

Dehydrogenase	Source	Binding* (mM KCl)	
		NAD	NADP
L-Malate	Pig heart	45	0
L-Lactate	Pig heart	325	0
"	Rabbit muscle	500	110
Alcohol	Horse liver	130	0
"	Yeast	415	0
D-Glyceraldehyde 3-Phosphate	Rabbit muscle	1M‡	60
β-hydroxy-butyrate	Rhodopseudomonas spheroides	250	165
L-Glutamate	Bovine liver	‡	‡
D-Glucose 6-Phosphate	Yeast	125	125
threo-D_S-Isocitrate	Pig heart	220	230
Glutathione Reductase	Yeast	120	400
L-Xylulose Reductase	Yeast	150	515

*'Binding' refers to a measure of the strength of the enzyme - immobilised cofactor interaction and is the concentration of KCl required to elute the enzyme.

‡ Elution could be effected by a 200µl pulse of 5mM NADH

‡ No enzyme was recovered from these columns.

These differences in binding between NAD and NADP-specific dehydrogenases are also apparent in their behaviour towards the two types of AMP polymer shown in Table II.
In general, NAD-specific enzymes interact strongly with N^6-Aminohexyl-AMP-sepharose, whereas NADP-dependent enzymes exhibit little affinity for this polymer. The NADP-specific

Table II. The binding of NAD(P)-linked dehydrogenases to two types of AMP polymer.

Dehydrogenase	Source	Binding (mM KCl)* N^6-aminohexyl AMP	5'-AMP
L-Malate	Pig heart	90	10
L-Lactate	Pig heart	1M‡	290
"	Rabbit muscle	1M‡	270
Alcohol	Horse liver	450	40
"	Yeast	405	525
D-Glyceraldehyde 3-Phosphate	Rabbit muscle	315	150
β-Hydroxybutyrate	Rhodopseudomonas sphaeroides	250	255
L-Glutamate	Bovine liver	130	‡
D-Glucose 6-phosphate	Yeast	0	50
threo-D_S-Isocitrate	Pig heart	0	370
Glutatione Reductase	Yeast	0 & 115§	160

*‡ As in Table I.

§ Major peak (70%) eluted in void volume; minor peak (30%) eluted at 115 mM KCl.

enzymes do, however, bind to immobilised 5'-AMP.

The most important application of the data presented in these tables is as a guide to the solution of specific problems in dehydrogenase separation and purification. Thus, separations can be effected by consulting the relative tightness of binding listed in the tables and then using the appropriate insolubilized nucleotide. This approach is illustrated by the resolution of alcohol dehydrogenase and D-glucose 6-phosphate dehydrogenase by chromatography of a

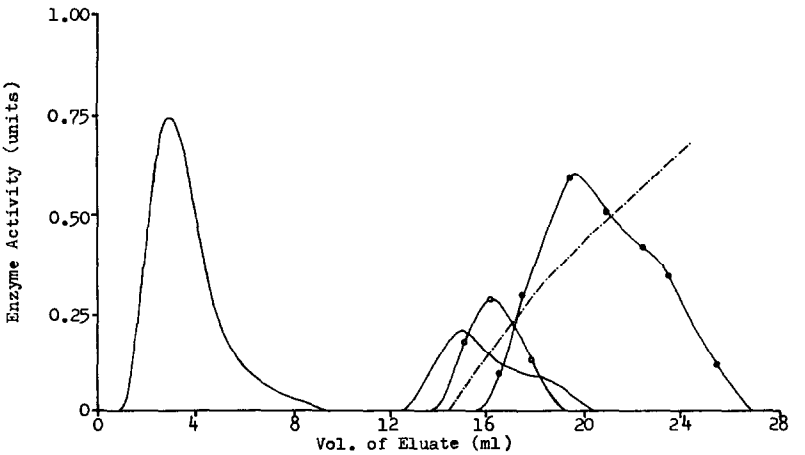

Fig.1: Affinity chromatography of a dialysed crude yeast extract on ε-amino-caproyl-NAD-sepharose prepared by the method of Larsson & Mosbach (1971). A sample (100µl) of a dialysed yeast extract was applied to a 5mm x 20 mm column of ε-aminocaproyl-NAD-sepharose equilibrated with 10mM phosphate buffer, pH 7.5. Non-absorbed protein was washed off with 10 ml of the same buffer and the enzymes eluted with a KCl gradient (0 - 1.0M; 20 ml total) in 10 mM phosphate buffer, pH 7.5. D-Glucose 6-phosphate dehydrogenase (o), alcohol dehydrogenase (•) and protein (-) were assayed in the effluent.

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crude yeast extract on ε-Amino-caproyl-NAD-sepharose (Fig.1) and N^6-Aminohexyl-AMP-sepharose (Fig.2). The one-step purification of alcohol dehydrogenase can readily be achieved by chromatography of the crude yeast extract on N^6-Aminohexyl-AMP-sepharose, but this polymer is of little value for the isolation of glucose 6-phosphate dehydrogenase from the same source. Chromatography of the yeast extract on ε-amino-caproyl-NADP-sepharose effects the significant purification of this enzyme (Lowe & Dean, unpublished observations) with a complete resolution from alcohol dehydrogenase that is not possible with the NAD polymer. Thus, the broad specificity of insolubilised cofactors enables one to separate complex mixtures of the same group of enzymes in a readily predictable manner.

There is an excellent correlation between the predicted and

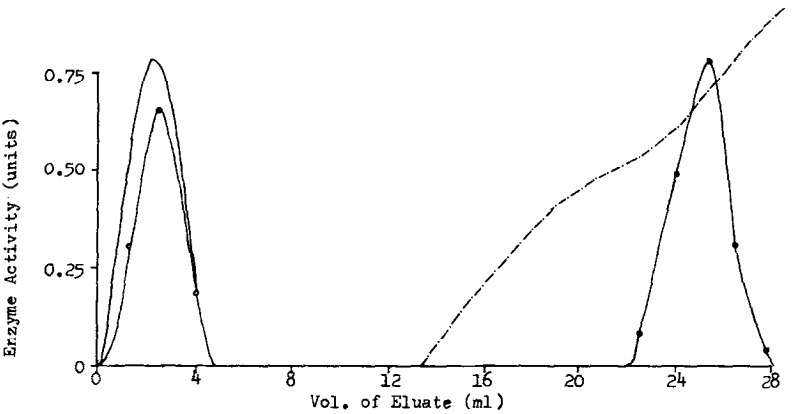

Fig.2: Affinity chromatography of a crude yeast extract on N^6 Amino-hexyl-AMP-sepharose. All other details are as given in the legend to Fig.1.

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actual strength of binding of these two enzymes, although the binding of yeast alcohol dehydrogenase to the N^6-Aminohexyl-AMP-sepharose is a little stronger than expected. This agreement is eminently satisfactory in view of the complex nature of the interactions between proteins from a crude mixture under chromatographic conditions. It should be noted however, that the results in Tables I and II were obtained with purified dehydrogenases from several sources and extension of these data to enzymes from other sources may not be generally applicable.

A more tentative comment on the applicability of insolubilized nucleotides relates to their potential as active site probes. Since these types of immobilised bio-molecules interact specifically with a wide range of enzymes, some information on the mode of binding of the nucleotide to NAD and NADP-specific enzymes might be obtained. The recent model for the coenzyme binding site of lactate dehydrogenase (12) would indicate that the binding of the adenine part of NAD is relatively unimportant

for this, and perhaps other, NAD-specific dehydrogenases. On the other hand, the difference in binding between NAD and NADP dependent enzymes suggests that the adenine moiety might play a critical role in the interaction of the cofactor with NADP-linked dehydrogenases. The interference of the extension arm with this important area of the cofactor involved in the interaction with the enzyme may explain why NADP enzymes do not bind to N^6-Aminohexyl-AMP-sepharose. This approach is the subject of further investigation.

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