Tetrahedron. Vol. 32, pp 1761 to 1765 Pergamon Press 1976. Printed in Great Britain

SPECTROSCOPIC CHARACTERIZATION OF A FUNCTIONALIZED NAD$^+$ ANALOGUE AND ITS POLYETHYLENEIMINE-BOUND DERIVATIVE

ELVIO MANTOVANI,* LUIGI SETTEMBRI and PIERGIORGIO ZAPPELLI
Snamprogetti S.p.A., Laboratory for Biochemical Processes, 00015 Monterotondo, Rome, Italy

(*Received in the UK* 31 *December* 1975; *Accepted for publication* 17 *February* 1976)

Abstract—The ^{1}H and ^{13}C NMR spectra of deuterium oxide solutions of two NAD$^+$ derivatives, i.e. nicotinamide-6-(2-hydroxy-3-carboxypropylamino)purine dinucleotide and 4(NAD$^+$–N^6)-3-hydroxybutyryl-polyethylememine, have been examined over a range of pH, as well as the fluorescence spectra of their reduced forms. The results have been compared with those obtained with NAD$^+$. Small shifts have been observed in both the proton and carbon magnetic resonance spectra in passing from low to high pH. This behaviour, as well as the fluorescence studies, indicate that, analogously to NAD$^+$, the two NAD$^+$ derivatives exist in solution as a pH dependent equilibrium of folded and unfolded forms, with the folded form prevailing at basic pH. As consequence of the folding, an intramolecular interaction takes place between the adenine and nicotinamide rings. The variation of proton and carbon resonances with pH change has been utilized to evaluate the strength of this intramolecular interaction.

INTRODUCTION

During the last few years NAD$^+$ and related compounds have been widely studied by ^{1}H[1-3] and ^{13}C NMR[4-7] spectrometry, as well as by fluorometric measurements.[8-10] In fact, the conformation of pyridine dinucleotides in aqueous solution is of interest in the study of the transition complexes formed in the course of enzymatic reactions. The fluorescence measurements and NMR studies on NAD$^+$ and NADH have demonstrated that, depending on pH, an intramolecular interaction between the adenine and pyridine rings can take place. This behaviour was interpreted in terms of a folding of the molecule at basic pH with the two dinucleotide rings staked nearly in parallel.[1,2,7,9]

Recently, in our Laboratories, hydrosoluble macromolecularized NAD$^+$ derivatives[11] were prepared in order to extend the use of immobilized enzymes to those cases in which readily dissociable cofactors are required.

For this purpose a suitable functionalized NAD$^+$ analogue, i.e. nicotinamide-6 - (2 - hydroxy - 3 - carboxypropylamino)purine dinucleotide (**1**) was prepared and coupled to polyethyleneimine and polylysine.

All derivatives retained coenzymic activity with various enzymes.[11,12] In this paper we report our investigation on the behaviour in solution, in comparison to NAD$^+$, of two of these new compounds, i.e. nicotinamide-6 - (2 - hydroxy - 3 - carboxypropylamino)purine dinucleotide (**1**) and 4(NAD$^+$–N^6) - 3 - hydroxybutyryl - polyethyleneimine (**2**).

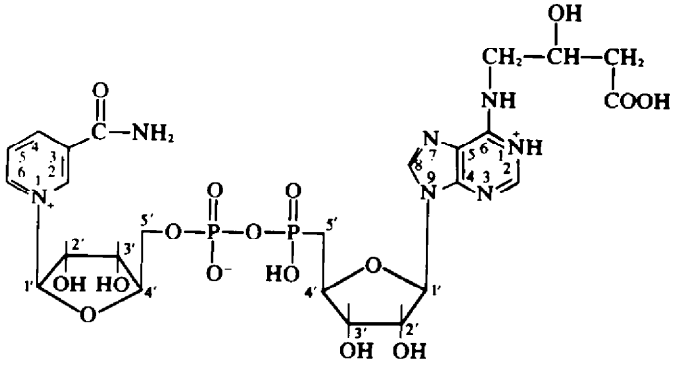
RESULTS AND DISCUSSION

^{1}H NMR measurements. In Table 1 are listed the chemical shifts as well as the coupling constant of various proton resonances of the dinucleotides used in this study, measured at pH 2.5 and 7.5.†

The resonance positions for NAD$^+$ agree with those previously reported in the literature[1,2] and the difference of chemical shift values shown by the two NAD$^+$ derivatives, in respect to NAD$^+$, can be taken as an indication of conformation changes as consequence of substitution. However, the fact that the coupling constant between ribosyl 1′ and 2′ protons does not change after substitution (Table 1), rules out an intromolecular interaction between the ribose and alkyl side chain, as otherwise observed for 8-substituted adenine nucleotide derivatives.[13]

The resonances observed at high field in the spectra of **1** were assigned to the aliphatic side chain. The same resonances could not be identified in the spectra of **2** as the presence of polyethyleneimine blurs the part of the spectrum where these peaks are located.

†The pH arises from deuterium ion, but the values reported are those obtained directly from a pH-meter. The corresponding pD values are 0.40 unit higher [P. K. Glaose and F. A. Song, *J. Phys. Chem.* **64**, 188 (1960)].



N 1 A

Table 1. pH dependence of chemical shifts (Hz) and coupling constants of various proton resonances of NAD^+, and NAD^+ derivatives, 1 and 2

Compound	pH	A_8	A_2	A'_1	N_2	N_6	N_4	N_5	N'_1	CH_2–COOH	CH_2–NH–	CH–OH	Coupling Constant (a) J 1'–2'
NAD^+	2.5	485.1	468.1	237.5	571.7	558.3	523.3	454.2	236.7	—	—	—	5.0
But–NAD^+(1)	2.5	470.1	470.2	243.4	570.1	556.7	522.5	465.2	243.4	−103.0	7.0	87.1–41.6	5.0
PEI–NAD^+(2)	2.5	458.0	447.1	—	558.5	539.5	514.5	452.1	240.1	—	—	—	—
NAD^+	7.5	465.0	433.1	235.8	560.1	543.1	512.5	441.7	226.7	—	—	—	5.0
But–NAD^+(1)	7.5	455.0	435.2	226.2	555.0	537.0	505.9	445.2	230.9	−115.8	−9.9	82.2–41.6	5.0
PEI–NAD'(2)	7.5	451.1	441.1	222.1	544.5	522.0	500.0	434.0	227.1	—	—	—	4.5

The chemical shifts (Hz) are measured from dioxane as internal reference. All measurements were made on a Varian XL-100 spectrometer using the Fourier transform technique. A and N refer to the adenine and nicotinamide rings respectively. A' and N' refer to ribose linked to adenine and nicotinamide rings respectively.

(a) Measured from adenine ribosyl 1' and 2' protons.

But–NAD^+ = nicotinamide–6-(2-hydroxy-3-carboxypropylamino)purine dinucleotide (1)

PEI–NAD^+ = 4-(NAD^+-N^6)-3-hydroxybutyryl-polyethylenimine (2).

Table 2. pH dependence of ^{13}C chemical shifts (Hz) of NAD^+, and NAD^+-derivative, I

	pH	A_2	A_4	A_5	A_6	A_8	^{13}C–NH_2	A'_1	A'_2	A'_3	A'_4	A'_5	$^{13}CH_2$–COO	$^{13}CH_2$–NH	^{13}CH–OH	^{13}COOH
NAD^+	2.5	2002.9	2053.4	1298.1	2113.1	1893.8	2521	531.4	202.7	91	439.2	−37.9				
But–NAD^+(1)	2.5	2062.5	2048	1310.6	2141.8	1866.0	2486.2	519.3	196.8	92.2	433.3	−34.6	−687.6	−503.3	−19.2	2739.5
NAD^+	7.5	2165.4	2067.5	1297.5	2225	1844.3 1838.3	2505	507.9	187.5	95.3	436.6	−29.1				
But–NAD^+(1)	7.5	2170.4	2054.2	1308.8	2215	1828.4 1842.5	2475.0	508.9	187.5	94.5	432.5	−29.6	−608.7	−515.5	37.4	2843.0
		N_2	N_3	N_4	N_5	N_6		N'_1	N'_2	N'_3	N'_4	N'_5				
NAD^+	2.5	1845.3	1690.7	1998.0	1562.2	1911.2		840.0	278.1	105.5	519.3	−37.9				
But–NAD'(1)	2.5	1845.7	1690.8	1996.7	1563.1	1910.8		840.4	276.8	102.9	524.4	−35.2				
NAD^+	7.5	1844.3 1838.3	1687.5	1993.5	1562.0	1907.3		842.0	277.5	100.3	516.4	−40.6				
But–NAD^+(1)	7.5	1842.5 1838.4	1687.3	1991.3	1562.3	1906.4		841.6	277.5	100.1	508.9	−43.5				

The chemical shifts (Hz) are measured from dioxane as internal reference. A and N refer to the adenine and nicotinamide rings respectively. A' and N' refer to ribose linked to adenine and nicotinamide rings respectively.

But–NAD' = nicotinamide–6-(2-hydroxy-3-carboxy propylamino) purine dinucleotide (1).

^{13}C NMR measurements. The low concentration of dinucleotide bound to polyethyleneimine prevented ^{13}C measurements on the macromolecularized NAD^+, and therefore in Table 2 only the ^{13}C resonances of NAD^+ and 1 are listed.

In Figs. 1 and 2, the proton noise decoupled spectrum of 1 is shown at pH 2.5 and 7.5 to monitor the effects of possible pH-dependent structural variations. The differing intensity of the peaks in each spectrum arises primarily from partial saturation. In fact, the signals due to the quaternary carbons C-4,-5,-6 of the adenine ring, as well as those arising from the carboxylic and carbonilic atoms, are less intense than any other. The lower peak height of the 4′ and 5′ carbons of the ribose rings, instead, is assumed to be due to splitting caused by the P atoms of the phosphate backbone.

The assignment of the ^{13}C resonances was done according to the data previously given in the literature for NAD^+ and related compounds.[4–7] Only the resonances due to C-8 of the adenine ring and C-2 of nicotinamide were not unambiguously assigned.

The extra peaks observed in the spectra of 1 (Figs. 1 and 2) have been assigned to the aliphatic side chain introduced in the molecule.

The carboxyl carbon resonance was identified taking into account, besides its position, also its increasing intensity with increasing pulse delay (PD). The long relaxation time the carboxyl carbons usually have, in fact,

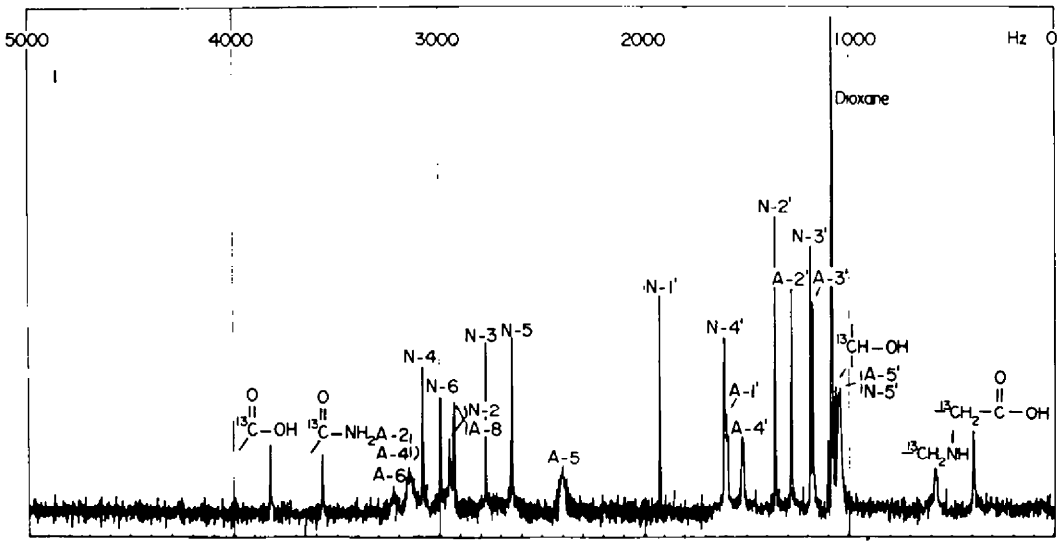


Fig. 1. Natural abundance proton-noise-decoupled ^{13}C nuclear magnetic resonance spectrum of nicotinamide-6-(-hydroxy-3-carboxy-propylamino) purine dicleotide (1) (40,000 transients, 50 μs pulse width, AT = 0.8 s, PD = 5.2 s). Samples concen. 10% (W/V) in ^2H_2O at pH 2.5, internal reference dioxane. A and N refer to the adenine and nicotinamide rings respectively; A′ and N′ refer to ribose linked to the adenine and nicotinamide rings respectively.

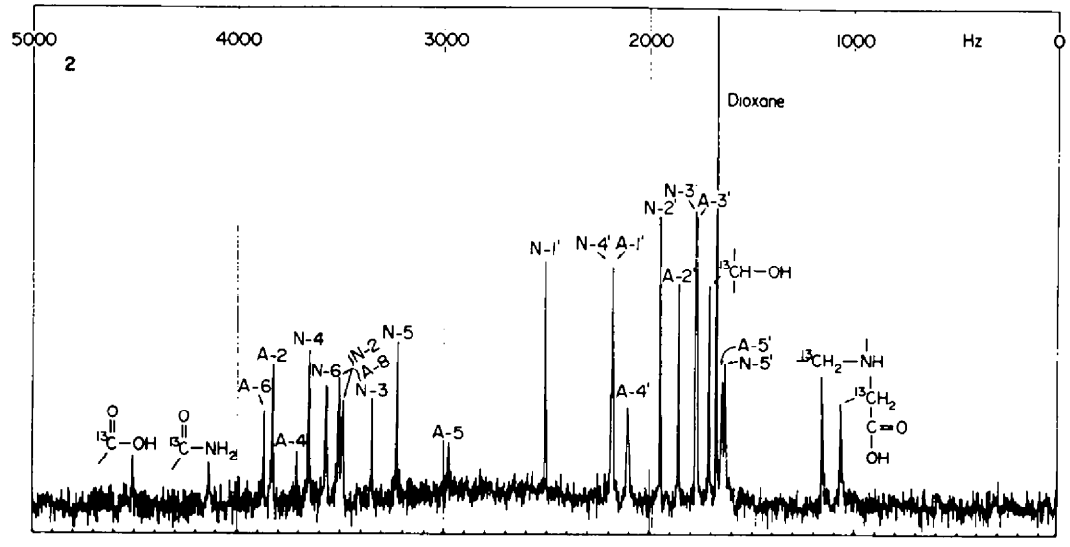


Fig. 2. Natural abundance proton-noise-decoupled ^{13}C nuclear magnetic resonance spectrum of nicotinamide-6-(2-hydroxy-3-carboxy-propylamino) purine dinocleotide (1). (8285 transients, 50μs pulse width AT = 0.8 s, PD = 2 s); Samples concen. 10% (W/V) in ^2H_2O at pH 7.5, internal reference dioxane. A and N refer to the adenine and nicotinamide rings respectively; A′ and N′ refer to ribose linked to the adenide and nicotinamide rings respectively.

would justify such a behaviour. Moreover, this resonance moves downfield at low pH, ($\Delta\nu$ = 103.5 Hz), analogously to the resonances attributed to the C atoms linked to the carboxylic group ($\Delta\nu$ = 78.9 Hz) and OH group ($\Delta\nu$ = 56.6 Hz), and this also supports the validity of our assignment.

Fluorescence spectra. Fluorescence studies carried out with NADH by Weber[8] hinted the existence of an intramolecular complex between the adenine and dihydropyridine rings, in order to account for the pyridine fluorescence at 468 nm following excitation at the absorption of the adenine moiety at 260 nm. Extending the fluorescence studies, Velik[9,10] attributed this intramolecular interaction to the existence of a folded conformation of NADH, with the two bases stacked nearly in parallel, which favors an energy transfer process from the adenine to the dihydropyridine ring. In fact, the cleavage of the molecule by nucleotide pyrophosphatase, to separate the adenine and reduced nicotinamidemononucleotide moieties, was found to abolish the effectiveness of excitation at 260 nm, while excitation at 340 nm remained effective. Therefore, the failure of 260 nm excitation to cause fluorescence, as well as a decrease of it, will suggest a relatively open conformation of the molecule with consequent reduction of the efficiency of the energy transfer mechanism.

In the light of these results, we applied the fluoresdence technique also to the reduced form of the NAD derivatives, comparing their fluorescence spectra to those of NADH. The UV spectra of the reduced forms of **1** and **2**, show, at 340 nm, the absorption due to the dihydropyridine ring, unchanged from NADH. The adenine moiety, otherwise, which absorbs at 260 nm in the case of NADH, has a max at 267 and 270 nm, for the reduced forms of **1** and **2**, respectively. In Table 3 are summarized the relative fluorescence intensity of the above mentioned compounds, obtained by excitation at the two absorptions.

Table 3. Fluorescence-emission spectral data for NADH, But-NADH and PEI-NADH. The solution concentration was always 7×10^{-5} M in Tris -HCl, pH 8.8

Excitation wavelength (nm)	Emission at 460 nm		
	NADH	But-NADH	PEI-NADH
260	55.0	—	—
267	—	82.0	—
270	—	—	64.0
340	47.0	57.5	74.3

Fluorescence intensity in scaled in arbitrary units

But-NADH = reduced nicotinamide-6 - (2 - hydroxy - 3 - carboxy - propylamino) purine dimecleotide

PEI-NADH = 4 - (NADH-N^6) - 3 - hydroxybutyril - polyethyleneimine.

As it can be seen, the two NAD derivatives exhibit an intense fluorescence, comparable to that of NADH, by excitation at both the dihydropyridine and adenine absorptions. Since the fluorescence of NADH after adenine excitation has been interpreted as due to the folding of the dinucleotide molecule at basic pH, the same supposition can be made to explain the fluorescence of **1** and **2**.

In conclusion, on the basis of all results so far reported, there is little doubt that, though substitution may cause structural variations in **1** and **2**, their behaviour in solution is not very different from that of NAD$^+$. In fact, analogously to NAD$^+$, the two NAD$^+$ derivatives show an upfield shift of their ^{1}H and ^{13}C resonances with increasing pH. In the case of the former compound, this was taken as further evidence, besides the fluorescence measurements, of the existence in solution of a pH dependent mixture of folded and unfolded forms of the dinucleotide molecule, with the folded form prevailing at basic pH.[1-3,7] By folding, nicotinamide moves directely over the adenine moiety and therefore the adenine ring current will exert a shielding influence on nicotinamide atoms determining the observed upfield shift of their resonances.

The same supposition can be made to explain the shifts observed in the NMR spectra of **1** and **2**, and it is also possible to utilize the NMR measurements for comparing the strength of the intramolecular interaction taking place as consequence of the folding of these dinucleotides at basic pH.

In fact, if one accepts the assumption[14] that the small shifts shown by nicotinamide resonances passing from low to high pH are due to the existence of an intramolecular complex, the magnitude of the shifts could be taken as a measure of the stability of the complex itself.

Considering the proton results, it is found that passing from pH 2.5 to 7.5, the upfield shift of nicotinamide resonances is, in the case of **1**, 15.1 Hz for N_2, 19.7 Hz for N_6, 16.6 Hz for N_4 and 20.0 Hz for N_5, while for the same protons of **2**, the shifts are 14.0, 17.5, 14.5, 18.1 Hz, respectively. In the case of NAD$^+$ the upfield shifts are comparatively smaller being 11.6 Hz for N_2, 15.2 Hz for N_6, 10.8 Hz for N_4 and 12.5 Hz for N_5.

The ^{13}C resonances show a similar pattern with increasing pH and for **1** we observe an upfield shift of 3.5 Hz for N_3, 5.4 Hz for N_4, 0.8 Hz for N_5 and 4.4 Hz for N_6, while for the same resonances of NAD$^+$ the shifts are 3.2, 4.5, 0.2, 3.9 Hz respectively.

Therefore, being the magnitude of the shifts the measure of the strength of the intramolecular interaction, it could be concluded that this interaction is in the NAD$^+$ derivatives somehow stronger than in NAD$^+$. As a consequence it can be assumed that the existence of an intramolecular complex more stable in **1** and **2** than in NAD$^+$, might contribute to the usually lower coenzymic efficiency of the former compounds[11,12] by hindering more strongly the formation of the transition complex between enzyme and coenzyme during the enzymatic reactions.

EXPERIMENTAL

NAD$^+$ and NADH grade I were from Boehringer Ltd. (Mannheim, Germany). The NAD$^+$ derivatives and their reduced forms were prepared in our Laboratories.[13] All other chemicals used were of the highest purity commercially available and further purified whenever necessary.

The ^{1}H and ^{13}C NMR spectra were taken at 28° on a high resolution Varian XL-100-15D spectrometer, equipped with a Varian 620-f computer, using the Fourier transform technique. The samples concentration in ^2H_2O was 10% (w/v) and all samples were lyophilized twice from ^2H_2O to remove exchangeable hydrogens.

For the ^{13}C spectra, proton noise decoupling was used in all experiments and conditions were such that all peaks were observed, though those with fairly long relaxation times were partially saturated.

The chemical shifts values were directly from the computer and dioxane was used as internal standard and lock signal. The values

given for both the ^1H and ^{13}C NMR resonances are the average of two runs performed on different days.

In the pH studies, the pH was adjusted by addition of ^2HCl or KO^2H solns and the values reported are direct pH-meter readings; the pH was rechecked after each measurement and only those with a constant value were taken into account.

The excitation fluorescence spectra were measured on a Perkin–Elmer MPF-2A spectrofluorometer; all experiments were carried out at room temp. using a Tris-HCl buffer to keep the pH at 8.8. The excitation spectra of NADH and of the two reduced NAD derivatives were obtained exciting at the absorptions of the reduced pyridine ring and of the adenine ring, the former absorption being located at 340 nm for all three compounds and the latter at 260 nm for NADH, and at 267 and 270 nm for the reduced forms of **1** and **2**, respectively.

The absorption spectra were obtained with a Cary 14 recording spectrometer.

Acknowledgements—The authors wish to thank Mr. M. Barbini and Mr. C. Marinensi for the helpful technical assistance during this work.

REFERENCES

[1]O. Jardetsky and N. G. Wade-Jardetsky, *J. Biol. Chem.* **241**, 85 (1966); and refs. therein.

[2]W. A. Catteral, D. P. Hallis and C. F. Walter, *Biochemistry* **8**, 4033 (1966).

[3]N. J. Oppenheimer, L. J. Arnold and N. O. Kaplan, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 3200 (1971).

[4]B. Birdsall, N. J. M. Birdsall and J. Feeney, *Chem. Comm.*, 316 (1972).

[5]B. Birdsall and J. Feeney, *J. Chem. Soc.* Perkin II, 1643 (1972).

[6]R. H. Sarma and R. J. Mynott, *J. Am. Chem. Soc.* **95**, 1641 (1973).

[7]M. Blumenstein and M. A. Raftery, *Biochemistry* **12**, 3585 (1973).

[8]G. Weber, *Nature* **180**, 1409 (1957).

[9]S. F. Velik, *J. Biol. Chem.* **233**, 1455 (1958).

[10]S. F. Velik, in *Light and Life* (Edited by W. D. McElroy and G. Glass). p. 108. Johns Hopkins Press, Baltimore (1961).

[11]P. Zappelli, A. Rossodivita and L. Re, *Eur. J. Biochem.* **54**, 475 (1975).

[12]W. Marconi, G. Prosperi, S. Giovenco and F. Morisi, *J. Mol. Catalysis* **1**, in press.

[13]C. Y. and N. O. Kaplan, *Arch. Biochem. Biophys.* **168**, 665 (1975).

[14]N. O. Kaplan and R. H. Sarma, *Pyridine Nucleotide-Dependent Dehydrogenase* (Edited by H. Sund), p. 39. Springer, Berlin (1970).