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Synthesis of Coenzymically Active Soluble and Insoluble Macromolecularized NAD^+ Derivatives

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Alkylation at N-1 of the NAD^+ adenine ring with 3,4-epoxybutanoic acid, followed by chemical reduction to the alkali-stable NADH form and alkaline Dimroth rearrangement, gave the NADH derivative alkylated at the exocyclic adenine amino group. Enzymic reoxidation of the latter derivative gave nicotinamide–6-(2-hydroxy-3-carboxypropylamino)purine dinucleotide, a functionalized NAD^+ analogue carrying an ω-carboxyalkyl side-chain at the exocyclic adenine amino group. Carbodiimide coupling of the latter derivative to high-molecular-weight water-soluble (polyethyleneimine, polylysine) and insoluble (aminohexyl-Sepharose) polymers gave the corresponding macromolecularized NAD^+ analogues. These derivatives have been shown to be enzymically reducible. The polyethyleneimine and polylysine analogues showed a substantial degree of efficiency relative to free NAD^+ with rabbit muscle lactate dehydrogenase (60 and 25% respectively) but a lower one with yeast alcohol dehydrogenase and *Bacillus subtilis* alanine dehydrogenase (2–7%). The polyethyleneimine derivative entrapped in cellulose triacetate fibres together with the lactate dehydrogenase was operationally stable during repetitive use.

Industrial, analytical or biomedical applications of enzymes immobilized by physical entrapment in porous structures (like polyacrylamide or starch gel, microcapsules and fibrous matrices), permeable to low-molecular-weight substrates and products, represent a promising fast developing new branch of applied enzymology (see *e.g.* [1]). Yet this technique is not economically applicable to enzymes requiring readily dissociable cofactors because the coenzyme, being of low molecular weight, cannot be retained by the porous matrix together with the enzyme (or multienzyme system). However, the availability of active, hydrosoluble and stable macromolecularized derivatives of these cofactors would make such applications possible. In fact, entrapment of a derivative of this type in porous matrices together with enzymes would preclude its loss by diffusion. An important class of enzymes requiring a dissociable cofactor is represented by the NAD^+-dependent dehydrogenases. The purpose of the present investigation was therefore to prepare hydrosoluble macromolecularized NAD^+ derivatives by attachment of the cofactor molecule to high-molecular-weight soluble polymers through a suitable linkage, in such a way as to assure retention of coenzymic activity and stability of bonding between cofactor and polymer.

This paper describes the synthesis of an NAD^+ analogue functionalized with a carboxyl group, suited for binding to polymers carrying amino groups. The new functionalized NAD^+ derivative synthesized, nicotinamide–6-(2-hydroxy-3-carboxypropylamino)-purine dinucleotide (Fig. 1, III), was then coupled to polyethyleneimine and polylysine to give the macromolecularized hydrosoluble derivatives IV. The coenzymic efficiency relative to NAD^+ of the derivatives III and IV with three enzymes (rabbit muscle lactate dehydrogenase, yeast alcohol dehydrogenase, *B. subtilis* alanine dehydrogenase) was also tested, as well as the operational stability of the polyethyleneimine IV derivative entrapped in cellulose triacetate fibres together with lactate dehydrogenase.

While the present work was already in progress the only other paper describing a coenzymically active and hydrosoluble macromolecularized NAD^+

Enzymes. Alcohol dehydrogenase (EC 1.1.1.1); lactate dehydrogenase (EC 1.1.1.27); alanine dehydrogenase (EC 1.4.1.1).

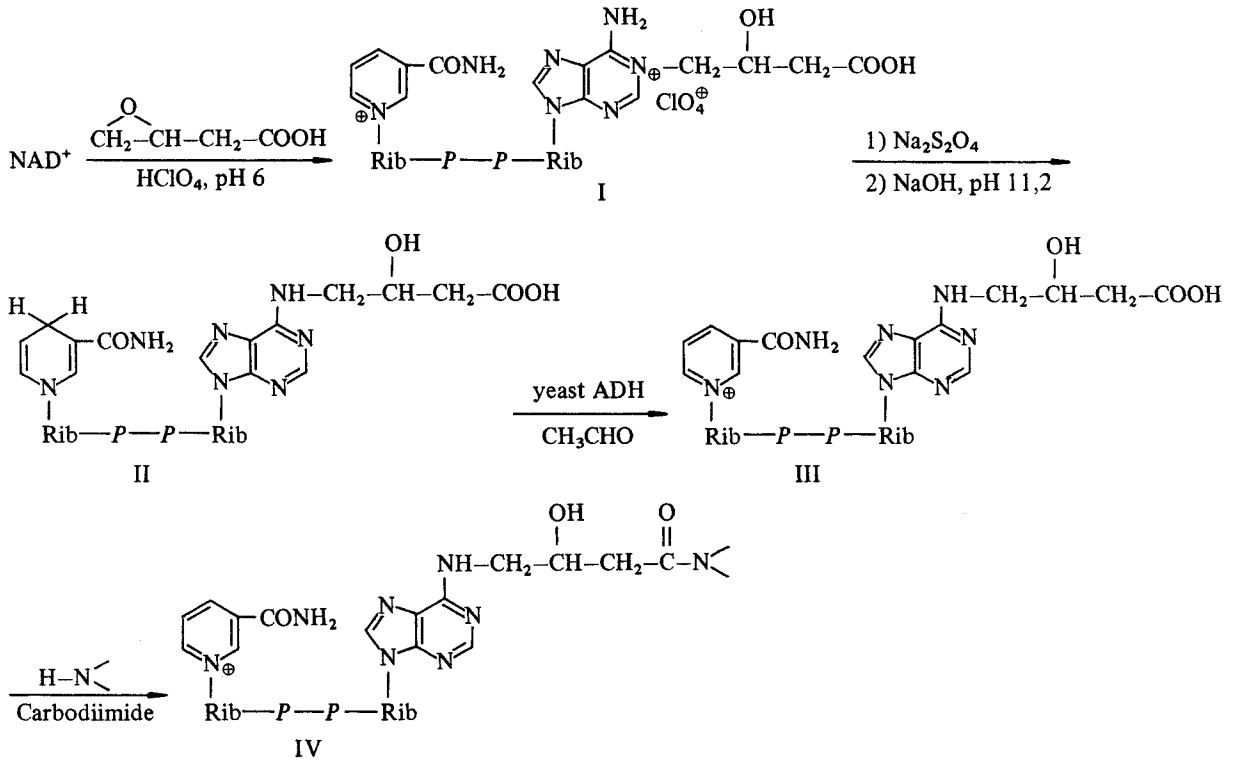

Fig. 1. *Synthesis of polymer-bound NAD+ derivatives.* Rib = ribose; *P* = phosphate; ADH = alcohol dehydrogenase; H − N< = polymer

derivative (polyethyleneimine-succinyl-NAD+) appeared [2]. Yet this analogue appears to be of limited applicability, due to hydrolytic instability of the succinyl link to the NAD+ molecule.

Finally, the present paper describes the coupling of the functionalized NAD+ analogue III to a water-insoluble polymer, *i.e.*, aminohexyl-Sepharose 4B (a Sepharose functionalized with 1,6-diaminohexane by the cyanogen bromide method), to afford a water-insoluble NAD+ derivative whose potential use in affinity chromatography [3] is under investigation.

MATERIALS AND METHODS

Materials

Semicarbazide hydrochloride was purchased from Carlo Erba (Milano, Italy); 3,4-butenoic acid, Dowex 21K and *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride were obtained from Fluka A.G. (Buchs, Switzerland); Tris, silica gel 60 F_{254} (0.25 mm) and cellulose F (0.1 mm) thin-layer chromatography plates were purchased from Merck A.G. (Darmstadt, Germany); polyethyleneimine (M_r 40000–60000) 33% aqueous solution was obtained from Polysciences Inc. (Warrington, U.S.A.); polylysine hydrobromide (M_r about 50000) was from Koch-Light Laboratories Ltd (Colnbrook, Bucks., England); Sephadex G-10 and G-50 and aminohexyl-Sepharose 4B were purchased from Pharmacia (Uppsala, Sweden); DEAE-cellulose was purchased from Whatman Biochemicals Ltd (Springfield Mill, Maidstone, Kent, England); Polyox WSR 301 was from B.D.H. Chemicals Ltd (Poole, Dorset, England); the enzymes (yeast alcohol dehydrogenase, rabbit muscle lactate dehydrogenase, *B. subtilis* alanine dehydrogenase) and NAD+ grade I were obtained from Boehringer (Mannheim, Germany).

Analytical Procedures

Thin-layer chromatography was carried out on silica gel and cellulose; the solvent systems and the spot-location method are given in the legend to Table 1. Ultraviolet spectra were obtained with a Unicam SP 1800 recording spectrophotometer and fluorescence excitation spectra with a Perkin-Elmer MPF-24 spectrophotofluorimeter.

High-voltage paper electrophoreses were carried out with a Shandon L-24 apparatus on 23 × 55-cm Whatman 3MM paper, using a 0.02 M ammonium acetate pH 5.0 buffer and applying a potential of 4000 V for 30 min; polyethyleneimine and polylysine were visualized under ultraviolet light (254 nm),

NAD^+ and NAD^+ derivatives by their fluorescence in ultraviolet light (340 nm) after spraying with 5 M NaOH and heating for 10 min at 100 °C [4].

Automatic titrations were performed with a Metrohm Combi-Titrator 3D.

Phosphate was determined by the method of Fiske and SubbaRow [5].

The ^1H nuclear magnetic resonance spectrum of methyl-3,4-epoxybutanoate in $C_6{}^2H_6$ was recorded with a Varian T-60 instrument using tetramethylsilane as internal reference.

^{13}C nuclear magnetic resonance spectra were obtained with an XL – 100 – 15D Varian instrument operating at 25.1 MHz; experimental conditions are given in the legend to Fig. 3.

The gas chromatography – mass spectrometry analysis of methyl-3,4-epoxybutanoate was taken with a Varian Mat 111 instrument under the following conditions: 1.83 m × 4-mm stainless-steel column packed with 3% OV-1 on Chromosorb W; column temperature, 60–220 °C (15 °C/min); injector temperature, 240 °C; ionizing energy, 70 eV.

Optical rotations were measured with a 181 Perkin-Elmer polarimeter.

Gel-filtration eluates were monitorized with a LKB Uvicord II instrument.

Determinations of enzymically reducible polymer-bound NAD^+ were carried out by exhaustive reduction with yeast alcohol dehydrogenase in a reaction mixture containing 0.15 M Tris buffer, pH 9.0; 0.5 M ethanol; 0.0075 M semicarbazide · HCl. The nucleotide content was determined by spectrophotometric measurement of the absorbance at 340 nm of the NADH derivative formed, assuming for the polymer-bound NADH an absorption coefficient equal to that of free NADH.

Determinations of the total polymer-bound NAD^+ were carried out by spectrophotometric measurement of the absorbance at 266 nm, assuming an absorption coefficient equal to that of NAD^+ derivative III.

Preparation of 3,4-Epoxybutanoic Acid

An 80% peracetic acid solution (10.5 ml, corresponding to 110 mmol of peracetic acid) prepared according to [6] was added dropwise to a solution of 3,4-butenoic acid (9.46 g, 110 mmol) in methylene chloride (30 ml) under stirring and ice-bath cooling. The bath was allowed to reach room temperature and the stirring continued for 4 days. Keeping the same temperature, more peracetic acid (25 mmol) was added and the stirring continued for an additional 24 h. The solvent was evaporated at 50 °C, 20 mm Hg (2.7 MPa), and the acetic acid removed from the residue by stripping with toluene at 50 °C, 20 mm Hg (2.7 MPa) giving 3,4-epoxybutanoic acid (10.27 g) as a colorless oil. The product was used without further purification for the preparation of III.

Preparation of Nicotinamide – 6-(2-Hydroxy-3-carboxypropylamino)purine Dinucleotide (III)

3,4-Epoxybutanoic acid (4.3 g, 42 mmol) was brought, at 0 °C, to pH 6.0 with 6 M NaOH and added to a solution of NAD^+ (1.0 g, 1.51 mmol) in water (6 ml). The resulting mixture was readjusted to pH 6.0 and stirred at room temperature, maintaining the pH value at 6.0 with an automatic titrator charged with 1 M $HClO_4$. After 8 days most of the NAD^+ had been converted to I (thin-layer control, for R_F values see Table 1). Acidification to pH 4.0 with 1 M $HClO_4$, precipitation with acetone (10 volumes) keeping the mixture overnight at −20 °C, and collection of the precipitate by centrifugation gave crude I. The nucleotide I was converted to the alkali-stable reduced form by heating (boiling-water bath) the crude preparation for 5 min with $Na_2S_2O_4$ (0.4 g) in 1% $NaHCO_3$ (60 ml) and the excess $Na_2S_2O_4$ was oxidized by bubbling air for 15 min through the cooled (25 °C) solution. Addition of 1 M NaOH to pH 11.2 and heating at 75 °C for 1 h gave the rearranged product II. The cooled (25 °C) solution was enzymically oxidized by addition of 3 M Tris (3 ml), 6 M HCl to pH 7.5, acetaldehyde (0.5 ml) and yeast alcohol dehydrogenase (100 µl of an enzymic suspension in 2.4 M $(NH_4)_2SO_4$, containing 29.4 mg of alcohol dehydrogenase/ml). Incubation was for 40 min (until no further decrease in absorbance at 340 nm occurred). Acidification to pH 3.0 with 6 M HCl followed by acetone (10 volumes) precipitation (−20 °C, overnight) and centrifugation gave crude III. The precipitate was dissolved in water (100 ml), the solution adjusted to pH 8.0 with 1 M NaOH and chromatographed over Dowex 21K ($HCOO^-$) (40 ml). After elution with water (100 ml) and 0.075 M HCOOH until no more absorbance around 260 nm was detected in the eluates, the derivative III was eluted with 0.20 M HCOOH (1.5 l). Concentration of this fraction to 1/10 of the original volume and acetone precipitation as above gave nicotinamide – 6-(2-hydroxy-3-carboxypropylamino)-purine dinucleotide (III) (190 mg). For characterization purposes, a sample of the product was further purified by the following sequence of operations: (1) chromatography over DEAE-cellulose eluting with a linear 1–500 mM gradient of Tris · HCl buffer, pH 7.0, and collecting the 0.15–0.2 M fraction; (2) concentration to 1/5 of the original volume and acetone precipitation after acidification to pH 1.5 with 2 M HCl; (3) desalting over Sephadex G-10, eluting

Table 1. *Thin-layer chromatography*
Solvent systems: (A) isobutyric acid – H_2O – 32% aqueous NH_3 (66:33:1.7, by vol.); (B) 1 M ammonium acetate – ethanol (2:5, v/v); (C) 0.5 M Tris adjusted to pH 8.0 with conc. HCl. The dinucleotides were detected by ultraviolet light (254 nm)

Compound	R_F values in chromatography systems				
	silica gel F_{254}			cellulose F	
	A	B	C	A	C
NAD$^+$	0.37	0.27	0.65	0.55	0.81
Nicotinamide – 1-(2-hydroxy-3-carboxypropyl)adenine dinucleotide (I)	0.17	–	–	–	–
Nicotinamide – 6-(2-hydroxy-3-carboxypropylamino)purine dinucleotide (III)	0.23	0.21	0.71	0.42	0.98

with water; (4) lyophilization of the eluates, followed by drying under 0.02 mm Hg (0.27 Pa) at room temperature for 3 days. Thin-layer chromatography R_F values in various systems are given in Table 1.

Preparation of 4-(NAD$^+$-N^6)-3-hydroxybutyryl-polyethyleneimine
(IV, –N< = polyethyleneimine residue)

To 0.38 ml of a 33% (w/v) aqueous solution of polyethyleneimine adjusted to a pH of about 6 with conc. HCl were added aqueous solutions (0.5 ml each) of III (40 mg) and *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride (40 mg). The mixture, adjusted to pH 5.5 with 1 M NaOH, was stirred for 48 h at room temperature. After dilution with water (to 10 ml), the polymer was purified by precipitation with 1 M phosphate buffer (10 ml), pH 6.0, followed by centrifugation (39000 × *g*). The precipitated polymer was collected and redissolved in a solution (2 ml) 2 M in NaCl and 0.05 M in acetate buffer, pH 5.5. More water (8 ml) was added and the above purification procedure was repeated five times. The final precipitate was collected, redissolved as before and dialyzed against a solution (250 ml) 2 M in NaCl and 0.1 mM in HCl for 24 h, then against 0.1 mM HCl for 4 days changing the HCl solution daily (250-ml portions). Lyophilization of the dialysis residue gave 4-(NAD$^+$-*N*^{6})-3-hydroxybutyryl-polyethyleneimine (IV, –N< = polyethyleneimine residue) (85.7 mg).

Preparation of 4-(NAD$^+$-N^6)-3-hydroxybutyryl-polylysine
(IV, –N< = polylysine residue)

The coupling of III (40 mg, dissolved in 0.5 ml water) to polylysine hydrobromide (100 mg, dissolved in 1 ml water) with the carbodiimide reagent (40 mg, dissolved in 0.5 ml water) was performed in the same way as described for polyethyleneimine. The polymer was purified by chromatography of the reaction mixture over Sephadex G-50 (1.8 × 60-cm column), eluting with 0.9% NaCl and collecting the first eluted peak (40 ml), followed by dialysis against water for 3 days daily changing the water (until negative chloride test). The lyophilized solution gave 4-(NAD$^+$-*N*^{6})-3-hydroxybutyryl-polylysine (IV, –N< = polylysine residue) (63 mg).

Preparation of 4-(NAD$^+$-N^6)-3-hydroxybutyryl-aminohexyl-Sepharose 4B
(IV, –N< = aminohexyl-Sepharose 4B residue)

Aminohexyl-Sepharose 4B (500 mg) was allowed to swell out with a 0.5 M NaCl solution, and after filtration washed with 0.5 M NaCl and water. To this gel (final volume about 2 ml) a solution of III (92 mg) in water (2 ml) was added and the mixture adjusted to pH 5.0 with 1 M NaOH. To the mechanically stirred suspension a solution of *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride (75 mg) in water (2 ml) was added dropwise at room temperature. The stirring at room temperature was continued for 24 h keeping during the first 5 h the pH at about 5 by additions of 1 M HCl. Washing of the filtered gel with a solution (200 ml) 1 M in NaCl and 0.1 mM in HCl and with water gave 4-(NAD$^+$-*N*^{6})-3-hydroxybutyryl-aminohexyl-Sepharose 4B (IV, –N< = aminohexyl-Sepharose residue) (about 1.8 ml wet gel).

Enzyme Assays

The experimental conditions for the enzymic reductions with rabbit muscle lactate dehydrogenase, yeast alcohol dehydrogenase and *B. subtilis* alanine dehydrogenase of NAD$^+$ and the soluble NAD$^+$ derivatives III and IV are given in the legend to Table 2.

RESULTS

STRUCTURAL ASSIGNMENTS

The structure of the epoxybutanoic acid was confirmed by nuclear magnetic resonance and mass

Table 2. *Rates relative to NAD$^+$ of enzymic reduction of the soluble NAD$^+$ derivatives*
Incubation mixtures (1.0 ml): (a) For lactate dehydrogenase: Tris · HCl, 150 μmol; L-lactic acid, 60 μmol; cofactor, 0.2 μmol (expressed as bound NAD$^+$ in the case of the NAD$^+$ derivatives); enzyme, 0.2 μg; pH 8.9. (b) For alcohol dehydrogenase: Tris · HCl, 150 μmol; ethanol, 500 μmol; semicarbazide · HCl, 75 μmol; cofactor, as for (a); enzyme, 0.67 μg; pH 9.0. (c) For alanine dehydrogenase: sodium carbonate, 200 μmol; L-alanine, 100 μmol; cofactor, as for (a); enzyme, 1.6 μg; pH 10.0. Incubations were run at 25 °C and rates of reduction determined from the increase in absorbance at 340 nm

NAD$^+$ derivative	Relative rate of enzymic reduction (NAD$^+$ = 1)		
	lactate dehydrogenase (rabbit muscle)	alcohol dehydrogenase (yeast)	alanine dehydrogenase (*B. subtilis*)
Nicotinamide–6-(2-hydroxy-3-carboxypropyl-amino)purine dinucleotide (III)	0.83	0.65	0.32
4-(NAD$^+$-N^6)-3-hydroxybutyryl-polyethyleneimine (IV, $-N\lessgtr$ = polyethyleneimine residue)	0.60	0.06	0.02
4-(NAD$^+$-N^6)-3-hydroxybutyryl-polylysine (IV, $-N\lessgtr$ = polylysine residue)	0.25	0.07	0.02

spectrometry of its methyl ester (diazomethane): δ 2.43 (1 H,dd, γ-CH), 2.46 (2 H,d, α-CH_2), 2.68 (1 H,dd, γ-CH), 3.12 (1 H,m, β-CH), 3.62 (3 H,s, CH_3); *m/e* 115, 85, 74, 59, 43. The structures assigned to the dinucleotide intermediates and products on the basis of the method of synthesis (see Discussion) were confirmed by the following analytical support:

Derivatives I and II

Derivative I, its reduced form and derivative II had ultraviolet spectra in 0.1 M Na_2CO_3 in agreement with the literature data for analogue derivatives [3,7]: I (crude precipitate) showed maximum at 259 and shoulders at 266 and around 300 nm; the reduced form of I (not isolated) had an additional maximum at 340 nm; II (not isolated) showed maxima at 266 and 340 nm and disappearance of maximum and shoulder at 259 and around 300 nm respectively.

Derivative III

The purified derivative III showed the following characteristics:

Ultraviolet Spectra (Fig. 2). (a) Absorption maxima at 211 (ε 29600 M^{-1} cm^{-1}) and 266 (ε 23600 M^{-1} cm^{-1}), minimum at 235 nm (ε 8100 M^{-1} cm^{-1}), in agreement with the literature data for NAD$^+$ analogue derivatives [3,7]; for comparison, the spectrum of NAD$^+$ is also given in Fig. 2. (b) After enzymic reduction the maximum at 266 nm shifted slightly to 268 nm (*cf.* also [7]) and the one at 340 nm appeared. (c) The cyanide adduct showed the expected maximum around 330 nm.

^{13}C Nuclear Magnetic Resonance Spectrum (Fig. 3). The assignments for the NAD$^+$ moiety of III coincided with those made, based on the literature data [8], in a control with NAD$^+$; the presence of the four additional signals of the aliphatic side-chain gave an unambiguous picture of derivative III.

High-Voltage Electrophoresis. Greater mobility was observed towards the anode as compared with NAD$^+$, consistent with the presence of the carboxyl group.

Fluorescence Excitation Spectrum of the Yeast Alcohol Dehydrogenase Reduced, Not Isolated, Form. The spectrum was similar to the one obtained in a reduction of NAD$^+$ under identical conditions; this similarity has been seen also in the case of an analogously alkylated NAD$^+$ derivative [7].

$[\alpha]_D^{22}$, 1% in water: $-34.5°$, the value for NAD$^+$ is $-34.8°$ [9]. Found: C, 36.88; H, 4.60; N, 11.85; P, 7.10; $C_{25}H_{33}N_7O_{17}P_2$. 3 H_2O (M_r 819.58) requires C, 36.63; H, 4.79; N, 11.96; P, 7.55.

Polyethyleneimine IV Derivative

The purified polyethyleneimine IV derivative had an ultraviolet spectrum in water with a maximum at 266 nm; that this absorbance was due uniquely to covalently bound III was assured by the following facts. (a) High-voltage electrophoresis showed the purified polymer IV to be free of III or carbodiimide-activated III, while these last two derivatives appeared neatly separated from the polymer IV in an electrophoresis performed before purification. (b) In a blank assay where a mixture of III and polyethyleneimine with no added carbodiimide was submitted to the same sequence of operations used in the coupling reaction, the product obtained showed no different absorbance from a control with polyethyleneimine alone.

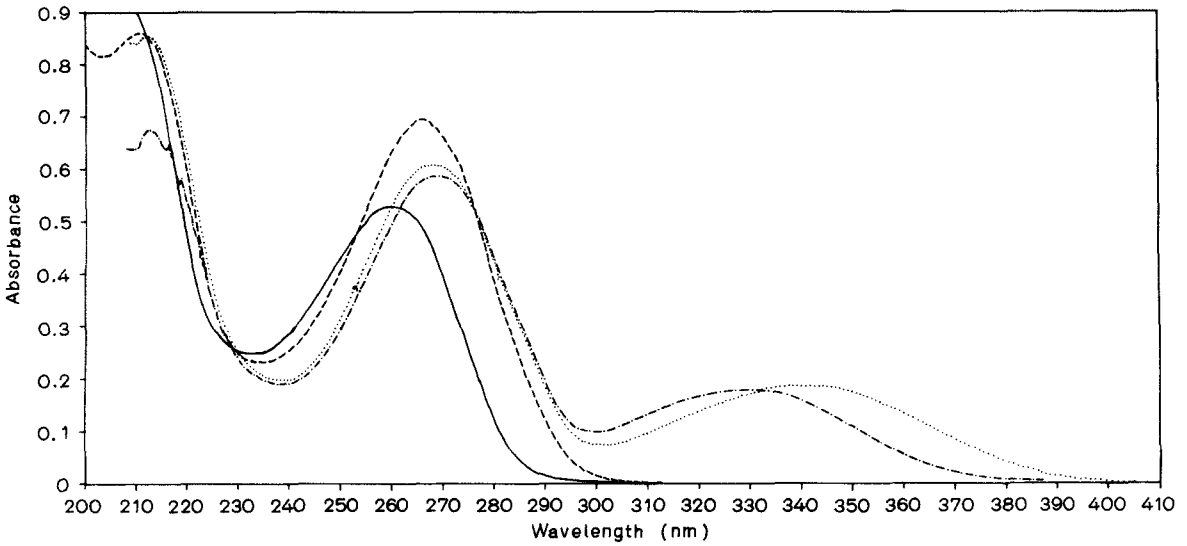

Fig. 2. *Ultraviolet spectra of NAD$^+$ and nicotinamide–6-(2-hydroxy-3-carboxypropylamino)purine dinucleotide (III) and its derivatives.* NAD$^+$ in water (——); III in water (– – – –); III reduced enzymically with yeast alcohol dehydrogenase (2.58 µg/ml) in 0.5 M ethanol and 0.1 M sodium pyrophosphate buffer, pH 9 (·····); III-cyanide adduct in 1 M KCN (–·–·–). All the samples were 29.4 µM in nucleotide and measured with reference to corresponding blank solutions

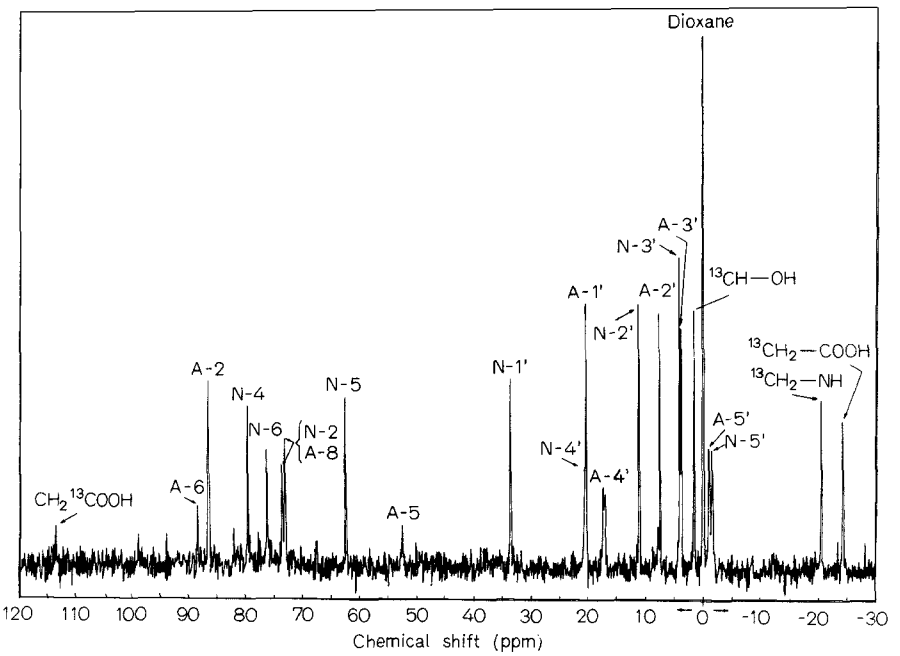

Fig. 3. *Natural abundance, proton-noise-decoupled ^{13}C nuclear magnetic resonance spectrum of nicotinamide–6-(2-hydroxy-3-carboxypropylamino)purine dinucleotide (III).* Sample concn, 10% (w/v) in ^2H_2O, pH 7.5 (NaO^{2}H); internal reference, dioxane; pulse width (US), 80 µs; spectral width, 5000 Hz; acquisition time, 0.8 s; accumulation time, 2 h, using the Fourier transform technique; down field shifts denoted as positive. A and N refer to the adenine and nicotinamide rings respectively, A' and N' refer to ribose linked to adenine and nicotinamide rings respectively

The amount of enzymically reducible polyethyleneimine-bound NAD$^+$ was 125 µmol/g of polymer; this value corresponded also to the one of total polymer-bound NAD$^+$.

Polylysine IV Derivative

The purified polylysine IV derivative had an ultraviolet spectrum in water with a maximum at 266 nm; the absence in the product of any unbound III or

carbodiimide-activated III was shown by high-voltage electrophoresis.

The amount of enzymically reducible polylysine-bound NAD^+ was 32.4 μmol/g of polymer, corresponding also to the total polymer-bound NAD^+.

Sepharose IV Derivative

The purified Sepharose IV derivative had an ultraviolet spectrum (suspension in 1% aqueous Polyox WSR 301) with a maximum at 266 nm; storage of the gel in a solution 1 M in NaCl and 0.1 mM in HCl for several days showed no ultraviolet absorbance in the supernatant, indicating the absence of diffusible III or carbodiimide-activated III in the polymer.

The amount of enzymically reducible Sepharose–bound NAD^+ was 3.45 μmol/g of dry product, corresponding to about 55% of the total polymer-bound NAD^+.

ENZYME ASSAYS

The rates of enzymic reduction with rabbit muscle lactate dehydrogenase, yeast alcohol dehydrogenase and *B. subtilis* alanine dehydrogenase of III and the soluble polyethyleneimine and polylysine IV derivatives were measured. The relative rates of these reductions, as compared with NAD^+ reduction under identical conditions, are given in Table 2. These data can be summarized as follows. (a) With all three derivatives the coenzymic activity relative to NAD^+ increases in the order: alanine dehydrogenase, alcohol dehydrogenase, lactate dehydrogenase. (b) Of the three derivatives, III shows the highest degree of relative efficiency with all three dehydrogenases (32–83%). (c) The polyethyleneimine and polylysine IV derivatives show still a substantial degree of activity relative to NAD^+ with lactate dehydrogenase (60 and 25% respectively), but a considerably lower one with the other dehydrogenases (2–7%).

A preliminary experiment in which the soluble polyethyleneimine IV derivative was entrapped together with lactate dehydrogenase in cellulose triacetate fibres showed also that the immobilized enzyme · coenzyme system is operationally stable during repetitive use, demonstrating that the macromolecularized cofactor is retained in the fibre and is chemically stable.

DISCUSSION

The choice of preparing a functionalized NAD^+ analogue like III for the synthesis of active and stable macromolecularized NAD^+ derivatives was made for the following reasons:

a) Among the alkylated or acylated NAD^+ derivatives known to date [2,3,7,10,11] (compare compounds 20, 47, 48, 58, 59 of Table 3 in [10]), the most active with a number of dehydrogenases have been shown to be those substituted at the exocyclic adenine amino group. In addition, for lactate dehydrogenase, structural studies of the NAD^+ · enzyme complex indicate the adenine ring as being the portion of the cofactor molecule less embedded in the active site of the enzyme [12]. That this may be true also in the case of other dehydrogenases arises from more recent studies [13]. These studies, in fact, revealed striking structural similarity between the coenzyme-binding regions of several dehydrogenases (lactate dehydrogenase included) and possibly of all the dehydrogenases.

b) An alkylic rather than an acylic link to the exocyclic adenine amino group of NAD^+ was considered more favorable. In fact, from mechanistic considerations (see, for example, [14]) the electron-attracting character of the purine ring was expected not to guarantee prolonged hydrolytic stability of the amide linkage under neutral and especially basic conditions, which are also those required by the NAD^+-dependent dehydrogenases. This hypothesis is also substantiated by published work on cleavage of various 6-acylamino purine derivatives [2,15–17]. Furthermore, an alkylation of the amino group would have altered its electron density to a lesser degree than an acylation. This alteration, *a priori*, could have further affected the coenzymic activity of the macromolecularized cofactor, in view of the known role played in this respect by the said amino group in NAD^+ [18–20].

c) Functionalization of the NAD^+ molecule with a carboxyl group is suited for carbodiimide coupling to polymers carrying amino groups, affording a stable amide linkage.

d) Windmueller and Kaplan [7] have shown that ethylene oxide and propylene oxide are selective N-1-alkylating agents of the NAD^+ adenine ring and that subsequent alkaline Dimroth rearrangement gives the corresponding NAD^+ derivatives alkylated at the exocyclic adenine amino group. They have also shown that chemical reduction ($Na_2S_2O_4$) to the alkali-stable NADH N-1 derivative prior to the rearrangement gives better yields of the rearranged product and that enzymic (yeast alcohol dehydrogenase) reoxidation permits, finally, the reconversion of the latter to the NAD^+ form. In view of this, it was expected that application of the above reaction sequence to an epoxyacid would afford a NAD^+ analogue carrying at the exocyclic adenine amino group an ω-carboxyalkyl side-chain. Indeed, using 3,4-epoxybutanoic acid as starting material the syn-

thesis of III was achieved. Reaction of NAD^+ with 6,7-epoxyheptanoic or 10,11-epoxyundecanoic acid gave respectively lower or no conversion of NAD^+ (thin-layer chromatography control).

The validity of the approach made for the preparation of active and stable macromolecularized NAD^+ analogues was demonstrated in the present investigation. However, while the coenzymic efficiency of the two soluble IV analogues with lactate dehydrogenase was quite satisfactory, that with alcohol dehydrogenase and alanine dehydrogenase was low. In order to investigate the influence of the nucleotide carrier on the coenzymic efficiency with these and other enzymes the coupling of III to soluble polymers of different kinds is now in progress. More extensive enzymic studies with the soluble derivatives described in the present paper, including coenzymic activity assays with the polyethyleneimine IV derivative in a recycling two-enzyme system, will be reported in a forthcoming publication (W. Marconi *et al.*).

Recently, in a preliminary experiment in which higher III and carbodiimide : NAD^+ ratios were used, a polyethyleneimine IV derivative with a higher content of enzymically reducible NAD^+ (295 μmol/g) was also prepared. In this case the product was purified by gel filtration, since it turned out to be soluble in phosphate buffer. The coenzymic efficiency of the latter NAD^+ analogue will be investigated in the near future.

Since writing this paper a paper describing another coenzymically active and soluble macromolecularized NAD^+ derivative (dextran-NAD^+) appeared [21]. This derivative was prepared by coupling cyanogen-bromide-activated dextran to a NAD^+ analogue carrying an ω-amino side-chain at the exocyclic adenine amino group. Yet this dextran-NAD^+, like the previously [2] described polyethyleneimine-succinyl-NAD^+, was shown to undergo release of nucleotide in aqueous media, albeit at a much slower rate than the latter derivative. The instability of the dextran analogue is probably due to hydrolysis of the linkage to the polymer molecule. In fact, it has now been demonstrated [22] that ligands of the R-NH_2 type, which are covalently bound to polysaccharides by the cyanogen bromide method, are slowly detached hydrolytically from their supports in aqueous media at pH above 5.

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Note Added in Proof (May 5, 1975). In recent experiments, in which III and the soluble polyethyleneimine and polylysine IV derivatives were tested with the yeast alcohol dehydrogenase under modified conditions, the last two derivatives gave striking higher rates of enzymic reduction relative to NAD^+ (1.0 and 0.23 respectively; $NAD^+ = 1$), while for III the rate remained unchanged. Details on these experiments too will be reported in the cited forthcoming publication (W. Marconi *et al.*).

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