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PURIFICATION AND CHARACTERIZATION OF COMMERCIAL NADH AND ACCOMPANYING DEHYDROGENASE INHIBITORS

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SUMMARY

The anion-exchange chromatography of commercial NADH using a potassium bicarbonate solution as eluent yields highly pure NADH with good stability. Twelve compounds are also separated which act as dehydrogenase inhibitors. The main impurities are further characterized. The compound mainly responsible for residual optical density in commercial NADH preparations is probably a stereoisomer of NADH which is in reversible equilibrium with NADH at pH values in the range 5–7. A method of thin-layer chromatography, to check commercial NADH preparations for impurities, is described.

INTRODUCTION

Accurate measurements of the activities of enzymes requiring NADH as coenzyme depend largely on the purity of the NADH. The formation of inhibitory compounds in NADH preparations was originally reported by Lowry *et al.*[6] and Dalziel[1] and subsequently confirmed by many other authors. A review on this subject by Gerhardt *et al.*[2] has recently appeared. Difficulties are encountered in purifying the coenzyme and assuring its stability. The unfavourable influence of humidity, freezing or acidification on its stability is well established. In previous papers we reported a new chromatographic procedure with potassium bicarbonate solution as an easily removable eluent[3,4]. The present paper deals with the application of this method to the purification of commercial NADH which results in a highly purified product with excellent stability. More than ten compounds are removed during the purification procedure, all of which inhibit several NADH-dependent dehydrogenases. Possible structures for the main impurities are discussed. Conditions leading to their formation from NADH were investigated.

MATERIAL AND METHODS

NADH was purchased from Arzneimittelwerk Dresden (Dresden, G.D.R.) and Boehringer (Mannheim, G.F.R.). DEAE-Sephadex A-25, particle size 40–120 μm (Pharmacia, Uppsala, Sweden) was prepared according to the manufactor's instruc-

tions and transformed into the bicarbonate form by equilibration with 1 *M* potassium bicarbonate. Dowex 50W-X8, 200–400 mesh, was obtained from Serva (Heidelberg, G.F.R.). Enzymes used: alcohol dehydrogenase (EC 1.1.1.1) (Arzneimittelwerk Dresden), lactic acid dehydrogenase (EC 1.1.1.27) from rabbit muscle (Boehringer).

Separation on DEAE-Sephadex columns

The columns (5 × 100 cm) were packed with DEAE-Sephadex A-25 and equilibrated with 50 m*M* potassium bicarbonate. After samples of about 5 g commercial NADH dissolved in 50–100 ml of 50 m*M* potassium bicarbonate had been drained into the gel, the column was washed with about 2 l of 50 m*M* potassium bicarbonate. For elution, a gradient from 50–800 m*M* potassium bicarbonate was applied using 150 cm water pressure (for profile, see Fig. 2). Separation was carried out at 4°. The eluate was fractionated (10 ml each) and the peaks detected by continuous measurement of light transmission at 254.7 nm (Uvicord, LKB). The extinction at 260 nm and 340 nm was also measured in each fraction.

Thin-layer chromatography

Glass plates (20 × 40 cm) were coated with a 1-mm-thick layer of DEAE-Sephadex A-25 (HCO_3^-). 5-μl samples containing 0.5–1.0 μmoles of the nucleotide were applied directly on the gel surface. For 20-μmole samples, the NADH solution was applied in a line on the gel surface. The eluent was carried to the gel by paper (FN 18; Filtrak, Niederschlag, G.D.R.) ensuring continuous contact between paper and gel and avoiding any overlapping of the paper on the gel. Development was carried out at 4° with 200 m*M* potassium bicarbonate, the plates being inclined at an angle of 15°. A water-saturated chamber is not necessary. The spots were detected by ultraviolet (UV) absorption or fluorescence. For quantitative determination the corresponding areas of gel were removed and eluted by 2 × 2.5 ml of 400 m*M* potassium bicarbonate.

Removal of potassium bicarbonate from the eluate

The potassium bicarbonate was removed from the eluate with Dowex 50W-X8, 200–400 mesh (H^+). The resin was stirred into the eluate in small portions at 4° and the pH was carefully controlled. The procedure was performed under vacuum (10 torr) to allow carbon dioxide to escape. The set-up is shown in Fig. 1. With eluates containing NADH, the pH must be carefully controlled to avoid a value below 6.5, where rapid degradation of NADH takes place.

The resin was added to the eluate until a final pH in the range 6.8–6.5 was attained. The resin was then removed by filtration and the solution evaporated to about one half the original volume. This procedure was carried out 2 or 3 times, after which the filtrate was dried in a rotary evaporator. The potassium bicarbonate content of the dried product amounts to about 15% (w/w). The dried compounds were stored in the darkness in a desiccator containing phosphorus pentoxide at 4°.

Spectrophotometric measurements

$A_{260\ nm}/A_{340\ nm}$ ratio. The absorbances at 260 nm and 340 nm of NADH and the separated impurities were measured in a 200 m*M* potassium bicarbonate solution with a VSU II spectrophotometer (Carl Zeiss Jena).

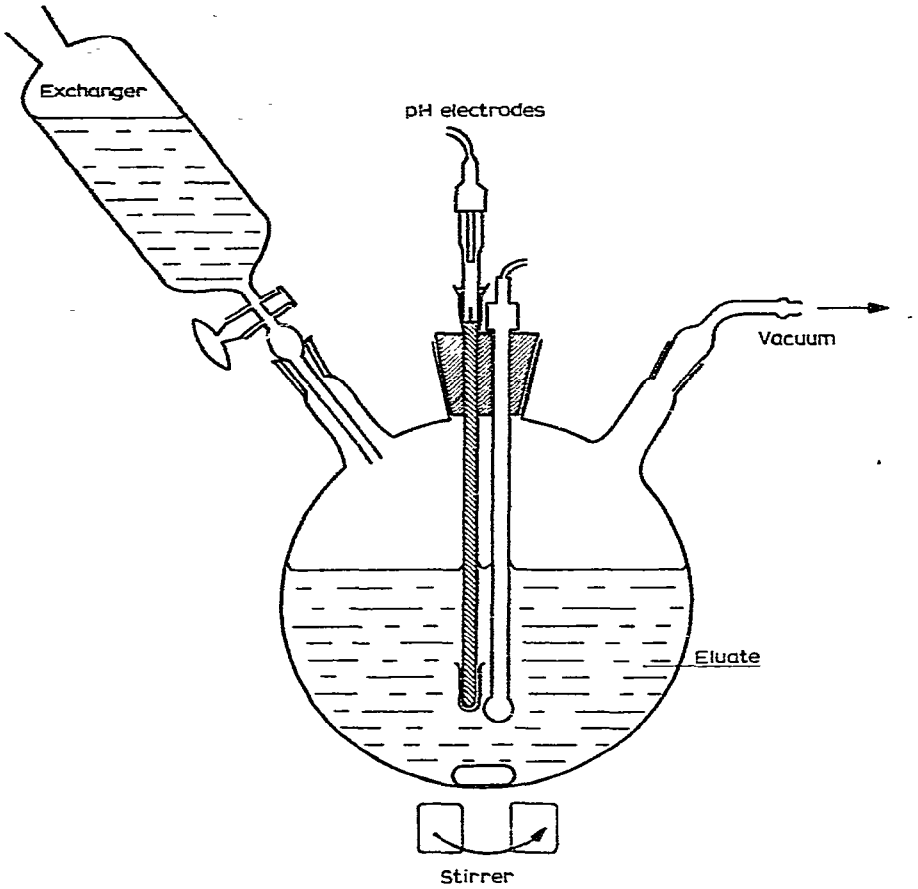

Fig. 1. Set-up for removing potassium bicarbonate from eluates after chromatographic separation of NADH and related compounds. To avoid freeing potassium chloride into the eluate, the calomel electrode was connected to the eluate by a membrane-coated glass tube containing saturated potassium bicarbonate solution. For details, see text.

Residual absorbance at 340 nm. The estimation of the residual absorbance was carried out after complete oxidation of NADH by the lactic acid dehydrogenase (LDH) reaction with the following final concentrations: phosphate buffer 100 m*M*, pH 7.5; pyruvate, 0.6 m*M*; NADH, 0.2 m*M*; LDH 5 μl. The residual absorbance is given by:

$$\text{Residual absorbance [\%]} = \frac{\text{final } A_{340\text{ nm}}}{A_{340\text{ nm}} \text{ before the addition of the enzyme}} \cdot 100$$

Measurements of the activity of lactic acid dehydrogenase (LDH) and alcohol dehydrogenase (ADH). In all cases NADH was dissolved in 200 m*M* potassium

bicarbonate. ADH reaction: phosphate buffer 100 m*M* pH 7.5; NADH ($A_{340\ nm}$ is ~0.45); acetaldehyde, 0.4% (v/v); ADH (1:2000, v/v, dissolved in 300 m*M* phosphate buffer, pH 7.5 containing 1 m*M* mercaptoethanol). LDH reaction: phosphate buffer, 100 m*M* (pH 7.5); NADH ($A_{340\ nm}$ is ~0.45); 0.1 ml pyruvate (1 mg/ml); LDH (1:1000, v/v, dissolved in 100 m*M* phosphate buffer pH 7.5).

All enzymatic assays were carried out with an Eppendorf photometer, 366 nm, 1 cm light path, 25°.

Measurements of optical rotation

Measurements were carried out with the Polamat (Carl Zeiss Jena).

Circular dichroism spectra

Circular dichroism spectra were monitored with a spectropolarimeter (Cary 60) at 25°.

UV spectra

The UV spectra were monitored with a Unicam SP-800 spectrophotometer in quartz cuvettes of 1 cm light path (25°). The substances were dissolved in 200 m*M* potassium bicarbonate and measured against blanks of the same solution.

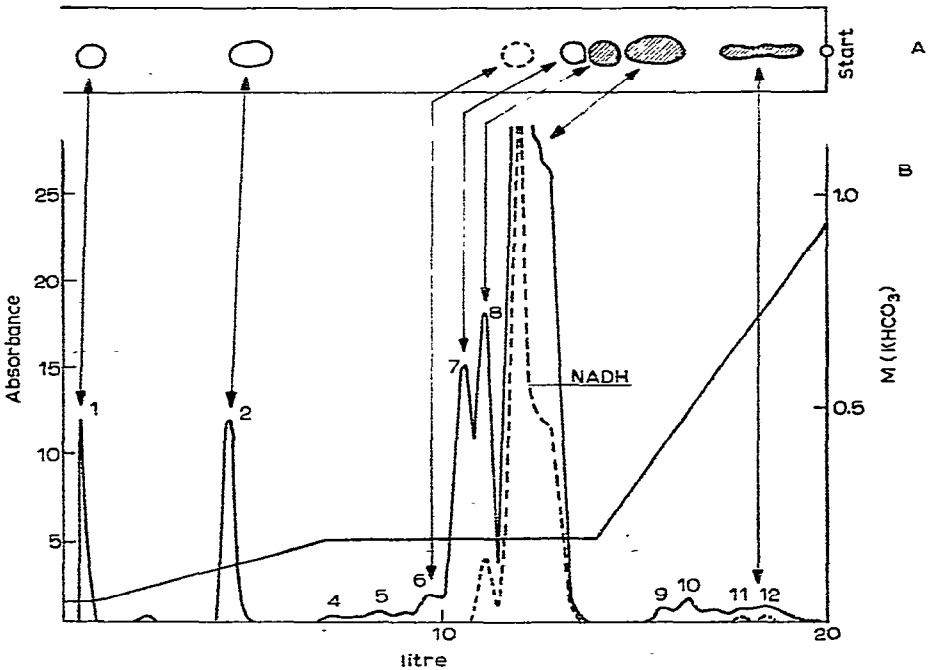

Fig. 2. Analytical and preparative chromatography of commercial NADH with DEAE-Sephadex A-25. A typical thin-layer chromatogram of about 1 μmole NADH is shown in the upper part. The lower part shows the elution profile after separation of 5 g NADH on a DEAE-Sephadex column (5 × 100 cm) using potassium bicarbonate as eluent. The peaks were detected by measuring the absorbance at 260 nm (———) and 340 nm (— — —). The compounds separated are numbered 1–12. Compound number 3 shows no absorbance at 260 nm and 340 nm but shows UV fluorescence.

TABLE I

$A_{260\ nm}/A_{340\ nm}$ RATIO AND RESIDUAL ABSORBANCE AT 340 nm OF THE PRODUCT BEFORE, DURING, AND AFTER PURIFICATION*

	$\frac{A_{260\ nm}}{A_{340\ nm}}$	*Residual* $A_{340\ nm}$ *(%)*
Before chromatography	2.87 ± 0.05 (10)	10.3
After chromatography	2.26 ± 0.04 (10)	1.2
After removal of the potassium bicarbonate	2.28 ± 0.04 (5)	
After drying	2.30 ± 0.04 (10)	1.3

* Number of estimations in parentheses.

UV fluorescence spectra

Excitation and emission spectra were recorded in 200 m*M* potassium bicarbonate with a Perkin-Elmer 204 fluorescence spectrophotometer at 25°.

RESULTS

Fig. 2 shows the results of preparative column chromatography and thin-layer chromatography (TLC) of commercial NADH. The elution profile of the column shows twelve compounds in addition to NADH. The re-chromatography of the purified and dried NADH in the same system shows only one peak, suggesting that formation of any of the twelve compounds does not occur during removal of the potassium bicarbonate. The same result is obtained by TLC. Therefore, TLC may be suitable for rapid detection of impurities in commercial NADH. The main impurities are peaks 1, 2, 7 and 8. Peaks 1, 2 and 7 correspond to nicotinamide, NAD and adenosine-5′-diphosphate–ribose (ADP–ribose), respectively, which were detected by reference substances, UV spectra and phosphorus analysis. Spectrophotometer measurements of the purified NADH show that the $A_{260\ nm}/A_{340\ nm}$ ratio decreases to the theoretical value of 2.27 (ref. 15) as the result of the chromatography (Table I). Similarly, there sidual optical density decreases from 10% to about 1%. These parameters do not alter during removal of the eluent and evaporation to dryness.

The influence of both commercial and purified NADH on the activity of LDH was studied (Table II). For comparison the enzyme velocity obtained with purified NADH is taken as 100%. With impure NADH only 80% residual activity was ob-

TABLE II

COMPARISON OF THE INFLUENCE OF COMMERCIAL AND PURIFIED NADH ON LDH ACTIVITY*

Time (days)	*NADH*			
	Impure		*Purified*	
	Stored as dried product	*Stored in 0.2 M $KHCO_3$*	*Stored as dried product*	*Stored in 0.2 M $KHCO_3$*
0	79.9 (6)	79.9 (6)	100	103 (4)
5	—	59.7 (2)	100 (2)	99.6 (2)
73	45.6 (2)	—	100 (4)	91.5 (4)

* Number of estimations in parentheses.

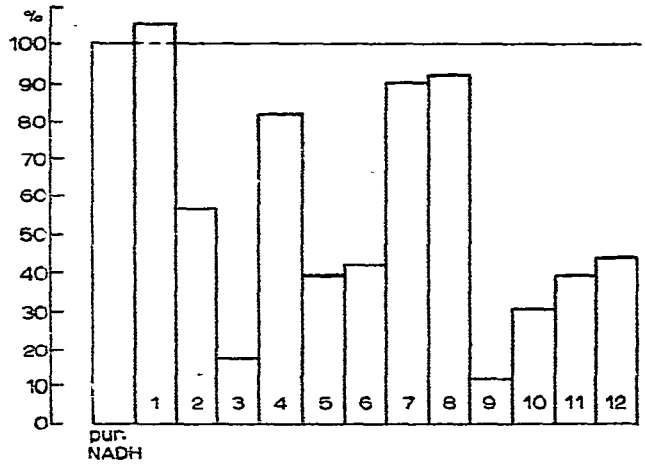

Fig. 3. LDH activity compared in purified NADH and in the presence of impurities (compounds 1–12) separated from commercial NADH. Each compound is added separately to the purified NADH in nearly equimolar concentrations, using the absorbance at 260 nm as the standard of reference.

tained. The purified NADH shows no loss of activity with respect to LDH for 73 days, the period under investigation. In contrast, the impure product shows a further loss in activity during this time, emphasizing that the stability depends largely on the degree of purity. Further, Table II shows the suitability of the eluate containing

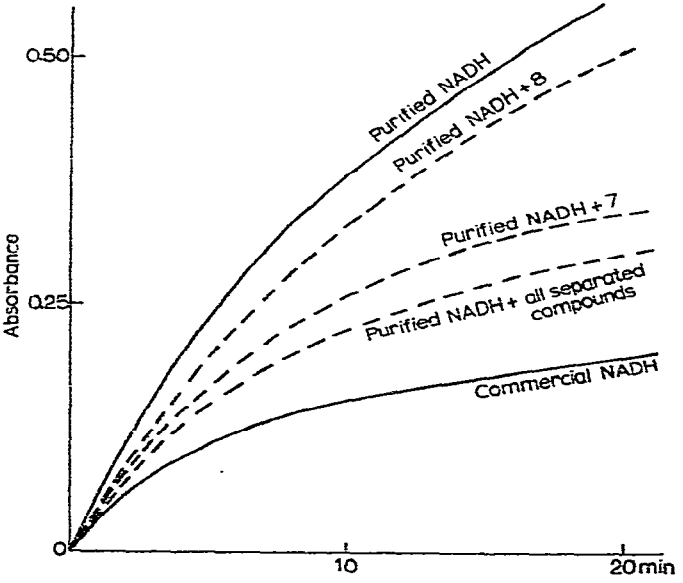

Fig. 4. Influence of NADH and impurities on ADH. Solid lines: comparison of commercial and purified NADH. Broken lines: A, purified NADH plus all twelve separated impurities in the same amounts as in the eluate after column chromatography (see Fig. 2); B, purified NADH plus equimolar amounts of compounds 7 and 8, respectively.

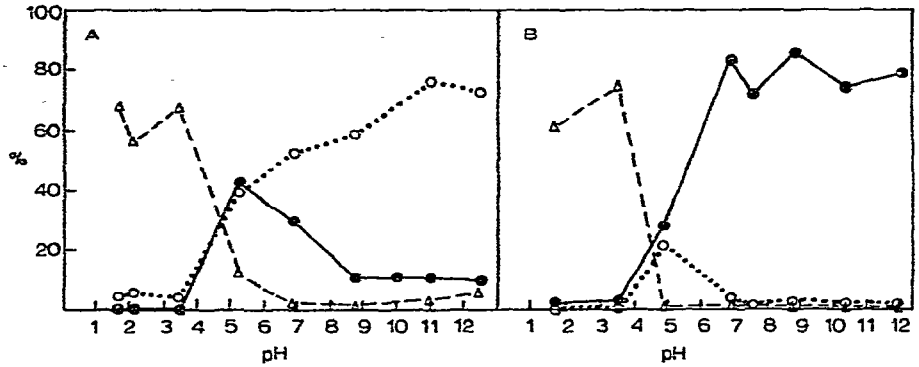

Fig. 5. Dependence of the interconversion of NADH (●—●), compound 7 (△—△) and compound 8 (○···○) on the pH value of the solution. pH values were adjusted with 0.1 *N* hydrochloric acid or 0.1 *N* sodium hydroxide. The compounds were then incubated for 2 h at 25° and separated by TLC. Conversion between the three compounds during chromatography can be excluded. After separation, the spots were eluted with 2 × 2.5 ml of 400 m*M* potassium bicarbonate and the absorbance was measured at 260 nm. The absorbance before incubation was defined as 100%. A: Incubation of compound 8; B: Incubation of purified NADH.

NADH for enzyme measurements when it is used within 5 days after chromatography, *i.e.* as there is no detectable loss in activity, it is unnecessary to remove the potassium bicarbonate.

Next, the influence on LDH activity of all twelve compounds separated from commercial NADH was tested. Fig. 3 shows the results after addition of each compound to purified NADH with the same absorbance at 260 nm. With the exception of compound 1, nicotinamide, all the separated compounds behave as inhibitors of LDH activity. The most pronounced effect was found with compounds 3 and 9. All twelve compounds were then added to purified NADH in the same quantities present in commercial NADH. Under these conditions the same LDH activity was obtained as with the impure product.

Similar results were obtained with ADH (Fig. 4). The velocity of the enzyme is

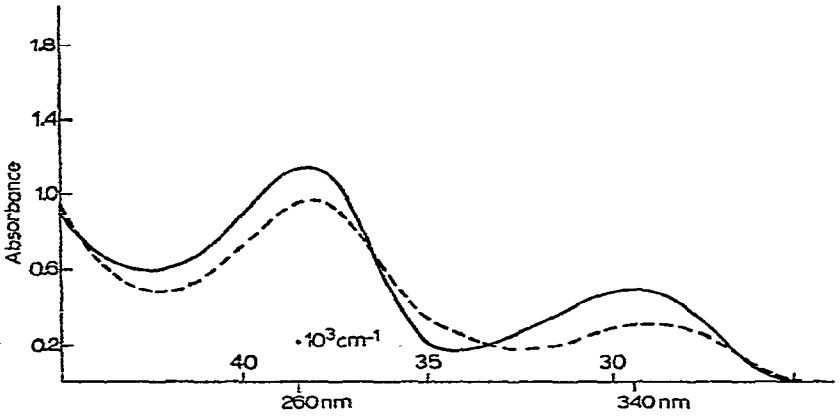

Fig. 6. UV spectra of purified NADH (———) and compound 8 (– – –).

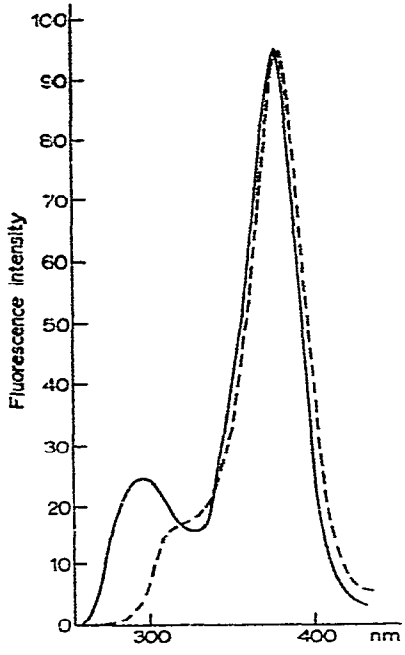

Fig. 7. UV fluorescence spectra of purified NADH (———) and compound 8 (– – –).

not linear with time. The effect of purification of NADH is much greater on ADH than on LDH. The difference between commercial and purified NADH becomes more marked as the time of the test increases.

The main impurities are at peaks 7 (ADP–ribose) and 8. Fig. 5 shows that compounds 7 and 8 derive from purified NADH, depending on the pH of the solution. Therefore, it is interesting to characterize these compounds in more detail.

At pH values below 5, both NADH and compound 8 are irreversibly converted into ADP–ribose. Between pH values 5–7 a reversible conversion between NADH and compound 8 can be observed. The conversion seems to be very rapid, since the same picture was obtained after 5 min incubation under the same conditions. Only

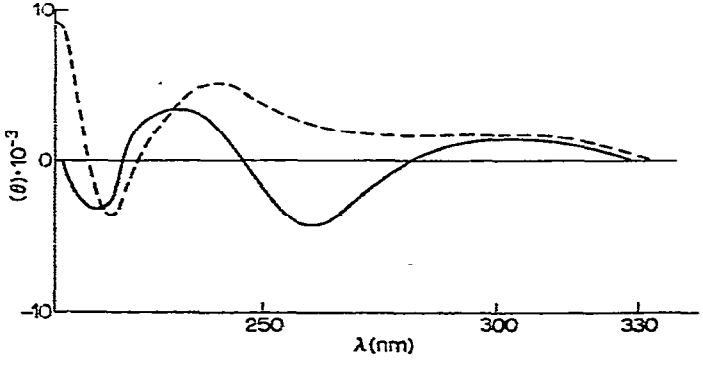

Fig. 8. Circular dichroism measurements of purified NADH (———) and compound 8 (– – –).

preliminary data on the optical properties of compound 8 are available. There are differences in the optical rotation between NADH and compound 8:

$[\alpha]_D^{25} = -47.5$ (NADH)

$[\alpha]_D^{25} = -26.2$ (compound 8)

Small differences are visible in the UV spectra and UV fluorescence spectra (see Figs. 6 and 7). The greatest differences are obtained in circular dichroism measurements (Fig. 8). The negative Cotton effect is lacking in the case of compound 8.

The findings suggest that compound 8 probably is a stereoisomer of NADH. To find the exact structure, further investigations are necessary.

DISCUSSION

The chromatography of commercial NADH using DEAE-Sephadex columns and potassium bicarbonate as eluent yields a highly purified product and involves relatively low expense and effort. The necessity of eluents with pH values greater than 7.5 for NADH purification is well established[5]. The advantage of using potassium bicarbonate solution is that it permits quick removal of the eluent after chromatographic purification. To avoid the formation of inhibitory compounds from NADH while removing potassium bicarbonate, a simple set-up is used which permits the pH to be checked (Fig. 1). At pH values below 6.5, dehydrogenase inhibitors form immediately which is in agreement with the literature[5–9]. The re-chromatography of the purified NADH after temporary acidification to below 6.5 shows that mainly ADP–ribose (compound 7) and a stereoisomer of NADH (compound 8) are formed (Fig. 5A). The potassium bicarbonate method gives a product with a lower residual optical density at 340 nm and a lower $A_{260\ nm}/A_{340\ nm}$ ratio than is attained with other purification methods[10].

The stability of the NADH purified by this method is excellent (Table II). During storage in a desiccator with phosphorus pentoxide at 4° in the darkness no impurities are formed within a ten week period. A further advantage is the possibility of storing and using the NADH-containing eluate from the column without removing the potassium bicarbonate. The number of impurities detected did not vary with the different commercial NADH preparations (Arzneimittelwerk Dresden, Boehringer Mannheim I and II) investigated, except for differences in the relative amounts of the compounds.

Fig. 3 shows that eleven of the twelve compounds separated from NADH inhibit LDH activity. In all the preparations investigated, compounds 7 and 8 constitute the main impurities. Compound 7, the ADP–ribose, is well established as an inhibitor of various dehydrogenases. Our results correspond to the data recorded by other authors[9,11]. In addition, we were able for the first time to separate completely compound 8 from NADH. The UV spectrum of this compound shows an absorption maximum at 340 nm similar to that of NADH. Therefore, it might be responsible for the residual absorbance at 340 nm in crude preparations, the $A_{260\ nm}/A_{340\ nm}$ ratio failing to reflect the presence of this impurity. To detect all possible impurities simple TLC, as outlined here, is a suitable method.

Compound 8 seems to be identical with the "340-nm compound" of the literature, the existence of which was assumed from residual optical density at 340 nm[8,12,13]. These authors investigated the properties of compound 8 using differential measurements in preparations having varying amounts of the "340-nm compound" while lacking any method of separation from NADH. Some of these findings, *e.g.* similarity of the UV spectrum to that of NADH, the inhibitory effect on ADH and sensitivity to acidification agree with our results on pure compound 8. The results in Fig. 5, showing the conversion of compound 8 into ADP ribose at pH values below 5, explain the differential spectra of this compound with and without addition of hydrochloric acid[12].

Of further interest is the reversible conversion of NADH and this compound at pH values below 7. It may be necessary to consider possible formation of this compound under *in vivo* conditions during acidification, resulting in inhibition of NADH-dependent reactions.

Initial attempts to elucidate the structure led to the conclusion that compound 8 is probably a stereoisomer of NADH. The circular dichroism spectrum of compound 8 shows some similarities to that of α-NADH[14]. From optical rotation measurements and its very rapid formation from NADH, however, the identity of compound 8 with α-NADH seems improbable.

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