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Carbanicotinamide Adenine Dinucleotide: Synthesis and Enzymological Properties of a Carbocyclic Analogue of Oxidized Nicotinamide Adenine Dinucleotide†

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ABSTRACT: The dinucleotide carbanicotinamide adenine dinucleotide (carba-NAD), in which a 2,3-dihydroxycyclopentane ring replaces the β-D-ribonucleotide ring of the nicotinamide ribonucleoside moiety of NAD, has been synthesized and characterized enzymologically. The synthesis begins with the known 1-aminoribose analogue (±)-4β-amino-2α,3α-dihydroxy-1β-cyclopentanemethanol. The pyridinium ring is first introduced and the resultant nucleoside analogue specifically 5′-phosphorylated. Coupling the racemic carbanicotinamide 5′-mononucleotide with adenosine 5′-monophosphate produces two diastereomeric carba-NAD analogues which are chromatographically separable. Only one diastereomer is a substrate for alcohol dehydrogenase and on this basis is assigned a configuration analogous to D-ribose. The reduced dinucleotide carba-NADH was characterized by fluorescence spectroscopy and found to adopt a "stacked" conformation similar to that of NADH. The analogue is reduced by both yeast and horse liver alcohol dehydrogenase with K_m and V_{max} values for the analogue close to those observed for NAD. Carba-NAD is resistant to cleavage by NAD glycohydrolase, and the analogue has been demonstrated to noncovalently inhibit the soluble NAD glycohydrolase from *Bungarus fasciatus* venom at low concentrations (≤100 μM).

Nicotinamide adenine dinucleotide (NAD) is essential to all cellular metabolism as a cosubstrate in biological hydride-transfer reactions. More recently it has become clear that NAD has a second role in metabolic regulation. NAD is known to serve as a substrate for a series of related enzymatic reactions in which the adenosine diphosphate ribose (ADP-ribose) moiety is transferred to a nucleophilic acceptor or to water with concomitant release of nicotinamide (Ueda & Hayaishi, 1985; Hayaishi & Ueda, 1982). The reaction is driven by the exothermic hydrolysis of the pyridinium–N-glycosidic bond of NAD (Hayaishi & Ueda, 1977). The prototypical enzymes in this family are the NAD glycohydrolases, which catalyze the hydrolysis of NAD to yield nicotinamide, ADP-ribose, and a proton. The NAD glyco-

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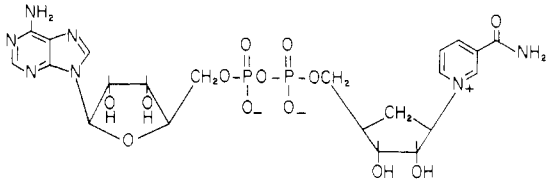
hydrolases are ubiquitously distributed and have been purified from prokaryotic as well as from eukaryotic sources. Additionally, mono- and poly(ADP-ribose) transferases are now known that catalyze the posttranslational modification of a variety of proteins. Although the function of these reactions is still unknown, evidence has begun to accumulate suggesting that this class of modification constitutes an important regulatory mechanism (Vaughan & Moss, 1981).

One strategy with which to illuminate the biological function of this important yet poorly understood class of enzymes is to produce potent and highly specific inhibitors for individual enzymes in the family. Such inhibitors could be used as tools with which to probe the active site structure and the regulation of a purified ADP-ribosyl transferase. They could serve as stable ligands for the development of affinity purification techniques and from which photoaffinity reagents could be designed. Finally, inhibitors can potentially be used to explore the role of these enzymes both in vitro and in vivo by enabling the experimentor to specifically inhibit the target enzyme and thereby directly evaluate proposed biological functions.

The carbocyclic dinucleotide carba-NAD (**1**) in which a



Carba-NAD, I

2,3-dihydroxycyclopentane ring replaces the β-D-ribonucleotide ring of the nicotinamide ribonucleoside moiety of NAD is the first of a series of such analogues which are predicted to be inhibitors of ADP-ribosyl transfer. The structure is modified to cause the C–N bond to be resistant to cleavage. Otherwise, the analogue is expected to resemble NAD in that its overall charge and shape will be maintained and in that it will be able to function as a hydride acceptor. We expect therefore that carba-NAD and its relatives will constitute an important set of mechanism-based inhibitors for NAD glycohydrolases as well as for the related ADP-ribosyl transferases.

Carba-NAD will additionally contribute to the study of the relation of nucleotide structure to reactivity in hydride-transfer reactions catalyzed by dehydrogenases. Although many chemically modified NAD analogues have been synthesized and examined in this regard, relatively few of these either are modified in the carbohydrate moiety or are less oxidizing than NAD (Anderson, 1982). Carba-NAD will therefore be a useful addition to the existing set of NAD analogues that will enable us to extend the structure–activity relations for this important family of enzymes.

Experimental Procedures

General Methods. Melting points were determined in open capillaries with a Thomas-Hoover apparatus and are uncorrected. The proton NMR spectra were recorded either at 90 MHz on a JEOL FX 90-Q spectrometer or at 300 MHz on a General Electric GN 300 spectrometer. Carbon NMR spectra were determined at 22.5 or at 75 MHz, respectively. Chemical shifts are reported in ppm from an internal standard of tetramethylsilane (TMS) (for organic solutions) or sodium 3-(trimethylsilyl)[2,2,3,3-$^2H_4$]propionate (TSP) (for aqueous solutions) unless otherwise indicated. Assignment of the ^1H and ^{13}C signals was accomplished with 2D NMR techniques (Benn & Gunther, 1983) and the attached proton test (Patt & Shoolery, 1982).

High-pressure liquid chromatography (HPLC) was performed on a system consisting of two LDC/Milton Roy constaMetric metering pumps, a LDC/Milton Roy Model 1601 gradient controller and dynamic mixer, and a Model 1203 A UV Monitor III fixed-wavelength detector operated at 254 nm. Products were analyzed on an RSIL AN, 10-μm anion-exchange column (4.6 × 250 mm, Alltech, Deerfield, IL) with a mobile phase of 50 mM KH_2PO_4, pH 3.5, at a flow rate of 1 mL/min (system A). The second anion-exchange system utilized a 10-μm DEAE Si 100 Polyol (4.6 × 250 mm, Serva, Heidelberg) column with a mobile phase consisting of a linear gradient from 50 to 250 mM KH_2PO_4, pH 3.5, formed over 10 min and at a flow rate of 2 mL/min (system B). Analysis of products by reversed-phase chromatography was performed on a DYNAMAX 8-μm C18 column (4.6 × 250 mm, Rainin, Woburn, MA) developed isocratically at a flow rate of 1 mL/min with 20 mM NaH_2PO_4 that had been adjusted to pH 6.0 with tetrabutylammonium hydroxide (system C).

Absorption spectra were determined on a Varian-Cary 219 spectrophotometer. Kinetic measurements were made on a Beckman DU-50 spectrophotometer equipped with a thermostated cell at 30 °C.

Fast atom bombardment mass spectra were acquired on a Finnigan-MAT 212 mass spectrometer in combination with an INCOS 2200 data system. An Ion Tech saddlefield atom gun operating at 8 kV was used with xenon gas. The ion source temperature was approximately 60 °C, and the accelerating voltage was 3 kV. Samples were dissolved in water (10 mg/mL). Thioglycerol (2.5 μL) was first applied to the copper probe tip; then, the sample was added and mixed thoroughly with the matrix. The contribution from the matrix was subtracted from the spectrum.

Methyl 4β-amino-2α,3α-dihydroxy-1β-cyclopentanecarboxylate hydrochloride was prepared from the dihydroxy lactam *exo-cis*-5,6-dihydroxy-2-azabicyclo[2.2.1]heptan-3-one as described by Cermak and Vince (1981). The product methyl ester was isolated and crystallized from methanol and ethyl ether to produce a colorless solid: mp 148.5–150.5 °C [lit. mp 151–153 °C (Cermak & Vince 1981), 146–147 °C (Kam & Oppenheimer, 1981)]; ^1H NMR (300 MHz; D_2O, 15% w/w) δ 1.88 (dt, J = 13.7 and 9.2 Hz, 1 H), 2.59 (dt, J = 13.8 and 8.6 Hz, 1 H), 3.04 (dt, J = 5.06 and 9.0 Hz, 1 H), 3.60 (dq, J = 8.3 Hz, 1 H), 3.76 (s, 3 H), 4.11 (dd, J = 5.4 and 7.1 Hz, 1 H), 4.32 (t, J = 5.4 Hz, 1 H); ^{13}C NMR (75 MHz; D_2O, 15% w/w) δ 30.02, 50.39, 55.65, 57.33, 75.40, 76.95, 178.13.

4β-Amino-2α,3α-dihydroxy-1β-cyclopentanemethanol (**2**) was produced by reduction of the unprotected amino ester with lithium triethylborohydride [Aldrich, 1 M solution in tetrahydrofuran (THF)] following the procedure of Kam and Oppenheimer (1981), to produce the amine triol as a ninhydrin-positive colorless oil, R_f 0.47 [silica; 1-butanol–acetic acid–water (5:2:3)]. To isolate the amine free from salt, the unpurified product from the reduction of 2.11 g (10 mmol) of starting ester was dissolved in 20 mL of water and applied to a column of Dowex 50 H^+ form (50 mL). The resin was washed with water until the effluent was free from chloride ion. The free amine was then eluted with 1 M ammonium hydroxide and the solvent removed in vacuo to yield the salt-free amorphous amine (1.4 g, 9.5 mmol, 95% yield). This material was directly used for the next reaction.

1-(2,4-Dinitrophenyl)-3-carbamoylpyridinium chloride (**3**) was prepared from 3-carbamoylpyridine and 2,4-dinitrochlorobenzene as described by Slama et al. (1984), with the

exception that the chloride salt was purified by crystallization from 1-butanol.

1-[2β,3β-Dihydroxy-4α-(hydroxymethyl)cyclopent-1α-yl]-3-carbamoylpyridinium Chloride (**4**, *Carbanicotinamide Nucleoside*). A solution of carbocyclic amine (**2**, 1.2 g, 8.16 mmol) in 25 mL of water was treated with a solution of 1-(2,4-dinitrophenyl)-3-carbamoylpyridinium chloride (**3**, 2.25 g, 6.94 mmol) in 10 mL of water, added dropwise with a Hershberg addition funnel. The addition of the pyridinium salt was conducted over a period of several hours. After the addition was complete, the reaction was stirred overnight at ambient temperature. The next day, precipitated 2,4-dinitroaniline was removed from the highly colored mixture by filtration, and the resulting supernatant was extracted 3 times with 50-mL portions of chloroform. The aqueous layer was evaporated to yield 1.58 g of a yellow glass. Analysis of this material by HPLC (anion exchange, system A) shows three major components with retentions of 2.8 min (nucleoside), 3.0 min [1-(2,4-dinitrophenyl)-3-carbamoylpyridine], and 4.7 min (unidentified). Although demonstrably impure, this material can be used successfully in the subsequent phosphorylation.

Highly pure nucleoside can be obtained by preparative liquid chromatography on a reversed-phase column (Rainin Dynamax C-18, 21.4 mm × 250 mm, with additional guard column). Nucleoside (ca. 50 mg) was applied to the column as a solution in 50 mM aqueous potassium phosphate at pH 3.5. Chromatography was developed with this same buffer at a flow rate of 3 mL/min. The nucleoside was eluted as a single peak after 18–20 min. Highly colored hydrophobic impurities were removed from the column before the next injection by application of a linear gradient of acetonitrile (0–20%) and phosphate buffer. Washes containing the nucleoside were evaporated to dryness, and the product was extracted from the salt by trituration with methyl alcohol. The methanolic extracts were evaporated, and the residue was dissolved in a small quantity of water and applied to a column of Dowex-50 W-X4, H^+ form (column volume 50 mL, diameter 10 mm). The resin was washed with 2 bed volumes (50 mL) of distilled water to remove the remaining potassium phosphate. The nucleoside as the chloride salt was eluted into 1 M HCl. Evaporation of the solvent resulted in the isolation of 1.22 g (4.24 mmol, 52% of theory) of **4** as a colorless, amorphous solid; ^1H and ^{13}C NMR are reported in Tables I and II. ^1H NMR is an agreement with that reported for this compound by Sicsic et al. (1986). Fast atom bombardment mass spectrum. Calcd for $C_{12}H_{17}O_4N_2$: m/z 253. Found: m/z 253 (M^+).

The specific complexation of the *cis*-glycol of the nucleoside can be used in an alternative purification. The nucleoside (340 mg) is dissolved in 5 mL of ammonium acetate (pH adjusted to 8.8 with ammonium hydroxide). A dark brown precipitate forms immediately and is removed by centrifugation. [This is caused by the reaction of excess (2,4-dinitrophenyl)-nicotinamide with ammonia to produce highly colored 2,4-dinitroaniline. It is not indicative of any decomposition of the nucleoside.] The solution was applied to a 30-mL column of an affinity absorbant containing covalently bound (3-aminophenyl)boronic acid (6 g, Bio-Rad Affi-Gel 601, Bio-Rad Laboratories, Richmond, CA), which had been equilibrated and packed in 1 M ammonium acetate, pH 8.8. The column was washed with this buffer until no more yellow color was observed (ca. 160 mL) and washed with 1 bed volume of water to remove the salts. The nucleoside was eluted in 30–40 mL of 50 mM ammonium acetate, pH 4.5. Repeated lyophilization produced salt-free nucleoside.

Carbanicotinamide Mononucleoside 5′-Phosphate (**5**). Carbocyclic nucleoside (**4**, 816 mg, 2.83 mmol) was dissolved in 3 mL of trimethyl phosphate (Aldrich, freshly distilled in vacuo from above BaO). The resulting solution was cooled in an ice/water bath, stirred magnetically, protected from moisture under a blanket of nitrogen, and treated with 1.6 mL of a 1:1 (v/v) mixture of phosphoryl chloride and trimethyl phosphate (1.34 g, 8.7 mmol of $POCl_3$, distilled immediately prior to use). The phosphoryl chloride solution was prepared at 0 °C under nitrogen and added to the solution of the nucleoside in a single portion. After 3 h of stirring, the mixture was examined by HPLC (anion exchange, system B). The analysis showed that the starting nucleoside which eluted as a single peak at 2.5 min had been completely converted to a single new product with a retention time of 3.3 min. The reaction was quenched by the addition of 1 mL of water and several small pieces of ice. The product was precipitated from this mixture by the addition of 80 mL of ice-cold acetone. After this was allowed to stand 1 h at 0 °C, the precipitate was collected by centrifugation, dissolved in water, and lyophilized. The 5′-monophosphate was purified by ion-exchange chromatography on Whatman DE-52 cellulose (column dimensions 16 mm × 75 cm; column had been preequilibrated by adjustment of the resin pH to 7.5 prior to pouring the column and subsequently washing the column with 1000 mL of low ionic strength buffer before the chromatography). The sample was applied dissolved in 10 mM ammonium carbonate buffer, pH 7.5 (adjusted with CO_2), and the column washed with 1 bed volume of the same buffer to remove any nonbinding materials. The column was developed with a linear gradient formed between 300 mL of 10 mM ammonium phosphate buffer and 300 mL of 80 mM ammonium phosphate buffer, both adjusted to pH 7.5 with gaseous CO_2. Fractions containing the mononucleotide were combined and lyophilized repeatedly to yield 566 mg (1.7 mmol, 60% yield) of a colorless amorphous solid. The electronic absorption spectrum of the nucleoside 5′-phosphate dissolved in neutral water exhibited a maximum at 267 nm ($\epsilon = 4.92 \times 10^3$ M^{-1} cm^{-1}). Carbanicotinamide mononucleoside 5′-phosphate formed a cyanide adduct when dissolved in 1 M aqueous KCN, exhibiting a maximum at 342 nm ($\epsilon = 3.19 \times 10^3$ M^{-1} cm^{-1}); ^1H and ^{13}C NMR spectra are reported in Tables I and II. Fast atom bombardment mass spectrum. Calcd for $C_{12}H_{17}N_2O_7P$: m/z 332. Found: m/z 333 (MH^+) and 252 ($M - HPO_3$).

1′,2′-Di-O-acetylcarbanicotinamide Mononucleoside 5′-Phosphate (**6**). A solution of 10 mL of pyridine (dried by distillation from BaO) and 10 mL of acetic anhydride (distilled from above diphosphorus pentoxide) was stirred magnetically in a water bath at 10–15 °C. Carbanicotinamide mononucleotide (**5**, 180 mg, 0.5 mmol) was dissolved in 0.5 mL of water and added to the vigorously stirred mixture of pyridine and acetic anhydride rapidly and in a single portion with a syringe. After 30 min of stirring, the precipitated nucleotide dissolved completely. Stirring was continued an additional 30 min at which time the reaction was worked up by evaporation of the reactants in vacuo. The resulting oil was evaporated 3 times from 5-mL portions of dimethylformamide to ensure the complete removal of the reactants. The residue was next dissolved in 5 mL of water and 5 mL of pyridine for 5 h at ambient temperature to hydrolyze any remaining 5′-anhydride. Solvent was removed in vacuo, and the residue was dissolved in water and lyophilized. The product was purified by size-exclusion chromatography on a column of Sephadex LH-20 (1.5 × 120 cm, 200-mL volume) developed with methanol. The diacetylated nucleotide was eluted in the

fraction between 70 and 112 mL. The fractions containing the product were pooled, and the solvent was evaporated and lyophilized from water to produce 195 mg (0.47 mmol) of a white powder: ^{1}H NMR (90 mHz, D_2O, referenced to HDO at 4.57) δ 2.02 (s, 3 H), 2.19 (s, 3 H), 2.1–2.9 (m, 3 H), 4.1 (m, 2 H), 5.4–5.6 (m, 3 H), 8.25 (t, J = 8 Hz, 1 H), 8.98 (d, J = 8 Hz, 1 H), 9.23 (d, J = 6 Hz, 1 H), 9.45 (s, 1 H). The diacetyl nucleotide is unstable and must be used immediately in the next reaction.

Di-n-butylphosphinothioyl bromide was prepared by bromination of tetra-*n*-butyldiphosphine disulfide following the procedure of Furusawa et al. (1976). The product was isolated as a liquid: bp 110–120 °C at 1–2 mmHg [lit. bp 143–144 °C at 6 mmHg (Furusawa et al., 1976)]; ^{13}C NMR (22.5 MHz, benzene-d_6) 13.60 (s), 23.16 (d, J = 18.3 Hz), 25.46 (d, J = 4.9 Hz), 41.8 (d, J = 50.0 Hz).

Adenosine 5′-(phosphoric di-n-butylphosphinothioic anhydride) (**7**) was prepared from adenosine monophosphate and the above phosphinothioyl bromide following the procedure of Furusawa et al. (1976) with modification in the isolation. Adenosine monophosphate (1.73 g, 5.0 mmol) and morpholino-*N*,*N*′-dicyclohexylcarboxamidine (1.47 g, 5.0 mmol, Aldrich) were suspended in dry pyridine (distilled from above BaO) and dissolved by brief heating on a steam bath. On cooling to room temperature, a suspension formed, which was stirred magnetically and treated with the phosphinothioyl bromide (2.55 g, 10 mmol), added dropwise. By the end of the addition a solution had formed. The flask was stoppered and stirred at room temperature for an additional 3 h, when the solvent was evaporated under reduced pressure to recover an oil. The oil was dissolved in water and extracted 3 times with ether. The combined ether layers were back-extracted 3 times with water and the organic layers discarded. The combined aqueous extracts were evaporated to produce an amorphous solid, to which acetonitrile was repeatedly added and evaporated in vacuo. After several such treatments, a solid resulted, which was triturated with acetonitrile 2 times and dried under vacuum. The result was isolation of a colorless solid, which was homogeneous by reversed-phase TLC (Analtech reversed-phase plate, developed with 20% acetonitrile in water; R_f 0.29; AMP R_f 0.9).

Carbanicotinamide Adenine Dinucleotide (**1**, *Carba-NAD*) *and Pseudocarbanicotinamide Adenine Dinucleotide* (**8**, *ψ-Carba-NAD*). Racemic diacetylcarbanicotinamide mononucleotide (**6a** and **6b**, 100 mg, 0.24 mmol) and adenosine 5′-phosphoric di-*n*-butylphosphinothioic anhydride) (**7**, 252 mg, 0.48 mmol) were dissolved in 1 mL of dry DMF and 2 mL of dry pyridine, and the solvent was removed under reduced pressure to remove traces of moisture. The process was repeated 2 times. The resulting oil was dissolved in 1 mL of dry DMF (distilled from BaO at reduced pressure and stored above 4A molecular sieves) and 4 mL of pyridine (distilled from above BaO and stored above 4A molecular sieves). It is important to dissolve the oil in the DMF before the addition of the pyridine. Finely powdered silver nitrate (326 mg, 1.92 mmol) was next added to the resulting solution. The flask was immediately capped with a septum, purged with nitrogen, wrapped in Al foil to protect the contents from light, and allowed to stir magnetically at ambient temperature for 36 h. At the end of this time the mixture was dissolved in 15 mL of water and hydrogen sulfide bubbled through the solution vigorously for 15 min. The precipitated silver sulfide was removed by filtration (Buchner funnel through celite filter aid) and the filtrate extracted 3 times with 25-mL portions of chloroform. The organic extracts were combined, washed once with 25 mL of water, and discarded. The combined aqueous portions were lyophilized and the lyophilizate was suspended in 60 mL of methanolic ammonia (3 N) and stirred magnetically at 5–6 °C for 6 h to complete the removal of the acetyl groups from the dinucleotide. The solvent was evaporated under reduced pressure at room temperature to leave an amorphous solid. Analysis of this material by HPLC (anion exchange, system B, run as a linear gradient from 50 to 250 mM KH_2PO_4, pH 3.5, over a period of 10 min) shows three major components identified as pyridine (retention time 3 min), carba-NAD (6.5 min), and P^1,P^2-bis(5′-adenosyl)diphosphate (13 min). The crude product was purified by ion-exchange chromatography on DE-52 cellulose (Whatman, 16 mm × 75 cm, 190-mL column volume), equilibrated with 10 mM ammonium carbonate buffer, pH 7.5. The sample was applied in low ionic strength buffer. The chromatography was developed starting with the application of 1 bed volume (190 mL) of low ionic strength buffer and was followed by the application of a linear gradient formed between 400 mL of 10 mM ammonium carbonate, pH 7.5, and 400 mL of 200 mM ammonium carbonate at the same pH. The single major UV-absorbing component which eluted at the approximate center of the gradient was isolated. Desalting with ion exchange followed by repeated lyophilization from distilled water produced 80 mg (0.12 mmol, 50% yield) of a fine white powder. This material produced a single peak when examined chromatographically by HPLC system B. When examined by reversed-phase HPLC (system C), the product was resolved into two equal peaks, which eluted at 19 and 22 min. The electronic absorption of the mixture of carba-NAD and its diastereomer [0.5 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), 0.5 M ethanol, pH 10] exhibits a maximum at 260 nm (ϵ = 15.4 × 10^3 M^{-1} cm^{-1}). Upon reduction (yeast alcohol dehydrogenase), a second absorption appears at 360 nm (ϵ = 3.1 × 10^3 M^{-1} cm^{-1}; A_{260}/A_{342} = 4.56). The spectrum of the dinucleotide in 1 M KCN exhibits maxima at 260 and 342 nm (ϵ = 3.98 × 10^3 M^{-1} cm^{-1}; A_{260}/A_{342} = 2.94). ^{1}H NMR (300 mHz, D_2O): signals attributed to the 2,3-dihydroxycyclopentane ring, δ 2.20–2.27 (m, 1 H), 2.46–2.48 (m, 1 H), 2.69–2.80 (m, 1 H), 4.05–4.11 (m, 1 H), 4.15–4.20 (m, 1 H), and 5.10 (J = 9 Hz, 1 H); signals assigned to the pyridinium, 8.24 (t, J = 6 Hz, 1 H), 8.92 (d, J = 7 Hz, 1 H), 9.19 (d, J = 7 Hz, 1 H), and 9.41 (s, 1 H); signals assigned to the adenosyl anomeric protons, δ 6.13 (d, J = 5.4 Hz, 0.5 H) and 6.15 (d, J = 5.4 Hz, 0.5 H); signals attributed to the adenine, δ 8.40 (s, 1 H) and 8.61 (s, 1 H). The region between 4.2 and 4.9 ppm is complex and contains the signals of the remaining five ribosyl protons, the signals of the remaining two cyclopentane protons, and an intense HDO absorption. ^{13}C NMR (75 MHz, D_2O): signals attributed to the 2,3-dihydroxycyclopentane ring, δ 31.71, 45.99, 68.06, 74.98, 78.71, and 79.90; signals attributed to the β-D-ribonucleotide ring, 69.67, 73.01, 77.54, 86.86 (d, J = 5.9 Hz), and 90.55; signals attributed to the 3-carbamoylpyridinium, 131.53, 136.74, 145.78, 147.79, 148.42, and 168.20; signals attributed to the adenosyl, 120.94, 144.62, 149.24, 151.00, and 153.47. Fast atom bombardment mass spectrum: Calcd for $C_{22}H_{29}N_7O_{13}P_2$: m/z 661. Found: m/z 662 (MH^+) and 333 ($M - C_{10}H_{11}N_5O_6P$).

Separation of the Dinucleotide Diastereomers: *Carba-NAD* (**1**) *and Pseudocarba-NAD* (**8**). The mixture of dinucleotide diastereomers could be separated by reversed-phase HPLC with two Rainin Dynamax C-18 columns (10-mm diameter × 250 mm) connected in series and equipped with a guard column module (10 mm × 50 mm). The column was equil-

ibrated with a buffer of 20 mM sodium dihydrogen phosphate and 2 mM tetrabutylammonium phosphate adjusted to pH 6.0 with tetrabutylammonium hydroxide. The mixture of dinucleotide (7 mg) was applied to the column and the chromatography developed isocratically at a rate of 3 mL/min. After 45 min, a linear gradient of the above buffer and methanol (from 0 to 10% methanol, formed over 10 min) was applied to the column. Development was continued isocratically with the 10% methanol and buffer mixture. The product carba-NAD eluted first, 15–17 min after the start of the gradient, and pseudocarba-NAD eluted immediately thereafter, at 17–19 min.

Desalting of synthetic dinucleotides can be accomplished by ion-exchange chromatography. Carba-NAD or any related dinucleotide (100 mg) is applied as a solution in distilled water to a column (7 mm × 75 mm, 3-mL bed volume) of Dowex 1X-2, 200–400 mesh, in the formate form. The resin is washed with 3 bed volumes of water to remove the salts. Nucleotide is eluted with 6 bed volumes of 0.2 N formic acid. Lyophilization yields a flocculent white material.

Determination of Extinction Coefficients of Carba-NAD and Carba-NADH. Solutions of carba-NAD were prepared by weight and dissolved in the appropriate solvent. Exact concentrations were determined by measurement of the quantity of organic phosphate according to the Fisk–Subba-Row method (Ames, 1966). A set of potassium phosphate standards covering the expected concentration range and prepared in a buffer identical with that used for the nucleotide was used for calibration of the analysis. To establish the nucleotide concentration, the measured concentration of inorganic phosphate was subtracted from the measured total phosphate and adjusted according to the stoichiometry of the nucleotide. Extinction coefficients were determined on a Cary-Varian 219 spectrophotometer, in a buffer of 0.5 M Tris containing 0.5 M ethanol at pH 10. The spectrum of the oxidized nucleotide was first determined, and yeast alcohol dehydrogenase (20 μL of the 9000 units/mL suspension supplied by Boehringer) was added to obtain the reduced dinucleotide. The increase in the absorbance was complete instantly. The reduction was determined to be complete under these conditions since neither changes in the pH nor trapping the product aldehyde (i.e., by adding hydroxylamine) led to further increases in the dihydronicotinamide absorbance.

Fluorescence absorption and emission spectra were determined on a Perkin-Elmer MPF-44A fluorescence spectrophotometer. Carba-NADH was prepared by enzymatic reduction of the mixture of the diastereomeric carba-NAD and pseudocarba-NAD in a 0.5 M Tris buffer at pH 10 containing 0.5 M ethanol by the addition of yeast alcohol dehydrogenase (10 μL, 90 units of the ammonium sulfate suspension). Enzyme was removed prior to measurement by ultrafiltration (filter centrifugation, Amicon YMT membrane). The concentration of carba-NADH was adjusted to ca. 8–9 μM for measurement (as determined by absorbance at 260 nm). Where appropriate, nucleotide pyrophosphatase (10 μL of a 2 mg/mL solution; Sigma) was added directly to the solution in the fluorescence cuvette (3 mL) to enzymatically cleave the pyrophosphoryl bond.

NAD glycohydrolase from Bungarus fasciatus venom was purified according to the three-step procedure of Yost and Anderson (1981). After the final step of the purification, an Amicon Matrix Gel Blue A affinity purification, the fractions containing the enzyme were pooled, concentrated (using an Amicon ultrafiltration apparatus), and washed free of salt by successive ultrafiltrations. The enzyme was stored frozen in 0.005 M potassium phosphate buffer, pH 7.5, at a concentration of 1–2 units/mL and was found to be stable indefinitely.

Substrate Activity of Carba-NAD for NAD Glycohydrolase. The mixture of carba-NAD (**1**) and ψ-carba-NAD (**8**) was tested as substrate for the NAD glycohydrolase from *B. fasciatus* venom. The reaction contained 479 μmol of the equimolar mixture of **1** and **8** in 0.9 mL of 33 mM KH_2PO_4, pH 7.5. The solution was incubated at 37 °C, and the experiment was initiated by addition of 0.11 unit of the glycohydrolase as a solution in 0.1 mL of 5 mM phosphate buffer, pH 7.5. Aliquots were removed after 30 min, 1 h, 2 h, 4 h, and 24 h and analyzed by HPLC (DEAE Si Polyol, system B, 1 mL/min). There was no evidence either for the release of nicotinamide or for other reaction of the mixture of dinucleotides.

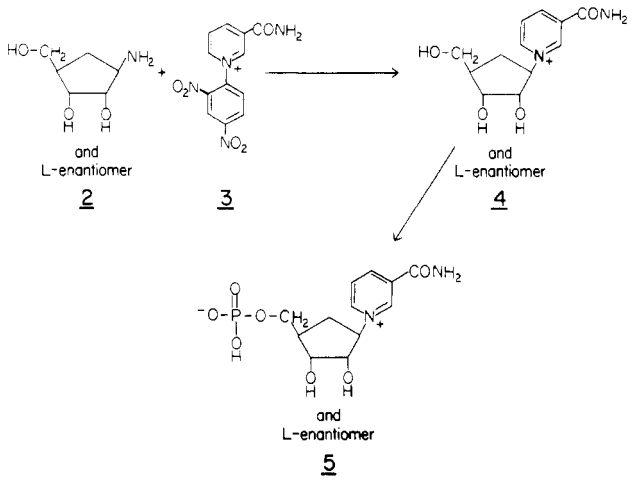
The NAD glycohydrolase was demonstrated to be active by assay immediately after addition to the nucleotide mixture and again after the 24-h incubation. Ther was no decrease in NAD glycohydrolase activity during incubation.

Assay of NAD Glycohydrolase Activity. The activity of the NAD glycohydrolase was measured by determining the rate of release of [*carbonyl*-^{14}C]nicotinamide from [*carbonyl*-^{14}C]NAD according to the procedure described by Moss and Vaughan (1984) [see also Moss et al. (1976)]. A typical assay was conducted in 0.3 mL of potassium phosphate buffer, pH 7.5, containing 0.015 μmol of NAD, 30 000–50 000 dpm [*carbonyl*-^{14}C]NAD and 0.35 milliunit of NAD glycohydrolase and at a temperature of 37 °C. The assay was initiated by the addition of the substrate and incubated for 30 min. At the end this time a 0.2-mL sample was removed and applied to a small column (1 mL) of ion-exchange resin (Bio Rex 1–X2, 100–200 mesh, chloride form, purchased from Bio-Rad Laboratories, Richmond, CA) packed in a 6-in. Pasteur pipet. The column was allowed to drain into a scintillation vial and was washed 5 times with 1-mL portions of 20 mM Tris buffer, pH 7.5. A liquid scintillant was added to the combined effluent (10 mL, Liquiscint, National Diagnositcs, Sommerville, NJ) and the radioactivity released as [*carbonyl*-^{14}C]nicotinamide determined by counting the homogeneous mixture.

Yeast alcohol dehydrogenase was purchased from Boehringer-Mannheim (Indianapolis, IN, 300 units/mg) as a suspension in ammonium sulfate. In order to determine the values of K_m and V_{max} for the dinucleotide coenzymes, assay was conducted at 30 °C in 3.0 mL of 0.025 M sodium pyrophosphate, pH 8.8, containing 0.33 M ethanol. For NAD, the dinucleotide concentration was varied from 0.18 to 0.73 mM (that is, 0.55–2.2 μmol of NAD per assay). The reaction was initiated by addition of 0.04 unit of enzyme, and the resulting increase in absorbance at 340 nm due to the production of NADH was measured spectrophotometrically for 5 min. The linear portion of this increase was used to determine the initial rate of reduction. The number of micromoles of NADH produced was obtained with the extinction coefficient $\epsilon = 6.2 \times 10^3$ M^{-1} cm^{-1}. For the mixture of carba-NAD and ψ-carba-NAD, an identical assay was conducted except that a 1-mL volume was used. Total dinucleotide concentration was varied from 0.57 to 1.42 mM (0.57–1.42 μmol of total dinucleotide per assay). The reaction was initiated by the addition of 0.036 unit of enzyme, and the linear increase in absorbance at 360 nm was determined. The number of micromoles of carba-NADH produced was obtained with the extinction coefficient $\epsilon = 3.1 \times 10^3$ M^{-1} cm^{-1}.

The initial reaction rates were plotted according to the method of Lineweaver and Burk, and in all cases satisfactory

Scheme I[a]



[a] For the sake of clarity, only the D enantiomer has been drawn for compounds **2**, **4**, and **5**. These are actually present as the racemate.

straight lines were obtained. The final published values for K_m and V_{max} were determined by fitting initial rates to a hyperbolic V vs [S] plot with a computer program supplied by Dr. F. J. Kezdy (Department of Biochemistry, University of Chicago) adapted from the procedure described by Yamaika et al. (1981).

The rate of reduction of NAD and carba-NAD changed by less than 4% when the concentration of ethanol was increased from 0.33 to 1.0 M, indicating that the ethanol concentration was saturating. The concentrations of NAD and carba-NAD employed for this determination were 4.4 and 1.7 mM, respectively.

Alcohol dehydrogenase from horse liver was supplied by Sigma Chemical Co. (St. Louis, MO). In order to determine the values of K_m and V_{max} for the nucleotide coenzymes, assay was conducted at 30 °C in 3.0 mL of 0.025 M sodium pyrophosphate buffer, containing 0.33 M ethanol. For NAD, dinucleotide concentration was varied from 9.2 to 46.3 μM (that is, from 28 to 139 nmol per assay). The reaction was initiated by the addition of 8 milliunits of enzyme. The initial rate of reduction was determined by recording the linear increase in fluorescence emission (excitation 340 nm, emission 450 nm) due to the production of NADH. The relative fluorescence was related to the quantity of NADH by calibration at 0% and at 100% reduction.

For the mixture of carba-NAD and ψ-carba-NAD, an identical assay was conducted except that total dinucleotide concentration was varied from 6.7 to 33.3 μM (20.1 to 100 nmol per assay), and a fluorescence excitation of 360 nm was employed.

The initial rates of reaction were analyzed by the method of Lineweaver and Burke and subsequently fit to a hyperbolic V vs [S] plot as described above.

The rate of reduction of NAD and carba-NAD changed by less than 6% upon increasing the concentration of ethanol from 0.33 to 1.0 M, indicating that ethanol concentration was saturating for all of the measured rates. The concentrations of NAD and carba-NAD employed for this determination were 0.94 and 0.90 mM, respectively.

RESULTS

Synthesis and Characterization of Carba-NAD. The overall synthetic scheme for preparation of carba-NAD (**1**) is shown in Scheme I and in Scheme II. Our synthetic effort to **1** begins with the carbocyclic analogue of 1-aminoribose: *dl*-4β-amino-2α,3α-dihydroxy-1β-cyclopentanemethanol (**2**). This compound was originally described from a lengthy synthesis (Shealy & Clayton, 1969). Subsequently, several shorter and more efficient routes were devised (Bindu Madhavan & Martin, 1986). Through combining elements of two of these conversions (Kam & Oppenheimer, 1981; Cermak & Vince, 1981), we are now able to start with cyclopentadiene and tosylcyanide and produce **2** in four steps in an overall yeild of 39%, and in multigram quantities.

Primary amine **2** was converted to the nicotinamide mononucleoside analogue **4** by treating it with 1-(2,4-dinitrophenyl)-3-carbamoylpyridinium chloride (**3**). This conversion is an application of the well-known Zincke reaction, in which pyridinium salts are synthesized from a primary amine on reaction with any one of a variety of 1-(2,4-dinitrophenyl)-pyridinium salts (Lettre et al., 1953; de Gee et al., 1974). The yield and purity of **4** proved troublesome. We initially conducted the conversion using the fluoroborate salt of **3** and in a solvent of acetonitrile and alcohol, a system that had proved superior in previous work (Slama et al., 1984). Although an apparently crystalline fluoroborate salt of **4** was isolated and satisfactory spectroscopic as well as analytical data were obtained, it was shown by HPLC analysis that this material was

Scheme II

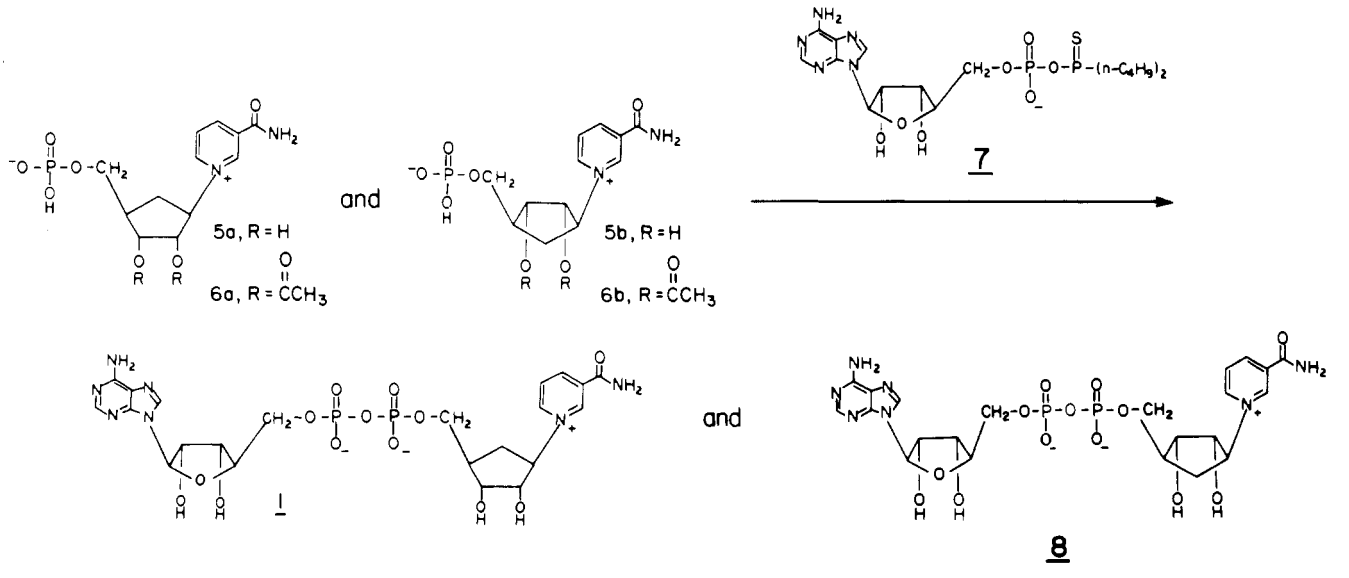
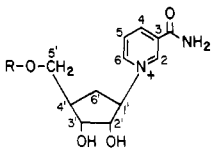


Table I: ^{1}H NMR Chemical Shifts (ppm) and Assignments[a]



and

L-enantiomer

4, R = H

5, R = PO_3H^-

Nucleoside 4

	δ from DSS	mult	coupling constants (Hz)	int
aliphatic carbon no.[b]				
1′	5.10	ddd	8.1, 9.3, and 10.6	1 H
2′	4.45	dd	6.0 and 9.4	1 H
3′	4.13	dd	3.1 and 6.0	1 H
4′	2.36	m		1 H
5′	3.76	d	6.0	2 H
6′a	2.00	ddd	8.7, 10.8, and 13.1	1 H
6′b	2.71	dt	13.1 and 8.2	1 H
aromatic carbon no.				
2	9.40	s		1 H
4	8.96	dt	1.2 and 8.2	1 H
5	8.27	dd	6.4 and 8.1	1 H
6	9.16	dt	1.1 and 6.3	1 H

Nucleotide 5

	δ from TSP	mult	coupling constants (Hz)	int
aliphatic region carbon no.				
1′	5.10	q (br)	≃9	1 H
2′	4.57	dd	5.4 and 9.5	1 H
3′	4.21	dd	1.9 and 5.4	1 H
4′	2.45	br		1 H
5′a	3.93	m		1 H
5′b	3.99	m		1 H
6′a	2.18	ddd	7.3, 10.3, and 13.6	1 H
6′b	2.77	dt	8.9 and 13.6	1 H
aromatic region carbon no.				
2	9.45	s		1 H
4	8.95	d	7.6	1 H
5	8.26	dd	6.5 and 7.4	1 H
6	9.22	d	6.2	1 H

[a] Spectra were obtained at 300 MHz in 5-mm tubes and with D_2O. [b] The carbocyclic rings in the nucleoside and nucleotide analogues have been numbered by analogy to the ribosyl-containing compounds. Carbon 6 designates the bridging methylene which has been substituted for oxygen.

highly impure. Subsequently, we determined that the production of **4** is best accomplished with the chloride of **3**, with water as a solvent, and with a slight excess of starting amine **2**. Purification of nucleoside **4** can be accomplished either with a semipreparative reversed-phase HPLC system (C-18, isocratic development using pH 3.5 aqueous sodium phosphate) or through specific complexation of the *cis*-glycerol to a polymeric absorbant containing covalently bound (*p*-aminophenyl)boronic acid.

Selective monophosphorylation of **4** was accomplished in high yield with phosphorus oxychloride dissolved in trimethyl phosphate (Yoshikawa et al., 1967, 1969; Kusashio et al., 1968), to produce the carbanicotinamide 5′-mononucleotide **5**. In our hands this system proved to be superior to that using phosphoryl chloride in mixtures of acetonitrile, pyridine, and water (Sowa & Ouchi, 1975). The nucleotide 5′-phosphate was purified by ion-exchange chromatography on DEAE-cellulose with gradient elution (10–80 mM ammonium carbonate, pH 7.5). A careful characterization of the product **5**, was particularly important since phosphorylation under these conditions can produce significant quantities of 2′- and 3′-phosphates, as well as diphosphates (Dawson et al., 1977).

Analysis of the ^{1}H and ^{13}C NMR spectra of nucleoside **4** not only supports its assigned structure but enables us to unambiguously deduce the site of phosphorylation on reaction of nucleoside **4** with $POCl_3$. The ^{1}H NMR of **4** in D_2O at 300 MHz consists of seven aliphatic and four aromatic signals (Table I). Of the aliphatic resonances, the two-proton doublet at 3.76 ppm can unambiguously be assigned to the 5′-(hydroxymethyl). The aliphatic absorption found furthest downfield at 5.10 ppm is reasonably assigned to the hydrogen attached to carbon 1′, which also bears the strongly deshielding pyridinium. The patterns for these and the remaining signals are pseudo first order, and consideration of the chemical shifts, coupling constants, and splitting permits the assignment of signals presented in Table I. The assignments are confirmed by two-dimensional NMR techniques for homonuclear chemical shift correlation [the so-called COSY experiment; see Benn and Gunther (1983)]. Once a set of proton assignments is made, assignment of the ^{13}C signals can be accomplished by the two-dimensional NMR technique for heteronuclear correlation [the ^{13}C–^{1}H correlation experiment; see Benn and Gunther (1983)] in conjunction with the attached proton test (Patt & Shoolery, 1982). The resulting ^{13}C assignments are presented in Table II.

Treatment of nucleoside **4** with $POCl_3$ leads to the production of a single product, purified by ion-exchange chromatography on DEAE-cellulose. Such material was homogeneous when examined by two different HPLC systems. The spectroscopic properties of this material enabled us to assign the site of phosphorylation. In the ^{1}H NMR, the two-hydrogen doublet at 3.76 ppm of **4** disappeared upon phosphorylation and was replaced by two single proton multiplets centered at 3.93 and 3.99 ppm. This result is explicable if we assume that the 5′-hydrogens, equivalent in **4**, are converted to a set of magnetically nonequivalent signals in **5**, due to hindered rotation about the 4′–5′ carbon–carbon bond. The complexity of the signals is further increased by spin coupling of the protons with each other and with phosphorus. Neither the pattern nor the chemical shifts of the signals assigned to the 2′- or 3′-hydrogens change significantly upon phosphorylation, again supporting the exclusive formation of the 5′-phosphate **5**. The analysis of the ^{13}C NMR confirms this conclusion. A signal at 65.51 ppm assigned to the 5′-CH_2OH of **4** was shifted

Table II: ^{13}C NMR Chemical Shifts (ppm) and Assignments

	aliphatic region carbon no.					
compd	1′	2′	3′	4′	5′	6′
4	78.40	74.40	79.49	47.58	65.51	32.03
5	78.84	75.55	80.13	46.03 (d, J = 8 Hz)	68.12 (d, J = 3.4 Hz)	31.95

	aromatic region carbon no.					
compd	2	3	4	5	6	carboxamide
4	145.85	136.91	147.53	131.50	148.13	168.44
5	145.76	136.76	147.71	131.26	148.30	168.45

downfield to 68.12 ppm and was split into a doublet upon phosphorylation. This splitting is the expected result of coupling of the carbon to phosphorus. Splitting due to coupling to phosphorus was also observed in the signal (at 46.03 ppm) of the adjacent 4′-carbon of **5**. The 2′ and 3′ signals of the nucleoside at 74.4 and 79.5 ppm, respectively, were shifted only slightly and remained singlets upon phosphorylation. We therefore conclude that the product of phosphorylation is the 5′-mononucleotide **5**.

Coupling of the racemic 5′-phosphate **5a** and **5b** to adenosine monophosphate to produce the dinucleotide **1** (Scheme II) proved to be the most difficult step in the synthesis. Enzymatic coupling of **5** with ATP catalyzed by bovine liver NMN adenylyltransferase failed to produce any dinucleotide. We next turned to the commonly cited method for synthesis of nucleotide anhydrides: the anion-exchange procedure of Michelson (1964). In this method, the nucleotide phosphate is activated by reaction with diphenyl phosphorochloridate to form the "activated" mixed anhydride. This is treated in a separate step with a phosphate monoanion, which displaces the more acidic diphenyl phosphate to produce the desired anhydride. In our hands, the Michelson procedure produces only low (e.g., less than 9%) yields of dinucleotide, despite repeated efforts to purify reagents and solvents and to optimize the reaction.

An alternative coupling using adenosine 5′-(phosphoric di-*n*-butylphosphinothioic anhydride) (**7**) (Furusawa et al., 1976) was found to be more efficacious. This anhydride is stable even in the presence of water, and therefore, it can be easily purified, characterized, and stored indefinitely. The anhydride can be coupled to NMN or to carba-NMN, **5a** and **5b**, after activation with silver salts in mixed solvents of formamide and pyridine. The reaction is very sensitive to the quality of formamide (purification and drying immediately prior to use is necessary), but with care moderate yields can consistently be obtained.

Both the yield and the reproducibility of dinucleotide formation have been improved significantly by protecting carba-NMN as the 2′,3′-di-*O*-acetyl derivative, **6a** and **6b**, prior to its use in the coupling. The diacetylated mononucleotides **6a** and **6b** were appreciably more soluble in relatively nonpolar DMF/pyridine mixtures than was either the unprotected nucleotide or the nucleotide protected as the acetonide. First, the increased solubility eliminated the necessity to use formamide, a substance that is difficult to reproducably obtain both dry and free of ammonium ion. Second, solvent mixtures containing higher percentages of pyridine can now be employed for the reaction. The coupling proceeds more rapidly and with fewer side reactions in a medium that contains higher percentages of pyridine. In this respect the reaction using phosphinothioic anhydrides resembles the Michelson anion-exchange procedure, where pyridine is also the preferred solvent and where a recent study supports the direct participation of pyridine in the coupling mechanism (Richard & Frey, 1983).

Coupling of the acetylated, racemic carba-NMN (**6a** and **6b**) to the activated adenosine 5′-monophosphate moiety of **7**, leads to the production of two diastereomeric dinucleotides, **1** and **8**, which are separated by reversed-phase chromatography (C-18, using a mobile phase of aqueous sodium phosphate, pH 6, containing tetrabutylammonium ion). One of these diastereomers is a substrate for yeast alcohol dehydrogenase. A configuration in the cyclopentane ring analogous to D-ribose is assigned to the carbocyclic sugar analogue in this diastereomer. The unreduced diastereomer, **8**, containing the L-ribose analogue is designated pseudocarbocyclic NAD (ψ-carba-NAD).

Table III: Reduction of Carba-NAD and NAD by Dehydrogenases

substrate and enzyme	K_m (M)	V_{max} (μmol min^{-1} unit^{-1})
carba-NAD[a] with yeast ADH	$(1.7 \pm 0.4) \times 10^{-3}$	$(2.8 \pm 5) \times 10^{-1}$
NAD with yeast ADH	$(4.5 \pm 0.3) \times 10^{-4}$	$(3.8 \pm 0.1) \times 10^{-1}$
lit.[b]	3.9×10^{-4}	
carba-NAD[a] with equine liver ADH	$(0.76 \pm 0.1) \times 10^{-5}$	$(1.8 \pm 0.7) \times 10^{-1}$
NAD with equine liver ADH	$(2.4 \pm 0.3) \times 10^{-5}$	$(7.4 \pm 0.3) \times 10^{-1}$
lit.[b]	8.0×10^{-5}	

[a] Carba-NAD was supplied as a 1:1 mixture of diastereomers. [b] Anderson et al., 1963.

Spectroscopic Properties. Carba-NAD can be reduced either chemically or enzymatically to the dihydropyridine dinucleotide, carba-NADH. Carba-NADH has an electronic absorption spectra with maxima at 260 and 360 nm. The band at 360 nm, associated with the dihydropyridine chromophore, appears at a longer wavelength than does the corresponding absorption of NADH (at 340 nm). The position of the absorption is in accord with our expectation based upon the behavior of simple 1-alkylated 1,4-dihydronicotinamides, where the absorption maxima occurs at 360 nm (Slama et al., 1984).

The reduced dinucleotide, carba-NADH, exhibits a broad fluorescence emission with a maxima at 456 nm characteristic of the dihydropyridine chromophore (emission maxima for NADH is 454 nm). The fluorescence excitation spectrum of the carbocyclic analogue shows the expected maxima at 360 nm as well as a second weaker maxima at 260 nm. The relative intensities of these bands are roughly the same as is observed for NADH when its fluorescence excitation spectrum is determined under identical conditions. That the 260-nm band is due to an intramolecular fluorescence energy transfer is supported by the observation that the 260-nm excitation of NADH and carba-NADH disappears when the intramolecular pyrophosphoryl linkage is cleaved by nucleotide pyrophosphatase (Weber, 1968; Shifrin & Kaplan, 1959). The observation of this fluorescence energy transfer band in carba-NADH indicates that this analogue is capable of forming an intramolecular complex between the adenine and the dihydropyridine rings in which efficient energy transfer is possible. The similarities in the ratio of the 260-nm excitation to the longer wavelength excitation between carba-NADH and NADH itself further indicated that the amount of "stacked" conformer present in both nucleotides is equivalent. Thus by the criterion of fluorescence spectroscopy, the presence of the carbocyclic ribose does not detectably perturb the solution conformation of the dinucleotide.

Dehydrogenase Reactions. The dinucleotide analogue carba-NAD is recognized as an efficient cosubstrate in the oxidation of ethanol to acetaldehyde catalyzed by either yeast alcohol dehydrogenase or by equine liver alcohol dehydrogenase. The kinetic constants measured for the reduction are presented in Table III. The K_m value obtained for carba-NAD is some 30-fold higher than is the K_m of the natural coenzyme for the yeast enzyme, but is threefold lower than the K_m of NAD for the equine enzyme. The maximal velocities for reduction of carba-NAD by yeast dehydrogenase are approximately the same as those for reduction of NAD, while the rate of reduction of carba-NAD by the equine enzyme is fourfold less than the rate of reduction of NAD.

The diastereomeric pseudocarba-NAD (**8**) is reduced neither by the yeast enzyme nor by the equine liver enzyme The

Table IV: Equilibrium Constant for Ethanol Oxidation by Carba-NAD

pH	K_{eq} of carba-NAD[a]
7.5	2.64×10^{-13}
8.0	2.86×10^{-13}
8.5	2.66×10^{-13}
9.0	4.07×10^{-13}
av K_{eq}:	3.06×10^{-13}
pH	**K_{eq} of NAD[b]**
7.5	1.45×10^{-11}
8.0	1.27×10^{-11}
av K_{eq}:	1.36×10^{-11}
lit. K_{eq}:[c]	1.15×10^{-11}

[a] Conducted in 3.0 mL of 30 mM sodium pyrophosphate adjusted to the indicated pH (NaOH or HCl) containing 147 mM ethanol, 0.148 μmol of carba-NAD, and 45 units of yeast alcohol dehydrogenase. After reaction had ceased, the absorbance at 360 nm was measured to determine the amount of reduced nucleotide. [b] Conducted under conditions identical with that used in footnote *a* except that the ethanol concentration was 16.2 mM and the absorbance was measured at 340 nm. [c] Racker, 1950.

inability of this material to serve as a substrate was first established by reduction of an equimolar mixture of the two diastereomeric nucleotides **1** and **8**. The reduction stopped after consumption of 50% of the oxidized nucleotide, and analysis of the resulting mixture by HPLC revealed that one of the two diastereomeric nucleotides had reacted completely, while the other was not consumed. The inability of the pseudocarba-NAD to serve as a cosubstrate for either dehydrogenase was confirmed by separation of the diastereomeric nucleotides by preparative HPLC. When purified, pseudocarba-NAD (**8**) was unable to serve as a cosubstrate for either dehydrogenase, even when long reaction times under forcing conditions were used.

Equilibrium Constant for Carba-NAD. The substitution of a methylene for an oxygen in the ribosyl ring of NAD is expected to alter the equilibrium value for the reaction

$$\text{ethanol} + \text{NAD} \Leftrightarrow \text{acetaldehyde} + \text{NADH} + \text{H}^+$$

To determine the new position of the equilibrium for carba-NAD, a mixture of ethanol and oxidized nucleotide was equilibrated at constant pH with an excess of yeast alcohol dehydrogenase (Racker, 1950). The concentration of reduced nucleotide present at equilibrium was measured from the absorption at 360 nm. The concentration of acetaldehyde was assumed to be equal to this value by the known stoichiometry. The results of measurement are presented in Table IV. The equilibrium constant for carba-NAD is approximately fortyfold smaller than that of NAD, indicating that the carba-NAD analogue is less oxidizing than the parent coenzyme.

Inhibition of NAD Glycohydrolase. Carba-NAD was tested as a substrate and as an inhibitor of the soluble NAD glycohydrolase from *B. fasciatus* venom. Neither release of nicotinamide nor any other reaction of the dinucleotide could be detected when an equimolar mixture of carba-NAD (**1**) and ψ-carba-NAD (**8**) containing 479 μmol of nucleotide was incubated with 0.15 unit of glycohydrolase at 37 °C for 24 h. The activity of the glycohydrolase was unchanged immediately after addition to the dinucleotide mixture and throughout the incubation, indicating that no time-dependent process of enzyme inactivation occurs.

Carba-NAD was next tested as a noncovalent inhibitor of the *B. fasciatus* venom glycohydrolase. To do this, increasing concentrations of the equimolar mixture of carba-NAD and the diastereomeric pseudocarba-NAD were added to the assay mixtures of the glycohydrolase, and the activity was measured.

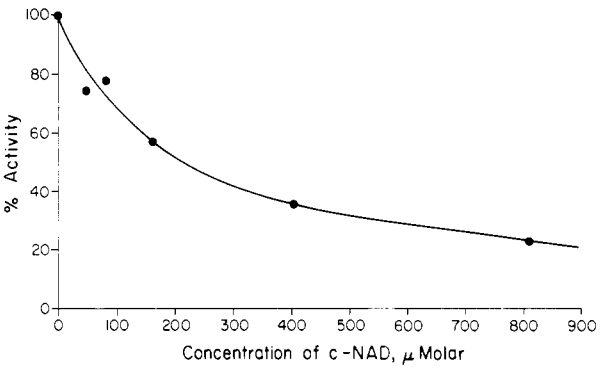


FIGURE 1: Inhibition of the NAD glycohydrolase from *B. fasciatus* by a mixture of carba-NAD and its diastereomer. The assay contained 33 mM KH_2PO_4 (pH 7.5), 50 μM [*carbonyl*-^{14}C]NAD (60000 dpm), and 0.06 milliunit of enzyme in a volume of 0.3 mL. The assay was conducted as described under Experimental Procedures.

The results (Figure 1) show a decrease in enzyme activity with increasing carbocyclic nucleotide, with 50% inhibition achieved at 0.2 mM dinucleotide.

Discussion

Carba-NAD was designed to be an analogue of NAD in which the pyridinium sugar bond was rendered resistant to cleavage by NAD glycohydrolases and ADP-ribosyl transferases. This was accomplished through replacement of the ribosyl ring oxygen with a methylene group. The effect of this substitution is to eliminate the stabilization of the intermediate oxocarbonium ion, which is provided by the ring oxygen, and therefore to raise the activation barrier for pyridinium cleavage. Due to the small structural perturbation involved in replacing a ribosyl oxygen with a methylene group, it was envisioned that carba-NAD would be recognized by NAD-specific binding sites. The replacemet of the ribosyl oxygen with a methylene carbon should have pronounced effect only upon the ability of the dinucleotide to serve as a substrate for an NAD glycohydrolase, in that the pyridinium carbon bond should be more stable and resistant to enzymatic cleavage. If the analogue were bound to the active site of such an enzyme, it would therefore function as an inhibitor.

The properties of carba-NAD have been found to closely resemble those of the parent coenzyme NAD. It is reduced to carba-NADH with dithionite or enzymatically with alcohol dehydrogenase. The reduced nucleotide exhibits the expected electronic absorption spectrum for an alkylated dihydropyridine with a maxima at 360 nm. It has fluorescence emission and excitation spectra that are both qualitatively and quantitatively similar to those of NADH. The fluorescence excitation spectrum is particularly significant in this regard, since the intensity of the excitation at 260 nm has been interpreted as indicating the relative amount of the "stacked" conformation of dinucleotide present at equilibrium. This is the conformation in which the dinucleotide is folded so that the adenosyl ring and the dihydropyridine moiety are in contact. The similarity of the fluorescence emission spectra of carba-NADH to those of NADH indicates therefore that the same amount of folded conformer is present at equilibrium and that the presence of the carbocyclic ribose does not alter the conformation of the dinucleotide.

Carba-NAD has further been shown to be recognized by the binding sites of yeast and equine liver alcohol dehydrogenase. The K_m values for the dinucleotide analogue differ by less than a factor of 20 from that of NAD. Indeed, the K_m value for carba-NAD and the liver enzyme is twofold smaller than that of NAD. The maximum velocities of re-

duction are also identical to within a factor of four. We infer from the similarity of these values that the carbocyclic dinucleotide can occupy the dinucleotide binding site of these enzymes and make all of the intermolecular contacts which are necessary for substrate recognition. Although carba-NAD is significantly less oxidizing than is NAD, this difference in oxidation potential is apparently not reflected in the maximal velocity of reduction for these enzymes. This apparent anomaly is likely due to rate-limiting product release, which has been previously observed with these dehydrogenases (Cleland, 1975).

The substitution of the β-D-ribonucleotide of the nicotinamide ribonucleoside moiety of NAD with *n*-alkyl groups of varying length has previously been explored as a means to produce functional NAD analogues and affinity labels (Göbbeler & Woenckhaus, 1966; Woenckhaus, 1974; Jeck et al., 1973). The best of these derivatives exhibit only weak enzymatic activity when tested as substrates for dehydrogenases. This is attributed both to the changed redox potential and to the decreased binding of the derivative to the protein. Presumably the hydroxyl groups of the ribose play an important role in the interaction of the dinucleotide with the binding site (Woenckhaus, 1974; Jeck et al., 1973). Carba-NAD preserves these important interactions and is demonstrated to be efficiently reduced by both liver and yeast alcohol dehydrogenase, showing saturation at low concentrations of the dinucleotide substrate. Carba-NAD is therefore expected to be a more effective starting point for the development of pyridine nucleotide binding site specific affinity and photoaffinity labels.

Carba-NAD (**1**) and the diastereomeric ψ-carba-NAD (**8**) are not substrates for the soluble venom NAD glycohydrolase. The mixture of **1** and **8** effectively inhibits the enzyme, however. When assayed with NAD concentrations close to the K_m (14 μM; Yost & Anderson, 1981), the concentration of 50% inhibition was 0.2 mM. We expect that carba-NAD itself will be shown to be a more effective inhibitor when sufficient quantities of the pure diastereomer are available for measurement.

The recognition of this series of carbocyclic nucleotides by enzymes is not universal, since bovine liver nicotinamide mononucleotide adenylyltransferase (NAD pyrophosphorylase) is unable to use the carbocyclic mononucleotide analogue as a substrate and so synthesize carba-NAD. None the less, we conclude on the basis of its physical and enzymological properties that carba-NAD resembles NAD closely and is recognized by NAD-specific protein binding sites. Carba-NAD should therefore prove to be a useful tool for the specific inhibition of NAD glycohydrolases and ADP-ribosyl transferases. It will also serve as a lead structure for the design of affinity and photoaffinity labels specific for these enzymes. Last, the activity of the analogue in enzymatic hydride-transfer reactions will enable it to be used in the ongoing studies of the function of the ribosyl sugar in catalysis (Oppenheimer, 1986). An interesting facet of this research will be to explore the effects of the removal of the furanose ring oxygen on dehydrogenase activity and stereochemistry. Specific proposals have been advanced to explain the stereochemical preference of dehydrogenases on the basis of an interaction between the furanose ring oxygen and the ring nitrogen of the reduced nucleotide (Nambiar et al., 1983). Carba-NAD and related carbocyclic nucleotides should prove useful in testing this hypothesis.

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Activation of the $O_2^{\cdot-}$-Generating Oxidase in Plasma Membrane from Bovine Polymorphonuclear Neutrophils by Arachidonic Acid, a Cytosolic Factor of Protein Nature, and Nonhydrolyzable Analogues of GTP†

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ABSTRACT: A reconstitution system for activation of the $O_2^{\cdot-}$-generating oxidase from bovine polymorphonuclear neutrophils (PMN) is described. This system consisted of three components, namely, a particulate fraction enriched in plasma membrane, a supernatant fluid (cytosolic fraction) recovered by high-speed centrifugation from sonicated resting bovine PMN, and arachidonic acid. The pH optimum (7.8) and the K_M value for NADPH (45 μM) of the activated oxidase were virtually the same as those found in the purified enzyme. All three components had to be present during the preincubation for elicitation of oxidase activity. A further enhancement of oxidase activity was observed with the addition of nonhydrolyzable GTP analogues, such as guanosine 5'-*O*-(3-thiotriphosphate) (GTP-γ-S) and guanosine 5'-(β,γ-imidotriphosphate) (GMP-PNP), to the preincubation medium. In contrast, GDP-β-S drastically decreased oxidase activation. In a two-stage experiment, a 9-min preincubation of PMN membranes with arachidonic acid and GTP-γ-S followed by a 1-min contact with the cytosolic fraction led to a more marked activation than did preincubation of the cytosol with arachidonic acid and GTP-γ-S for 9 min followed by a 1-min contact with membranes, suggesting the presence of a G-protein in the membrane fraction. In the absence of added cations, the reconstitution system exhibited a substantial oxidase activity which was totally prevented by ethylenediaminetetraacetic acid (EDTA). Mg^{2+} added at a concentration of 0.5-1 mM enhanced oxidase activation by about 30%, indicating that endogenous Mg^{2+} or other activating cations were sufficient to ensure 70% of maximal activation. ATP was not required, and all assays carried out to check whether protein phosphorylation occurred during the activation process were negative. In particular, protein kinase C did not appear to be directly involved. The ratio of arachidonic acid to the amount of membrane material was the critical factor in the activation process. Maximal activation occurred with a ratio of 1 mol of arachidonic acid per 4 mol of membrane phospholipids. Long-chain unsaturated fatty acids of the cis and trans conformation were as effective as arachidonic acid. The protein nature of the factor present in the cytosolic fraction was demonstrated by loss of activity upon trypsin and proteinase K treatment. The cytosolic factor was found in PMN and not in other tissues, such as brain and heart; in contrast, its species specificities were rather broad, as cytosol from bovine PMN could replace cytosol from rabbit PMN to activate the oxidase from rabbit PMN membranes. The maximal rate of $O_2^{\cdot-}$ production depended on the amount of cytosol protein in contact with the membrane fraction, indicating that activation was not a catalytic process but involved a stoichiometric interaction between the cytosolic factor and a strategic component of the membrane fraction. Oxidase activation was temperature-dependent. The plateau of activation was attained in about 5 min at room temperature (22-25 °C) and in more than 30 min at 0 °C.

The NADPH-specific oxidase producing superoxide ions, $O_2^{\cdot-}$, is a characteristic enzyme of the phagocytosing blood cells. In the last few years increasing interest has been paid to this intriguing enzyme, but neither its structure nor its function or regulation has yet been unambiguously clarified [see Rossi (1986)]. The discovery of a technique for the in vitro activation of the NADPH oxidase in isolated membranes, originally described by Bromberg and Pick (1983, 1984) for guinea pig macrophages, was an important step in the investigation of the mechanism of activation. This cell-free activation system, consisting of arachidonic acid and cytosol, has been successfully applied to neutrophil granulocytes (Heyneman & Vercauteren, 1984; McPhail et al., 1985; Curnutte, 1985), and some properties of this system have been

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