The concentration of free sulfalene in the blood over a period of 96 h after intramuscular injection exceeded the minimum effective concentration for the free preparation by 2.8-fold. This permits the time interval between administration of maintenance doses to be increased to 48 h.

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SYNTHESIS AND PROPERTIES OF A NEW NAD ANALOG: AMINOETHYLNICOTINAMIDE ADENINE DINUCLEOTIDE

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UDC 615.355:577.152.1'133

In recent years considerable advances have been made in the study of NAD-dependent dehydrogenases, an important group of metabolic enzymes, yielding information on the structures and complexes of apoenzymes [1] and kinetic reaction mechanisms [2].

The active centers of the most thoroughly studied dehydrogenases – glyceraldehyde-3-phosphate dehydrogenase (GAPD), alcohol dehydrogenase (ADH), and lactate dehydrogenase (LDH; E.C. 1.1.1.27) – contain charged groups, which seem to be involved in binding the coenzyme and substrate [1]. Coenzyme analogs containing additional positively or negatively charged centers can be used to examine the nature of these groups. The presence of an extra phosphate group in the adenine ribose moiety of NAD (NADP), for example, is known to weaken binding with dehydrogenases, probably because of the nonpolar environment or of contact with the anionic locus.

Knowledge of the nature of the functional groups of the active center in the region of the nicotinamide part of the coenzyme has considerable significance for our understanding of the mechanism of enzyme reactions, since these groups can take a direct part in catalysis. The amide group of the coenzyme has a specific role in LDH-catalyzed reactions [3]. The microenvironment of this substituent in the active center can be examined by using NAD analogs with charged groups on the C^3 carbon atom of the pyridine ring. Our intention in the work described here was to synthesize aminoethylnicotinamide adenine dinucleotide (AE-NAD) and examine its properties and ability to interact with yeast GAPD, yeast ADH, and the pig muscle M_4LDH isoenzyme.

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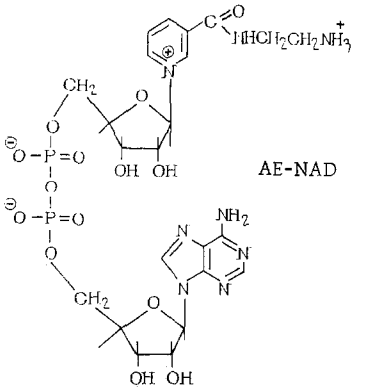


AE-NAD

Synthesis of AE-NAD. We synthesized AE-NAD by the exchange reaction of transglycosylation from NAD and N^7-aminoethylnicotinamide in the presence of pig spleen β-NAD-transglycohydrolase. This method has been extensively used [4] for the synthesis of NAD analogs.

During the incubation we monitored the content of NAD by reaction with ethanol catalyzed by yeast ADH and the quantity of pyridine dinucleotides (NAD + AE-NAD) by reaction with potassium cyanide, assuming that the NAD and AE-NAD adducts have similar molar extinctions. The resulting kinetic plots are shown in Fig. 1. The formation of AE-NAD is implied by the different time dependences of the concentration of NAD and dinucleotides. The quantity of the analog at any time corresponds to the difference between the curves. Figure 1 shows that the maximum quantity of AE-NAD (about 40%) is present in the solution during the first 20-25 min of incubation. Its concentration thereafter diminishes, apparently as a result of hydrolysis by β-NAD-transglycohydrolase.

Precipitation of the proteins from the solution left a mixture of pyridine bases and nucleotides in the solution, which we were able to separate by precipitation with acetone followed by column chromatography (32 × 2 cm) on DEAE-cellulose (OH^- form) in 0.02 M glycine buffer solution, pH 7.4. Elution with the buffer solution gave successively aminoethylnicotinamide and AE-NAD (Fig. 2) and then NAD and ADP-ribose in an increasing sodium chloride gradient. Desalting was carried out on Sephadex G-10; the yield was 10-12%.

In conformity with its structure, AE-NAD gave a positive reaction with ninhydrin and migrated to the cathode in paper electrophoresis at pH 7.6.

We verified its structure spectrocopically. Figure 3 shows the PMR spectrum of AE-NAD. The spectrum of NAD resembled that published earlier [5]. The spectrum of AE-NAD shows the protons of the pyridine, purine, and pentafuranose rings, together with those of methylene groups of the 3-substituent of the pyridine ring.

The absorption spectrum of AE-NAD has a maximum at 260 nm, which is typical of the purine and pyridine chromophores.

Among the products of the acid hydrolysis of AE-NAD in 5.7 N hydrochloric acid at 110°C we detected aminoethylnicotinamide, thus providing further support for the structure.

Properties of AE-NAD. To get a reasonably sound estimate of the coenzyme activity of the NAD analog we need information on the affinity of the pyridine ring for nucleophiles and on the conformation of the dinucleotide in solution. Addition to a solution of AE-NAD of sodium sulfite or potassium cyanide causes intense bands to appear in the long-wavelength region of the absorption spectrum as a result of the formation of adducts at position 4 of the pyridine ring (Fig. 4). We found the dissociation constants (K_{diss}) of the (AE−NAD)·SO_3^{2-} adduct by titration, monitoring the change in solution optical density at 320 nm. Our results are shown in Fig. 5. The point of inflection on this curve corresponds to K_{diss} [6] and is $1.2 \pm 0.2 \times 10^{-2}$ M. For the NAD·SO_3^{2-} complex K_{diss} is $2.0 \pm 0.2 \times 10^{-2}$ M, which is in agreement with literature data [6]. This ratio of K_{diss} of the adducts implies that the electrophilicity of position 4 of the pyridine ring is much the same in AE-NAD as in NAD.

Pyridine dinucleotides are known to exist in solution as a set of conformers, some of which have a folded conformation as a result of the interaction of the heterocyclic bases [5]. When they bind at the active center of the enzyme, some of the binding energy is expended in unfolding the dinucleotide molecule [1].

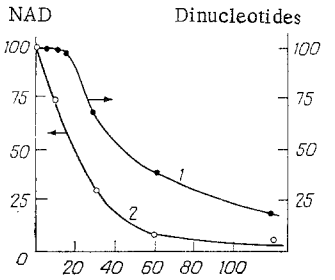


Fig. 1

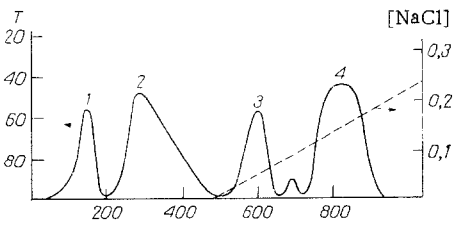


Fig. 2

Fig. 1. Change in dinucleotide concentration during the synthesis of AE-NAD; the abscissa shows the time (min); the ordinate the concentration (%); 1) NAD concentration; 2) total NAD and AE-NAD concentration.

Fig. 2. Separation of the components of the reaction mixture after the synthesis of AE-NAD on a column of DEAE-cellulose; the abscissa shows the consumption of the buffer solution (ml), the ordinate the transmittance (%) and sodium chloride concentration (M); 1) pyridine bases; 2) AE-NAD; 3) NAD; and 4) ADP-ribose.

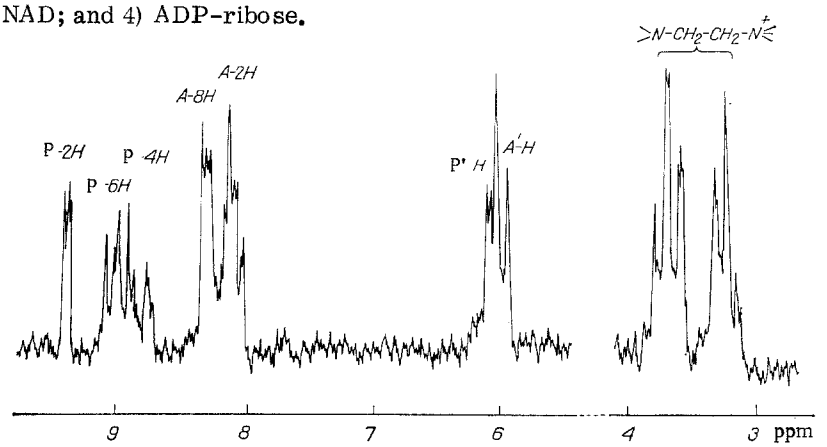


Fig. 3. PMR spectrum of AE-NAD in D_2O (pH 7.0, 34°C, AE-NAD concentration 0.05 M).

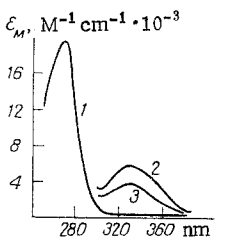


Fig. 4

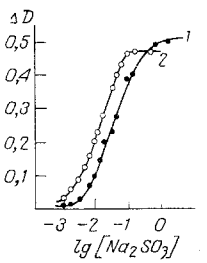


Fig. 5

Fig. 4. Absorption spectra of AE-NAD (20°C); 1) 0.1 M phosphate buffer solution, pH 7.0; 2) 2 M potassium cyanide solution, pH 10.8; and 3) 1.5 M sodium sulfate solution, pH 8.0.

Fig. 5. Dependence of the optical density of solutions of AE-NAD and NAD on the molar concentration of sodium sulfite and 0.1 M Tris buffer solution, pH 8.0 (21°C); 1) AE-NAD (1.3×10^{-4} M, λ 320 nm); 2) NAD (1.1×10^{-4} M, λ 325 nm).

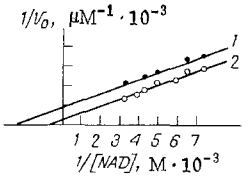


Fig. 6. Inhibition by AE-NAD of LDH-catalyzed oxidation of lactate, 0.1 M phosphate buffer solution, pH 7.0 [35°C, concentration of DL-lactate 0.2 M and LDH 1.77×10^{-9} M (active centers)]; 1) (1.2×10^{-3} M); 2) no inhibitor.

We can examine the interaction of the heterocycles and consequently deduce the conformation of the dinucleotide from the magnitude of the hypochromism accompanying the cleavage of the molecule to a mixture of mononucleotides by phosphodiesterase.

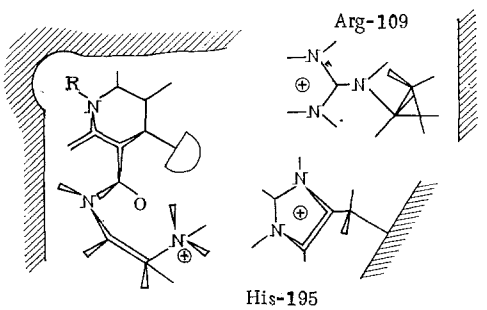
We measured the hypochromism by the method of [9]. At pH 8.7 phosphodiesterase causes almost no change in the absorption spectrum. The hypochromism is approximately 1%, implying that the interaction of the heterocycles is weak and that the analog favors the unfolded conformation. In the case of NAD the hypochromism is approximately 12%. The partially unfolded structure of AE-NAD should facilitate the interaction with the dehydrogenase active center.

Thus the electrophilicity of the pyridine ring of AE-NAD and the degree of folding of the molecule promote the enzyme reactions.

Interaction of AE-NAD with Dehydrogenases. The reduced form of the analog is not formed in a solution containing 3-phosphoglyceraldehyde (4×10^{-4} mole), AE-NAD (1.5×10^{-4} mole), and sodium arsenate (1×10^{-2} mole) in the presence of GAPD, as is implied by the absence of absorption in the 340 nm region. Nor is AE-NAD reduced by ethanol in the presence of ADH or by LDH in the presence of lactate. This could be due to the disruption of dinucleotide binding at the active center of the enzyme by comparison with NAD. To clarify this point we examined the effect of AE-NAD on the rate of LDH-catalyzed reactions. We found that the analog has a weak inhibitory effect. Figure 6 shows the dependence of the reaction velocity on NAD concentration. The inhibition is noncompetitive [10]; K_i is $1.4 \pm 0.3 \times 10^{-3}$ M. For the LDH·NAD complex K_{diss} is 4×10^{-4} M [11]. The LDH·(AE-NAD) complex is therefore considerably weaker, apparently as a result of the presence of positively charged or nonpolar groups in the microenvironment of the 3-substituent of the pyridine ring of the coenzyme.

We got the same conclusion regarding the nature of the active centers of GAPD and ADH.

We examined the possible interactions of the aminoethyl residue of AE-NAD at the active center of LDH with an atomic model of the LDH : NAD-pyruvate complex, constructed on the basis of the coordinates of the α-carbon atoms [1]. We followed [10] in choosing the position of the pyridine ring of the coenzyme. We analyzed the most probable modes of orientation of the aminoethyl group relative to the amino acid residues of the protein by freely rotating the substituent about the C3–C7 bond. The results of our analysis are shown in the diagram



Orientation of AE-NAD at the active center of LDH relative to the positively charged groups of the His-195 and Arg-109 residues

which reveals that the ammonium group of the substituent lies in the immediate vicinity of the imidazole ring of the His-195 residue and the guanidinium group of the Arg-109 residue. The electrostatic repulsion at a distance of 2-4 Å therefore weakens the binding.

EXPERIMENTAL METHOD

The materials were NAD, sodium pyruvate, and crystalline pig muscle LDH (all Reanal), and snake venom phosphodiesterase (Worthington Biochemical Corporation). The M_4 isoenzyme was isolated from commercial LDH by chromatography on CM-cellulose [8]. The microsomal fraction of pig spleen was prepared and used as the source of β-NAD-glycohydrolase by the method of [4]. The GAPD was isolated from baker's yeast by Kreb's method [12].

$N^{7'}$-Aminoethylnicotinamide. To a stirred solution of ethylenediamine hydrochloride (52 g, 0.3 mole) in 85% ethanol (60 ml) was added dry potassium hydroxide (43.7 g, 0.6 mole). The reaction mixture was filtered off, water (60 ml) was added, and the alcohol was stripped off. To the resulting ethylenediamine solution at pH 11.3, cooled to 4°C, was added nicotinic acid azide (0.03 mole) portionwise with stirring. The mixture was left for 10 h. The solution was then mixed with silica gel (30 g, Woelm, 100 mesh) and put on the top part of a column of silica gel and n-butanol. Elution was carried out in n-butanol–25% ammonia (5 : 1) at 20 ml/h. Fractions were analyzed by thin-layer chromatography on Silufol plates in the same system with visualization by UV and reaction with ninhydrin. The solvent was removed from the eluates by azeotropic distillation with water at 40°C. The oily residue of aminoethylnicotinamide was dissolved in water (10 ml) and converted to the hydrochloride by adding 1 N hydrochloric acid to pH 2.5. After evaporation of the reaction mixture, traces of hydrochloric acid were removed by repeated distillation with water. Recrystallization was carried out from 95% ethanol. The yield was 10%, mp 230°C.

Found, %: C 40.8, H 5.40, Cl 29.6, N 17.4. $C_8H_{13}Cl_2N_3O$ Calculated %: C 40.4, H 5.46, Cl 29.8, N 17.6.

Aminoethylnicotinamide Adenine Dinucleotide (AE-NAD). The mixture of NAD (1.4×10^{-3} mole) and aminoethylnicotinamide (9.0×10^{-3} mole) was incubated at 36°C for 60 min in 0.05 M phosphate buffer solution, pH 8.0 (480 ml) in the presence of β-NAD-glycohydrolase (520 kcat/ml). The progress of the reaction was monitored from the disappearance of NAD and from the total dinucleotide content in the incubation mixture. The proteins were precipitated with 5% trichloroacetic acid and the nucleotides were isolated from the supernatant liquid by precipitation with acetone (1 : 5) at 4°C. The precipitated nucleotides were chromatographed on a column (35 × 2 cm) of DEAE-cellulose and 0.02 M glycine buffer solution, pH 7.4. The lyophilized AE-NAD preparation was desalted by gel fitration on a Sephadex column (100 × 1.8 cm). The yield of AE-NAD was 17%. The synthetic preparation was homogeneous as shown by paper electrophoresis and by paper chromatography in 0.1 M acetic acid–ethanol (1 : 1), R_f 0.55.

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