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Dimers of Nicotinamide Adenine Dinucleotide: New Evidence for the Structure and the Involvement in an Enzymatic Redox Process[1]

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The composition and the structure of the product from the known electrochemical dimerization of the NAD^+ have been conclusively demonstrated. A detailed analysis of the ^1H and ^{13}C nmr spectra has in fact led to the conclusion that the product contains three diastereoisomeric dimers of the 4,4′-tetrahydrobipyridyl type. Furthermore, the cytoplasmic fraction obtained from a standard mitochondrial preparation of rat liver has been shown to catalyze the oxygen uptake by the dimers. A 1 : 1 molar ratio of the reagents in the redox process is indicated by manometric data on oxygen uptake complemented by spectrophotometric analysis of the oxidized substrates, suggesting that H_2O_2 is the reduction product. NAD^+ was identified as the oxidation product by an enzymatic method.

The basic electrochemical reduction pathway of nicotinamide adenine dinucleotide (NAD^+) has been well established and extensively reviewed (*1–3*). It has been consistently found that one- or two-electron reduction products are formed. The uptake of two electrons leads to the formation of two dihydropyridine species, namely, NADH and its 1,6-isomer (*4*), whereas the uptake of one electron leads to the formation of a radical that undergoes irreversible dimerization. The dimeric product has been interpreted as a 4,4′-tetrahydrobipyridyl structure but only on the grounds of preliminary evidence (*5, 6*), and no information has been reported concerning the number of stereoisomers obtained. It has been reported that the dimeric product does not display the activity of NADH when tested for coenzyme activity using lactic dehydrogenase or yeast alcohol dehydrogenase (*4, 5, 7*). However, it has been observed that preparations from seedlings of various plants, as well as commercial mushrooms tyrosinase oxidize the dimeric product to NAD^+ (*8*).

We now present conclusive evidence concerning the structure and the number

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[1] Paper V in a series on the electrochemical reduction of pyridinium salts.

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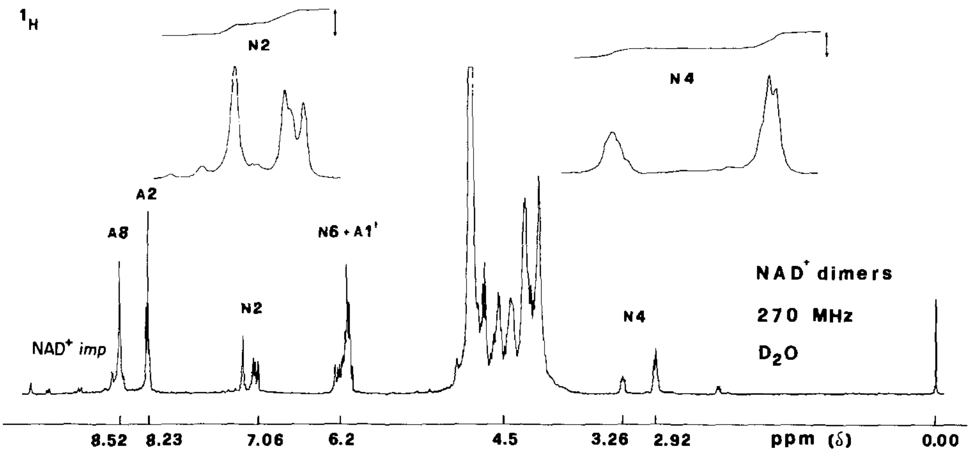

FIG. 1. ¹H nmr spectrum of the mixture of the diastereoisomeric dimers 1.

of the dimeric species from electrochemical reduction of NAD^+, and furthermore we report the uptake of oxygen by the dimeric product in the presence of biological materials.

RESULTS AND DISCUSSION

The previously reported polarographic behavior of NAD^+ (9) has been confirmed by our experiments. It is essentially characterized by two reduction waves A and B, both monoelectronic. Electrolysis of NAD^+ has been performed at different potentials within the wave A plateau in about 1.5×10^{-2} *M* aqueous solutions, buffered in the pH range 9–10. The electrolyzed solution was lyophilized and the residue chromatographed on a Sephadex column, which allowed the separation of a fraction containing the dimeric product. The uv spectrum of the dimeric product does not exhibit absorption bands beyond 400 nm, which excludes structures involving dimerization at position 2. ¹H nmr spectrum (Fig. 1) shows five groups of signals comparable to those observed for NADH under the same experimental conditions, viz. the A-8 and A-2 adenine protons[3] at 8.51–8.54 and 8.23–8.25 ppm, respectively (both patterns appear as the sum of more than two overlapped signals); the N-2 protons as four well-separated signals at 7.22, 7.12, 7.11, and 7.06 ppm with similar intensities; the N-6 protons together with the A-1′ ribose protons as a complex pattern at 6.1–6.3 ppm; the ribose region between 4.0 and 4.9 ppm, which includes also the N-5 protons and is partially overlapped by the HOD peak; finally the N-4 protons as two broad peaks at 3.26 and 2.92 ppm. The spectrum shows also the presence of impurities (ca. 5%), which were regarded in part as oxidation products. Their amount depends on the particular preparation and increases with time. Five preparations were checked

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[3] A and N indicate the carbon atoms of the adenine nucleoside and those of the nicotinamide nucleoside moiety, respectively.

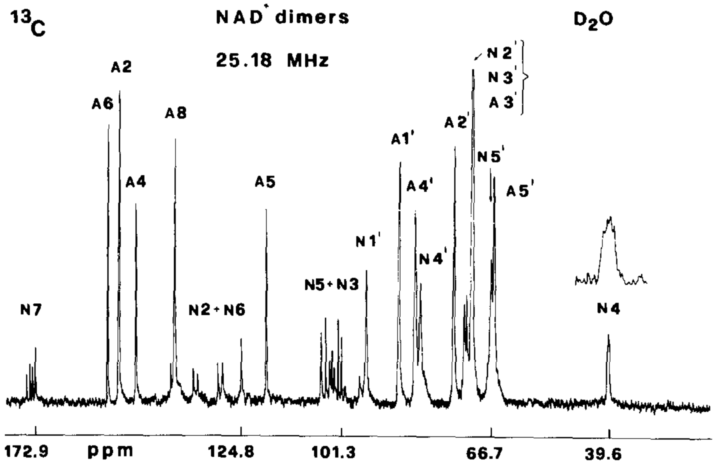


FIG. 2. ^{13}C nmr spectrum of the mixture of the diastereosomeric dimers **1**.

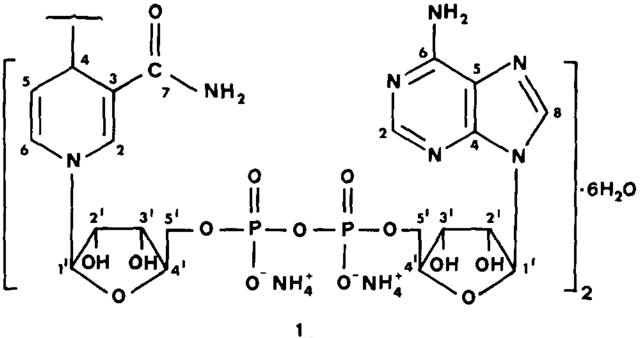
by nmr; the spectra also show a slight variation in the intensity ratios for the peak at 7.22 with respect to the other ones in the 7-ppm region (N-2), but the total integrated area remains constant with respect to the protons at 8, 6, and 3 ppm, i.e., in the ratio 1 : 2 : 2 : 1.

The ^{13}C nmr spectrum (Fig. 2) shows the quadrupling of the signals of the

TABLE 1

^{13}C NUCLEAR MAGNETIC RESONANCE CHEMICAL SHIFT ASSIGNMENTS FOR DIMERS **1** AND NADH

	Dimers **1**	NADH
N-7	174.9, 174.1, 173.5, 172.9	173.3
A-6	155.8	156.1
A-2	153.3	153.4
A-4	149.3	149.6
A-8	140.3	140.4
N-2	141.2, 135.9, 135.0, 130.2	139.1
N-6	129.1, 124.8	124.7
A-5	119.0	119.3
N-5	106.1, 104.1, 103.0	106.1
N-3	105.0, 103.6, 102.1, 101.3	100.8
N-1′	97.1, 95.6	95.9
A-1′	88.1, 87.9	88.0
A-4′	84.3	84.4
N-4′	83.1, 82.8	83.1
A-2′	75.2	75.3
N-2′ N-3′ A-3′	72.9, 72.4, 71.2	71.6, 71.3, 71.0
N-5′	66.7	66.7
A-5′	66.1	66.0
N-4	40.0, 39.8, 39.7, 39.6	22.7



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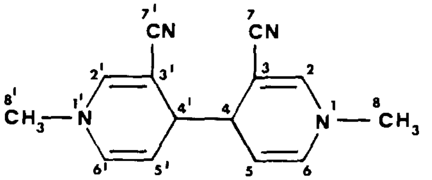
dihydropyridine carbon atoms. The assignment of all the carbon frequencies (Table 1) was made on the basis of the multiplicity of the signals and our assignments for NADH, which agree with those reported by Birdsall *et al.* (*10*).

Four peaks are clearly visible for each of the N-7, N-3, and N-4 carbons, whereas some peaks are missing, probably overlapped, for carbon N-5 (one missing) and for the undistinguished pair of carbons, N-2 and N-6 (which lacks two peaks). The adenine and ribose carbon atoms give broader signals (width ca. 10 Hz). The five dimerization reactions checked gave the same ^{13}C results.

The chemical shift values for the N-4 carbons, as well as for the N-4 protons, indicate that the dimerization occurs at the 4 position of the pyridine ring, as depicted in the structure 1. The accurate measurements of the integrated areas in the 270-MHz spectrum and the complete analysis of the ^{13}C nmr spectrum allowed the exclusion, for the dimeric product in our hands, of possibilities such as 4,6′- or 6,6′-junctions. Actually, the presence of 1,6-dihydropyridine moieties should decrease the integrated area of the proton pattern at 3 ppm (the signal for the CH-6 methine proton lies at 4.1 ppm (*11*)) and moreover should give signals in the 60 to 65-ppm region of the ^{13}C spectrum, as proved by complete analysis of the two model compounds 2 and 3[4] (Table 2). The substituents and solvent effects are very small, as is shown by the chemical shift values of C-4 carbons for the model dimer 2 (39.7 ppm in $CDCl_3$, 39.0 ppm in DMSO-d_6), for two diastereoisomeric 1,1′-dibenzyl-3,3′-dicarbamoyl-1,1′,4,4′-tetrahydro-4,4′-bipyridyls (38.9 and 39.2 ppm in DMSO-d_6 (*12*)) and for the dimeric product 1 (40–39.6 ppm in D_2O). Thus the expected chemical shift value for the C-6′ carbon in a 4,6′-linked dimer is 63–64



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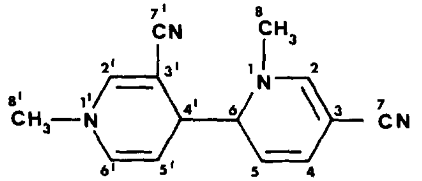
[4] The analyzed dimers 2 and 3 are the diastereoisomers which melt at 143–145 and 161–163°C, respectively, cf. Ref. (*11*).



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ppm, but in this region of the spectrum no signals have been detected (Fig. 2 and Table 1).

In any molecule arising from the electrochemical dimerization of NAD$^+$, two additional chiral centers must be considered, in our case the N-4 carbon atoms. Since the configuration of the adenosine diphosphatoribose moieties (A) is retained, neither enantiomers nor mesoforms are allowed; and a maximum of three diastereoisomers is possible: ARRA, ASSA, ARSA. Corresponding nuclei in the two halves are stereochemically equivalent by internal comparison for dimers ARRA and ASSA and must thus show chemical shift equivalence[5] in nmr spectroscopy; whereas for the dimer ARSA they are diastereotopic and may give different nmr signals (*13*). The nmr spectrum of a mixture of the above three diastereoisomers can thus exhibit a maximum for four signals for each proton or carbon.

Different conformers interconverting slowly on the nmr time scale can in principle show different signals. The problem of the conformation in solution for dinucleotides, in particular whether they are folded or unfolded, has not yet been solved (*14–21*). The observation of two nmr signals for the single nicotinamide C-4 proton of NADH, stereospecifically deuterated at this position, has been presented as evidence for significant populations of both right and left helices (*16*) or as an indication that any other slow conformational process (*18*), for instance hindered rotation about the dihydropyridine-ribose glycosidic linkage, may occur. The results of recent studies on some mononucleotides (*21*) indicate that they are best described as being in a rapid equilibrium between *syn* and *anti* conformation.

Actually, the ^{13}C spectrum of NADH (Table 2) shows a single resonance for each carbon atom, whereas the dimers **1** under the same experimental conditions exhibit the quadrupling of the nicotinamide ^{13}C and ^{1}H signals. Although the possibility of slow conformational processes cannot be completely excluded in the present case,[6] we can reasonably conclude that the quadrupling of the ^{13}C and ^{1}H nmr signal is due to the presence in the dimeric product of the three above diastereoisomers.

The biological preparations assayed for stimulation of oxygen uptake by the dimeric product were the sonicated precipitate (mitochondrial fraction) and the dialyzed supernatant (cytoplasmic fraction) of a standard mitochondrial preparation from rat liver. Figure 3 shows that the sonicated mitochondrial precipitate

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[5] The two N-4 protons, although they have the same chemical shift, may from the symmetry have a different coupling to each of the other nuclei in the molecule (i.e., are magnetically inequivalent or spin coupling nonequivalent). In fact the broad patterns at 3.26 and 2.92 ppm in Fig. 1 must be considered as the AA' part of second-order AA'XX'YY' . . . spin system.

[6] The nmr spectra at +60°C were unsuccessful because the dimers are unstable at this temperature.

TABLE 2

^{13}C NUCLEAR MAGNETIC RESONANCE CHEMICAL SHIFT ASSIGNMENTS FOR 2 AND 3

	2		3	
	$CDCl_3$	DMSO-d_6	$CDCl_3$	DMSO-d_6
C-2	144.6	145.3	148.3	149.6
C-2′			145.1	146.0
C-6′	130.4	131.0	131.0	131.2
C-6			63.0	62.3
C-7′	121.1	120.8	{121.2	{120.7[a]
C-7			120.5}	121.3}
C-5	102.8	101.0	110.0	109.7
C-5′			100.5	99.9
C-3′	78.5	76.2	{78.0	{74.9
C-3			76.3}	74.2}
C-8	40.8	39.9	42.1	41.2
C-8′			40.9	40.0
C-4	39.7	39.0	123.1	122.4
C-4′			37.7	36.7

[a] Similar values in braces may be interchanged.

rapidly catalyzes the oxidation of NADH with the stoichiometry expected for H_2O formation but does not stimulate oxygen uptake by the diastereoisomeric mixture 1. On the other hand the cytoplasmic fraction is active on both substrates. Figure 3 reports data concerning short incubation times. Actually the final value (not shown in the figure) was reached after 6–8 hr and is in favor of H_2O_2 formation. A complementary spectrophotometric analysis of the incubated mixtures at various times support this stoichiometry, since the amounts of oxidized substrate parallel the oxygen consumption with an approximate 1 : 1 molar ratio (Table 3). Samples of cytoplasmic fraction, denaturated by heating at 100°C for 15 min, are no longer able to catalyze the uptake of oxygen, thus demonstrating the involvement of an enzyme. NAD^+ was identified as the oxidation product by an enzymatic method. Its amount was found to represent only a fraction of the oxidized substrates, and could be detected only at initial incubation times. Therefore, some independent biological degradation of NAD^+ must occur. In fact, a comparable decrease was also observed in parallel control experiments, where NAD^+ was incubated with the cytoplasmic fraction (Table 3).

The results clearly indicate that dimers 1 as well as NADH are oxidized by a liver cytoplasmic preparation to yield NAD^+. Work is in progress to localize and purify the enzyme.

In conclusion, the results reported in this paper constitute the first unequivocal demonstration that reaction of dimers 1 with oxygen can be stimulated by biological catalysts. This calls for a more comprehensive investigation of the possible involvement of these dimers in redox enzymatic processes and stimulates new ideas in current theories of electron transfer by pyridine coenzymes.

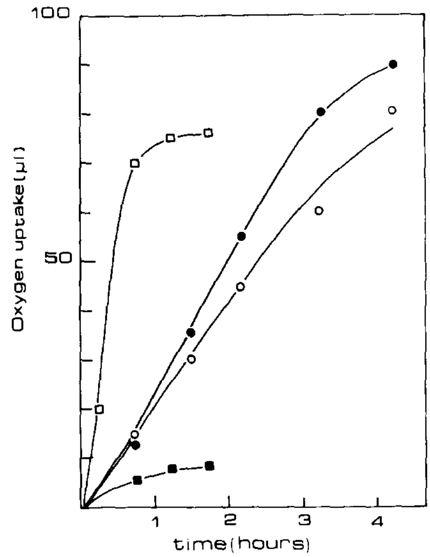

FIG. 3. Oxygen uptake by NADH and dimers 1; (□) mitochondrial fraction from rat liver (5.3 mg total protein) with NADH; (■) the same with dimers 1; (○) cytoplasmic fraction from rat liver (4.8 mg total protein) with NADH; (●) the same with dimers 1.

EXPERIMENTAL

Macroscale electrolyses were carried out using an AMEL MODEL 551 potentiostat. A water-jacketed three-compartment cell, kept at 25.0 ± 0.1°C, containing a magnetically stirred mercury pool as a working electrode (apparent area 20 cm^2), was used. Agar salt bridges were inserted at the counter and reference sides of the medium porosity glass frits separating the compartments. The reference compartment contained a saturated calomel electrode and the counter compartment contained a platinum gauze cylinder immersed in a satu-

TABLE 3

STOICHIOMETRY OF OXIDATION OF DIMERS 1 AND NADH BY THE CYTOPLASMIC FRACTION OF RAT LIVER

Substrate (7.5 μmol)	Incubation time (min)	μmol of O_2 consumed	μmol of oxidized product	μmol of NAD$^+$ detected
NADH	60	1.2	1.5	0.4
	240	4.5	5.4	—
Dimers 1	90	1.2	0.9	0.2
	240	3.3	3.3	—
NAD$^+$	60	—	—	0.4
	240	—	—	—

rated KCl solution. Buffer solutions were preelectrolyzed at the same potential of electrolysis. Nitrogen or argon (99.99% purity), equilibrated by bubbling through buffer solution, was continuously passed through the cell during the electrolyses. ^{1}H nmr and ^{13}C nmr spectra were recorded with a Bruker WH-270 and a Varian XL-100-15 spectrometer, respectively. ^{1}H shifts were measured from sodium 3-trimethylsilyl-tetradeuteropropionate and are accurate to ±0.005 ppm. ^{13}C shifts were measured from internal tetramethylsilane for $CDCl_3$ and DMSO-d_6 solutions, and from internal dioxane for D_2O solutions; these latter values were then converted to the tetramethylsilane scale by using the observed (67.4 ppm (*22*)) difference between dioxane and internal tetramethylsilane; they are accurate to ±0.1 ppm. ^{13}C spectra of NADH and dimers **1** were measured in D_2O (200 mg/2 ml) containing NH_3 (pH ca. 9); the presence of NH_3 does not significantly affect the chemical shift values. The ^{13}C assignments for models **2** and **3** were performed by ^{1}H single-frequency selective decoupling.

Spectrophotometric measurements were carried out in a Beckmann uv 5230 spectrophotometer, and in a Perkin–Elmer uv 575 spectrophotometer.

Electrochemical Reduction of NAD⁺ and Isolation of Dimers 1

In a typical run, 1.0 g of NAD^+ was dissolved in 100 ml of 0.1 *M* NH_3–0.1 *M* NH_4Cl buffer and electrolyzed at −1.20 V. The electrolysis took about 1 hr to reach completion, as inferred from the constant value of the current, equal to that obtained after preelectrolysis at the same potential of a solution containing only supporting electrolyte. During the electrolysis the deviation of the voltage was ±1 mV with respect to the applied potential. The electrolyzed solution was lyophilized and the residue (0.78 g) dissolved in 5 ml of 1 *M* ammonia was applied to the top of a Sephadex G-10 column (32 × 800 mm) and eluted with distilled water containing 0.1% (v/v) of 25% ammonia. A flow rate of 0.5 ml/min was maintained and the effluent monitored up to 340 nm. The fraction eluted in the volume range from 200 to 250 ml exhibits a 4.9 : 1 ratio between the absorbance values measured at 260 and 340 nm, respectively, and contains the dimeric product practically free of NAD^+ and chlorides. The product was dried overnight *in vacuo* over P_2O_5, yielding 0.30 g.

Anal. Calcd for $C_{42}H_{52}N_{14}O_{28}P_4(NH_4)_4 \cdot 6H_2O$: C, 33.51; H, 5.36; N, 16.75. Found: C, 33.75; H, 5.06; N, 16.24.

$\lambda_{max}(H_2O)$, 260 nm (ϵ = 31,600) and 340 nm (ϵ = 6400).

Oxygen Uptake Measurements

Washed and roughly cut rat liver was manually homogenized in a Potter–Elvehjem apparatus with 20 m*M* Tris buffer at pH 7.6 containing 0.32 *M* sucrose and 3 m*M* $MgCl_2$ (10 : 1 v/w). The homogenate was filtered through a cheese cloth, diluted 1.25 : 2 with 0.25 *M* sucrose, layered on the top of equal volume of buffer, and then centrifuged at 700*g* for 10 min. The supernatant was centrifuged again for 10 min at 7000*g*. The pellet so obtained was washed twice with 0.25 *M* sucrose, resuspended in 0.1 *M* phosphate buffer at pH 7.4, and then disrupted by sonication. This fraction is referred to as the mitochondrial fraction. The supernatant was extensively dialyzed against the same buffer before use (cytoplasmic fraction). Oxygen uptake was measured in a conventional Warburg apparatus at 37°C in 0.1 *M* phosphate buffer at pH 8.0 (3-ml final incubation volume). The reported values represent averages of duplicate samples after subtraction of appropriate blanks. Seven and one-half micromole of dimers 1 or of NADH was used as substrate in all experiments. Incubations were performed in the presence of 200,000 units of penicillin to prevent bacterial growth. NAD^+ was measured by an enzyme assay, using alcohol dehydrogenase as detector enzyme (*23*), under the following conditions: 0.1 to 0.3 ml of sample was incubated in 3 ml of 75 m*M* pyrophosphate buffer at pH 8.7 containing 1% ethanol and 0.84% semicarbazide in the presence of 0.01 ml of yeast alcohol dehydrogenase (6000 IU/ml) at 25°C. The absorbance value at 340 nm was read against a reference solution without alcohol dehydrogenase.

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