USE OF IMMOBILIZED ALCOHOL DEHYDROGENASE FOR THE REDUCTION OF NAD TO NADH

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The possibility was examined of preparative NAD to NADH reduction in the presence of an excess of ethanol by alcohol dehydrogenase immobilized on a copolymer of glycidylmethacrylate with ethylenebismethacrylate. The effect of pH, ethanol concentration and of compounds reacting with acetaldehyde on the conversion and on the purity of NADH formed is described. Under optimal conditions of the reduction the NADH is preferentially bound to a DEAE ion exchanger. The best results as regards conversion degree and purity of product were obtained by cyclic flow of the reaction mixture, containing NAD and ethanol in glycine buffer (pH 8·5) of low ionic strength, through a column of immobilized enzyme and a column of DEAE-cellulose. NADH picked up by the ion exchanger can be eluted by concentrated ammonium carbonate buffer and the final product obtained by lyophilization of the effluent. The purity of NADH formed as determined spectrophotometrically, by HPLC, and measurement of the coenzyme activity using lactate dehydrogenase, is comparable to the purity of the commercial product.

Reduced nicotinamide-adeninedinucleotide (NADH) is an important biochemical especially employed in clinical biochemistry, enzymology, bioenergetics, and in other fields of biochemistry. It has been utilized as coenzyme of NAD-dependent dehydrogenases, such as lactate dehydrogenase, glutamate dehydrogenase, malate dehydrogenase and in reactions coupled to these enzymes (*e.g.*, in assays of aminotransferase activity).

The reduced coenzyme, NADH, can be prepared from the more readily available form (NAD) by enzymatic reduction. The chief advantage of this method of reduction over other methods, such as chemical or electrochemical reduction, is its specificity: it results in the desirable enzymatically active 1,4-dihydro-NADH whereas a mixture of 1,4- and 1,6-dihydro-NADH is obtained by other methods[1] in the best case.

This study reports on the use of immobilized horse liver alcohol dehydrogenase (EC 1.1.1.1, ADH) for the reduction of NAD to NADH. The enzyme was chosen mainly because of its relative availability and possibility of easily removing the excess

of the substrate and of the second reaction product (alcohol and aldehyde) by, *e.g.* lyophilization. The chief advantage of using the immobilized enzyme represents the higher purity of NADH obtained, the higher stability of the enzyme, and the possibility of its repeated use.

EXPERIMENTAL

Alcohol dehydrogenase (ADH) was isolated from horse liver[2], lactate dehydrogenase was purchased from Boehringer (Mannheim, FRG). The copolymer of glycidylmethacrylate and ethylenebismethacrylate G-60 (particle size 150–250 μm, surface area 60·2 m^2/g) was prepared in the Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences, Prague. It was modified by ammonia and hydrazine; the reactive group content after the modification was 1·4–1·8 mequiv./g dry weight, ref.[3]). DEAE-cellulose (DE-32) was from Whatman (U.K.), DEAE-Sephadex A-50 from Pharmacia, (Uppsala, Sweden), DEAE-Spheron from Lachema (Czechoslovakia). NAD was purchased from Imuna (Czechoslovakia), NADH, AMP and ADP from Reanal (Hungary), glutaraldehyde from Serva (FRG); the remaining chemicals were purchased from Lachema (Czechoslovakia).

ADH was coupled to the ammonia-modified support G-60 by glutaraldehyde[3]. The activity of the immobilized enzyme was measured according to[3] (30 mmol/l ethanol and 3 mmol/l NAD in 0·1 mol/l glycine buffer, pH 10, at 20°C). The incubation time was 8 min and the absorbance at 340 nm was measured after the polymer had been removed. The activity of the preparations used was similar to that described in ref.[3] (3·0–3·5 U/g wet gel).

The purity of NADH formed was determined spectrophotometrically from the A_{340nm} to A_{268nm} ratio ($\varepsilon_{340} = 6{\cdot}2\ cm^2/\mu mol$, $\varepsilon_{260} = 14{\cdot}1\ cm^2/\mu mol$, ref.[4]). The initial concentration of NAD was determined spectrophotometrically ($\varepsilon_{260} = 18{\cdot}0\ cm^2/\mu mol$, ref.[4]). The HPLC analyses were carried out in an apparatus consisting of Model VCM-300 pump (Instrument Development Workshops, Czechoslovak Academy of Sciences), a stainless steel column (150 × 4 mm) packed with Silasorb C_{18} (Lachema, Czechoslovakia), and a model LCD 254 UV-detector (Laboratorní přístroje, Czechoslovakia). The mobile phase was prepared from 0·1 mol/l Na-phosphate buffer, pH 6, containing 4% (v/v) of methanol as recommended in ref.[5]. The separation was allowed to proceed at a pressure of 4·8 MPa at a flow rate of 1·1 ml/min. The sample (5 μl) was applied through a silicon septum.

The coenzyme activity of NADH was tested with a reaction mixture containing 0·6 mmol/l pyruvate in 0·05 mol/l Na-phosphate buffer, pH 7·5, to which the NADH tested was added, so that its concentration was 0·18 mmol/l. The reaction was triggered by the addition of lactate dehydrogenase and was allowed to proceed at 25°C (ref.[6]). The enzymatic activity was determined from a decrease of absorbance at 340 nm. The spectrophotometric measurements were made in Cary 118 (USA) and Beckman DU (USA) spectrophotometers. Lyophilization was carried out in the apparatus manufactured by Virtis (USA).

RESULTS AND DISCUSSION

Effect of Various Factors on the NAD to NADH Conversion

The equilibrium of the reaction between an alcohol (ethanol) and NAD catalyzed by ADH does not favor under physiological conditions the formation of NADH

(ref.[7]). Principally, the degree of conversion can be increased in several manners. The first one is an increase in pH: the NAD→NADH conversion at a pH higher than 10 at an about 10-fold higher concentration of ethanol is more than 60% (ref.[7]), the relatively low stability of NAD and ADH in alkaline media[1,7] represents, however, a problem. The second possibility is an increase of the concentration of substrate (ethanol); concentrations exceeding 100 mmol/l cannot be used, however, since substrate inhibition takes place[7]. Another possibility leading to an increase of the conversion degree could represent the removal of the reduction product (acetaldehyde), *e.g.*, by binding to hydrazine or semicarbazide[8]. Still another possibility is the bonding of the reaction product (NADH) to an ion exchanger. It has been known[9] that NADH has a higher affinity for weak ion exchangers than NAD.

We have examined all the possibilities described above; the results of the first three methods of increasing the conversion degree are given in Table I. The increasing of the pH-value has a positive effect on the conversion which corresponds to the effect postulated on the basis of the knowledge of the equilibrium constant (K) characterizing NADH formation from NAD with the participation of ethanol as substrate ($K = 8 . 10^{-12}$ mol/l, ref.[7]). As shown by HPLC of the product, however, an increased quantity of degradation products of NAD is formed during the reaction. In spite of the relatively low conversion pH 8·5 was chosen for the additional experiments. We found that the conversion is maximal at ethanol concentrations

TABLE I

Effect of reaction conditions on NAD to NADH conversion catalyzed by immobilized alcohol dehydrogenase. The experiments were carried out with 3 ml of reaction mixture containing 2 mmol/l NAD in 0·1 mol/l buffer (Na-phosphate buffer was used for pH 7·5 and 8·0, NaOH--glycine buffer for the remaining pH-values) and 200 mg of wet slurry of the G-60 copolymer with immobilized ADH. The reaction was allowed to proceed 50 min at room temperature with gentle shaking; the absorbance at 340 nm was measured after the polymer had been separated. The data are means obtained in three parallel experiments

pH	7·5	8·0	8·5	8·5	8·5	8·5	9·0	10·0
Ethanol mmol/l	100	100	20	50	100	200	100	100
Conversion %	5	9	12	14	15 14[a] 18[b]	14	24	53

[a] The reaction was allowed to proceed in the presence of 50 mmol/l of semicarbazide in the reaction mixture; [b] the reaction was allowed to proceed in the presence of 200 mg (wet weight) of the G-60 copolymer modified by hydrazine.

of 50–100 mmol/l (Table I), as expected, and decreases at higher concentrations. Similar results were also obtained in experiments where the initial NAD concentration was higher almost by one order. The concentration of ethanol chosen for the subsequent experiments was 100 mmol/l. We have also made an effort to remove the acetaldehyde produced. As shown in Table I neither the presence of semicarbazide nor the presence of a matrix with hydrazine groups attached contributed any significantly to an increase of the conversion.

Considerably more successful was the binding of the product required (NADH) to ion exchangers. We were able to show in orienting experiments that the use of DEAE ion exchangers (on the basis of cellulose, agarose or Spheron) is most suited if an increase of the conversion is required. We determined in model experiments with a mixture of NAD and NADH the optimal conditions (*i.e.* the pH and the ionic strength of the buffer used) of *a*) the binding of NADH (to obtain minimal binding of NAD) and *b*) the elution of NADH (to obtain a quantitative displacement).

The "working" stage *a*) during which NADH, formed by the enzymatic reaction, is strongly bound by the column packed with the DEAE ion exchanger while NAD is bound only a little, can be performed virtually in any weakly alkaline buffer (pH 8·5–8·0) of low ionic strength ($I < 0{\cdot}1$). We have tested glycine, borate and ammonium carbonate buffer. A higher pH is more suitable for the separation of NADH yet we worked at pH 8·5 because of the lability of NAD and ADH in alkaline media. NADH is quantitatively bound to the DEAE ion exchange column (1 ml of 4 mmol/l

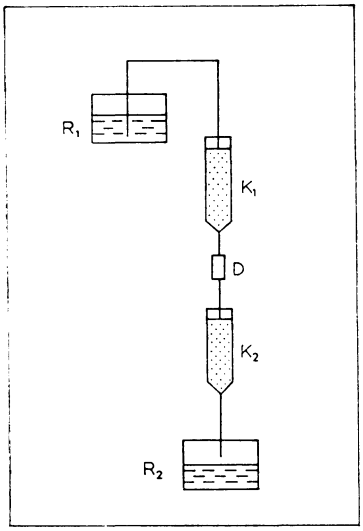

FIG. 1

Experimental arrangement of NADH production in "two-column" system. R_1, R_2 — reservoirs with reaction mixture (the initial composition is given in Table II), K_1 column with immobilized ADH, K_2 column with DEAE-ion exchanger (the packing of these columns is specified in Table II). A through-flow photometer cell D was placed between the two columns and the absorbance at 340 nm was recorded. The subsequent working cycle started with the replacement of the reservoirs

NADH solution per 3 ml of wet resin) at this pH-value; however, about 20% out of the 4 mmol/l concentration of NAD in the NADH–NAD mixture applied is also picked up. NAD can be displaced by an excess of NADH since the latter is preferentially bound.

A buffer of high ionic strength ($I > 0{\cdot}3$) at pH 7·0–8·5 is suitable for stage *b*) during which NADH is displaced from the column. We used either phosphate or ammonium carbonate buffer. The former was used mainly because it is most commonly used in lactate dehydrogenase assays in clinical practice, the latter buffer because its components can easily be removed from NADH by lyophilization of the ion exchange column effluent. The elution of NADH is practically quantitative under these conditions.

The efficiency of the ion exchange resins tested was alike except for DEAE-Spheron; with this resin we observed (even at pH 8·5) a relatively extensive formation of degradation products derived from the coenzyme. These products were eluted only together with NADH from the ion exchanger. The formation of these undesired admixtures was detectable at a first glance because the DEAE column acquired a weak yellowish tinge. Likewise, HPLC of the effluent of this material showed the presence of minor fractions.

Design of Enzyme Reactor

We have tested three types of reactors which contained an anion exchanger with DEAE groups (cellulose, Sephadex, Spheron) in addition to the matrix with the immobilized enzyme. The arrangement was the following: *a*) separate polymers, the DEAE ion exchange resin only was in the column. *b*) separate materials, both polymers are in columns, *c*) mixed materials in one column.

We observed that all three procedures are usable in principle. The testing experiments of reactor types *a*) and *b*) were carried out in 5–8times repeated "working" cycles always consisting of enzymatic generation of NADH (20 min incubation with mild shaking with immobilized ADH in case *a*) or a slow flow of the reaction mixture through a column packed with this material in case *b*)) and of binding of the NADH formed to the column packed with the DEAE resin. The quantity of NADH was always determined by measurement of its absorbance at 340 nm before its binding to the column. The arrangement used in case *a*) was the direct measurement of the absorbance of the reaction mixture after the separation of the immobilized enzyme; in case *b*) the effluent from the first column was passed through the spectrophotometer cell before it was applied to the second column (Fig. 1). In case *c*) both phases merged to one and the effluent was checked for the presence of any significant quantities of NADH by through-flow measurement. The working cycles were terminated by the "elution" phase in all cases. The residual NAD was eluted first from the

DEAE-column by 0·02 mol/l NaOH-glycine buffer at pH 8·5 and water and subsequently by 0·5 mol/l ammonium carbonate buffer at pH 7·0–8·5; the application of the latter resulted in quantitative displacement of NADH.

The worst results as regards the conversion and the purity of NADH were obtained with procedure *a*) whereas procedure *b*) was the best one. The third procedure (variant *c*)) using one column only seems the most suitable from the practical point of view; likewise, the total length of the working cycles (*c.* 1·5 h) is shorter than with procedure *b*) (*c* 2–2·5 h) and procedure *a*) (*c.* 4 h). Variant *c*), however, is essentially disadvantageous compared to *b*) in several respects. The main disadvantage is, in addition to the lower conversion degree and slightly lower purity, the lower stability of the enzyme: the column packed with the mixture of immobilized ADH and the DEAE ion exchanger cannot be used more than 10 times (it is practically inactive after ten experiments already). The decrease of the stability of the immobilized enzyme is most likely due to the fact that immobilized ADH is slightly denatured in media of high ionic strength (during washing of the column with concentrated ammonium carbonate buffer). The preparation of the material for repeated use is also more complicated: the column has to be washed after the elution of NADH for a relatively long time by the "working" buffer (*e.g.*, with 0·02–0·05 mmol/l NaOH-glycine buffer, pH 8·5) in the cold until the effluent from the column has the proper pH and conductivity value. The column cannot be regenerated by the efficient procedure

TABLE II

Production of NADH from NAD in the "two-column" system. The first column contained 1·5 g (wet weight) of the G-60 copolymer with *c.* 2 mg of immobilized active ADH, the second column contained 4 g (wet weight) of DEAE-cellulose; both columns were washed with NaOH-glycine buffer, pH 8·5, before use. The reaction mixture (8 ml) contained 28 μmol (*c.* 20 mg) of NAD and 1 mmol of ethanol in 0·02 mol/l NaOH-glycine buffer, pH 8·5. The rate of flow through the columns was 0·5–0·7 ml/min, the reaction was allowed to proceed at room temperature; the experimental arrangement can be seen in Fig. 1. The values given are the best values obtained n four experiments which gave analogo us results

Working cycle	1	2	3	4	5	6	7	8
NADH formed in individual cycles μmol	4·4	4·1	3·9	2·9	1·6	1·0	0·3	0·1
Total quantity of NADH formed μmol	18·3 (*i.e.* 65% of NAD used)							
Eluted quantity of NADH μmol	16·8 (*i.e.* 60% of NAD used)							

described in ref.[10], *i.e.* washing with 0·5 mol/l HCl and 0·5 mol/l NaOH with the subsequent equilibration in the "working" buffer because of the presence of the immobilized enzyme. If the two-column arrangement (variant *b*)) is chosen the used column can be coupled to a new ion exchange column when the process is repeated. Another advantage of the two-column procedure represents the possibility of determination of the activity of ADH immobilized on the first column (this determination is carried out as described under "Methods"). This simple method cannot be used for the testing of the viability of the mixed reactor (variant *c*)).

We studied in more detail the second procedure (variant *b*)) where separate polymers in two columns were used. The results of a typical experiment are presented for illustrative purposes in Table II. This procedure of NAD reduction yielding a relatively large quantity of NADH of relatively high purity (96%) according to the spectrophotometric measurement described under "Methods") can be used repeatedly. The enzyme column is thoroughly washed after use with phosphate buffer of ionic strength 0·05–0·1 and pH 7–8 and is stored in this medium at 4°C until applied again. The DEAE ion exchanger can be regenerated by recycling[10]. When the procedure was applied ten times during two weeks a negligible decrease only of the reduction efficiency was observed (the conversion degree decreased by about 10%).

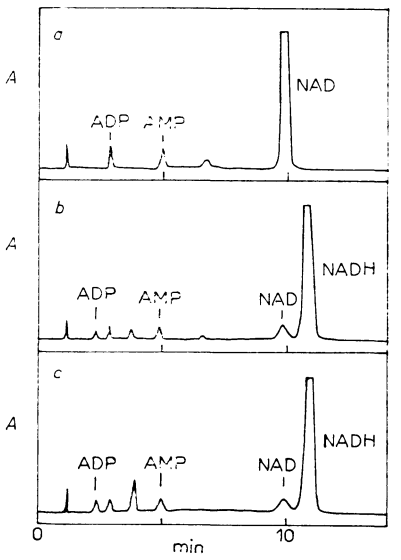

FIG. 2

HPLC analysis of NAD (Imuna, ČSSR) *a*, NADH produced *b*, and of commercial NADH from Reanal *c*. The experimental conditions of the separation are described under "Methods"; the samples of the corresponding preparations, whose concentrations had been determined spectrophotometrically (*cf.* "Methods") were applied as 1 mmol/l solutions (5 µl). The identification of AMP and ADP was carried out by standard additions of these compounds; additional minor fractions were not identified

It is probable that the procedure described, which has been tested on a microscale, would be suitable for the preparation of larger quantities of NADH from NAD. The product obtained, *i.e.* the effluent from the DEAE ion exchange column containing practically NADH only (in a concentration of 1–2 mmol/l), ethanol, and acetaldehyde in ammonium carbonate buffer, can be lyophilized easily. Almost pure reduced coenzyme NADH is obtained by this approach; the preparation obtained contains negligible amounts only of other products in addition to traces of contaminants of nucleotide character.

Assay of Purity of NADH *Produced*

We were able to show by the spectrophotometric method, which expresses the purity of NADH as the ratio of absorbance of the fraction at 340 nm to "total nucleotide concentration" absorbing at 280 nm (see "Methods") that the purity of the preparation obtained especially by variant *b*), *i.e.* using separate columns, is relatively high (96%). Two other methods were used for a more exact assay of the purity of the preparation obtained, namely HPLC, and an enzymatic assay based on lactate dehydrogenase. The chromatograms of the starting material (NAD, from Imuna), of the lyophilized product obtained by variant *b*), and of NADH (from Reanal) are shown in Fig. 2. These results provide additional evidence in favor of the fact that the purity of the material obtained is sufficient and can be compared to the purity of commercial products. The occurrence of impurities, *i.e.* of fragments of coenzyme AMP and ADP, of residual NAD, and of unidentified minor fractions absorbing at 254 nm is negligible. The coenzyme activity is the most important criterion from the viewpoint of the possible practical application of NADH as coenzyme of dehydrogenases. We have compared the obtained lyophilized preparation of NADH with the commercial product of Reanal as regards its coenzyme activity in the presence of lactate dehydrogenase. We have carried out 5 measurements with both materials. The relative rate of the enzymatic reaction obtained with the commercial product was 100 ± 4% and relative values of 98 ± 4% were obtained with our preparation. These results show that NADH produced as described above is suitable for enzymologic applications and that its usability is comparable to that of a commercial preparation of Reanal.

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