Inhibition of NAD Glycohydrolase and ADP-ribosyl Transferases by Carbocyclic Analogues of Oxidized Nicotinamide Adenine Dinucleotide†

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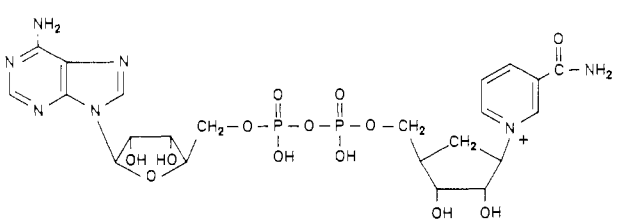
ABSTRACT: Analogues of oxidized nicotinamide adenine dinucleotide (NAD^+) in which a 2,3-dihydroxycyclopentane ring replaces the β-D-ribonucleotide ring of the nicotinamide riboside moiety of NAD^+ have recently been synthesized [Slama, J. T., & Simmons, A. M. (1988) *Biochemistry 27*, 183]. Carbocyclic NAD^+ analogues have been shown to inhibit NAD glycohydrolases and ADP-ribosyl transferases such as cholera toxin A subunit. In this study, the diastereomeric mixture of dinucleotides was separated, and the inhibitory capacity of each of the purified diastereomers was defined. The NAD^+ analogue in which the D-dihydroxycyclopentane is substituted for the D-ribose is designated carba-NAD and was demonstrated to be a poor inhibitor of the *Bungarus fasciatus* venom NAD glycohydrolase. The diastereomeric dinucleotide pseudo-carbocyclic-NAD (Ψ-carba-NAD), containing L-dihydroxycyclopentane in place of the D-ribose of NAD^+, was shown, however, to be a potent competitive inhibitor of the venom NAD glycohydrolase with an inhibitor dissociation constant (K_i) of 35 μM. This was surprising since Ψ-carba-NAD contains the carbocyclic analogue of the unnatural L-ribotide and was therefore expected to be a biologically inactive diastereomer. Ψ-Carba-NAD also competitively inhibited the insoluble brain NAD glycohydrolase from cow (K_i = 6.7 μM) and sheep (K_i = 31 μM) enzymes against which carba-NAD is ineffective. Sensitivity to Ψ-carba-NAD was found to parallel sensitivity to inhibition by isonicotinic acid hydrazide, another NADase inhibitor. Ψ-Carba-NAD is neither a substrate for nor an inhibitor of alcohol dehydrogenase, whereas carba-NAD is an efficient dehydrogenase substrate. Both compounds are competitive inhibitors of the NAD glycohydrolase activity of cholera toxin A subunit; carba-NAD is the superior cholera toxin inhibitor, but Ψ-carba-NAD is only slightly less active. Cholera toxin A subunit is apparently less able to discriminate among the diastereomeric dinucleotides than are the active sites of either the vertebrate NAD glycohydrolases or the dehydrogenases. Stereochemical differences between these inhibitors are therefore capable of discriminating between closely related NAD^+ binding sites. They will therefore be useful for selective enzyme inhibition in vivo and in vitro and in the design of isozyme-selective affinity or photoaffinity labels.

ADP-ribosyl transferases and NAD glycohydrolases catalyze the transfer of the adenosine diphosphate ribose (ADP-ribose) portion of NAD to a variety of acceptor proteins or to water (Hayaishi & Ueda, 1977). Mono(ADP-ribosyl) transferases are components of microbial exotoxins (cholera toxin, diphtheria toxin, pertussis toxin), and endogenous mammalian mono(ADP-ribosyl)transferases are now known for which a cell regulatory function is envisioned (Moss & Vaughan, 1988). Poly(ADP-ribose) synthetase is a related enzyme that is found associated with chromatin in most mammalian cells. Synthesis of poly(ADP-ribose) catalyzed by this enzyme is thought to function in the modulation of chromatin structure and regulation of nuclear activity (Ueda & Hayaishi, 1985; Hayaishi & Ueda, 1982).

An appreciation of the role of NAD^+ in such nonredox metabolism has led to a renaissance in interest in a related family of enzymes, the NAD glycohydrolases (NADases), which catalyze the hydrolysis of the nicotinamide–ribosyl bond of NAD, releasing nicotinamide, ADP-ribose, and a proton (Price & Pekala, 1987; Anderson, 1982). Regulation of the concentration of NAD^+ by NADase action may itself be an important cellular control mechanism. Alternately, the salvage of nicotinamide or adenosine through the hydrolysis of NAD^+ may be important to the cellular economy.

Despite the apparent importance of ADP-ribosyl transfer and NAD^+ hydrolysis to regulation of the cellular economy, little is known about the mechanisms of these enzymes, their protein architecture, or their regulation. In most cases the physiological function is obscure or is controversial. Understanding of ADP-ribosylation would therefore benefit by the production of specific and potent inhibitors for individual enzymes in this class. Such inhibitors are now available for only one class of ADP-ribosyl transferase—in the inhibition of poly(ADP-ribose) synthetase by 3-substituted benzamides (Purnell & Whish, 1980; Jacobson et al., 1989).

The dinucleotide analogue carbanicotinamide adenine dinucleotide (carba-NAD, **1**), in which a 2,3-dihydroxycyclopentane ring replaces the β-D-ribotide ring of the nicotinamide riboside moiety of NAD^+, is the first of a series of NAD^+



Carbanicotinamide Adenine Dinucleotide, 1

analogues designed to be inhibitors of ADP-ribosyl transfer

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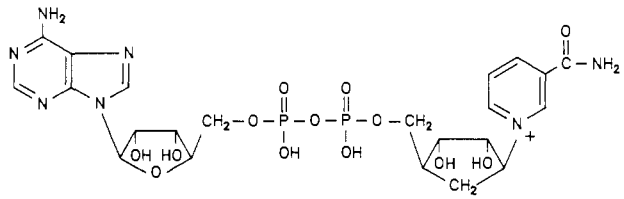
(Slama & Simmons, 1988, 1989). In this and related compounds the structure of the dinucleotide has been modified to cause the glycosidic pyridinium–ribotide bond to be resistant to cleavage. Otherwise, the analogue closely resembles NAD+ in its physical and enzymological properties. We expect therefore that carba-NAD and its relatives will constitute an important set of mechanism-based inhibitors for NAD glycohydrolases as well as for certain ADP-ribosyl transferases.

We have recently reported a successful synthesis of the diastereomeric pyridine dinucleotides carba-NAD (**1**) and pseudocarbanicotinamide adenine dinucleotide (Ψ-carba-NAD, **2**) (Slama & Simmons, 1988). Carba-NAD (**1**) was



Pseudocarbanicotinamide Adenine Dinucleotide, 2

shown to be recognized as a substrate by the alcohol dehydrogenases from yeast and horse liver and to be reduced to carba-NADH. Ψ-Carba-NAD (**2**), the dinucleotide containing the unnatural L-ribotide analogue, was not a substrate for either dehydrogenase. Neither dinucleotide was a substrate for the NADase from *Bungarus fasciatus* venom. The mixture of **1** and **2** effectively inhibited the enzyme by 50% at 0.2 mM. In this work, we have separated the mixture of diastereomers and have further explored the mechanism and the scope of the inhibition of ADP-ribosyl transfer reactions by these pyridine dinucleotide analogues.

Experimental Procedures

General Methods. Electronic absorption spectra and kinetic measurements were made by using a Beckman DU-50 spectrophotometer. Fluorescence measurements were made by using a SLM Aminco SPF-500C spectrofluorometer. HPLC was performed by using 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. System 1 consisted of an Alltech (Deerfield, Il) RSIL AN 10-μm anion-exchange column (4.6 × 250 mm), developed isocratically with 250 mM KH_2PO_4, pH 3.5, at a flow rate of 1.0 mL/min. System 2 consisted of an Alltech Versapack 10-μm C_{18} packing (4.6 × 300 mm) developed at a flow rate of 2 mL/min isocratically for 5 min with 95% buffer A and 5% buffer B, followed by a linear gradient of 5–60% buffer B formed over 15 min, and then isocratically at 60% buffer B for 5 min. Buffer A was 20 mM NaH_2PO_4 and 2 mM tetrabutylammonium phosphate, pH 6.0 (adjusted with tetrabutylammonium hydroxide). Buffer B was buffer A that contained 20% (v/v) methanol.

Separation of Carba-NAD (**1**) and *Ψ-Carba-NAD* (**2**). The 50:50 mixture of **1** and **2** was separated by preparative reversed-phase HPLC according to the procedure of Slama and Simmons (1988).

Desalting Purified Diastereomers. After separation by preparative reversed-phase HPLC, the dinucleotides were desalted by anion-exchange chromatography according to the procedure described in Slama and Simmons (1988).

Preparation of Cow, Sheep, and Rabbit Brain NAD Glycohydrolases. Brain tissues were prepared according to the method of Kaplan (1955). Cow brain tissue was purchased fresh from a local butcher. Sheep and rabbit brain tissue were purchased from Pel-Freez (Rogers, AR), shipped frozen, and stored at −20 °C. The diced brain tissue was blended with ice water (500 mL of ice water to 150 g of tissue) in a Waring blender and the resulting mixture filtered through a single layer of cheesecloth. The homogenate was centrifuged in a Sorvall centrifuge at 5 °C for 20 min at 20000*g* and the supernatant discarded. The pellet was resuspended to the original volume with ice water and homogenized, first by use of a rubber policeman and next with a tissue homogenizer (Thomas), and then centrifued for 40 min at 20000*g*. The supernatant was again discarded, and the pellet was resuspended to half the original volume with cold water. Finally, the suspension was sonicated by using a Heat Systems (Model W-375) sonicator equipped with the standard probe at 60% duty cycle for 4 min followed by three 1-min treatments at full power with cooling between each treatment. The resulting colloidal fluid was centrifuged at 20000*g* for 20 min, and the small amount of precipitate was discarded.

The protein content of the colloidal brain homogenate was measured according to the procedure of Bradford (1976) using the commercial Bio-Rad protein assay dye kit II, with bovine serum albumin as a standard. The standard assay contained 20–140 μg of protein in a total volume of 0.1 mL. Five milliliters of diluted dye was added, and after 5 min, the absorbance at 595 nm was read against a blank. Absorbance versus micrograms of albumin was plotted, and protein concentrations of unknowns were derived from the standard curve.

The enzymatic activity of the colloidal NADase was measured according to the method of Kaplan (1955). A typical assay contained 0.3 mL of 0.1 M phosphate, pH 7.2, 0.5 μmol of NAD, and 25 μL of the colloidal enzyme in a total volume of 0.6 mL. The assay was initiated by the addition of enzyme, incubated 8 min at 37 °C, and quenched by the addition of 3.0 mL of 0.1 M glycine–NaOH buffer, pH 9.5, containing 0.5 M ethanol and 0.02 M nicotinamide. The absorbance at 340 nm was determined, yeast alcohol dehydrogenase was added, and the absorbance was redetermined. The amount of NADH was determined by difference using the extinction coefficient for NADH (6.2×10^3 M^{-1} cm^{-1}). Specific activities for the colloidal brain homogenates were as follows: cow brain, 0.05 unit/mg of protein;[1] sheep brain, 0.036 unit/mg of protein; rabbit brain, 0.044 unit/mg of protein.

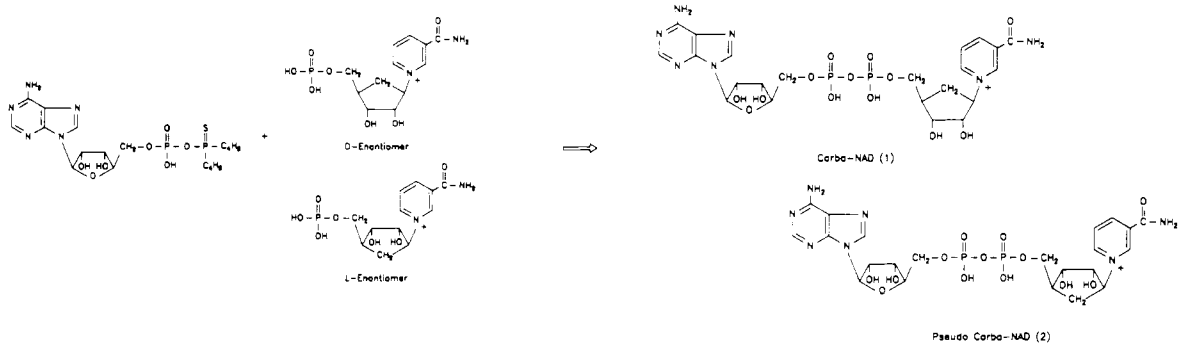
Pig Brain NAD Glycohydrolase. The NAD glycohydrolase from pig brain was purchased (Sigma Chemical Co., St. Louis, MO) as an insoluble acetone dried powder. The enzyme was resuspended and solubilized by trypsin treatment according to the procedure of Windmueller and Kaplan (1962) [see also Swislocki and Kaplan (1967)]. The solubilized NADase was stored at 5 °C as a precipitate in saturated ammonium sulfate solution.

NADase from Neurospora crassa was purchased from Sigma as a solid lyophilized from phosphate buffer at pH 7.0. Each vial was reported to contain 2.3 mg of protein with a specific activity of 0.7 unit/mg. When reconstituted with 1.6 mL water, the solution had an activity of 1.0 unit/mL[1] (as determined by the Kaplan assay).

Cholera toxin A subunit was purchased from List Biological Laboratories (Campbell, CA) as a lyophilized powder (0.25 mg of protein) that was reconstituted to 2.5 mL with water. The protein content was determined by the Lowry method (Lowry et al., 1951) using bovine serum albumin as a standard.

[1] A unit of NADase activity is that quantity of enzyme which catalyzes the hydrolysis of 1 μmol of NAD+/min.

Scheme I



NADase activity was measured by using the radiometric assay under the conditions specified for cholera toxin (Moss & Vaughan, 1984; Moss et al., 1976). The enzyme had a specific activity of 0.15 unit/mg.[1]

Purification of NAD glycohydrolase from B. fasciatus venom was accomplished by using the three-step procedure of Yost and Anderson (1981), as described in our previous publication (Slama & Simmons, 1988).

NAD Glycohydrolase Assay. The rate of hydrolysis of NAD by the variety of glycohydrolases was measured by the release of [*carbonyl*-^{14}C]nicotinamide from [*carbonyl*-^{14}C]-NAD (Moss et al., 1976; Moss & Vaughan, 1984; Slama & Simmons, 1988). The standard assay was conducted for 30 min at 37 °C and contained 33 mM potassium phosphate, pH 7.5, 50 μM [*carbonyl*-^{14}C]NAD (Amersham; 50 000 dpm), and enzyme (0.3 milliunits[1]) in a total volume of 0.3 mL.

Assay of the NAD glycohydrolase activity of cholera toxin A subunit was conducted in 200 mM potassium phosphate, pH 7.5, containing 20 mM dithiothreitol and 25 μg of bovine serum albumin as carrier protein.

Characterization of the Products of NADase Action. The products from hydrolysis of NAD^+ catalyzed by the crude brain homogenates as well as the NADase from *N. crassa* were identified by HPLC. Each assay contained 0.3 mL of 0.1 M KH_2PO_4, pH 7.2, 0.5 μmol of [*adenosine*-^{14}C]NAD (2 000 000 dpm), and enzyme (0.03 unit) in a total volume of 0.6 mL. The reaction was incubated at 37 °C for 8 min and then stopped by centrifugation through a YMT membrane (Amicon). The products were identified by HPLC using system 1, by comparison of their retention times to that of standards solutions of nicotinamide, nicotinamide mononucleotide, NAD^+, adenosine diphosphate ribose, AMP, and adenosine. After passing through the UV monitor, effluent was passed into a radiometric flow detector (Flo-One β radioactive flow detector, Model IC, Radiomatic Instruments and Chemical Co., Inc., Tampa, FL) to determine the specific radioactivity associated with each peak. In all cases, the major products of hydrolysis were nicotinamide and adenosine diphosphate ribose, along with unreacted NAD^+. The cow brain NADase showed a peak of adenosine accounting for 0.9% of the total counts; sheep brain, 3.7% as adenosine; rabbit brain, 1.1% as a mixture of AMP and adenosine; porcine brain, 0.6% as adenosine; and NADase from *N. crassa*, 0.6% as AMP. No unidentified ^{14}C-containing peaks were observed, and the activities of the known products accounted for >95% of the [^{14}C]NAD consumed.

Stability of Inhibitors **1** *and* **2** *to NADase.* The susceptibility of the inhibitors to the glycohydrolase activity of the brain preparations or *N. crassa* was determined on a 50:50 mixture of the diastereomers. The assay contained 0.3 mL of 0.1 M KH_2PO_4, pH 7.2, a 50:50 mixture of carba-NAD and Ψ-carba-NAD with a total dinucleotide concentration of 1.1 mM, and 0.03 unit of enzyme in a total volume of 0.6 mL. The reaction was incubated at 37 °C. Aliquots were removed and enzyme was removed by centrifugation through a YMT membrane (Amicon). HPLC analysis was performed by using both systems 1 and 2, and the products were idenitified by their absorptions at 254 nm. Products were quantitated by comparison of peak heights to a standard curve. The only products identified were traces of adenosine or AMP. After 30 min, the cow brain homogenate degraded 0.5% of the dinucleotides to give a new peak identified as adenosine; sheep brain degraded 0.75% to adenosine; rabbit brain degraded 0.83% to adenosine; porcine brain gave no detectable degradation of the dinucleotides; and *N. crassa* gave 1.95% degradation to a mixture of adenosine and AMP.

Analysis of Enzyme Inhibition Data. Kinetic parameters were determined by using the nonlinear regression analysis developed by Cleland (1979). Each data set was fitted first to COMP, which assumes linear competitive inhibition, and then to NONCOMP, in which the data are fit to a linear noncompetitive inhibition pattern, that is, one with both a slope and an intercept component. According to the criteria set forth by Cleland for the evaluation of a successful fit, all patterns of inhibition evaluated in this work are best described as competitive.

Inhibition of Yeast Alcohol Dehydrogenase by Ψ-Carba-NAD. Yeast alcohol dehydrogenase was purchased as an ammonium sulfate suspension from Boehringer-Mannheim (Indianapolis, IN; 300 units/mg). The spectrometric assay was described previously (Slama & Simmons, 1988).

Inhibition of Horse Liver Alcohol Dehydrogenase by Ψ-Carba-NAD (**2**). Horse liver alcohol dehydrogenase was purchased from Sigma. The fluorometric assay was described previously (Slama & Simmons, 1988).

Results

A synthesis of carba-NAD (**1**) and Ψ-carba-NAD (**2**) has recently been reported (Slama & Simmons, 1988). This work begins by producing a racemic carbanicotinamide 5′-mononucleotide. The racemic mixture of nucleotides is coupled with an activated AMP derivative (see Scheme I) to produce the dinucleotides. Coupling of the racemic carba-NMN to activated and optically pure adenosine 5′-monophosphate leads to the production of the two diastereomeric dinucleotides, carba-NAD (**1**) and pseudocarba-NAD (Ψ-carba-NAD, **2**). These diastereomeric dinucleotides are separable by reversed-phase chromatography. Only one of the two diastereomeric dinucleotides is a substrate for either yeast alcohol dehydrogenase or horse liver alcohol dehydrogenase. That diastereomer which is reduced by the dehydrogenase is assigned the configuration in which the D-hydroxycyclopentane derivative replaces the β-D-ribonucleotide ring of the nicotinamide ribonucleoside moiety. That is, the isomer which is

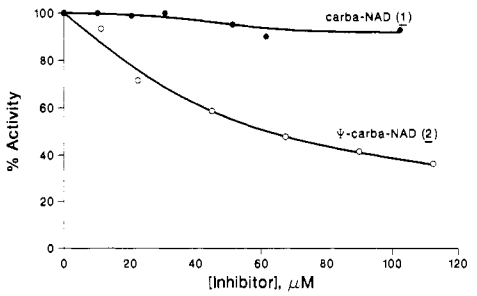


FIGURE 1: Inhibition of *B. fasciatus* venom NADase by purified carba-NAD (**1**) and Ψ-carba-NAD (**2**). The assay was conducted in 33 mM KH_2PO_4, pH 7.5, containing 35 μM NAD and 0.06 milliunit of enzyme in a total volume of 0.3 mL. The assay was conducted as described under Experimental Procedures. Concentrations of carba-NAD (**1**) were varied from 0 to 103 μM (●). Concentrations of Ψ-carba-NAD (**2**) were varied from 0 to 112.5 μM (○).

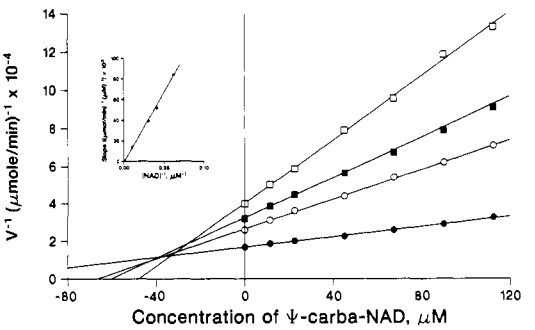


FIGURE 2: Effect of Ψ-carba-NAD (**2**) on the rates of hydrolysis of NAD by *B. fasciatus* venom NADase. The assay contained 33 mM KH_2PO_4, pH 7.5, and 0.06 milliunit of enzyme in a final volume of 0.3 mL. The concentration of Ψ-carba-NAD was varied from 0 to 112.5 μM. The reaction was initiated by the addition of NAD and allowed to incubate for 30 min at 37 °C. The concentrations of NAD were as follows: (□) 16.1 μM; (■) 24.2 μM; (○) 32.2 μM; (●) 96.7 μM. (Insert) Replot of the slopes versus the reciprocal of the substrate concentrations.

reduced by alcohol dehydrogenase is represented as formula **1**, whereas the isomer which cannot be enzymatically reduced is represented as formula **2**.

Although mixtures of **1** and **2** were shown to inhibit the NAD glycohydrolase from *B. fasciatus* venom, the stereospecificity of inhibition had not been determined (Slama & Simmons, 1988, 1989). Since the venom NADase is a stable and soluble enzyme that is in many respects representative of the vertebrate NAD glycohydrolases, we began by investigating the inhibition of this enzyme by our carbocyclic dinucleotides.

Inhibition of the NAD Glycohydrolase from B. fasciatus Venom. Addition of the mixture of carba-NAD (**1**) and Ψ-carba-NAD (**2**) to assays of the venom NADase was shown to inhibit the enzyme catalyzed NAD^+ hydrolysis competitively with an inhibitor dissociation constant, K_i, equal to 66 ± 4 μM (Slama & Simmons, 1989).[2] Previous work had further established that neither **1** nor **2** was a substrate for this enzyme and that no time-dependent process of enzyme inactivation occurred (Slama & Simmons, 1988).

To determine which dinucleotide in the diastereomeric mixture of **1** and **2** was the effective inhibitor, the diastereomeric dinucleotides were separated by using reversed-phase preparative HPLC. Although the capacity of even a large (i.e., 1 cm × 50 cm) reversed-phase column is low (5 mg/injection), a few milligrams of each pure diastereomer could be prepared by using multiple repetitive injections. Dinucleotide was subsequently desalted by ion-exchange chromatography on Dowex 1 anion-exchange resin. Increasing concentrations of each of the purified dinucleotides were added to the assay mixture of venom NAD glycohydrolase, and the activity was measured. The results (Figure 1) clearly established that the effective inhibitor was Ψ-carba-NAD (**2**), which inhibited the rate of hydrolysis by 50% at the concentration of 63 μM. Carba-NAD (**1**) was shown to be a relatively ineffective inhibitor, slowing the rates of NAD cleavage by only 4% at a concentration of 63 μM.

This result was surprising and the opposite of that which we had anticipated. Ψ-Carba-NAD (**2**) was apparently a very effective competitive inhibitor for the NAD^+ binding site in the venom NAD glycohydrolase, even though it was shown not to be a substrate for either the yeast or the horse liver alcohol dehydrogenase. Carba-NAD (**1**), demonstrated to be an effective dehydrogenase substrate, was poorly recognized by the NADase binding site.[3]

The inhibition of the venom NADase by purified Ψ-carba-NAD (**2**) was measured at constant substrate concentration as a function of increasing concentration of inhibitor. The enzyme activity measured at four concentrations of substrate, plotted according to the procedure of Dixon (1953), is shown in Figure 2. The mechanism of inhibition is linear competitive, and an inhibitor dissociation constant of 35 ± 1 μM is calculated for pure **2** from this data. Both the mechanism (competitive) and the dissociation constant (i.e., a K_i of roughly half that determined for the mixture of **1** and **2**) obtained from the Dixon plot using purified **2** agree well with that from the analysis using the mixed diastereomers. The dissociation constant for carba-NAD (**1**) must therefore be sufficiently high that it makes essentially no contribution to inactivation of the NADase over concentrations below about 400 μM.

Inhibition of Yeast and Horse Liver Alcohol Dehydrogenase by Ψ-Carba-NAD. Although we had previously determined that carba-NAD (**1**) was a dehydrogenase substrate and that Ψ-carba-NAD (**2**) was not, we had not determined if **2** was a competitive inhibitor of the dehydrogenase. Horse liver alcohol dehydrogenase was assayed by using a concentration of NAD^+ equal to its K_m (0.02 mM) and by using saturating alcohol (0.3 M) at pH 8.8. Ψ-Carba-NAD (**2**) added to this assay mixture at concentrations of up to 10 times that of the K_m for NAD^+ failed to inhibit the rate of reduction (data not shown). Yeast alcohol dehydrogenase was similarly insensitive to inhibition by **2**. When assayed by using a concentration of NAD^+ equal to its K_m (0.2 mM), the rates of enzymatic reduction were inhibited to the extent of 50% only at 8 mM Ψ-carba-NAD (**2**) (K_i = 4.2 mM estimated from the Dixon plot, not shown). Thus, Ψ-carba-NAD is neither a substrate nor an effective inhibitor for the NAD binding site in alcohol dehydrogenase.

Inhibition of Mammalian and Microbial NAD Glycohydrolase by Carba-NAD and Ψ-Carba-NAD. The efficacy and surprising stereospecificity of the carbocyclic dinucleotides as inhibitors of the venom NADase prompted us to determine the generality of the phenomenon by testing our dinucleotides as inhibitors for several other NAD glycohydrolases. A variety

[2] The data reported in Slama and Simmons (1989) were reanalyzed by using the statistical procedure of Cleland (1979), and a K_i of 66 ±4 μM was determined.

[3] The samples of **1** and **2** used in the inhibition assays were reanalyzed at this point and their electronic absorption spectra, their susceptibility to reduction by alcohol dehydrogenase, and their chromatographic purity redetermined. The identities of the samples were thus reestablished, confirming that **2** was the NADase inhibitor.

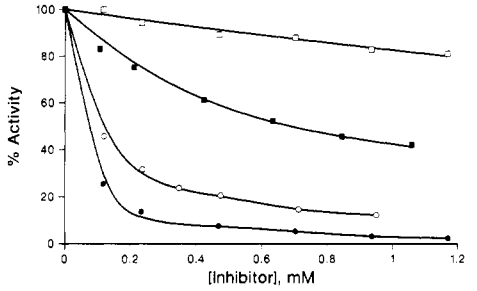


FIGURE 3: Inhibition of the insoluble brain NADase from various mammals by the mixture of carba-NAD (**1**) and Ψ-carba-NAD (**2**). The assay was conducted in 33 mM KH_2PO_4, pH 7.5, with concentrations of NAD equivalent to the K_m determined for each enzyme. Inhibitor was added over the range 0.1–1.0 mM. Approximately 0.3 milliunit of enzyme was added to each assay mixture to a final volume of 0.3 mL. Reactions were initiated by the addition of [*carbonyl*-^{14}C]NAD and incubated 30 min at 37 °C. The source of the NAD glycohydrolase was as follows: (●) cow brain; (○) sheep brain; (■) pig brain; (□) rabbit brain.

Table I: Sensitivity of NAD Glycohydrolases to Inhibition by Carba-NAD (**1**) or Ψ-Carba-NAD (**2**)

source of NAD glycohydrolase	K_m, NAD (μM)	% activity[a] carba-NAD (**1**)	% activity[a] Ψ-carba-NAD (**2**)
B. fasciatus venom	36	77	29
cow brain	74	88	8
sheep brain	55	83	23
pig brain	25	88	64
rabbit brain	48	96	90
cholera toxin A subunit	4000[b]	59[c]	82[c]

[a] Each assay contained 33 mM KH_2PO_4, pH 7.5, and [*carbonyl*-^{14}C]NAD$^+$ at a concentration equal to its K_m and enzyme (0.3 milliunit) in a volume of 0.3 mL. Inhibitor was present at a concentration of 0.5 mM. [b] Galloway and Van Heyningen (1987). [c] Assays of the NADase activity of cholera toxin A subunit were conducted as described under Experimental Procedures but with 0.1 mM NAD$^+$ to produce a greater relative inhibition. Inhibitor **1** or **2** was present at a concentration of 4.8 mM.

of NAD glycohydrolases are known, classified according to their origins, their ability to catalyze exchange reactions, and their sensitivity to inhibitors (Price & Pekala, 1987). The equimolar mixture of carba-NAD (**1**) and Ψ-carba-NAD (**2**) was tested as an inhibitor of the NAD glycohydrolase from bovine, sheep, porcine, and rabbit brains as well as against the NADase isolated from *Neurospora* mycelia. Increasing concentrations of the mixture of nucleotides were added to assay mixtures of the glycohydrolase, and the remaining activity was measured. To compare the sensitivity of the different enzymes to inhibition, the substrate concentration was adjusted in each case to equal the K_m determined for NAD$^+$ (shown in Table I). The results (Figure 3) show that the NADases differ significantly in their sensitivity to inhibition by these nucleotide analogues. The bovine brain and the sheep brain enzymes are most sensitive, inhibited by 50% at 60 and 100 μM concentrations of dinucleotide, respectively. The porcine enzyme is of intermediate sensitivity, inhibited by 50% at a nucleotide concentration of 700 μM. The rabbit enzyme is only weakly inhibited, while the *Neurospora* enzyme is insensitive to inhibitor at concentrations of 1 mM.

The stereospecificity of inhibition of the sensitive enzymes was determined by adding the purified nucleotides **1** or **2** to an assay at a concentration of 0.5 mM and measuring residual activity. The result (Table I) indicates that in each case Ψ-carba-NAD (**2**) was the superior inhibitor. Thus, Ψ-carba-NAD (**2**) was demonstrated to be the diastereomer that is recognized by the pyridine nucleotide-specific binding sites

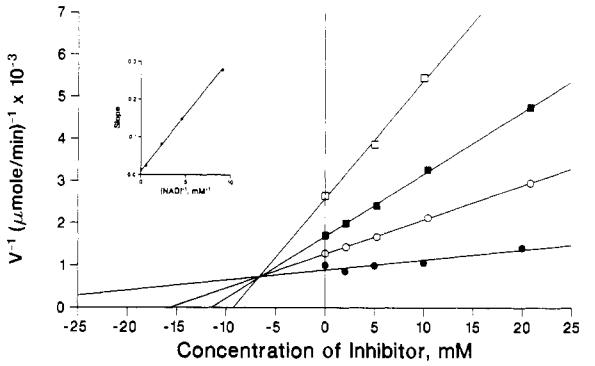


FIGURE 4: Effect of the mixture of carba-NAD (**1**) and Ψ-carba-NAD (**2**) on the rate of NAD hydrolysis catalyzed by cholera toxin A subunit. The assay contained 200 mM KH_2PO_4, pH 7.0, 20 mM dithiothreitol, 25 μg of bovine serum albumin, 4.1 μg of cholera toxin A subunit (as a solution in 25 μL of 50 mM Tris·HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, and 3 mM NaN_3) in a total volume of 0.3 mL. The concentration of the mixture of **1** and **2** was varied from 0 to 21 mM. The concentrations of NAD were as follows: (●) 1.8 mM; (○) 0.44 mM; (■) 0.22 mM; (□) 0.11 mM. (Insert) Replot of the slopes versus [NAD]$^{-1}$. We have determined that the nonzero intercept of the line shown in the insert is not statistically significant. The data were fit first to a model assumming strictly competitive inhibition and then to a model for noncompetitive inhibition using the nonlinear regression analysis of Cleland (1979). The model for noncompetitive inhibition used here includes both a slope and intercept component and therefore includes the condition of "mixed-noncompetitive" inhibition that the nonzero intercept of the insert could imply. The results indicate that competitive inhibition is the best model using the criteria outlined in the Cleland paper. That is, the value for K_{ii}, an additional constant required in the noncompetitive model, is not significantly different from zero (324 ± 719 mM) and its inclusion does not lead to a significantly better fit of the data.

of four different NAD glycohydrolases.

The mechanism of inhibition of the bovine and sheep brain enzymes by Ψ-carba-NAD (**2**) was determined by Lineweaver–Burk analysis of the rates of NAD hydrolysis as a function of NAD concentration conducted in the presence of Ψ-carba-NAD (**2**) (data not shown). In both cases **2** was found to be a linear competitive inhibitor. The values of the inhibitor dissociation constants (K_i) were determined to be 6.7 ± 0.3 μM for the bovine brain enzyme and 31 ± 1 μM for the sheep brain enzyme.

Inhibition of Mono(ADP-ribosyl) Transfer: Cholera Toxin A Subunit. Cholera toxin A subunit is a well-characterized and commercially available mono(ADP-ribosyl) transferase. A vertebrate enzyme that resembles cholera toxin in enzymatic properties and in its substrate specificity has recently been isolated (Moss & Vaughan, 1988). Thus, cholera toxin serves as a model not only of the microbial mono(ADP-ribosyl) transferases but also for a class of mammalian enzymes as well.

The NAD glycohydrolase activity of cholera toxin A subunit is sensitive to inhibition by the mixture of **1** and **2**. Analysis of the initial rates of enzyme-catalyzed hydrolysis as a function of increasing inhibitor concentration according to the procedure of Dixon (1953) is presented in Figure 4. The mechanism of inhibition was found to be competitive. An inhibitor dissociation constant of 5.8 ± 0.8 mM was determined from analysis of this data (Figure 4) for the mixture of **1** and **2**.

Although an inhibitor dissociation constant in the millimolar range is high, cholera toxin A subunit is known to exhibit an unusually high substrate dissociation constant (the K_m for NAD$^+$ is 4 mM; Galloway & Van Heyningen, 1987). Thus, the mixture of **1** and **2** has an affinity for the active site that is approximately equal to that of its substrate.

To assess the stereospecificity of inhibition, the NADase activity of cholera toxin was measured by using 0.1 mM

NAD^+. In the presence of 4.8 mM carba-NAD (**1**), the rate of NAD^+ hydrolysis was reduced by 41%, whereas in the presence of 4.8 mM Ψ-carba-NAD (**2**), the rate of NAD^+ hydrolysis was reduced by only 18%. Separated **1** and **2** were not available in sufficient quantitity to allow us to construct Dixon plots by using the purified diastereomers. However, it is clear from the experiment that the stereochemical preference is different from that of the vertebrate NADases. Carba-NAD (**1**) is the superior inhibitor of cholera toxin A subunit. The active site of this NADase is, however, less able to discriminate between the diastereomeric dinucleotides **1** and **2** than are either the active sites of the vertebrate NADase or the active sites of the alcohol dehydrogenases.

Discussion

Carbanicotinamide adenine dinucleotide (carba-NAD, **1**) was designed as a tool with which to explore the relationship between the structure of the ribonucleotide ring of the nicotinamide ribonucleoside moiety of NAD^+ and the function of the coenzyme. One aspect of coenzyme function is the role of NAD^+ as a dehydrogenase cosubstrate. A second important aspect of coenzyme function is the role of NAD^+ as a substrate in ADP-ribosyl transfer reactions and for NAD^+ hydrolysis. The replacement of the ribotide ring oxygen with a methylene CH_2 causes the pyridinium carbon bond to be more stable and therefore resistant to enzymatic cleavage by an NAD glycohydrolase or an ADP-ribosyl transferase. The substitution of a methylene for the oxygen produces a coenzyme analogue that otherwise should resemble NAD^+ in its overall charge and shape. It was therefore expected that the resultant carba-NAD would closely resemble the parent coenzyme in both chemical and enzymological properties. In accord with this expectation, the modified coenzyme carba-NAD (**1**) is an efficient substrate for alcohol dehydrogenase from yeast or from horse liver (Slama & Simmons, 1988). Its diastereomer, Ψ-carba-NAD (**2**) is neither a substrate for nor an inhibitor of either dehydrogenase reaction. This is also expected since the diastereomer should possess an inappropriate geometry for either binding or catalysis.

The mixture of carba-NAD (**1**) and Ψ-carba-NAD (**2**) has been shown to strongly inhibit the venom NAD^+ glycohydrolase. In this work we show that the effective inhibitor in this mixture is Ψ-carba-NAD (**2**). Carba-NAD (**1**) is in fact a very weak inhibitor of the NADase. This result was unexpected, since it is carba-NAD (**1**) that stereochemically resembles the parent coenzyme. Ψ-Carba-NAD contains the carbocyclic analogue of L-ribose in place of the D-ribonucleotide ring of the nicotinamide ribonucleoside moeity of NAD^+. Ψ-Carba-NAD (**2**) is therefore the carbocyclic analogue of an unnatural diastereomer of NAD^+. Ψ-Carba-NAD (**2**) was further shown to be a linear competitive inhibitor, with a dissociation constant of 35 ± 1 μM.

To explore the scope and limitation of this effect, we tested several NADases for sensitivity to inhibition by the diastereomeric dinucleotides **1** and **2**. The insoluble NAD^+ glycohydrolase from the brains of cattle and sheep was shown to be highly sensitive to inhibition by Ψ-carba-NAD (**2**). The enzyme from pig brain was significantly less sensitive and that from rabbit brain less sensitive still. The *Neurospora* NAD glycohydrolase, representative of the microbial enzymes (Everse et al., 1975) is quite insensitive to inhibition by either **1** or **2**.

The sensitivity of vertebrate NADases to inhibition by Ψ-carba-NAD (**2**) parallels their sensitivity to inhibition by isonicotinic acid hydrazide (INH), which had been studied previously by Kaplan (Zatman et al., 1954; Kaplan, 1955). The enzymes from *B. fasciatus* venom (Yost & Anderson, 1982), bovine brain, and sheep brain are all INH sensitive, while the pig and rabbit enzymes are INH insensitive. The inhibition of the INH-sensitive enzymes by **2** must indicate that the active-site geometry of these enzymes is related in some more fundamental way than simply by the ability to accommodate the 4-substituted pyridine of INH or the INH-substituted dinucleotide analogue. Sensitivity to inhibition by **2** is therefore a property through which closely related NADase binding sites can be distinguished. It will be interesting to see if such sensitivity can be used to distinguish between NAD glycohydrolase isozymes present within a single species or even within a single tissue.

Mono- and poly(ADP-ribosyl) transferases represent a further type of NAD-metabolizing enzyme. The development of potent and specific inhibitors for these would benefit both physiological studies and protein chemical studies on these important targets. The mixture of diastereomeric dinucleotides **1** and **2** was demonstrated to inhibit cholera toxin, the model mono(ADP-ribosyl) transferase, competitively with a K_i of 5.8 ± 0.8 mM. An inhibitor dissociation constant of this magnitude, although high, is comparable to the reported K_m for substrate NAD of 4 mM, indicating that the carbocyclic dinucleotides are recognized as well as is the substrate NAD^+. Although carba-NAD (**1**) is in this case the more effective inhibitor, the ability of cholera toxin to distinguish between the diastereomeric dinucleotides is low.

Carba-NAD (**1**), although a very efficient substrate for alcohol dehydrogenase (Slama & Simmons, 1988), is recognized poorly by the NAD^+ binding sites of NAD glycohydrolases or ADP-ribosyl transferases. This poor recognition might reflect an unfavorable steric interaction between the CH_2 of the carbocyclic derivative and the enzyme active site. Alternately, the replacement of the furanose ring oxygen by a methylene could distort the five-membered ring and force the nucleotide to adopt a conformation unfavorable for binding. Some support for the latter explanation is available from NMR studies on the carbocyclic analogue of nicotinamide mononucleotide (Oppenheimer, 1987), where coupling constants have been interpreted as supportive of a distorted conformation of the carbocycle relative to the furanose. How this distortion is manifest in the dinucleotide conformation will require extension of this work to carba-NAD and Ψ-carba-NAD. The superior NADase inhibitor, Ψ-carba-NAD (**2**), must either be able to adopt a shape compatible with the binding sites of the INH-sensitive NADases or have an alternate binding mode available to it that is not available to carba-NAD (**1**).

The efficacy of Ψ-carba-NAD (**2**) as an inhibitor of NADases and ADP-ribosyl transferases suggests that affinity labels and photoaffinity labels based upon this analogue will be of use in elucidating active-site structure in susceptible enzymes. The advantage of the carbocycle over alternate affinity labels is high selectivity combined with stability toward NADase activity. We have further demonstrated that changes in cofactor structure are capable of producing analogues that can finely discriminate among related pyridine dinucleotide binding sites. The synthesis of additional noncleavable NAD^+ analogues is therefore expected to produce new inhibitors with different specificities, which in turn will be applicable to the study of other NAD^+ metabolizing enzymes both in vitro and in vivo.

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Low-Affinity γ-Aminobutyric Acid Transport in Rat Brain†

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ABSTRACT: The low-affinity (K_m = 100–200 μM) γ-aminobutyric acid (GABA) transporter from membrane vesicles from rat brain has been characterized and found to be in many aspects similar to the well-known sodium- and chloride-coupled high-affinity γ-aminobutyric acid transporter (K_m = 2–4 μM). Influx by this system is sodium and chloride dependent and stimulated by an interior negative membrane potential. Steady-state levels obtained by both systems are lowered by the sodium channel openers veratridine and aconitine. However, while the channel blocker tetrodotoxin fully reverses this inhibition with the high-affinity system, this is not the case for its low-affinity counterpart. Furthermore, the toxin from the scorpion *Androctonus australis* Hector inhibited high-affinity transport only. Efflux of γ-aminobutyric acid taken up by the high-affinity system displayed a K_m of about 100 μM. Exchange catalyzed by the low-affinity system was observed in the absence of external sodium and chloride. Furthermore, both activities copurified in the fractionation procedure developed to purify the high-affinity transporter. All these observations are consistent with the idea that both activities are manifestations of only one γ-aminobutyric acid transporter. The high-affinity binding site represents the extracellular and the low-affinity site the cytosolic aspect of the transporter. In addition, it was found that right-side-out synaptosomes also contain a low-affinity GABA transporter. This apparently represents a different transport protein.

The role of high-affinity neurotransmitter transport is to terminate the overall process of synaptic transmission by removing the transmitters from the synaptic cleft (Iversen, 1975; Kuhar, 1973). These transporters are in fact sodium ion–neurotransmitter cotransporters and are able to accumulate the neurotransmitter against considerable concentration gradients by using the electrochemical gradient of sodium ions. Furthermore, many of these transporters are also absolutely

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