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LIQUID CHROMATOGRAPHIC SEPARATIONS OF DIMERS OF NICOTINAMIDE ADENINE DINUCLEOTIDE AND NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE

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SUMMARY

The dimers of nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) [$(NAD)_2$ and $(NADP)_2$, respectively] were prepared electrolytically from NAD and NADP and analysed by several chromatographic techniques. Gel permeation chromatography on UltroPac columns was more convenient for the separation of dimers from monomers than the currently used separation on Sephadex. The reversed-phase high-performance liquid chromatographic analysis of the products yielded results comparable to those obtained by anion-exchange chromatography on a Polyanion SI column. The $(NAD)_2$ preparation contained one main isomer and smaller amounts of other two isomers (probably of the 4,4′ type), whereas only one significant peak with the properties of the dimer was detected for $(NADP)_2$. Anion-exchange chromatography is suitable for preparative separations of isomeric forms of dimers.

INTRODUCTION

Electrochemical reduction of nicotinamide adenine dinucleotide (NAD) at about −1 V yields a mixture of isomeric tetrahydrobipyridine dimers, $(NAD)_2$[1]. These compounds are able to interact with several NAD-dependent dehydrogenases and act as inhibitors competitive with respect to the coenzyme[2,3]. $(NAD)_2$ can be reoxidized to NAD by chemical, electrochemical or enzymic methods[1,4]. The basic properties of $(NADP)_2$, the dimer of nicotinamide adenine dinucleotide phosphate (NADP), which can be prepared from NADP in an analogous way, are similar to those of $(NAD)_2$[3]. This compound is bound by several NADP-dependent dehydrogenases as a competitive inhibitor[3]. The ability of dimers to influence the activities of the metabolically important enzymes and their possible role in some *in vivo* redox reactions are relevant from the biological viewpoint and might be the reason for the current interest in these compounds[1–5]. $(NAD)_2$ or $(NADP)_2$ can be separated from

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the monomers by simple gel permeation chromatography on Sephadex G-10 or G-15 columns[1,3]. Reversed-phase high-performance liquid chromatography (HPLC) with increasing concentrations of ethanol in the elution buffers has been shown to be the most convenient method for the analysis of these products[4,5]. This method permits the separation of $(NAD)_2$ into isomers, although conflicting results regarding the number and relative amounts of the isomeric forms have been obtained[3–5].

The purpose of this work was to study the possibility of separating $(NAD)_2$ and $(NADP)_2$ by other rapid and efficient chromatographic methods. Gel permeation chromatography (GPC) on an UltroPac TSK 3000SW column was compared with separation on Sephadex columns and the results of anion-exchange chromatography on Polyanion SI HR5/5 were compared with those obtained by reversed-phase chromatography. In principle, both methods might be used analytically and on a semi-preparative scale (*i.e.*, GPC for separation of dimers from compounds of lower molecular weight and anion-exchange HPLC for their resolution into isomers). We analysed both $(NAD)_2$ and $(NADP)_2$ samples in parallel experiments, as data on the dimer formed from NADP are scarce[3]. Some of the data presented here have been briefly presented previously as preliminary results[3].

EXPERIMENTAL

Materials

$(NAD)_2$ was prepared according to the literature[1,3] from NAD (Boehringer, Mannheim, F.R.G.) using an OH105 potentiostat (Radelkis, Budapest, Hungary) and a 15-ml water-jacketed three-compartment cell at -1.1 V *vs.* SCE at 20°C. The lyophilized material was stored under vacuum at -60°C and dissolved just before use. The same conditions were used for the preparation of $(NADP)_2$ from NADP (Reanal, Budapest, Hungary). Glutamate dehydrogenase and nicotinamide mononucleotide (NMN) were obtained from Boehringer and adenosine 5′-monophosphate (AMP) from Reanal. Other chemicals were of analytical-reagent grade and used without further purification.

Chromatographic separations

The separation of dimers on a Sephadex G-10 column (800 × 16 mm I.D.) using 0.02% ammonia as the eluent was carried out as described[1]. Chromatography on an UltroPac TSK 3000SW column (600 × 7.5 mm I.D.) (LKB, Bromma, Sweden) attached to an anion-exchange HPLC system (Pharmacia, Uppsala, Sweden) was used as a more efficient variant of GPC. Sodium phosphate buffer (0.2 *M*) (pH 7.5) served as the mobile phase [the manufacturer does not recommend mobile phases with pH values above 7.5[6], although such solutions would be advantageous from the viewpoint of increased stability of dimers (see ref. 1 and below)]. The absorbance at 254 nm was recorded by means of a UV-1 monitor and an REC 482 recorder (Pharmacia), 50–100-μl samples being applied to the column (see below). The liquid chromatograph used for reversed-phase HPLC consisted of a VCM 300 pump (ČSAV Workshop, Brno, Czechoslovakia), a variable-wavelength monitor (LKB) and a glass column (150 × 4 mm I.D.) packed with Separon C_{18} (5 μm) (Laboratory Instruments, Prague, Czechoslovakia). The samples were loaded with a syringe sample injector with a 10-μl sample loop (Rheodyne, U.S.A.). The mobile phase used was

25 m*M* sodium phosphate buffer (pH 6.6), alone or containing 7% of methanol. The monitor wavelength was set at either 260 or 340 nm (to detect either all nucleotides or dimers only).

The anion-exchange HPLC apparatus (Pharmacia) with a Polyanion SI HR5/5 column (containing tertiary and secondary amino groups[7]) and a UV-1 monitor (254 nm) was used for the anion-exchange chromatography of dimers. The mobile phases had the following composition: solvent A, 10 m*M* sodium phosphate buffer (pH 7.5), and solvent B, 0.3 *M* sodium phosphate buffer (pH 7.5) or 1 *M* sodium phosphate buffer (pH 6). Samples of 50–200 μl were applied to the column with a V-7 valve (Pharmacia).

All mobile phases were degassed thoroughly with helium. The separations were carried out at 20°C (except for the chromatography on Sephadex, which was carried out at 5°C). The fractions containing compounds absorbing at 260 (254) or 340 nm were collected by means of a FRAC-100 collector (Pharmacia), their pH was adjusted to 9 (if necessary) and they were pooled, freeze-dried or analysed immediately. Identification of the dimers was performed spectrophotometrically (Cary 118; Varian, Palo Alto, CA, U.S.A.) either directly by scanning the spectra[1–5] or by measuring the absorbance changes at 340 nm during oxidation with 0.1 m*M* $K_3Fe(CN)_6$ to NAD(P) and subsequent enzymatic reduction to NAD(P)H in the presence of 10 m*M* glutamate and 0.1 μM glutamate dehydrogenase.

RESULTS AND DISCUSSION

Gel permeation chromatography

The separation of dimers on Sephadex G-10 or G-15 is a time-consuming procedure with only moderate resolution[3,5]. It takes several hours and yields fractions of dimers that are partially contaminated with monomers. However, it proved to be suitable for macroscale preparations of these compounds. The elution profile of a crude $(NAD)_2$ preparation on a Sephadex G-10 column, which was achieved by us (not shown), was essentially similar to that reported by Jaegfeldt[5], the resolution being slightly worse. The behaviour of crude $(NADP)_2$ on this column was similar, the retention time being slightly shorter. On the other hand, the use of a high-performance UltroPac column (with rigid microparticles allowing the application of higher pressures to ensure sufficient flow-rates) provided excellent resolution of both dimers from monomers (and other compounds of lower molecular weight), in spite of the relatively low molecular weights of the compounds under study (nearly at the lower limit of the fractionation range of this column, *i.e.*, 1000–100,000 for dextrans[6]). The separations were complete within 20 min (Fig. 1). The first peak in both instances (Fig. 1a and b) had the properties of dimers (*i.e.*, their A_{260}/A_{340} ratios were in the range 4.5–5.0[1,5]) and they were oxidizable with hexacyanoferrate(III) to catalytically active coenzymes. The catalytic efficiency of NAP or NADP formed in the reaction of dimers with hexacyanoferrate(III) was tested in the presence of glutamate and glutamate dehydrogenase, as this enzyme is able to use both NAD and NADP as coenzymes. The method described by Finazzi-Agrò *et al.*[2] was not suitable as it uses alcohol dehydrogenase, which is specific for NAD only.

The average purities of $(NAD)_2$ and $(NADP)_2$ obtained in several experiments (determined from the areas of the peaks shown in Fig. 1) were 85% and 80%, re-

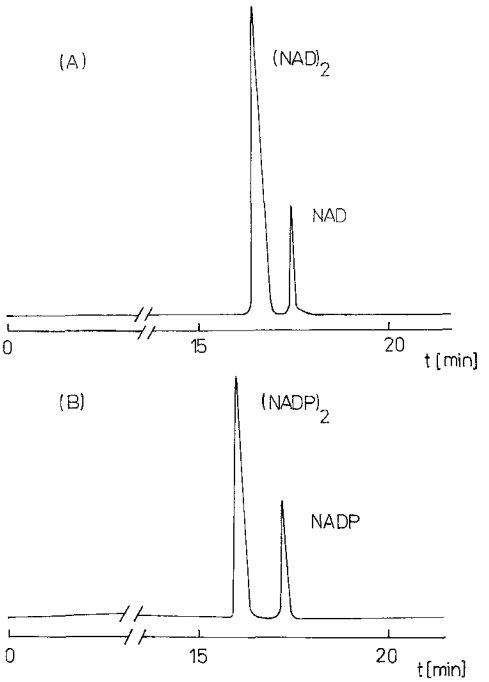

Fig. 1. Separation of electrolytically prepared samples of (A) $(NAD)_2$ and (B) $(NADP)_2$ on an UltroPac TSK 3000SW column. Mobile phase, 0.2 *M* sodium phosphate buffer (pH 7.5); flow-rate, 1.3 ml/min; λ = 254 nm.

spectively, if the residual current at the end of electrolysis was close to 0.1 mA. Shorter electrolysis times led to a decrease in the yields of both dimers. The yields obtained for $(NAD)_2$ were similar to those obtained by Jaegfeldt[5]. The GPC column used by us (*i.e.*, UltroPac TSK 3000SW) was able to resolve the samples even on a semi-preparative scale (up to *ca.* 6 μmol), *i.e.*, about 10 mg of the preparation were separated satisfactorily into two fractions. The application of large preparative columns packed with this material would be very convenient for the separation of tens to hundreds of milligrams of dimers from the reaction mixtures with a resolution surpassing that of Sephadex columns, the elution times being about one order of magnitude shorter.

Reversed-phase and anion-exchange HPLC analysis

Typical elution profiles of crude samples of $(NAD)_2$ and $(NADP)_2$ analysed by reversed-phase HPLC are shown in Fig. 2. The isocratic conditions used gave similar results with $(NAD)_2$ to the gradient elutions described by other workers[4,5]. The mobile phase containing 7% of methanol was suitable for the separation of $(NAD)_2$. With $(NADP)_2$, the mobile phase without methanol could be used owing to the higher hydrophilicity of this compound in comparison with $(NAD)_2$.

When the samples of dimers after the GPC step were analysed, the small peaks near the front of both elution profiles and the peaks of NAD and NADP disappeared. The same occurred if the absorbance at 340 nm instead of 260 nm was recorded. It

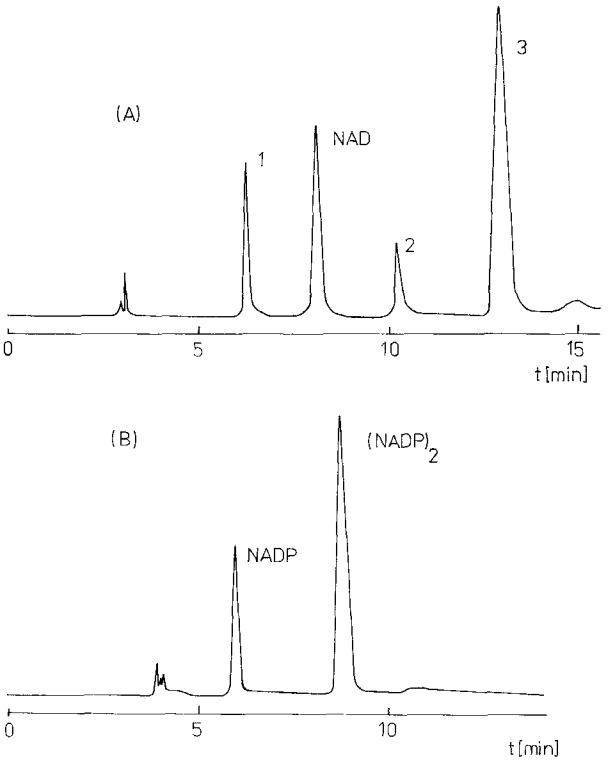

Fig. 2. Reversed-phase HPLC separation of (A) $(NAD)_2$ and (B) $(NADP)_2$. Column, Separon C_{18}; mobile phases, (A) 25 m*M* sodium phosphate buffer (pH 6.6) containing 7% of methanol and (B) 25 m*M* sodium-phosphate buffer (pH 6.6); flow-rate, 0.5 ml/min; λ = 260 nm. Peaks 1–3 had the properties of $(NAD)_2$ (see Experimental).

follows from Fig. 2b that $(NADP)_2$ appeared as the only significant isomeric form (λ_{max} = 339 nm); this might be due to the occurrence of only one $(NADP)_2$ isomer (probably of the 4,4′ type; see below) or to the inconvenience of the conditions used for the separation of $(NADP)_2$ isomers (see below). On the other hand, the $(NAD)_2$ preparation contained, in addition to one main isomer (peak 3 in Fig. 2a), three other minor components (peaks 1 and 2 and the small peak with a retention time of about 15 min in Fig. 2a).

Our results [as for the resolution of $(NAD)_2$] are comparable to those published by Avigliano *et al.*[4] and Jaegfeldt[5]. However, there are some differences in the relative amounts of $(NAD)_2$ isomers. The results in Fig. 2a were obtained with the preparation in which the abundance of minor fractions was the highest in all the experiments we carried out. In the other three preparations the predominance of peak 3 was more perceptible than in Fig. 2a. The average relative amount of isomer 3 was about 80% whereas isomers 1 and 2 constituted about 12% and 7%, respectively. The peaks (to which the numbers were assigned in Fig. 1a) were collected and analysed spectrophotometrically. All of them had the properties of $(NAD)_2$ (see Experimental) and their near-UV absorption maxima at 341 (1), 337 (2) and 342 nm (3) were close to the values reported by Jaegfeldt[5] for three 4,4′ structural isomers of

$(NAD)_2$. We did not detect significant amounts of other isomers (*e.g.*, those of the 4,6′ type, which constituted very small fractions with absorption maxima above 345 nm in ref. 5. It is not precluded that the small peak with a retention time of about 15 min (which was not analysed owing to the very small peak height) might be one of these isomeric forms. We were not able to explain the preferential formation of one of the 4,4′ isomers of $(NAD)_2$ (peak 3) in our experiments (which was already mentioned previously[3]). At variance with our data, the most abundant isomeric forms of $(NAD)_2$ reported by other workers[4,5] did not constitute more than 50–60% of the total amount of this dimer.

The anion-exchange chromatography of dimers on Polyanion SI was carried out both analytically (to complete the results obtained by reversed-phase HPLC) and semi-preparatively (in order to prepare larger amounts of individual isomeric forms). Several gradient elutions with phosphate buffers with increasing concentration and/or decreasing pH were tested. The pH range was limited to 6–8 as the dimers

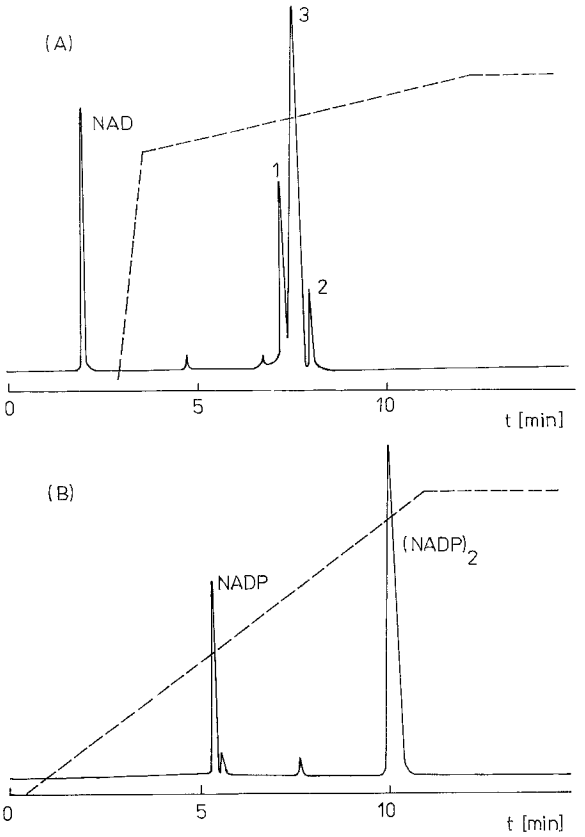

Fig. 3. Anion-exchange HPLC separation of (A) $(NAD)_2$ and (B) $(NADP)_2$. Column, Polyanion SI HR5/5; gradient elution (----), 10 m*M* sodium phosphate buffer (pH 7.5) (solution A in both instances), 0.3 *M* sodium phosphate buffer (pH 7.5) [solution B in $(NAD)_2$ separation], 1 *M* sodium phosphate buffer (pH 6) [solution B in $(NADP)_2$ separation]; flow-rate, 1.5 ml/min; λ = 254 nm. Peaks 1–3 are $(NAD)_2$ isomers (*cf.*, Fig. 2).

are unstable in acidic media (see below) and alkaline mobile phase are not compatible with the stability of the gel[7]. The results obtained with gradients optimized for $(NAD)_2$ and $(NADP)_2$ separations are shown in Fig. 3. The resolution of crude preparations of dimers by these methods were very good. The results achieved with anion-exchange HPLC confirm the conclusions drawn from the reversed-phase HPLC analysis. The preparation of $(NADP)_2$ contained only one isomeric form, which was eluted at the end of the applied gradient (Fig. 3b). Relatively low pH and high concentration of phosphate in buffer B had to be used owing to the anionic character of $(NADP)_2$. The small peaks eluted between NADP and $(NADP)_2$ might correspond to impurities, as they were observed even in the chromatogram of NADP.

Other gradient shapes and buffer combinations did not bring about any further resolution of the observed $(NADP)_2$ peak. Hence the formation of only one isomeric form of $(NADP)_2$ is very probable (*cf.*, the above results of the HPLC analysis). On the other hand, the $(NAD)_2$ preparation was resolved into three larger peaks (in addition to the small peaks that were eluted prior to peak 1 and were not analysed). The main peaks corresponding to $(NAD)_2$ isomers were collected, characterized spectrophotometrically as mentioned above and applied to the reversed-phase column. In this way, the elution order of $(NAD)_2$ isomers in Fig. 3a was established (the same numbers were assigned to the corresponding peaks in reversed-phase and anion-exchange HPLC separations; *cf.*, Figs. 2a and 3a). The correctness of this correspondence could easily be checked by comparing the heights of individual peaks in Figs. 2a and 3a.

Chromatography on Polyanion SI columns was also used to prepare larger amounts of $(NAD)_2$ isomers and of $(NADP)_2$. Samples containing 10–15 μmol of dimers (*i.e.*, about 20 mg) were separated using the small (50 × 5 mm I.D.) column with satisfactory resolution. These results were achieved with both the crude reaction mixture after electrolysis and the preparation partially purified by GPC. The anion-exchange HPLC method appears to be the most efficient technique suitable for semi-preparative purifications of both compounds under study. The main advantage of this method over GPC is that it permits not only the separation of dimers from monomers but is also convenient for obtaining pure $(NAD)_2$ isomers.

Stability of dimers

Anion-exchange HPLC on Polyanion SI and reversed-phase HPLC were used to test changes in the preparations of dimers kept under different conditions. Freeze-dried samples stored at −60°C were found to be stable for at least 6 months, only slight oxidation back to NAD and NADP and nearly negligible formation of degradation products (such as AMP and NMN) being detected. It was confirmed that the hydrolytic stability of $(NAD)_2$ at higher temperatures (5–25°C) depends on several factors, as mentioned elsewhere[1–5]. The main factors are the presence of oxygen (which oxidizes these compounds to NAD) and the pH of the medium (the oxidative reaction requires protons).

In agreement with Jaegfeldt[5], we found that solutions of purified samples of $(NAD)_2$ isomers were more stable than those of crude preparations. The stabilities of the three main $(NAD)_2$ isomers were comparable (see also ref. 5). The behaviour of $(NADP)_2$ solutions was very similar to that of $(NAD)_2$ solutions. The results of the stability tests with aerated solutions of $(NADP)_2$ (performed with neutral and

alkaline media at two temperatures) are shown in Fig. 4. The chromatographic analysis of $(NADP)_2$ samples exposed to oxygen showed that this dimer was oxidized to NADP, no significant production of other products being detected under the described conditions. The rate of the oxidation was increased at higher temperatures and at lower pH. In the absence of oxygen, $(NADP)_2$ solutions (purified by GPC or anion-exchange HPLC) were stable for several days.

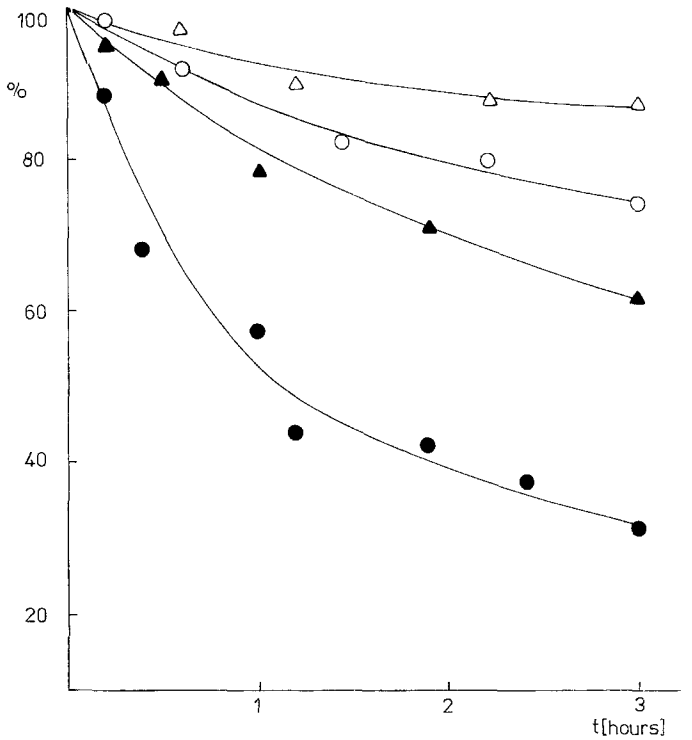

Fig. 4. Stability of aerated $(NADP)_2$ solutions tested by anion-exchange HPLC. Conditions as in Fig. 3; 1 m*M* solutions of crude $(NADP)_2$ preparation were kept under the following conditions: △, pH 9, 4°C; ○, pH 9, 25°C; ▲, pH 7, 4°C; ●, pH 7, 25°C; 100% corresponds to the initial $(NADP)_2$ concentrations in the samples; the data are averages of two measurements.

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