Biochimica et Biophysica Acta, 397 (1975) 277—287

BBA 67562

AFFINITY CHROMATOGRAPHY OF ENZYME COFACTORS: THE SEPARATION OF NAD ON IMMOBILISED DEHYDROGENASE COLUMNS

K. DAS, P. DUNNILL and M.D. LILLY

Biochemical Engineering Section, Department of Chemical Engineering, University College London, Torrington Place, London WC1 (U.K.)

(Received February 17th, 1975)

Summary

1. Alcohol dehydrogenase (EC 1.1.1.1) has been immobilised to aminoethyl-cellulose by glutaraldehyde, to DEAE-cellulose by an *s*-triazine derivative and to agarose using CNBr. Lactate dehydrogenase has been immobilised to the latter two supports.

2. Their use for affinity chromatography of NAD was compared and alcohol dehydrogenase immobilised to CNBr-activated agarose chosen for detailed study due to the efficient coupling of applied enzyme and the specific nature of binding.

3. The efficiency of coupling of alcohol dehydrogenase dropped from 94.5 to 72.2% when the applied load was increased from 18 to 54 mg/g activated agarose. Activity relative to free enzyme fell from 21 to 11%. The binding of NAD was maximal between pH 5.5 and 6. With the lowest loading of enzyme, NAD binding fell from 450 to 320 μg/g support when the linear flow rate was increased from 0.84 to 3.95 cm/min.

4. NAD was completely separated from a mixture with ATP, ADP and AMP. Separation from NMN and hydrolysed RNA and DNA was evidently possible. Immobilised alcohol dehydrogenase used for 34 binding experiments over a period of weeks maintained 60% of its original enzyme activity.

5. The method was applied to yeast NAD following mechanical disruption of yeast, clarification and either ultrafiltration or hollow-fibre dialysis to permit separate purification of macromolecules and nucleotides.

Introduction

Recently there have been many reports of the selective retention of enzymes on columns to which inhibitors, cofactors or other enzyme effectors

have been bound [1—4]. There are few reports as yet of the reverse procedure. Fritz et al. [5] used bound proteases to isolate inhibitors of the enzymes from biological fluids. Similarly bound biocytin has been applied to the isolation of avidin [6]. Recently Matuo et al. [7] have used immobilised CoA to bind proteins having an affinity for CoA. These proteins were then covalently linked to Sepharose 6B and used as an affinity column for the purification of CoA. ATP and ADP were also adsorbed to the column.

Umezawa [8] has pointed out that in the search for new chemotherapeutic agents molecular species which interact in one way or another with enzymes are likely to figure prominently. Affinity chromatography using immobilised enzymes can provide a means of detection and of purification of such substances.

The cofactor NAD has been immobilised to insoluble supports for use as an affinity medium to separate dehydrogenases [9—11] and to a soluble high molecular weight support [12] to act as an immobilised cofactor in an enzyme reactor. It was therefore of interest to examine the reverse situation using information on immobilised dehydrogenases [13] as a basis for the separation of molecules interacting with enzymes. As with other uses of affinity chromatography [14], the main concern for practical utilisation is with column capacity, resolving power, ease of product elution, throughput and column stability. The present paper considers these factors in four ways.

First, the properties of several immobilised forms of two dehydrogenase enzymes are compared with a view to their use as affinity chromatography supports. Secondly, the factors influencing binding of pure NAD to the selected form of immobilised dehydrogenase is examined. Thirdly, the degree of resolution of pure NAD from related pure compounds on such a column is studied. Finally, the recovery of NAD from clarified yeast homogenate following mechanical disruption, debris removal and ultrafiltration or dialysis is examined. We have previously discussed the possibility of simultaneously recovering small molecules and macromolecules from cell extracts [15]. The inclusion of the ultrafiltration or dialysis step was intended to permit this without destruction of either stream and to allow affinity chromatography of small molecules free from the column fouling associated with viscous protein and nucleic acid extracts.

Materials

Alcohol dehydrogenase (EC 1.1.1.1, yeast) and lactate dehydrogenase (EC 1.1.1.27, pig heart) were supplied by Boehringer Corp., London. The nucleotides NAD, AMP, ADP, NMN, and the nucleic acids RNA and DNA were obtained from Sigma Chemical Co., London, and ATP was from Kyowa Hakko Kogyo Co., Tokyo, Japan. Thin-layer chromatography plates, silica gel F-254 or cellulose F, were from E. Merck, Darmstadt, Germany. CNBr-activated Sepharose 4B was from Pharmacia Fine Chemicals, Uppsala, Sweden. AE-cellulose from Micro-Bio Laboratories Ltd, DEAE-cellulose (DE-52) from Reeve Angel Ltd, London, glutaraldehyde from Koch-Light Laboratories Ltd, Bucks, England, and cyanuric chloride from B.D.H. Ltd, Poole, Dorset. Analar grade reagents and buffers were used as far as possible.

Methods

Preparation of AE-cellulose-alcohol dehydrogenase

Immobilisation of alcohol dehydrogenase to glutaraldehyde-treated AE-cellulose was based on the method of Glassmeyer and Ogle [16]. In a typical experiment, 14 g (dry wt) AE-cellulose in 210 ml of 0.5 M NaOH were washed with water to remove all NaOH. The AE-cellulose was suspended in 200 ml of 0.1 M phosphate buffer, pH 7.0, to which 56 ml of 12% glutaraldehyde in water were added. The mixture was then covered and kept at room temperature for 2 h with stirring, after which it was centrifuged at 10 000 × *g* and the AE-cellulose derivative washed six times with the same buffer to dilute out the remaining glutaraldehyde. Glutaraldehyde-treated AE-cellulose was re-suspended in 200 ml of the same buffer. To 20 ml (1.4 g AE-cellulose) of this suspension, 20 mg of alcohol dehydrogenase were added for coupling and the mixture was kept overnight (16 h) at 4°C with stirring.

Preparation of DEAE-cellulose-linked lactate and alcohol dehydrogenases

The method for preparation of 2-amino-4,6-dichloro-*s*-triazine and its derivative of DEAE-cellulose was according to Kay and Lilly [17] and Wykes et al. [18]. 2 ml of enzyme suspension in $(NH_4)_2SO_4$ (10 mg/ml) were centrifuged for 10 min at 30 000 × *g* and 5°C. Supernatant was decanted and the residue was redissolved in 2 ml of 0.025 M borate buffer, pH 8.8. This was added to 1 g (dry wt) of *s*-triazine-activated DEAE-cellulose in 15 ml of the same buffer and incubated for 18 h at room temperature with stirring. The pH was then adjusted to 6.5. The mixture was poured into a column and washed with 100 ml of 0.05 M phosphate buffer containing 1 M NaCl, pH 6.5, and again with 100 ml of 0.1 M phosphate buffer, pH 6.5, containing 0.01 M 2-mercaptoethanol. The coupled enzyme was stored at 4°C in the same buffer with 2-mercaptoethanol.

Preparation of Sepharose 4B-linked lactate and alcohol dehydrogenase

CNBr-activated Sepharose 4B was treated with 10^{-3} M HCl to remove added dextran and lactose. The enzyme coupling procedure was the same as for DEAE-cellulose using 0.025 M borate buffer, pH 8.8. Unbound enzyme was removed by washing with the same buffer and any remaining active groups were reacted with 1 M ethanolamine at pH 8.8 for 1—2 h. Three washings (each 10 ml) with 0.1 M phosphate buffer, pH 6.5, containing 1 M NaCl, were used to remove non-covalently bound protein and this was followed by washing with 0.1 M phosphate buffer, pH 6.5, containing 2-mercaptoethanol.

Assay of enzymes and nucleotides

The activity of alcohol dehydrogenase was measured by following absorbance changes at 340 nm. The assay mixtures incubated at 30°C contained 2.8 ml of 0.1 M Tris, 0.2 M ethanol and 0.05 M semicarbazide · HCl, pH 6.5. and 0.01—0.05 ml of 10^{-1} M NAD in 0.01 M HCl, made up with water to 3 ml to which 1.5 μg of yeast alcohol dehydrogenase were added to start the reaction. The procedure was the same for lactate dehydrogenase except that the reaction mixture contained 2.8 ml of 10^{-2} M pyruvate in 0.1 M phosphate

buffer, pH 6.5, and 0.03—0.15 ml of 10^{-2} M NADH as a substrate, adjusted to 3 ml with water, to which 1.5 μg of lactate dehydrogenase were added. Using alcohol dehydrogenase and the same buffer containing ethanol as a substrate, NAD was determined by quantitative reduction to NADH, which was spectrophotometrically determined at 340 nm. For assay of immobilised alcohol dehydrogenase the reaction mixture was the same. 0.05—0.5 ml of 10^{-1} M NAD was added to 27.5 ml of this solution, adjusted with water to 29.5 ml and then incubated at 30°C. At zero time, 3 ml were withdrawn and 0.5 ml of alcohol dehydrogenase support suspension was added. At intervals of 20, 40, 60, 90, 120 and 150 s, 3-ml samples were removed through a sintered glass filter and cooled in an ice bath. The absorbance at 340 nm was noted. For coupled lactate dehydrogenase 10^{-2} M pyruvate was used instead of Tris buffer and 10^{-2} M NADH in place of 10^{-1} M NAD. Other procedures were as for alcohol dehydrogenase.

Protein attached to the solid support was estimated by difference using the Folin-Lowry method [19].

AMP, ADP, ATP, NMN, RNA and DNA were separated by thin-layer chromatography on cellulose F- and silica gel F-254-coated plates. The solvent was isobutyric acid/conc. ammonia solution (spec. gravity 0.38)/water (66 : 1 : 33, by vol). Each spot was assayed by measurement of its fluorescence with a Vitratron thin-layer densitometer (Fisons Scientific Co. Ltd, Loughborough). The amount of each nucleotide was calculated by comparison with standard samples.

Experimental procedures

All affinity columns were run with downward flow at 20°C. Bakers' yeast was disrupted in a Manton Gaulin homogeniser as previously described [20] and clarified in either a multichamber centrifuge (Model KDD 605, Westfalia Separator Ltd, Wolverton, Bucks) or a laboratory centrifuge (Model High Speed 18 000, MSE Ltd, Crawley, Sussex). The extracting solution was 0.15 M NaCl and 4 mM K_2HPO_4, pH 5.6. Ultrafiltration was done in a thin channel system (Model TC1, Amicon Corp., Lexington, Mass., U.S.A.). Amicon membranes were used in all ultrafiltration studies. Dialysis was done in a hollow-fibre dialyser with membrane type HDX1 (Amicon Corp.).

Results

(A) Studies with pure NAD

Choice of solid support and immobilisation method. Alcohol dehydrogenase and lactate dehydrogenase were immobilised by different procedures to several supports (Tables I and II). The data indicate a high enzyme uptake in all cases other than the coupling of alcohol dehydrogenase to glutaraldehyde-linked AE-cellulose. The retention of enzyme activity was highest for lactate dehydrogenase but for both enzymes retention compared favourably with previous studies [21]. The initial load of NAD applied to the columns of 1 g of absorbent was 5 mg in 5 ml of 0.1 M phosphate, pH 6.0, and the flow rate through the columns (6.5 cm × 1.1 cm diameter) was 3.5 ml/min. Unbound NAD was eluted by the same buffer.

TABLE I

PREPARATION OF IMMOBILISED ALCOHOL DEHYDROGENASE AND ITS CAPACITY FOR BINDING NAD

18 mg of alcohol dehydrogenase presented to 1 g of support.

Support	Enzyme coupled (%)	Activity relative to free enzyme (%)	NAD bound (μg)
Glutaraldehyde-linked AE-cellulose	55.5	18	2500
s-Triazinyl DEAE-cellulose	92.0	17	1270
CNBr-activated Sepharose 4B	94.5	21	381

Effect of applied enzyme load on the amount and activity of bound enzyme. The effect of increasing the applied load of alcohol dehydrogenase on the amount of enzyme attached and its activity was examined for CNBr-activated Sepharose 4B. The method of immobilisation was the same as described earlier. The lyophilised enzyme was dissolved in 0.025 M borate buffer, pH 8.8. Table III indicates the fall in efficiency of attachment at higher applied enzyme load and the reduced specific activity of the bound enzyme.

Effects of pH and flow rate on column capacity. The binding of NAD to alcohol dehydrogenase and lactate dehydrogenase attached to CNBr-activated Sepharose 4B was examined with equilibrating buffers of different pH values ranging from 5 to 7.5. The effect of pH on NAD binding capacity is shown in Fig. 1. The optimum pH for stability of both dehydrogenases lies between 6 and 8.5.

The effect of varying the flow rate of the solution containing NAD through the column of immobilised enzyme is shown in Table IV. The column and conditions are as before with 5 mg of NAD in 5 ml of buffer added. For this and subsequent experiments the preparation containing 17 mg of alcohol dehydrogenase per g of support was used.

(B) The separation of NAD from pure nucleotides

Separation of NAD. AMP, ADP and ATP were co-chromatographed with NAD (5 mg of each in 5 ml total volume) on the above column (6.5 cm × 1.1 cm diameter) of alcohol dehydrogenase immobilised to Sepharose 4B at a flow rate of 3.5 ml/min. The equilibration and subsequent washing buffer was 0.01 M phosphate buffer, pH 6.0. The first eluting buffer was 0.01 M phos-

TABLE II

PREPARATION OF IMMOBILISED LACTATE DEHYDROGENASE AND ITS CAPACITY FOR BINDING NAD

20 mg of lactate dehydrogenase presented to 1 g of support.

Support	Enzyme coupled (%)	Activity relative to free enzyme (%)	NAD bound (μg)
s-Triazinyl DEAE-cellulose	80	47	980
CNBr-activated Sepharose 4B	90	43	470

TABLE III

EFFECT OF THE WEIGHT OF ALCOHOL DEHYDROGENASE PRESENTED FOR COUPLING ON THE EFFICIENCY OF COUPLING AND IMMOBILISED ENZYME ACTIVITY

Enzyme added* (mg)	Enzyme bound (mg)	Enzyme coupled (%)	Activity relative to free enzyme (%)
18	17	94.5	21
27	23	85.1	16
54	39	72.2	11

* To 1 g of CNBr-activated Sepharose 4B.

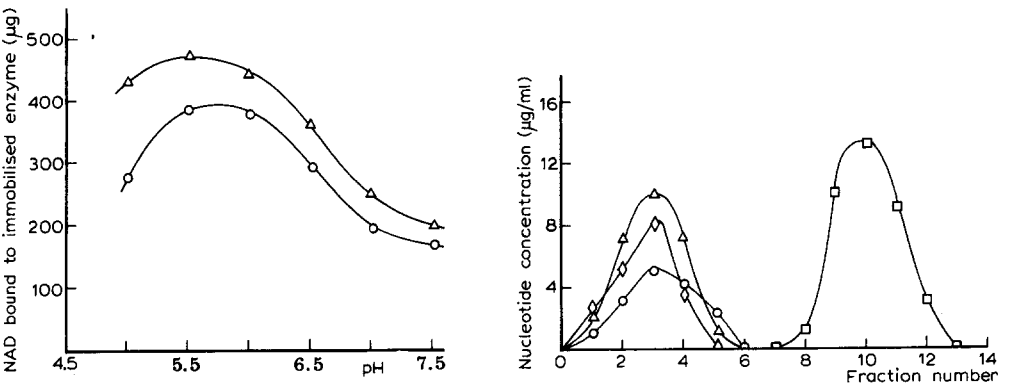

Fig. 1. The effect of pH on binding capacity of immobilised dehydrogenase for NAD. 5 ml of 0.1 M phosphate buffer containing 1 mg/ml of NAD was passed through a column (6.5 cm × 1.1 cm diameter) of immobilised alcohol dehydrogenase (○) or immobilised lactate dehydrogenase (△) at 3.5 ml/min. The column was then washed with more buffer to elute unbound NAD.

Fig. 2. The separation of NAD (□) from AMP (○), ADP (◇) and ATP (△) on a column (6.5 cm × 1.1 cm diameter) of alcohol dehydrogenase immobilised to Sepharose 4B, when eluted with 0.01 M phosphate buffer, pH 7.0 (fractions 1—7), and the same buffer containing 0.15 M sodium salicylate (fractions 8 onwards) at a flow rate of 3.5 ml/min.

TABLE IV

EFFECT OF FLOW RATE ON THE BINDING OF NAD TO IMMOBILISED ALCOHOL DEHYDROGENASE

Flow rates (ml/min)	NAD bound (μg)
0.8	450
1.5	437
2.5	400
3.5	381
3.75	320

TABLE V

THE ENZYME ACTIVITY OF IMMOBILISED ALCOHOL DEHYDROGENASE AFTER REPEATED USE FOR AFFINITY CHROMATOGRAPHY OF NUCLEOTIDES

Number of re-uses	Activity relative to free enzyme (%)	Retention of original activity (%)
10	19.8	94.3
15	19.1	91.0
25	15.8	75.2
34	12.5	60.0

phate, pH 7.0, for fractions 1—7 (each 5 ml) followed by 0.15 M sodium salicylate [22] in 0.01 M phosphate, pH 7.0, for fractions 8 onwards (Fig. 2). The concentrations of nucleotides in the column effluent were determined by fluorescence measurements after separation by thin-layer chromatography.

In other experiments NMN and hydrolysed RNA and DNA were separated from NAD in a similar way.

Column stability. When not in use, the affinity column of alcohol dehydrogenase immobilised to Sepharose 4B was stored at 4°C with buffer containing 2-mercaptoethanol. Between repeated re-use measurements of enzyme activity were made to determine operational stability. Each cycle of use involved loading, washing and eluting NAD from the column. In most cases a clear solution of pure NAD or clear solutions of NAD and nucleotides were used. Enzyme activities of the column after repeated use are shown in Table V.

(C) Purification of NAD from yeast extract

Ultrafiltration separation of small molecules from macromolecules. To examine the purification of NAD from small molecules in a separate process stream to that containing macromolecules, 1-l samples of clarified yeast extract were ultrafiltered in a thin channel system. In a series of experiments extracts of protein concentration, 40 mg/ml, and NAD concentration, 270 μg/ml, were ultrafiltered with membranes of three porosities of nominal cut-off 10 000 (PM-10), 100 000 (XM-100A) and 300 000 (XM-300). The original solution was then diluted 2-fold and 4-fold and the experiments repeated. The results are presented in Table VI. In each instance 0.75 l of ultrafiltrate was collected and the assay results represent the mean values for the whole ultrafiltrate sample. The re-cycle rate of retentate through the ultrafilter at 20 lb/inch^2 was 1.5—1.6 l/min. The variation of NAD in the ultrafiltrate with time was examined in further detail in the 4-fold diluted sample with the XM-300 membrane since this represented the system with greatest NAD yield. The pattern of variation is shown in Fig. 3. Two opposing factors are evident. The NAD level initially rose rapidly which may be a reflection of the increasing NAD concentration in the retentate. The later fall-off in throughput is associated with protein concentration polarisation which curtails ultrafiltration of smaller molecules. This effect is also reflected in the lower rate of ultrafiltration in more concentrated solutions.

Dialysis separation of small molecules from macromolecules. Dialysis does not suffer the acute concentration polarisation effects associated with ultrafil-

TABLE VI

EFFECT OF MEMBRANE TYPE AND PROTEIN CONCENTRATION ON THE RECOVERY OF NAD FROM CLARIFIED YEAST HOMOGENATE BY ULTRAFILTRATION

Membrane type	Initial dilution factor	Ultrafiltrate				Time to collect 0.75 l filtrate (min)
		Protein		NAD		
		mg/ml	%*	μg/ml	%*	
PM-10	1 X	0.1	0.2	15	4	130
	2 X	0.2	0.75	29	16	120
	4 X	0.3	2.2	31	35	107
XM-100A	1 X	0.4	0.75	22	6	125
	2 X	0.7	2.6	48	27	115
	4 X	0.6	4.4	42	45	92
XM-300	1 X	1.1	2.0	36	10	114
	2 X	1.5	5.6	99	55	100
	4 X	1.1	8.2	72	80	70

* Expressed as percent of concentration in clarified homogenate (after dilution).

tration and the availability of hollow-fibre dialysis modules offers the hope of low-cost large-scale units of high efficiency. A module of 1 ft^2 dialysis area was employed to dialyse a sample of 200 ml of clarified yeast extract of the same initial protein and NAD concentrations as employed in ultrafiltration experiments against 150 ml of 0.1 M phosphate, pH 6.0. The extract was pumped through the hollow fibres and the buffer around them by peristaltic pumps. The results are shown in Table VII. A yield of 59% of the initial NAD was obtained with a very low level of protein contamination.

Affinity chromatography for purification of NAD from ultra-filtered extract. Ultrafiltrate from the experiment employing an XM-300 membrane and a 4-fold diluted sample of extract was applied to the column of alcohol dehydrogenase immobilised to Sepharose 4B (1.1 cm diameter × 6.5 cm containing 17 mg enzyme) and used for previous experiments. When 25 ml of ultrafiltrate containing 1800 μg NAD were applied, 875 μg appeared in the effluent, 350 μg

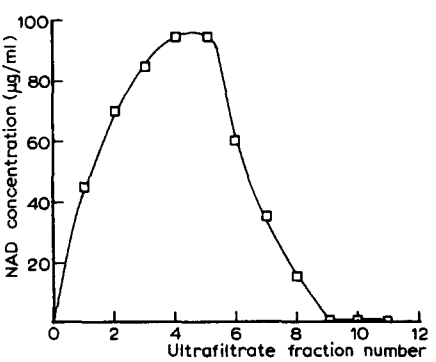

Fig. 3. The variation of NAD level in ultrafiltrate with time for a 4-fold diluted sample of clarified yeast homogenate and an XM-300 membrane in a TC1 ultrafilter. The volume of each fraction was 50 ml.

TABLE VII

RECOVERY OF NAD FROM CLARIFIED YEAST HOMOGENATE BY HOLLOW-FIBRE DIALYSIS

	Volume		Protein (mg/ml)	NAD (μg/ml)
	Initial	Final		
Sample	200	275	40	270
Dialysate	150	75	0.11	425
Yield (%)			0.001	59

could be eluted by 0.01 M phosphate, pH 7, and 600 μg were only eluted by 0.15 M sodium salicylate (3 × 5 ml).

Discussion

The results expressed in Table I indicate that, while alcohol dehydrogenase applied to CNBr-activated Sepharose 4B resulted in the highest efficiency of enzyme coupling, the enzyme derivatives of AE-cellulose-glutaraldehyde and DEAE-cellulose-triazine bound larger amounts of NAD per gram dry weight of derivative. However, on the basis of their molecular weights and assuming four active sites per molecular weight of 148 000 for alcohol dehydrogenase [23], the expected binding of NAD per 17 mg of enzyme is 305 μg. It is evident that the higher capacity of the cellulose derivatives must be associated with secondary binding and, since such non-specific binding is likely to occur with other intracellular material, these supports are less suitable for affinity chromatography.

The reduction in efficiency of enzyme binding and of specific activity relative to free enzyme on increasing the amount of alcohol dehydrogenase presented for coupling is in agreement with other studies of enzyme immobilisation in porous supports [21,24]. Generally an increased utilisation of deeper buried sites within the matrix with less favourable access of enzyme results in less effective coupling and diminished substrate access results in reduced effective activity.

The reduction in binding capacity for NAD with increased flow rate of the NAD loading step (Table IV) is an expected property of any chromatographic operation reflecting the need for a finite time for effective binding. Above a flow rate of 3.5 ml/min, i.e. 3.7 cm/min linear flow rate, there is a more rapid fall-off in binding, possibly due to flow channeling in the packed bed.

The data of Fig. 2 indicate effective separation of NAD from similar substances on immobilised dehydrogenase columns. However, the capacity for NAD is reduced by the presence of these substances. Whereas 17 mg alcohol dehydrogenase on 1 g Sepharose 4B in a column of 6.5 × 1.1 cm diameter bound 381 μg of NAD from 5 mg added in 5 ml at a flow rate of 3.5 ml/min, the capacity under identical conditions with AMP and NMN added in the same concentration was 170 μg. With the modified procedure in the presence of ATP, ADP and AMP the capacity was 180 μg. The 50% reduction in capacity may be accounted for by competitive binding which is consistent with kinetic

studies of dogfish muscle lactate dehydrogenase in free solution [25,26]. After elution the salicylate may be removed from the NAD by precipitation at low pH, ultrafiltration or molecular sieving.

The preservation of 60% of the activity of the column after 34 re-uses over a period of weeks suggests that aside from the additional problems of column fouling occurring with cell extracts the column life should be good. Though pig heart lactate dehydrogenase retains a higher proportion of the applied enzyme activity, yeast alcohol dehydrogenase is considerably cheaper as a commercial product.

The second major practical objective of this work was to study the simultaneous isolation of small molecules and macromolecules using NAD as the model for the former. Separation of the small and macromolecule streams was quite successful by ultrafiltration. A maximum recovery of 80% was attainable though with the disadvantage of contamination by 1.1 mg/ml protein with potential column fouling as a consequence at the subsequent affinity chromatography stage. Dialysis with a hollow-fibre unit gave a yield of 59% with 0.11 mg/ml protein contamination which may best be compared with the yield of 4% and 0.1 mg/ml protein by ultrafiltration using a membrane of comparable cut-off.

Affinity chromatography on the immobilised alcohol dehydrogenase column previously used for model studies was effective in binding NAD from the yeast cell extract. The binding of 600 μg of NAD from ultrafiltrate as against 381 μg for pure NAD and lower levels with other pure nucleotides present may be accounted for either by the larger volume of feed, 25 ml rather than 5 ml, permitting longer equilibration, or by lower levels of competing nucleotides.

The study shows that immobilised enzymes can represent a suitable affinity medium for the recovery and purification of cofactors and the approach may be extended to specific inhibitors and other effectors of adequate binding power. It is not suggested that the procedure provides a method currently competitive with classical production of NAD by methods which destroy other cell components. However, it does provide an indication of how simultaneous isolation of small molecules and macromolecules might be accomplished by procedures which are mild and not destructive of other cell components. Such an approach, particularly using affinity methods, may become important in fuller utilisation of biological materials [15].

Acknowledgements

We are grateful to the Delhi Committee for a fellowship awarded to one of us (K.D.) and indebted to Dr J.R. Wykes for comments on aspects of immobilisation. K.D. was a visiting scientist from the Department of Chemical Engineering, Indian Institute of Technology, Delhi, India.

References

1 Friedberg, F. (1971) Chromatogr. Rev. 14, 121—131
2 Cuatrecasas, P. and Anfinsen, C.B. (1971) Annu. Rev. Biochem. 40, 259—278
3 Reiner, R.H. and Walch, A. (1971) Chromatographia 4, 578—587

4 Turkova, J. (1974) J. Chromatogr. 91, 267—291
5 Fritz, H., Gebhardt, M., Meister, R. and Schult, H. (1970) Hoppe-Seyler's Z. Physiol. Chem. 351, 1119—1122
6 Cuatrecasas, P. and Wilchek, M. (1968) Biochem. Biophys. Res. Commun. 33, 235—246
7 Matuo, Y., Tosa, T. and Chibata, I. (1974) Biochim. Biophys. Acta 338, 520—528
8 Umezawa, H. (1972) Proc. IV IFS: Fermentation Technology Today (Terui, G., ed.), pp. 19—29, Soc. Ferment. Technol., Japan
9 Lowe, C.R. and Dean, P.D.G. (1971) FEBS Lett. 14, 313—316
10 Mosbach, K., Guilford, H., Ohlsson, R. and Scott, M. (1972) Biochem. J. 127, 625—631
11 Weibel, M.K., Doyle, E.R., Humphrey, A.E. and Bright, H.J. (1972) Biotechnol. Bioeng. Symp. 3, 167—171
12 Wykes, J.R., Dunnill, P. and Lilly, M.D. (1972) Biochim. Biophys. Acta 286, 260—268
13 Wykes, J.R., Dunnill, P. and Lilly, M.D. (1975) Biotechnol. Bioeng. 17, 51—68
14 Robinson, P.J., Dunnill, P. and Lilly, M.D. (1972) Biochim. Biophys. Acta 285, 28—35
15 Dunnill, P. (1972) Proc. IV IFS: Fermentation Technology Today (Terui, G., ed.), pp. 187—194, Soc. Ferment. Technol., Japan
16 Glassmeyer, C.K. and Ogle, J.D. (1971) Biochemistry 10, 786—792
17 Kay, G. and Lilly, M.D. (1970) Biochim. Biophys. Acta 198, 276—285
18 Wykes, J.R., Dunnill, P. and Lilly, M.D. (1971) Biochim. Biophys. Acta 250, 522—529
19 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
20 Hetherington, P.J., Follows, M., Dunnill, P. and Lilly, M.D. (1971) Trans. Inst. Chem. Eng. 49, 142—148
21 Melrose, G.J.H. (1971) Rev. Pure Appl. Chem. 21, 83—119
22 Hines, W.J.W. and Smith, M.J.H. (1964) Nature 201, 192—192
23 Boyer, P.D., Lardy, H. and Myrbäck, K. (1963) The Enzymes, 2nd edn, Vol. 7, Academic Press, New York
24 Lilly, M.D., O'Neill, S.P. and Dunnill, P. (1973) Biochimie 55, 985—990
25 McPherson, A. (1970) J. Mol. Biol. 51, 39—46
26 Adams, M.J., McPherson, A., Rossmann, M.G., Shevitz, R.W., Smiley, I.E. and Wonocott, A.J. (1970) Pyridine Nucleotide-Dependent Dehydrogenases (Sund, H., ed.), Springer, Berlin