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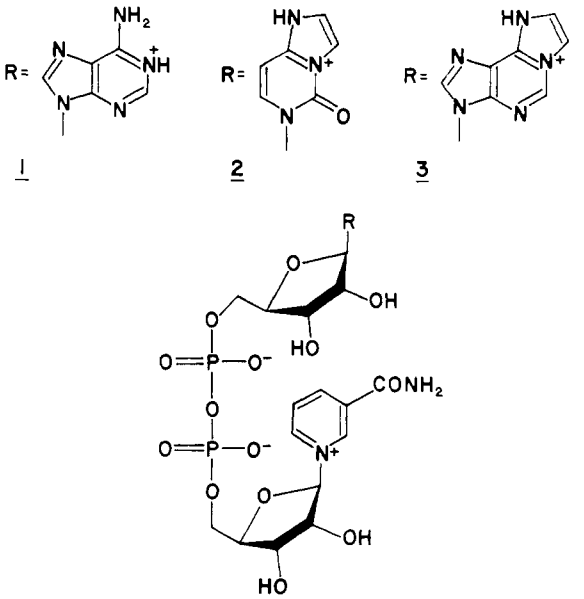
Nicotinamide 3,N^4-Ethenocytosine Dinucleotide, an Analog of Nicotinamide Adenine Dinucleotide. Synthesis and Enzyme Studies†

John C. Greenfield,‡ Nelson J. Leonard, and Richard I. Gumport*

ABSTRACT: A structural analog of NAD^+, nicotinamide 3,N^4-ethenocytosine dinucleotide (ϵNCD^+), has been synthesized, characterized, and compared in activity with the natural coenzyme in several enzyme systems. The V_{max} and apparent K_m values were determined for NAD^+, ϵNCD^+, and ϵNAD^+ (nicotinamide 1,N^6-ethenoadenine dinucleotide) with yeast alcohol, horse liver alcohol, pig heart malate, beef liver glutamate, and rabbit muscle lactate and glyceraldehyde-3-phosphate dehydrogenases. The V_{max} for ϵNCD^+ was as great or greater than that obtained for NAD^+ with three of the enzymes, 60–80% with two others, and 14% with one. ϵNCD^+ was found to be more active than ϵNAD^+ with all six dehydrogenases. ϵNCD^+ served as a substrate for *Neurospora crassa* NADase, but could not be phosphorylated with pigeon liver NAD^+ kinase. NAD^+ pyrophosphorylase from pig liver was unable to catalyze the formation of ϵNCD^+ from the triphosphate derivative of ϵ-cytidine and nicotinamide mononucleotide, but was able to slowly catalyze the pyrophosphorolytic cleavage of ϵNCD^+. The coenzyme activity of ϵNCD^+ with dehydrogenases can be discussed in terms of the close spatial homology of ϵNCD^+ and NAD^+, which may allow similar accommodations within the enzyme binding regions.

The prominent biological roles played by nicotinamide adenine dinucleotide (NAD^+) (**1**) and its reduced form NADH have generated considerable interest in these compounds and in the enzymes which utilize them as coenzymes or substrates. One investigative technique that has been employed in determining structure and function relationships of these compounds has been the preparation of NAD^+ analogs and the study of their interaction with dehydrogenases and other enzymes (Sund, 1968a; Colowick, *et al.,* 1966; Biellmann *et al.,* 1974; Chaykin, 1967; Suhadolnik *et al.,* 1974). Analogs have also been used in studies of the mechanisms of dehydrogenase action (Sund, 1968b), the detection of heterogeneity of enzymes having the same function (Kaplan, 1963a), and the evolution of enzyme structure (Kaplan, 1963b; Kaplan *et al.,* 1960; *cf.* Rao and Rossmann, 1973).



The close spatial outline and similar potential binding areas of the base moiety of ϵ-cytidine nucleotides and adenosine nucleotides (Barrio *et al.,* 1973), as shown in the overlay in Figure 1, has inspired the preparation of nicotinamide ϵ-cytosine dinucleotide (ϵNCD^+) (**2**) which should closely mimic the structural features of the natural coenzyme,

† From the Department of Biochemistry, School of Chemical Sciences, and School of Basic Medical Sciences, University of Illinois, Urbana, Illinois 61801. *Received September 9, 1974.* This work was supported in part by Research Grants from the National Institutes of Health, GM 05829 and GM 19442. R.I.G. is the recipient of a National Institutes of Health Career Development Award K04 GM 70,520.

‡ Present address: Laboratorium für Organische Chemie, Eidgenössische Technische Hoschschule, CH-8006 Zürich, Switzerland.

[1] Abbreviations following the IUPAC–IUB Commission on Biochemical Nomenclature recommendations (*J. Mol. Biol. 55,* 299 (1971)) are used throughout. The abbreviation "ϵ" stands for etheno, so that ϵCyd is 3,N^4-ethenocytidine or 5,6-dihydro-5-oxo-6-β-D-ribofuranosylimidazo[2,1-*c*]pyrimidine (Secrist *et al.,* 1972; Barrio *et al.,* 1972a); ϵCMP, 3,N^4-ethenocytidine 5′-monophosphate; ϵNCD^+, nicotinamide 3,N^4-ethenocytosine dinucleotide and ϵNCDH is the reduced form; ϵNAD^+, nicotinamide 1,N^6-ethenoadenine dinucleotide and ϵNADH is the reduced form (Barrio *et al.,* 1972b); NMN, nicotinamide mononucleotide, PP_i, pyrophosphate.

NAD+. It has been shown that nucleotides containing the ϵ-cytosine moiety, prepared by reaction of chloroacetaldehyde with cytidine compounds (Kochetkov *et al.*, 1971; Barrio *et al.*, 1972a), can replace adenosine nucleotides in enzymatic phosphorylation (Barrio *et al.*, 1973), and the dinucleotide derivatives are accepted as substrates by some nucleases (G. L. Tolman, J. R. Barrio, N. J. Leonard, submitted for publication). Furthermore, the ϵ-cytidine moiety is fluorescent at acidic pH (Barrio *et al.*, 1972a) and this property is potentially applicable to the study of inter- and intramolecular interactions under these conditions. In order to define functionally the spatial similarities between ϵNCD+ and NAD+, we have measured and compared the apparent K_m and V_{max} values of ϵNCD+ *vs.* NAD+ with six dehydrogenases, and have compared the ability of several other enzymes which utilize NAD+ as a substrate to use ϵNCD+. For purposes of quantitative comparison, kinetic data with the dehydrogenases were also determined with nicotinamide 1,N^6-ethenoadenine dinucleotide (ϵNAD+) (**3**), which has already been studied because of its strong fluorescence and has been shown to be less active than NAD+ with selected dehydrogenases (Barrio *et al.*, 1972b; Lee and Everse, 1973).

Our investigation relates to previous studies with NAD+ analogs and dehydrogenases in which the adenine portion of the coenzyme has been chemically modified, *e.g.*, by deamination (Stockwell, 1959) or by reaction with ethylene oxide (Windmueller and Kaplan, 1961), or has been replaced with another heterocyclic moiety, such as uridine (Fawcett and Kaplan, 1962), cytidine (Honjo *et al.*, 1962; Pfleiderer *et al.*, 1963), purine derivatives (Woenckhaus and Pfleiderer, 1965; Woenckhaus, 1964; Ward *et al.*, 1972), and with benzene (Woenckhaus and Volz, 1966). An analog of NAD+ having the adenine moiety attached at N-3 instead of N-9 also has been reported (Leonard and Laursen, 1965). These diverse analogs have shown gradations in ability to replace the natural coenzyme in the reaction with dehydrogenases.

Recent progress in the determination of the three-dimensional structure of enzymes (Liljas and Rossmann, 1974) has increased understanding of the mechanisms of enzyme action (Mildvan, 1974). It has been shown that the coenzyme binding domains of many dehydrogenases are very similar (Rossmann *et al.*, 1974) and that three-dimensional homology exists between lactate dehydrogenase (Adams, *et al.*, 1970; Rossmann *et al.*, 1971), liver alcohol dehydrogenase (Brändén *et al.*, 1973, Brändén, 1974), soluble malate dehydrogenase (Hill *et al.*, 1972), and glyceraldehyde-3-phosphate dehydrogenase (Buehner *et al.*, 1973). Similar nucleotide binding domains are also seen in phosphoglycerate kinase (Blake and Evans, 1974; Bryant *et al.*, 1974) and adenylate kinase (Schulz *et al.*, 1974), suggesting that these two kinases may be related in evolution to the dehydrogenases (Blake, 1974). Knowledge of the molecular structure of the coenzyme binding sites of the enzymes and the molecular dimensions of 3,N^4-ethenocytidine hydrochloride (Wang, 1974) should make possible a correlation of the coenzyme activity of ϵNCD+ and the spatial dimensions of the enzymes. We have examined the apparent interaction of structural models of ϵNCD+ with Kendrew models of dogfish lactate dehydrogenase and lobster muscle glyceraldehyde-3-phosphate dehydrogenase which have been constructed in Professor M. G. Rossmann's laboratory at Purdue University, and we can compare the experimental kinetic data in these terms.

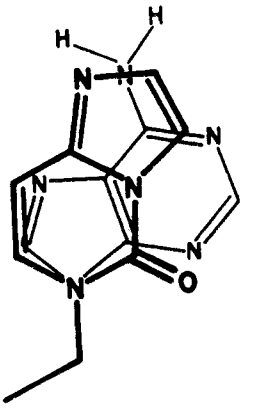


FIGURE 1: Overlay of representative structural formulas of N^9-substituted adenine and N^1-substituted 3,N^4-ethenocytosine.

Experimental Section

Materials. Cytidine 5′-monophosphate (CMP), nicotinamide mononucleotide (NMN), and nicotinamide adenine dinucleotide (NAD+) (ChromatoPure) were purchased from P-L Biochemicals, Inc. Yeast alcohol dehydrogenase (EC 1.1.1.1), *Neurospora crassa* NADase (EC 3.2.2.5), pig heart malate dehydrogenase (EC 1.1.1.37), and rabbit muscle lactate dehydrogenase (EC 1.1.1.27) were obtained from Sigma. Horse liver alcohol dehydrogenase (EC 1.1.1.1), beef liver glutamate dehydrogenase (EC 1.4.1.3), rabbit muscle aldolase (EC 4.1.2.13), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), pigeon liver NAD+ kinase (EC 2.7.1.23), snake venom phosphodiesterase (EC 3.1.4.1), pig liver NAD+ pyrophosphorylase (EC 2.7.7.1), and yeast pyrophosphatase (EC 3.6.1.1) were products of Boehringer-Mannheim Corp. Buffers, enzyme substrates, and other chemicals were of reagent quality. Nicotinamide ϵ-adenine dinucleotide (ϵNAD+) was prepared as previously described (Secrist *et al.*, 1972; Barrio *et al.*, 1972b). ϵ-Cytidine 5′-monophosphate (ϵCMP) was prepared as reported by Barrio *et al.* (1973). [^{14}C]ϵCTP was prepared by reaction of CTP with [1-^{14}C]chloroacetaldehyde (procedure submitted for publication, *J. Label. Compounds*). [γ-^{32}P]ATP was prepared by a modification of the method of Glynn and Chappell (1964) in which 1 mM dithiothreitol replaced 2 mM cysteine.

Thin-Layer Chromatography and Electrophoresis Systems. Thin-layer partition chromatography was performed on Eastman cellulose sheets using the following solvent systems: system A, isobutyric acid–concentrated NH_4OH–H_2O, 75:1:24, v/v; system B, 2-propanol–1% ammonium sulfate, 2:1; system C, 1-butanol–glacial acetic acid–water, 5:2:3. Thin-layer ion-exchange chromatography was performed on PEI-cellulose sheets obtained from Brinkman Instruments, Inc.

High voltage electrophoresis was carried out on Whatman No. 1 paper at 65 V/cm using a Savant flat-plate apparatus, Model FP-30A, in the following buffers: buffer A, 25 mM sodium citrate (pH 5.0); buffer B, 15 mM sodium citrate (pH 5.3)–1 mM EDTA; and buffer C, 15 mM sodium citrate (pH 3.0). Qualitative chromatograms and electropherograms were examined after development under a short wave (253.7 nm) lamp before and after treatment in a NH_3/2-butanone atmosphere to visualize quaternary nicotinamide compounds (Kodicek and Reddi, 1951).

*Synthesis of Nicotinamide ϵ-Cytosine Dinucleotide (ϵNCD+) (**2**).* Nicotinamide mononucleotide (100 mg, 0.27

Table I: Electrophoretic and UV Spectrophotometric Characterization of ϵNCD$^+$ and Its Hydrolytic Products.

Compd	R_m[a]	λ_{max}[b]	A_{280}/A_{260}[c]	A_{250}/A_{260}[c]	mol of ϵCMP/mol of NMN[d]
NMN	0.22	266 (266)	(0.26)	(0.84)	
ϵCMP	0.81	288 (288)	(2.19)	(0.84)	
ϵNCD$^+$	0.55	267			
ϵNCD$^+$	0.22	266	0.29	0.84	1.00 (1.00)
+ VPD[e]	0.83	288	2.13	0.83	
NAD$^+$	0.52	259 (259)			
AMP	0.71	259 (259)	(0.15)	(0.80)	
NAD$^+$	0.23	266			
+ VPD	0.71	258	0.14	0.81	

[a] Electrophoretic mobility relative to picric acid marker. [b] Observed λ_{max} after elution from developed electropherogram, literature values in parentheses. See Experimental Section for details. [c] Literature values in parentheses. [d] Theoretical value in parentheses. [e] VPD represents venom phosphodiesterase.

mmol) and ϵ-cytidine 5′-monophosphate (200 mg, 0.51 mmol) were treated with dicyclohexylcarbodiimide (DCC) in aqueous pyridine essentially as described by Hughes *et al.* (1957) except that twice the prescribed amount of DCC was used. The crude product after chloroform extraction was purified by chromatography on a 1.2 × 90 cm DEAE-Sephadex A-25 column (acetate form) using a 1000-ml linear gradient of triethylammonium acetate (0.01–0.20 M, pH 4.6). The fractions containing the product were combined and evaporated to dryness under reduced pressure at 30°, giving a slightly yellow gum. The product at this stage of purification was dissolved in water, and, based on the absorbance at 267 nm, the yield was calculated as 27%. By recovering the unreacted NMN and ϵCMP from the Sephadex column and recycling with further DCC treatment, the overall yield based on NMN was increased to 40%. The purified ϵNCD$^+$ at this point contained a trace of a fluorescent contaminant which was removed by further chromatography on Aminex A-28 (Bio-Rad) anion exchange resin with 0.05 M formic acid as the eluent. The combined fractions were reduced to dryness and dissolved in a minimum of water. Addition of methanol followed by ether produced a flocculent white precipitate which was collected by centrifugation after standing overnight at −20°. The use of acetone in the usual manner for the precipitation of the dinucleotide was avoided to prevent the formation of fluorescent products which are inhibitory to enzymes (Dolin and Jacobson, 1964). The precipitate was washed with two portions of cold, anhydrous ether and dried to give a white amorphous powder, mp, softens at 136°, decomposes at 149–151°. Elemental analysis (Midwest Microlab, Inc., Indianapolis, Indiana) was consistent with structure **2**. *Anal.* Calcd for $C_{22}H_{27}N_5O_{15}P_2 \cdot 2H_2O$: C, 37.78; H, 4.47; N, 10.01. Found: C, 38.07; H, 4.23; N, 9.77.

Nicotinamide ϵ-adenine dinucleotide (**3**) was purified on DEAE-Sephadex and Aminex A-28 as described above. The final purified product was homogeneous by thin-layer chromatography (solvent systems A, B, and C).

Characterization of ϵNCD$^+$. Thin-layer chromatography (solvent systems A, B, and C) and electrophoresis (buffer systems A, B, and C) showed a single spot for the final product. Treatment of 11.8 mM ϵNCD$^+$ in 60 mM glycine buffer (pH 9.2) containing 1.0 mM $MgCl_2$ for 30 min at 37° with 50 mU snake venom phosphodiesterase in a total volume of 50 μl followed by electrophoresis of the mixture gave two products which had electrophoretic mobilities identical with authentic samples of ϵCMP and NMN. The slower moving spot, and a blank of equal area, were quantitatively eluted with four successive 0.5-ml portions of 10 mM HCl and evaporated to dryness under reduced pressure at 30°. The residue was redissolved in potassium phosphate buffer (0.10 M, pH 7.0), and the uv spectrum was recorded. The second spot was similarly eluted, evaporated, redissolved in 0.05 M HCl, and the spectrum was recorded. The results are presented in Table I. The two products resulting from the treatment of ϵNCD$^+$ with venom phosphodiesterase were identified as NMN and ϵCMP by their electrophoretic mobilities and their observed spectral properties. Using the literature values for the molar extinction coefficients of ϵ-cytidine (Barrio *et al.*, 1972a) and NMN, the ratio of ϵCMP to NMN recovered after hydrolysis and electrophoresis was found to be 1.00.

Total phosphate analyses (Chen *et al.*, 1956) using the ashing procedure of Ames and Dubin (1960) and assuming 2 mol of phosphate/mol of ϵNCD$^+$ gave a calculated millimolar extinction coefficient of 17.0 at 267 nm in 0.05 M phosphate buffer (pH 7.0). A similar determination for NAD$^+$ gave a millimolar extinction coefficient of 17.9 at 260 nm in pH 7.0 buffer (lit. 18.0 (Siegel *et al.*, 1959)).

The uv spectra of ϵNCD$^+$ were determined with a Cary 15 recording spectrophotometer at the indicated pH's using the buffers described in the figure legends. The ultraviolet adsorption data are presented in Table II. The spectrum of the CN adduct was taken in 1.0 M KCN (pH 10.0). The spectrum of the reduced form of ϵNCD$^+$, namely ϵNCDH, was recorded after quantitative reduction of ϵNCD$^+$ by the addition of 2 mU of horse liver alcohol dehydrogenase to ϵNCD$^+$ in 0.1 M sodium glycine buffer (pH 8.72) containing 0.5 M ethanol, 0.075 M semicarbazide, and 0.05 M $MgCl_2$. Hypochromicity was determined by recording the uv spectrum of ϵNCD and ϵNCDH before and after a 20-min incubation at room temperature with 3 μg (*ca.* 150 mU) of snake venom phosphodiesterase. Technical fluorescence spectra of ϵNCD$^+$ (1×10^{-5} M), corrected for background, were taken in 0.05 M citrate buffer, at the indicated pH's, on a photon-counting spectrofluorometer (Jameson *et al.*, 1974). Excitation was at 300 nm.

Preparation of ϵNADH. The chloroacetaldehyde-modified coenzyme ϵNAD$^+$ could not be reduced quantitatively by yeast or horse liver alcohol dehydrogenases by published procedures (Ciotti and Kaplan, 1957; Siegel *et al.*, 1959), despite the use of several sets of reaction conditions. Instead, the equilibrium mixture produced by treatment of ϵNAD$^+$ with horse liver alcohol dehydrogenase (0.1 M Tris-HCl (pH 8.5); 0.5 M ethanol) was separated on an Aminex A-28 column by elution with 0.30 M NH_4Cl, adjusted to pH 8.3 with NH_4OH, containing 25% by volume of ethanol. The spectrum of the purified ϵNADH was recorded (0.05 M potassium phosphate buffer, pH 8.0, containing 0.5 M acetaldehyde), the ϵNADH was oxidized by the incubation with 2 μg (2mU) of horse liver alcohol dehydrogenase for 30 min at 25°, and the spectrum was redetermined.

Enzyme Kinetic Data. Coenzyme reduction catalyzed by various dehydrogenases was followed by recording the increase in absorbance at 340 nm *vs.* time on the 0.1 absorbance slidewire of a Cary 15 spectrophotometer equipped with a thermoregulated cell holder kept at 25.0 ± 0.1°. The initial slope of the resulting curves, taken during the first

minute of reaction and extrapolated to zero time, was used in the calculation of kinetic data. A reciprocal plot (Lineweaver and Burk, 1934) of rate (μmol/min) *vs.* coenzyme concentration was checked for linearity and was used for the estimation of the Michaelis constant. The coenzyme concentration was then varied with at least six values between 0.5 K_m to *ca.* 5 K_m. The final data were analyzed statistically, being fitted by the least-squares method directly to the hyperbolic rate expression $v = VS/(K + S)$, using the Fortran program described by Cleland (1967). The standard errors of the resulting fitted kinetic constants K_m and V_{max} were ≤10 and ≤5%, respectively.

The reaction conditions for the enzyme studies were chosen so as to give linear plots of $1/S$ *vs.* $1/v$, indicating that the normal Michaelis–Menton rate law obtained. The 1.0-ml solutions were equilibrated at 25° for 10 min before initiation of the reaction by the addition of the appropriately diluted enzyme in a 25 μl aliquot. Each commercial enzyme preparation was diluted immediately prior to use in 1 mM buffer containing 0.1% bovine serum albumin. Such solutions were stable at least 1 hr at 0°.

The following conditions were used in the kinetic assays (all numbers refer to concentrations in the cuvet): horse liver alcohol dehydrogenase, 6.3 μg of enzyme (17 mU), 50 mM ethanol, 0.25 M Tris-HCl, (pH 9.8); beef liver glutamate dehydrogenase, 2.5 μg of enzyme (220 mU), 25 mM L-glutamate, 25 mM Tris-acetate, (pH 8.0); rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, 1.3 μg of enzyme (130 mU), 0.1 mg of rabbit muscle aldolase (900 mU), 50 mM fructose 1,6-diphosphate, 10 mM sodium arsenate, 50 mM Tris-HCl, (pH 7.5); yeast alcohol dehydrogenase, 0.8 μg of enzyme (38 mU), 25 mM ethanol, 0.1 mM EDTA, 50 mM sodium pyrophosphate, (pH 8.5); beef muscle lactate dehydrogenase, 0.1 μg of enzyme (67 mU), 50 mM DL-lactate, 50 mM Tris-HCl (pH 8.0); beef heart malate dehydrogenase, 38 μg of enzyme (171 mU), 12.5 mM L-malate, 50 mM Tris-HCl (pH 8.0).

Reaction of ϵNCD+ with NAD+ Pyrophosphorylase. To determine whether ϵNCD+ could be synthesized by NAD+ pyrophosphorylase, which catalyzes the reaction NAD+ + $PP_i \rightleftarrows$ ATP + NMN, 39 mM [^{14}C]ϵCTP (1.73×10^5 cpm/μmol), 20 mM NMN, 200 mM glycylglycine buffer (pH 7.4), 10 mM $MgCl_2$, 6 mU of yeast inorganic pyrophosphatase, and 6.8 mU of pig liver NAD+ pyrophosphorylase were incubated in a final volume of 5 μl. At zero time 2 μl was spotted onto a PEI-cellulose coated thin-layer chromatogram onto which 25 nmol each of ϵCTP and ϵNCD+ had been previously applied. The remainder of the reaction mixture was drawn into a capillary tube, the ends were sealed, and the tube was incubated at 37° for 3 hr. From the contents, 2 μl was transferred onto another PEI-cellulose plate, prespotted with the same markers. Both tlc plates were developed in freshly prepared 0.4 M ammonium bicarbonate, and after the chromatograms were dried, the nucleotides were located by their quenching of uv-induced fluorescence and the fluorescence appearing after development in NH_3/2-butanone vapors The chromatograms were cut into eighteen 0.5-cm strips and each strip was counted in scintillation fluid containing 0.6% 2,5-diphenyloxazole and 0.025% 1,4-bis[2-(5-phenyloxazolyl)]benzene in toluene in a Nuclear Chicago Isocap 300. The counts obtained were plotted as percentages of total counts on the chromatogram and there was no difference in the profiles obtained at zero time and after 3 hr of incubation. When [^{3}H]ATP was incubated under similar conditions [^{3}H]NAD+ was obtained in 85–90% yield. Several different ratios of NMN/ϵCTP with varying amounts of $MgCl_2$ were tried with the same negative result.

Table II: Ultraviolet Absorption Data for Etheno Coenzymes.[a]

Compd	pH	λ_{max} (nm)	$\epsilon \times 10^{-3}$ [b]	λ_{min} (nm)	A_{250}/A_{260}	A_{280}/A_{260}
ϵNCD+	7.0	267	17.0	235	0.69	0.86
		270 (sh)				
		292 (sh)				
	4.5	270	12.3	235	0.71	1.00
		292 (sh)				
	(10 mM HCl)	275	12.8	235	0.84	1.21
		283 (sh)				
		290				
		303 (sh)				
ϵNCDH	8.7	272	14.1	245	0.72	1.12
		280 (sh)		303		
		293 (sh)				
		340	6.43			
ϵNCD-CN	10 (1 M KCN)	273	13.9	240	0.64	1.20
		283 (sh)		303		
		293 (sh)				
		327	6.00			
ϵNAD+[c]	7.0	258 (sh)		249	0.89	0.82
		265	10.5	272		
		275				
		295 (sh)				
ϵNADH	8.0	258 (sh)		251	0.89	0.79
		265	6.91	271		
		275	7.09	283		
		335	6.83			
		340	6.67			

[a] Determined in aqueous solution at concentrations on the order of 5×10^{-5} M using the buffers described in the figure legends. [b] At λ_{max}. [c] Barrio *et al.* (1972b).

The ability of ϵNCD+ to serve as a substrate for the enzyme was investigated by incubating 4.6 mM ϵNCD+ (or NAD+), 230 mM glycylglyine buffer (pH 7.4), 28 mM $NaPP_i$ (pH 7.5), 18.5 mM $MgCl_2$, and 19 mU of NAD+ pyrophosphorylase in a total volume of 25 μl at 37° for 30 min. Control incubation mixtures were made which lacked pyrophosphate in order to check for the possible hydrolytic cleavage of the nucleotides. The reactions were terminated by the addition of 200 μl of 1 M HCl, 0.1 M $NaPP_i$, 200 μl 1 M HCl, 0.1 M NaP_i, 20 μl of bovine serum albumin (5 mg/ml), 20 μl of concentrated HCl, and 50 μl of a suspension of Norit (100 mg/ml) in water. After standing on ice for 10 min the charcoal was collected by centrifugation and was washed with two successive 1.0-ml portions of H_2O. The nucleotides were eluted, by treatment at 0° for 10 min, with two successive 0.5-ml portions of cold 50% ethanol containing 0.15 M NH_3. The solutions were reduced to dryness by evaporation under reduced pressure at 25°, dissolved in 50 μl of the ethanol–ammonia solution, and spotted on Whatman #1 paper. Electrophoresis was carried out in buffer system B. The nucleotides were located as described except that the development in NH_3/2-butanone vapors was for 12–16 hr. In order to estimate the extent to which the nucleotides were pyrophosphorylized, reactions containing increasing amounts of NMN but no NAD+ or ϵNCD+ were incubated and worked up as described. Since

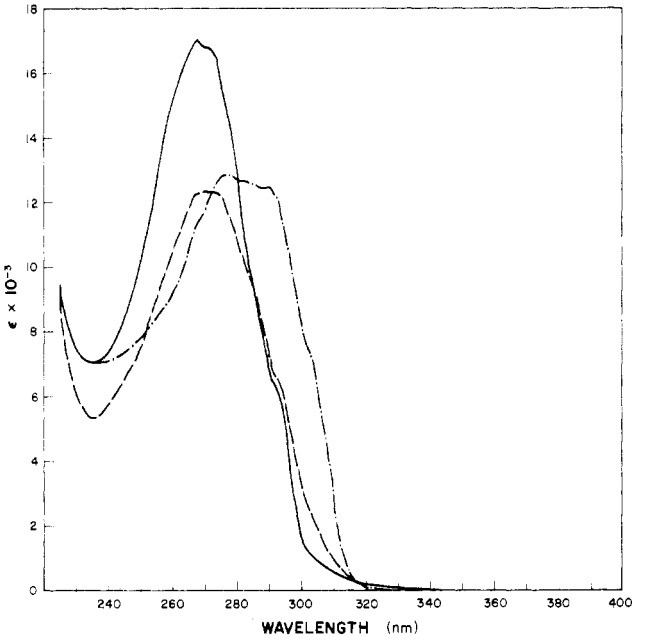


FIGURE 2: The ultraviolet absorption spectra of ϵNCD$^+$ in 0.025 M phosphate buffer (pH 7.0) (—), 0.025 M phosphate buffer (pH 4.5) (- - - - -), and in 0.01 M HCl (- · - · -).

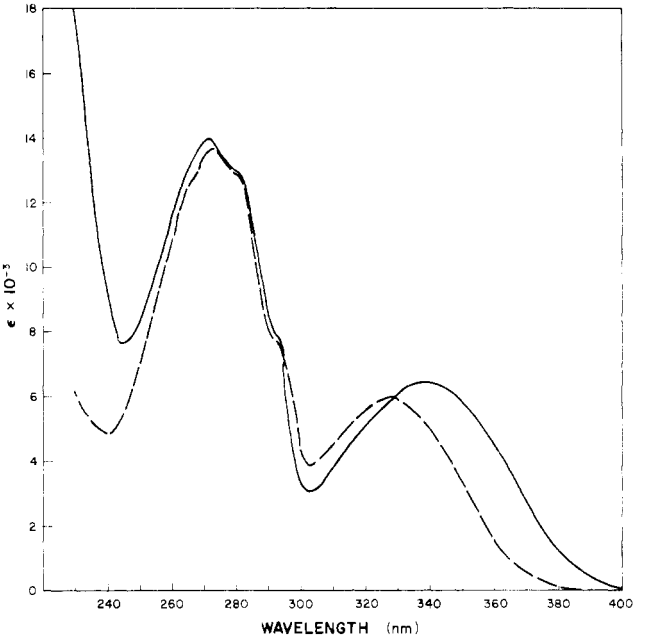


FIGURE 3: The ultraviolet absorption spectra of ϵNCDH in 0.10 M glycine–semicarbazide buffer containing 0.5 M ethanol (pH 8.72) (—) and of ϵNCD$^+$ in 1.0 M KCN (pH 10) (- - - - -).

the apparent equilibrium constant for the pyrophosphorolysis reaction is 2.2 (Kornberg, 1950), we would expect NAD$^+$ to be 93% cleaved under these reaction conditions. The electropherograms indicated that only a small portion of the NAD$^+$ remains after incubation with PP_i. Most of the fluorescence on the electropherograms was found at the position of migration of NMN and the amount was consistent with ≥80% of the NAD$^+$ being cleaved. When ϵNCD$^+$ served as a substrate, a small amount of NMN was formed. By comparison with the NMN standards it could be judged to be between 8 and 15% of the ϵNCD$^+$ added. The rate of ϵNCD$^+$ cleavage by pyrophosphorylase was not examined.

Reaction of ϵNCD$^+$ with NAD$^+$ Kinase. To test whether ϵNCD$^+$ could be converted to ϵNCDP$^+$, a reaction mixture of 1.0 mM ϵNCD$^+$ (or NAD$^+$), 10 mM [γ-^{32}P]ATP (2.97 × 10^6 cpm/mmol), 15 mM $MgCl_2$, 50 mM potassium phosphate (pH 6.95), and 50 μg of pigeon liver NAD$^+$ kinase was incubated in a total volume of 50 μl for 1 hr at 37°. The mixture was spotted onto Whatman #1 paper and electrophoresis was performed in buffer system C. The positions of the nucleotides and of added NADP$^+$ marker were determined as described, and the paper was cut into 1-cm strips. The radioactivity was quantitated by determining the Cerenkov irradiation from the dry strips of paper in a scintillation counter. The amount of NAD$^+$ phosphorylated was calculated from the proportion of the radioactivity shifted from the position of ATP on the electropherogram to the position of NADP$^+$ and from the known amount of [γ-^{32}P]ATP added to the reaction mixture. Under the reaction conditions described, the NAD$^+$ was quantitatively converted to [2′-^{32}P]NADP$^+$, and when the NAD$^+$ concentration was increased to 10 mM and the [γ-^{32}P]ATP to 20 mM, 82% of the NAD$^+$ was phosphorylated. No [^{32}P]ϵNCDP$^+$ could be detected under either of these reaction conditions when ϵNCD$^+$ was substituted for the natural coenzyme.

Reaction of ϵNCD$^+$ with NADase. The NADase assay of Kaplan (1955) which is based on the loss of ability to form a CN adduct characteristic of quaternary nicotinamide compounds was employed. ϵNCD$^+$ (10 mM) in 50 mM phosphate buffer (pH 7.5) was treated with 20 U/ml of NADase for at least 8 min at 37° in volume of 50 μl. The solution was diluted to 1 ml with 1.0 M KCN (pH 10) and it and a control prepared in the same way but lacking enzyme were scanned from 240 to 400 nm using a KCN blank. The control gave a spectrum identical with that obtained for ϵNCD$^+$ in KCN solution, while the solution treated with NADase showed no increase in absorbance at 327 nm, indicating complete hydrolysis by the enzyme; 8 min or longer was required for complete cleavage of ϵNCD$^+$. When NAD$^+$ was examined under the same conditions, complete cleavage occurred within 5 min, indicating a somewhat faster hydrolysis than that obtained for ϵNCD$^+$.

Results and Discussion

Preparations and Characterization of ϵNCD$^+$. The data presented in Table I, along with the thin layer and electrophoretic indications of homogeneity, established that the synthesized product was indeed a single dinucleotide containing ϵ-cytidine 5′-monophosphate and nicotinamide mononucleotide in 1:1 molar ratio. Since the final product obtained was chromatographically and electrophoretically homogeneous and free of inorganic ions, the column chromatographic methods employed (*cf.* Experimental Section) provided a rapid and efficient means of purification and can be generally recommended for the purfication of NAD$^+$ and its analogs. Experimental uv data are summarized in Table II.

The uv spectra of ϵNCD$^+$ as a function of pH (Figure 2) indicate the changes in absorption as the ϵ-cytidine moiety, with a pK_a of *ca.* 3.7 (Secrist *et al.*, 1972), becomes protonated in increasingly acidic solution. The uv spectra of ϵNCDH and of the cyanide adduct of ϵNCD$^+$ (Figure 3) showed new absorbance maxima at 340 and 327 nm, respectively, which are characteristic of changes in the nicotinamide moiety. Similarly, the reduced form of ϵNAD$^+$ (Figure 4) showed a new maximum at 335 nm. The hypochromicity of ϵNCD$^+$ (Figure 5) and of ϵNCDH (Figure 6)

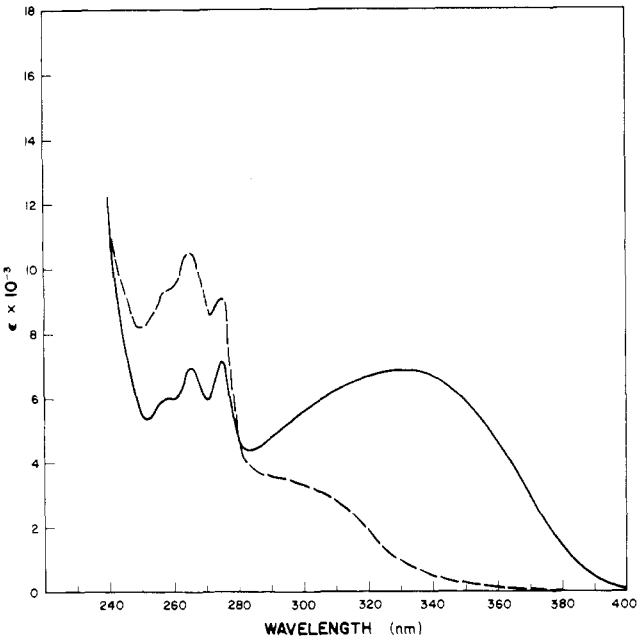


FIGURE 4: The ultraviolet absorption spectra of ϵNADH in 0.05 M phosphate buffer (pH 8.0) before (—) and after (- - - - -) complete oxidation by horse liver alcohol dehydrogenase.

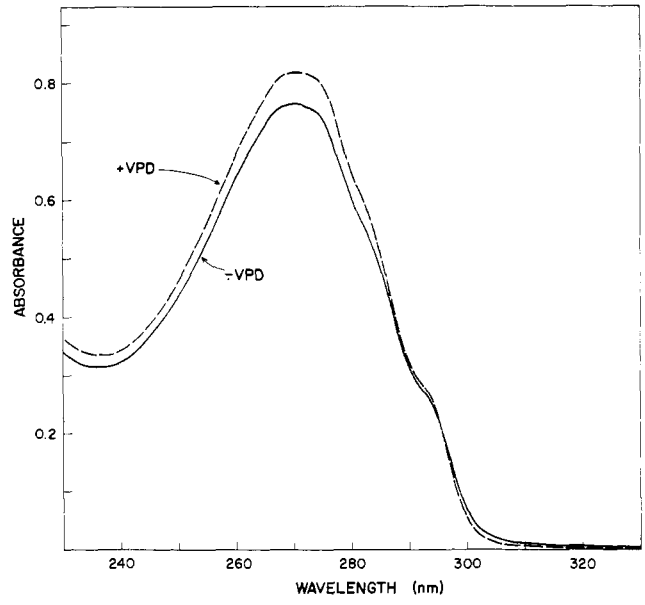


FIGURE 5: The ultraviolet absorption spectra of ϵNCD+ in 0.10 M Tris-HCl buffer containing 0.05 M $MgCl_2$ (pH 8.0) before (—) and after (- - - - -) complete enzymatic hydrolysis with snake venom phosphodiesterase (VPD).

upon hydrolytic cleavage with venom phosphodiesterase suggests that the oxidized and reduced forms of the coenzyme analog are predominantly in folded conformations. This observation is consistent with the hypochromicity observed for NAD+ (Scott *et al.*, 1970) and some of its analogs, *e.g.*, NMN-4′-thioAMP (Hoffman and Whistler, 1970), and NMN-3-isoAMP (Leonard and Laursen, 1965).

Although the ϵ-cytidine moiety showed moderate fluorescence in acidic solution (Barrio *et al.*, 1972a), the fluorescence of ϵNCD+ was very low (Figure 7) at pH *ca.* 3–4 and was not detectable at pH 6 when excited at 300 nm, the excitation maximum. The fluorescence quenching is further indication of interaction between the pyridinium and ϵ-cytosine moieties. Attempts at measuring the fluorescent lifetime of ϵNCD+ by the cross-correlation method (Spencer and Weber, 1969) indicated that the lifetime was extremely short, being less than 0.2 nsec. This combination of weak fluorescence intensity and short lifetime limits the utility of the oxidized form of ϵNCD+ as a fluorescent probe for the study of molecular dynamics and for the investigation of enzyme binding, which have proved so useful in studies with ϵNAD+ (Secrist *et al.*, 1972) and NADH.

Interaction of ϵNCD+ and ϵNAD+ with Dehydrogenases. The kinetic parameters apparent K_m and V_{max} were determined for ϵNCD+ and ϵNAD+, along with the natural coenzyme NAD+. These data are presented in Table III. The enzymes were chosen so as to reflect a wide spectrum of specificities with respect to NAD+ and its analogs (Sund, 1968) and with respect to substrates. Two of the oxidoreductases (glutamate and glyceraldehyde 3-phosphate) are known to exhibit B stereospecificity with respect to the transfer of the hydride ion to position 4 of the nicotinamide (Colowick *et al.*, 1966). The other four dehydrogenases exhibit the opposite stereospecificity of reduction. This stereospecificity has recently been shown to be retained in the enzymatic reduction of NAD+ analogs even though the nicotinamide moiety had been modified (Biellman *et al.*, 1974).

Because of the inherent problems associated with the linear transformations of the Michaelis–Menton rate law (Dowd and Riggs, 1965), the experimental data were analyzed statistically using v^4 weighting factors, being fitted by the least-squares method directly to the hyperbolic rate equation (Cleland, 1967). This method of data analysis has the additional advantage that the standard errors associated with the experimentally determined kinetic parameters can be estimated.

In general, the prediction that ϵNCD+ should serve as a functioning, structural analog of NAD+ was borne out by the kinetic data in Table III. With all six oxidoreductases under the conditions employed, ϵNCD+ was reduced, thus

Table III: Dehydrogenase Kinetic Data.

Dehydrogenase	Coenzyme	K_m[a]	$K_m/K_m^{NAD^+} \times 100$	V_{max}[b]	$V_{max}/V_{max}^{NAD^+} \times 100$
Glyceraldehyde 3-phosphate	NAD+	0.0763	100	6.79	100
	ϵNCD+	0.0651	85.3	5.30	78.1
	ϵNAD+	0.259	339	2.73	40.2
Malate	NAD+	0.0599	100	7.58	100
	ϵNCD+	0.174	290	7.99	105
	ϵNAD+	1.28	2140	1.57	20.7
Horse liver alcohol	NAD+	0.175	100	4.76	100
	ϵNCD+	0.170	97.1	5.97	125
	ϵNAD+	4.86	2780	1.27	26.7
Yeast alcohol	NAD+	0.128	100	19.5	100
	ϵNCD+	12.9	10100	2.76	14.2
	ϵNAD+	1.62	1270	2.30	11.8
Lactate	NAD+	0.148	100	21.5	100
	ϵNCD+	0.549	371	22.4	104
	ϵNAD+	1.08	730	15.9	74.0
Glutamate	NAD+	0.272	100	13.9	100
	ϵNCD+	0.138	50.7	8.42	60.6
	ϵNAD+	0.332	122	8.72	62.7

[a] Expressed as mM; standard error ≤10%. [b] Expressed as nmol min^{-1}; standard error ≤5%.

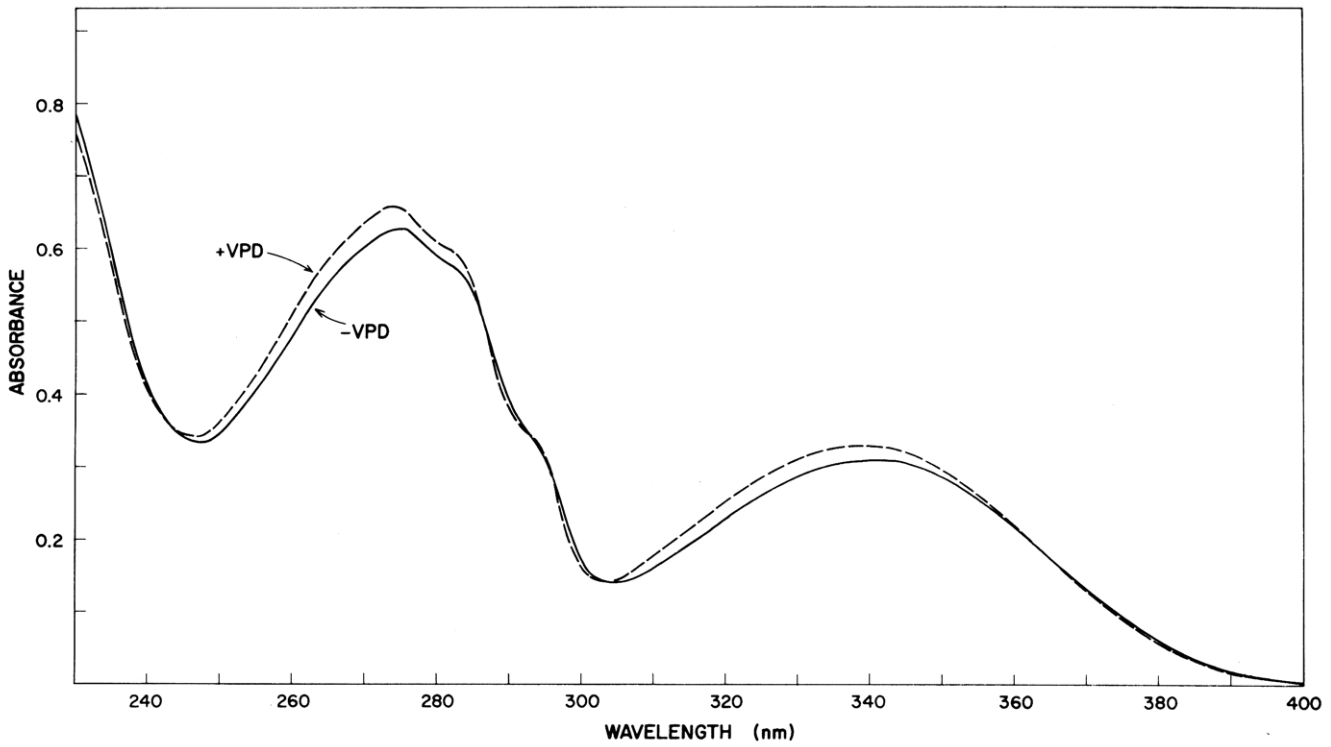


FIGURE 6: The ultraviolet absorption spectra of εNCDH in 0.10 M glycine–semicarbazide buffer containing 0.5 M ethanol and 0.05 M $MgCl_2$ (pH 8.0) before (—) and after (- - - - -) complete enzymatic hydrolysis with snake venom phosphodiesterase (VPD).

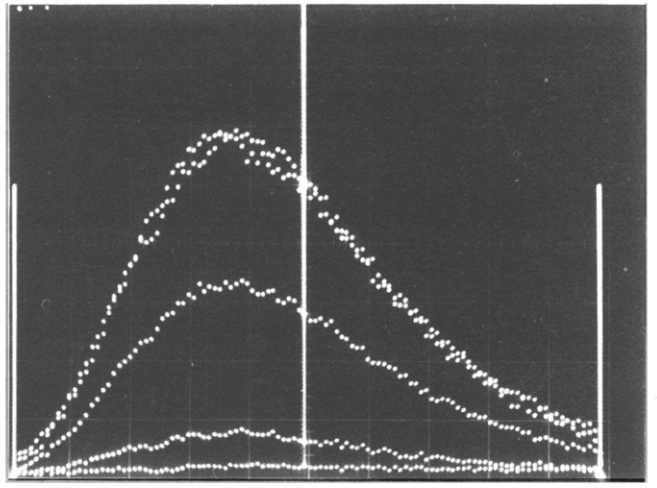


FIGURE 7: Technical fluorescence emission spectra (relative intensity *vs.* wavelength) of ϵNCD^+, corrected for background, in 0.05 M citrate buffers. Scanned between 306 and 415 nm with excitation at 300 nm. The emission maximum is 346 nm, with the highest relative intensity at pH 2.5 and pH 3.5, represented by the uppermost curves (superimposed). The relative intensity decreases successively at pH 4.0, pH 5.0, and pH 6.0, shown respectively. The vertical center marker represents 361 nm.

serving as a functioning coenzyme. In three cases (malate, horse liver alcohol, and lactate dehydrogenases) the V_{max} *was equal to or greater than that of the natural coenzyme.* With the glyceraldehyde 3-phosphate and glutamate dehydrogenases, the V_{max} determined for ϵNCD^+ was somewhat less than for NAD^+, and with yeast alcohol dehydrogenase, was only 14% of the NAD^+ reduction rate. This order of rate of reactivity supports and extends the earlier observations (Colowick *et al.*, 1966; Sund, 1968b) that yeast alcohol dehydrogenase is highly selective with respect to the structure of the coenzyme. In four cases, the maximum velocity found for the reduction of ϵNAD^+ was smaller than that for ϵNCD^+; with yeast alcohol and glutamate dehydrogenase the rates of reduction of the two analogs were equal. In general the maximal rate of reduction of ϵNAD^+ was smaller than the maximal rate obtained for NAD^+. This confirms the earlier studies of ϵNAD^+ reduction by selected dehydrogenases (Barrio *et al.*, 1972b; Lee and Everse, 1973).

The values of the apparent K_m obtained for ϵNCD^+ are smaller or equal to those determined for NAD^+ with glyceraldehyde-3-phosphate dehydrogenase, horse liver alcohol dehydrogenase, and glutamate dehydrogenase. With malate and lactate dehydrogenases, the apparent K_m values for ϵNCD^+ were three to four times larger than those of NAD^+, and with the yeast alcohol enzyme, the apparent K_m was 100 times larger. In five cases, the apparent K_m for ϵNAD^+ was from 3 to 28 times larger than that of NAD^+. With glutamate dehydrogenase, the apparent K_m of ϵNAD^+ was similar to that for NAD^+. Only in the experiments with yeast alcohol dehydrogenase was the apparent K_m of ϵNAD^+ smaller than that of ϵNCD^+.

Several earlier reports have appeared which presented relative rates of reduction of NAD^+ analogs having an *unmodified pyrimidine* replacing the adenine. Although these data cannot be compared directly and quantitatively with ours, the analogs containing cytosine (Pfleiderer *et al.*, 1963; Honjo *et al.*, 1962), uracil (Fawcett and Kaplan, 1962; Honjo *et al.*, 1962), and thymine (Honjo *et al.*, 1962) appear to be poorer than ϵNCD^+ in replacing NAD^+ in dehydrogenase reduction.

In general, the kinetic data support the hypothesis that ϵNCD^+ more satisfactorily approximates NAD^+ spatially than does the larger, more bulky ϵNAD^+. The ability of the εCyt moiety to functionally replace adenine in nucleotides was first shown with 3-phosphoglycerate kinase (Barrio *et al.*, 1973). These findings show that the selectivity of the

enzymes studied toward the nucleotides bound and used are similar. This correlates with the fact that the nucleotide binding domains of some dehydrogenases and kinases have been shown to be very similar (Schultz *et al.*, 1974; Brändén, 1974; Blake, 1974) and that these groups of enzymes may have evolved from a common ancestral binding protein (Rossmann *et al.*, 1974).

In order to help understand the selectivity of dehydrogenases with the modified coenzymes used in this study, we have examined the apparent ability of two dehydrogenase molecular models—dogfish lactate and lobster muscle glyceraldehyde-3-phosphate—to spatially accommodate models of NAD$^+$ in which A had been replaced with ϵC or ϵA. Access to the models at Purdue University was kindly provided by Professor M. G. Rossmann, who has previously reported the three-dimensional structures of these enzymes (Adams *et al.*, 1970; Rossmann *et al.*, 1971; Buehner *et al.*, 1973). In both the lactate and glyceraldehyde-3-phosphate holoenzymes, the replacement of the adenine of the bound NAD$^+$ with ϵ-cytosine produced no apparent structural constraints in the coenzyme binding sites. However, the kinetic data in Table III indicate that the K_m with lactate dehydrogenase is increased 3.7-fold and the V_{max} with the triose dehydrogenase is reduced to a value 0.8 of that with NAD$^+$. Furthermore, the lactate dehydrogenase model suggested that the replacement of adenine with ϵ-adenine could be accommodated within the coenzyme binding site and although the K_m is increased 7.3-fold the V_{max} is reduced by only 26%. The glyceraldehyde-3-phosphate dehydrogenase model, however, indicated a more crowded condition at the binding area with ϵ-adenine replacing adenine. This may be reflected in the 3.4-fold larger K_m and 2.5-fold reduced V_{max} although the dissociation constant for the rabbit muscle enzyme and ϵNAD$^+$ has been found to be similar to that for NAD$^+$ (Schlessinger and Levitzki, 1974). These observations suggest that the ability of ϵNCD$^+$ to replace NAD$^+$ as a coenzyme, as judged by V_{max} determinations with these two dehydrogenases, may be due to its relatively similar accommodation within the enzyme binding sites. Furthermore, the observed ability of ϵNAD$^+$ to be reduced by lactate dehydrogenase better, in terms of V_{max}, than by glyceraldehyde-3-phosphate dehydrogenase may be the result of greater steric restrictions within the coenzyme binding region of the latter enzyme.

The high degree of conservation of the tertiary structure of the coenzyme binding regions and the homology of amino acid sequences amon the the dehydrogenases (Rossmann *et al.*, 1974; Olsen, K. W., Moras, D., Rossmann, M. G., and Harris, J. I., in preparation) greatly minimize species differences in the lactate and glyceraldehyde-3-phosphate dehydrogenases used in this study and those represented in the molecular models.

Reaction of ϵNCD$^+$ with Other Enzymes. The pyrophosphorolytic cleavage of ϵNCD$^+$ by NAD$^+$ pyrophosphorylase was detectable, but much less complete (8–15%) than that of NAD$^+$ (>80%) under the same conditions (*cf.* Experimental Section). In the reverse reaction, the synthesis of ϵNCD$^+$ from ϵCTP and NMN was undetectable under conditions where ATP was 80–95% converted to NAD$^+$ (*cf.* Experimental Section). These results suggest that ϵNCD$^+$ is much less efficiently utilized by this enzyme, under these conditions, than is NAD$^+$. NAD$^+$ pyrophosphorylase has been reported to catalyze the formation of NAD$^+$ analogs from triphosphate derivatives of 3′-deoxyadenosine (Suhadolnik *et al.*, 1974), 2′-deoxyadenosine (Suhadolnik *et al.*, 1974), 7-deazapurine riboside (Ward *et al.*, 1972; Suhadolnik *et al.*, 1974), and 2-aminopurine riboside (Ward *et al.*, 1972). The triphosphate derivatives of formycin (Ward *et al.*, 1972), ϵ-adenosine, and sangivamycin (7-deaza-7-carboxamidoadenosine) (personal communication from R. J. Suhadolnik) are not converted to the corresponding NAD$^+$ analogs. Since the K_{eq} favors pyrophosphorolysis and the ratio of V_{max} for NAD$^+$ synthesis to the V_{max} for NAD$^+$ breakdown is 0.48 (Kornberg, 1950) perhaps other reaction conditions or longer reaction times might allow ϵCTP and the other analogs which are modified in the adenine portion to form the desired products.

Pigeon liver NAD$^+$ kinase did not catalyze the phosphorylation of ϵNCD$^+$ to form ϵNCDP$^+$ under the same reaction conditions in which NAD$^+$ was quantitatively converted to NADP$^+$ (*cf.* Experimental Section). The enzyme from pigeon liver has been reported to be unable to phosphorylate one other adenine modified analog, hypoxanthine nicotinamide dinucleotide (Wang and Kaplan, 1954). These results suggest that NAD$^+$ kinase is highly specific for NAD$^+$.

NADase cleaves ϵNCD$^+$ more slowly than NAD$^+$ (*cf.* Experimental Section). Nicotinamide hypoxanthine dinucleotide (Kaplan *et al.*, 1952) and ϵNAD$^+$ (Lee and Everse, 1973) have also been found to be cleaved more slowly than NAD$^+$ with *Neurospora crassa* NADase. This enzyme also appears to display specificity toward the adenine moiety of the coenzyme.

The inspection of the spatial relationships between the three-dimensional models of two coenzyme analogs and the coenzyme binding domains of two dehydrogenases has allowed a rationalization of the kinetic behavior of the analogs. These studies indicate that a systematic examination of the interactions of a large series of coenzyme analog models and dehydrogenase models, now that the latter are available from X-ray data, together with a comparison of their kinetic behavior, can provide additional information concerning the nature of the binding pocket of the functioning enzymes.

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