Carbon-13 Nuclear Relaxation Measurements in Nicotinamide Adenine Dinucleotide and Adenosine Monophosphate

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Abstract: Carbon-13 spin–lattice relaxation times (T_1's) have been measured on acidic and neutral solutions of nicotinamide adenine dinucleotide (NAD) at 0.2 and 0.4 *M* concentration and on neutral 0.2 and 0.4 *M* solutions of 5′-adenosine monophosphate (AMP). These were compared with existing AMP T_1 values at 1.0 *M*. Relaxation times are seen to increase with decreasing concentration. Also in AMP small differences in the relaxation rates between the base and sugar carbons are observed. These data are discussed in terms of known models of AMP base ring stacking. Small changes in the T_1's of NAD as a function of pH are noted at 0.2 *M* concentration. The separation of the nicotinamide and adenine rings in NAD with the protonation of the adenine N-1 position is proposed as an explanation of the data.

Nuclear magnetic resonance has made important contributions in the study of the conformation and properties of nicotinamide adenine dinucleotide (NAD) and related compounds in solution. While proton nmr has been the most actively used technique to date,[1–16] the narrow range of chemical shifts and the problem of overlapping and interfering lines have made the task of interpreting the data a difficult one. There have been some problems of conflicting data (see ref 2 and 3) and differences concerning the information that can be obtained from the data (see ref 3–6). Other workers using phosphorus[7,17] and carbon[17–19] magnetic resonance have attempted to avoid the complications of proton spectra mentioned above.

Most workers have relied upon chemical shift and coupling constant measurements for their data. However, the introduction of pulsed spectrometers with Fourier analysis packages has made relaxation (T_1) studies of carbon-13 and other magnetic nuclei relatively simple, even on fairly dilute samples. Relaxation times allow the rate of both over-all and differential segmental motion in molecules to be studied, and these data are expected to be quite useful in the study of biological compounds. The present study reports the results of ^{13}C relaxation time measurements made on NAD and 5′-adenosine monophosphate (AMP) and focuses on the molecular dynamics important to spin relaxation in these systems.

Experimental Section

Grade III NAD was purchased from Sigma Chemical Co. and used without further purification. AMP, disodium salt, was obtained from Miles Laboratories. Samples were prepared by dissolving NAD or AMP in doubly distilled water and adjusting the pH with dilute ammonia and dilute hydrochloric acid. Paramagnetic oxygen was removed by bubbling nitrogen gas through the samples. A D_2O capillary was inserted in a 12-mm sample tube and the tube was sealed with paraffin. All spectra were obtained with a Varian XL-100-15 spectrometer equipped with a Varian 620-f computer. A Xebec disk was used for storage of the free induction decay. For carbons with short T_1's (<1 sec) the inversion recovery technique[20] was employed while longer relaxation times were measured by progressive saturation.[21] The temperature of the samples was held at 40 ± 1°. T_1's were measured on NAD at pH 2 and 7 in 0.4 and 0.2 *M* solutions and on AMP at pH 7 in 0.4 and 0.2 *M* solutions.

Results and Discussion

The T_1 values for NAD and AMP (see Figure 1) are presented in Table I. The resonance positions for NAD are those reported by Birdsall and Feeney,[18] and the T_1 values for 1.0 *M* AMP are from Allerhand, *et al.*,[19] who also presented arguments which indicate that the dipolar mechanism dominates the relaxation of all protonated carbons in molecules such as AMP. From Table I, it is noted that the relaxation rates of the methylene carbons (A-5′ and N-5′ in NAD and A-5′ in AMP) are about one-half the relaxation rates of the methyne carbons as expected for the ^{13}C–^{1}H dipole–dipole dominated relaxation mechanism.

Major changes in the relaxation times are observed with variations in concentration. In AMP the T_1's approximately double as one goes from 1.0 to 0.4 *M*. As the concentration is reduced to 0.2 *M*, the T_1's, with the exception of the quaternary carbons, are seen to increase by another 50%. The scatter in the data for the quaternary carbons at 0.2 *M* is large and, therefore, the error in the reported T_1 values for these carbons is

(1) O. Jardetzky and N. G. Wade-Jardetzky, *J. Biol. Chem.*, **241**, 85 (1966).
(2) W. A. Catterall, D. P. Hollis, and C. F. Walter, *Biochemistry*, **8**, 4032 (1969).
(3) R. H. Sarma and R. J. Mynott, *Proc. Jerusalem Symposia Quantum Chem. Biochem.*, **5**, 591 (1973); *J. Amer. Chem. Soc.*, **95**, 1641 (1973); *J. Chem. Soc., Chem. Commun.*, 140 (1973); 975 (1972); 977 (1972); *Org. Magn. Resonance*, **4**, 577 (1972).
(4) R. H. Sarma and N. O. Kaplan, *Biochemistry*, **9**, 539 (1970).
(5) J. Jacobus, *Biochemistry*, **10**, 161 (1971).
(6) N. Oppenheimer, L. Arnold, and N. Kaplan, *Proc. Nat. Acad. Sci. U. S.*, **68** (12), 3200 (1971).
(7) R. H. Sarma, R. J. Mynott, F. E. Hruska, and D. J. Wood, *Can. J. Chem.*, **51**, 1843 (1973); *J. Chem. Soc., Chem. Commun.*, 140 (1973).
(8) D. P. Hollis, *Org. Magn. Resonance*, **1**, 305 (1969).
(9) R. H. Sarma, V. Ross, and N. O. Kaplan, *Biochemistry*, **7**, 3052 (1968).
(10) R. H. Sarma, P. Dannies, and N. O. Kaplan, *Biochemistry*, **7**, 4359 (1968).
(11) R. H. Sarma and N. O. Kaplan, *Biochem. Biophys. Res. Commun.*, **36**, 780 (1969).
(12) R. H. Sarma and N. O. Kaplan, *J. Biol. Chem.*, **244**, 771 (1969).
(13) R. H. Sarma, M. Moore, and N. O. Kaplan, *J. Biol. Chem.*, **9**, 549 (1970).
(14) R. H. Sarma and N. O. Kaplan, *J. Biol. Chem.*, **9**, 557 (1970).
(15) P. R. Griffith, M. J. Nye, and J. G. Alexander, *Can. J. Biochem.*, **48**, 947 (1970).
(16) D. Pattell, *Nature (London)*, **221**, 1239 (1969).
(17) M. Blumenstein and M. A. Raftery, *Biochemistry*, **11**, 1643 (1972).
(18) B. Birdsall and J. Feeney, *J. Chem. Soc., Perkin Trans. 2*, 1643 (1972).
(19) A. Allerhand, D. Doddrell, and R. Komoroski, *J. Chem. Phys.*, **55**, 189 (1971).
(20) R. Freeman and H. D. W. Hill, *J. Chem. Phys.*, **53**, 4103 (1970).
(21) R. Freeman and H. D. W. Hill, *J. Chem. Phys.*, **54**, 3367 (1971).

Table I. T_1's in Seconds

	NAD[a]				AMP		
	0.4 *M* pH 7	0.2 *M* pH 7	0.4 *M* pH 2	0.2 *M* pH 2	1.0 *M*[b]	0.4 *M*	0.2 *M*
A-2	0.16	0.29	0.15	0.26	0.15	0.30	0.46
A-4	4.3	7.0	3.9	7.8	5.3	10	12
A-5	4.7	7.8	4.2	8.2	4.7	8.2	12
A-6	2.3	1.6	1.9	2.1	2.6	4.7	6
A-8	0.18[c]	0.32[c]	0.20	0.30	0.16	0.26	0.47
N-2	0.18[c]	0.32[c]	0.26	0.45			
N-3	4.3	6.7	4.3	6.6			
N-4	0.20	0.33	0.22	0.29			
N-5	0.22	0.38	0.26	0.44			
N-6	0.18	0.34	0.15	0.24			
N-7	4.5	5.3	4.0	6.4			
A-1′	0.18[e]	0.32[e]	0.16	0.27	0.19	0.35	0.50
A-2′	0.22	0.40	0.20	0.37	0.22	0.35	0.49
A-3′	0.23	0.37	0.22	0.34	0.23	0.37	0.46
A-4′	0.23	0.30	0.16	0.29	0.19	0.29	0.44
A-5′	0.12	0.19	0.13[d]	0.18[d]	0.11	0.20	0.33
N-1′	0.19	0.34	0.20	0.44			
N-2′	0.26	0.44	0.28	0.39			
N-3′	0.25	0.42	0.26	0.37			
N-4′	0.18[e]	0.32[e]	0.21	0.39			
N-5′	0.13	0.20	0.13[d]	0.18[d]			

[a] The spectral assignments are those reported by Birdsall and Feeney, ref 18. [b] Data reported by Allerhand, *et al.*, ref 19. [c] Lines A-8 and N-2 were not resolved at pH 7. [d] Lines A-5′ and N-5′ were not resolved at pH 2. [e] Lines A-1′ and N-4′ were not resolved at pH 7.

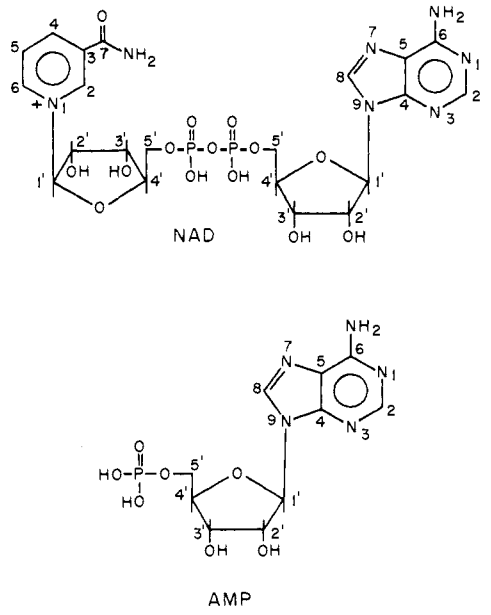

Figure 1. Structure and numbering scheme of NAD and 5′-AMP.

probably on the order of 20%. The tendency of AMP to form molecular aggregates due to the stacking of the base rings was reported by Schweizer, *et al.*,[22] and the observed increase in relaxation times with decreasing concentration is interpreted in terms of this model as a decrease in molecular aggregate size. One notes that at 1.0 and 0.4 *M* AMP, carbons A-2 and A-8 have slightly shorter T_1's than those observed on the average for the ribosyl methyne carbons, whereas this difference is no longer discernible at 0.2 *M*. While this difference in T_1's at the higher concentrations is not large, it is reproducible and beyond experimental error. Although AMP is aggregated at these higher concentrations, the ribosyl portion of the molecule appears to exhibit a slightly greater degree of freedom through segmental motion as evidenced by the ribosyl T_1's being slightly longer than those observed for the heterocyclic ring. These data lend support to the model proposed by Schweizer, *et al.*,[22] in which the aggregates are visualized as a stack of base rings in the middle of the aggregate with the ribosyl rings around the outside where they are able to undergo more extensive torsional motion about the glycosyl bond. As the AMP concentration decreases and the aggregates become smaller and tumble at a faster rate, the difference in the rate of motion between the base carbons and the ribosyl carbons apparently decreases. At 0.2 *M* no significant difference is noted in the T_1's by the base and sugar moieties, and extensive differential segmental motion need not be invoked to explain the data.

The relaxation times of NAD follow many of the same general patterns observed in AMP. One exception in NAD is at A-6 where the T_1 does not appear to change with concentration. Its relaxation time is less than that for other nonprotonated carbons and is probably dominated by the motion of the attached amine group. The torsional motions of the NH_2 group would be relatively insensitive to concentration in agreement with the concentration-independent T_1 data. NAD is believed to form molecular aggregates similar to AMP,[2,3] and therefore the increase in T_1's in NAD with decreasing concentration is attributed to the break-up of molecular aggregates at lower concentration.

The effect of pH on the relaxation rates in NAD is much less than observed for concentration changes. This leads one to conclude that protonation of the adenine ring apparently has only minor effects on either the degree of aggregation or the reorientation of the molecule as both would affect the relaxation time. It is interesting that a positive charge at position 1 on the adenine ring should have so little effect on the structural and dynamical features affecting spin relaxation.

Sarma, *et al.*,[3,4] as well as other investigators,[2,4,6,8,11,16] have proposed a model for NAD in which the adenine

(22) M. P. Schweizer, A. D. Broom, P. O. P. Ts'o, and D. P. Hollis, *J. Amer. Chem. Soc.*, 90, 1042 (1968).

and the nicotinamide rings are stacked, one above the other. The T_1 data for NAD at 0.4 and 0.2 *M* at pH 7 corroborates this model. In both cases the relaxation rates of corresponding carbons in the adenine and nicotinamide portions of the molecule are similar, suggesting that the molecule reorients as a whole with little differential segmental motion between the two bases. It has been suggested that a lowering of the pH would diminish the intramolecular base stacking[9] through repulsion of the two positively charged rings which would provide the nicotinamide and adenine moieties the opportunity to move independently of each other and thus to have different effective correlation times. The data taken from the 0.4 *M*, pH 2 sample of NAD show no evidence of such independent segmental motion of the two rings confirming that NAD exists in aggregates of sufficient size to preclude a detectable amount of differential motion at the 0.4 *M* concentration. The data taken at 0.2 *M* and pH 2, however, do give some evidence for differential segmental motion. Note that the values for T_1 at N-2 and N-5 are somewhat longer than the T_1 values of the corresponding protonated carbons in the adenine rings (A-2 and A-8). Likewise, the T_1's for N-1′ and N-4′ are longer than for A-1′ and A-4′. These data suggest that there is greater ring separation with protonation at the lower concentration and that differential motion of the two rings becomes important as the aggregation of molecules decreases.

The concentration and pH data obtained by means of relaxation time studies on NAD and AMP have revealed details on molecule aggregation and differential segmental motion in two important biological compounds. Extrapolation of these data to lower and more typical biological concentrations suggests that even more significant results on segmental motion might be obtainable. Such studies would require isotopic labeling such as used by Blumenstein and Raftery[17] or improved instrumental techniques.[23] It is anticipated that the structure and dynamics of substrate binding to enzymes would also be amenable to T_1 studies.

Acknowledgment. We gratefully acknowledge the support of U. S. Public Health Services Grants No. RR-574-02 and GM08521-12. One of the authors (W. D. H.) is indebted to the National Science Foundation for support in the form of a predoctoral fellowship. We also want to thank Dr. Sidney Velick for helpful information and for supplying some of the NAD for this study.

(23) Such data have been obtained as specifically labeled enzymes in this laboratory and these data will appear shortly. In addition, A. Allerhand, R. F. Childen, R. A. Goodman, E. Oldfield, and X. Ysern, American Laboratory, November, 1972, p 19, have demonstrated the utilities of employing large sample tubes in the investigation of biological compounds.

Determination of the Fluxional Barrier in Semibullvalene by Proton and Carbon-13 Nuclear Magnetic Resonance Spectroscopy

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Abstract: The ^1H and ^{13}C nmr spectra of semibullvalene are temperature dependent in the range of -100 to $-170°$. Rate constants were obtained at seven different temperatures by comparisons of calculated with experimental ^1H spectra. The free energy of activation for the degenerate Cope rearrangement in semibullvalene is 5.5 $\pm$ 0.1 kcal/mol at $-140°$; the enthalpy and entropy of activation are 4.8 $\pm$ 0.2 kcal/mol and -5.4 ± 3 eu, respectively. Semibullvalene has the lowest barrier of any presently known compound capable of undergoing the Cope rearrangement.

Nuclear magnetic resonance (nmr) has proved to be a powerful tool for the determination of barriers in molecules with fluctuating bonds. Fluxional molecules include the norcaradiene–cycloheptatriene system,[2] various annulenes,[3] and homotropilidenes.[4] The bridged homotropilidenes, such as bullvalene (I),[5] dihydrobullvalene (II),[6] barbaralane (III),[7] and semibullvalene (IV),[8] are of particular interest. In these

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(2) E. Ciganek, *J. Amer. Chem. Soc.*, **87**, 1149 (1965); **93**, 2207 (1971); G. E. Hall and J. D. Roberts, *ibid.*, **93**, 2203 (1971); E. Vogel, W. Wiedemann, H. D. Roth, J. Eimer, and H. Günther, *Justus Liebigs Ann. Chem.*, **759**, 1 (1972).

(3) R. C. Haddon, V. R. Haddon, and L. M. Jackman, *Fortschr. Chem. Forsch.*, **16**, 103 (1971).

(4) (a) W. von E. Doering and W. R. Roth, *Tetrahedron*, **19**, 715 (1963); (b) H. Günther, J. B. Pawliczek, J. Ulmen, and W. Grimme, *Angew. Chem., Int. Ed. Engl.*, **11**, 517 (1972).

(5) (a) Reference 4a; (b) G. Schröder and J. F. M. Oth, *Angew. Chem., Int. Ed. Engl.*, **6**, 414 (1967); (c) M. Saunders, *Tetrahedron Lett.*, 1699 (1963); (d) A. Allerhand and H. S. Gutowsky, *J. Amer. Chem. Soc.*, **87**, 4092 (1965).

(6) (a) R. Merenyi, J. F. M. Oth, and G. Schröder, *Chem. Ber.*, **97**, 3150 (1964); (b) ref 5b.

(7) W. von E. Doering, B. M. Ferrier, E. T. Fossel, J. H. Hartenstein, M. Jones, Jr., G. Klumpp, R. M. Rubin, and M. Saunders, *Tetrahedron*, **23**, 3943 (1967).

(8) (a) H. E. Zimmerman and G. L. Grunewald, *J. Amer. Chem. Soc.*, **88**, 183 (1966); (b) H. E. Zimmerman, R. W. Binkley, R. S. Givens, G. L. Grunewald, and M. A. Sherwin, *ibid.*, **91**, 3316 (1969); (c) J. Meinwald and D. Schmidt, *ibid.*, **91**, 5877 (1969); (d) H. E. Zimmerman, J. D. Robbins, and J. Schantl, *ibid.*, **91**, 5878 (1969).