to the hydrophobic stack. A tautomeric change of N_7H to N_9H for the purine would cause an upfield shift of the C_4 resonance and a downfield shift of the C_5 resonance. Therefore, it is important to assign C_4 and C_5 resonances properly. It should be noted that under certain conditions the solvent effect, i.e., the transfer of nuclei from aqueous environment to hydrophobic environment, on ^{13}C NMR shift, particularly for sp^2 carbon, can be substantial. This effect should be kept in mind in the interpretation of ^{13}C NMR of nucleic acids.

Acknowledgment. This research was supported in part by Grant PCM 77 25226 from the National Science Foundation and by the National Institutes of Health (GM 166066-11). We wish to thank Dr. A. A. Bothner-By for several useful discussions and Mr. J. Kast as well, for aid in the computer programming.

References and Notes

(1) (a) The Johns Hopkins University. (b) Institut de Biologie Physico-Chimique.
(2) (a) Johnson, C. E., Jr.; Bovey, F. A. *J. Chem. Phys.* **1958**, *29*, 1012–1014. (b) Giessner-Prettre, C.; Pullman, B. *J. Theor. Biol.* **1970**, *27*, 87–95.
(3) Borer, P. N.; Kan, L. S.; Ts'o, P.O.P. *Biochemistry* **1975**, *14*, 4847–4863. Kan, L. S.; Borer, P. N.; Ts'o, P. O. P. *Ibid.* **1975**, *14*, 4864–4869.
(4) DuVernet, R.; Boekelheide, V. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 2961–2228.
(5) Ts'o, P. O. P.; Melvin, I. S.; Olson, A. C. *J. Am. Chem. Soc.* **1963**, *85*, 1289–1296.
(6) Ts'o, P. O. P.; Chan, S. I. *J. Am. Chem. Soc.* **1964**, *86*, 4176–4181.
(7) Schweizer, M. P.; Chan, S. I.; Helmkamp, G. K.; Ts'o, P. O. P. *J. Am. Chem. Soc.* **1964**, *86*, 696–700.
(8) Cross, B. Thesis, University Microfilm 75-6982, Ann Arbor, Mich., 1974.
(9) Pugmire, R. J.; Grant, D. M.; Rubins, R. K.; Rhodes, G. W. *J. Am. Chem. Soc.* **1965**, *87*, 2225–2228.
(10) Thorpe, M. C.; Coburn, W. C., Jr.; Montgomery, J. A. *J. Magn. Reson.* **1974**, *15*, 98–111.
(11) Chenon, M. T.; Pugmire, R. J.; Grant, D. M.; Panzica, R. P.; Townsend, L. B. *J. Am. Chem. Soc.* **1975**, *97*, 4636–4642.
(12) Fischer, P.; Lösch, G.; Schmidt, R. R. *Tetrahedron Lett.* **1978**, *17*, 1505–1508.
(13) Live, D. H.; Chan, S. I. *Org. Magn. Reson.* **1973**, *5*, 275–276.
(14) Mantsch, H. H.; Smith, I. C. P. *Can. J. Chem.* **1973**, *51*, 1384–1391.
(15) Giessner-Prettre, C.; Pullman, B. *Biochem. Biophys. Res. Commun.* **1976**, *70*, 578–581.
(16) Barfield, M.; Grant, D. M.; Ikenberry, D. *J. Am. Chem. Soc.* **1975**, *97*, 6956–6961.
(17) Vogler, H. *Tetrahedron Lett.* **1979**, 229–232.
(18) Giessner-Prettre, C.; Pullman, B. *C. R. Acad. Sci.* **1969**, *268*, 1115–1117.
(19) Giessner-Prettre, C.; Pullman, B. *C. R. Acad. Sci.* **1968**, *266*, 933–936.
(20) Chertkov, V. A.; Sergeyev, N. M. *J. Am. Chem. Soc.* **1977**, *99*, 6750–6751.
(21) Chan, S. I.; Schweizer, M. P.; Ts'o, P. O. P.; Helmkamp, G. K. *J. Am. Chem. Soc.* **1964**, *86*, 4182–4188.
(22) Live, D. H.; Chan, S. I. *Anal. Chem.* **1970**, *42*, 791–792.
(23) Ellis, P. D.; Ditchfield, R. In "Topics in Carbon-13 NMR Spectroscopy", Levy, G. C., Ed.; Wiley: New York, 1976; Vol. II, pp 433–476.
(24) Alderfer, J. L.; Ts'o, P. O. P. *Biochemistry* **1977**, *16*, 2410–2416.
(25) Voet, D.; Rich, A. *Prog. Nucleic Acids Res. Mol. Biol.* **1970**, *10*, 183–244.
(26) Barfield, M.; Grant, D. M. *Adv. Magn. Reson.* **1965**, *1*, 149–193.
(27) Bothner-by, A. A. *Adv. Magn. Reson.* **1965**, *1*, 195–316.
(28) Ts'o, P. O. P. In "Basic Principles in Nucleic Acid Chemistry", Ts'o, P. O. P., Ed.; Academic Press: New York, 1974; Vol. I, pp 453–584.
(29) Pullman, B.; Pullman, A. *Adv. Heterocycl. Chem.* **1971**, *13*, 77–159.
(30) Pullman, B. *Jerusalem Symp. Quantum Chem. Biochem.* **1970**, *2*, 292–300. Pullman, B.; Bergmann, E. D.; Weiler-Feilchenfeld, H.; Neiman, Z. *Int. J. Quantum Chem., Suppl.* **1969**, *3*, 103–111.
(31) Jones, A. J.; Winkley, M. W.; Grant, D. M.; Robins, R. K. *Proc. Natl. Acad. Sci. U.S.A.* **1970**, *65*, 27–30.
(32) Iwahashi, H.; Kyogoku, Y. *J. Am. Chem. Soc.* **1977**, *99*, 7761–7765.

Proton Magnetic Resonance of NADH in Water–Methanol Mixtures. Conformational Change and Behavior of Exchangeable Proton Resonances as a Function of Temperature

James Tropp and Alfred G. Redfield*

Contribution No. 1286 from the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154. *Received April 2, 1979*

Abstract: The proton magnetic resonance spectrum of NADH (nicotinamide adenine dinucleotide, reduced form) was studied in H_2O-methanol mixtures over the temperature range 35 to −20 °C using long pulse Fourier transform methods to suppress the solvent resonances. Addition of methanol to 40% by volume disrupted the ring stacking of NADH at 20 °C. Lowering the temperature caused restacking; the stacking enthalpy was estimated to be −3.5 to −4.2 kcal/mol. In 40% methanol, the amide resonance of NADH broadened and split as temperature was lowered from 35 °C. This behavior indicated the expected hindered rotation of the amide, but the measured rotation rates were unusually large (680/s at 20 °C) and the maximum peak separation at low temperature was unusually small (0.28 ppm). Possible explanations of these anomalies are discussed, including the possibility of hindered rotation of the entire carboxamide group. Several new resonances, due to exchangeable protons, appeared in the region 1.0–1.5 ppm downfield of H_2O as temperature was lowered from 20 °C. These were assigned to the ribose hydroxyl protons of NADH.

We have studied the proton magnetic resonance spectrum of NADH in H_2O-methanol mixtures, using long pulse and analogue filtering techniques[1] to suppress the solvent resonances. Our motivation was twofold: to study the behavior of exchangeable proton resonances and the amide group of NADH, and to investigate the effects of organic cosolvent and temperature upon its solution conformation.

We shall present evidence that addition of methanol to solutions of NADH at room temperature disrupts the ring stacked conformation of NADH, as recently observed by Oppenheimer et al.,[2] while lowering the temperature reestablishes the stacking. We find that lowering temperature causes broadening and splitting of the amide resonance, which we tentatively attribute to a hindered rotation about the amide carbonyl-dihydronicotinamide ring C-3 bond. Finally, we report the appearance, at low temperature, of several new resonances, in the range 1.0–1.5 ppm downfield of H_2O, which we assign to the ribose hydroxyl protons of NADH. Apart from our interest in the spectrum and properties of NADH, certain aspects of the present work are of technical interest. These

0002-7863/80/1502-0534$01.00/0

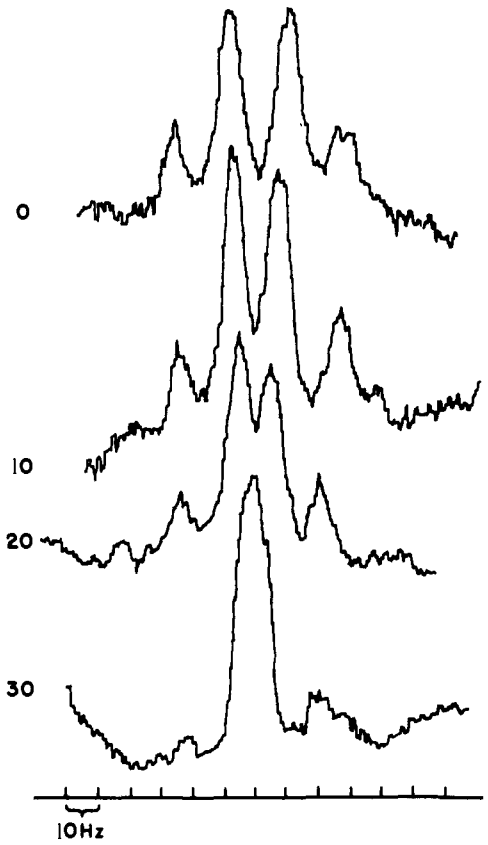


Figure 1. The dihydronicotinamide C_4-H resonance as a function of methanol concentration at 20 °C. The volume percent deuterated methanol (d_4) is given alongside each spectrum. The horizontal scale has been shifted to align the spectra.

include the observation of low-field exchangeable protons within 1.5 ppm of H_2O by pulsed NMR in a mixed solvent consisting of 60% H_2O–40% CH_3OH and the use of saturation transfer to elucidate a complex spectrum.

Experimental Section

Materials. NADH was purchased from Sigma Chemicals, and used without purification. Methanol-d_4 was purchased from Stohler Isotope Chemicals; all other chemicals were reagent grade.

NMR Samples. Samples for NMR consisted of NADH (5–15 mM), dissolved in aqueous methanol or deuteriomethanol (volume percent methanol noted in figure legends), with 5 or 10% D_2O included for field frequency lock. The pH was adjusted with minimal HCl to an apparent meter reading of 6.80–6.95 (Radiometer pH M 26 meter, GK 232K combination electrode). That these apparent readings gave a close indication of the molar lyonium ion activity was verified following Bacarella et al.[3] The constant E_0 in the equation

$$\text{pH} = -\log a_H = \frac{E - E_0}{2.303RT/F}$$

was determined for various dilute solutions of HCl (10^{-2}–10^{-3} M) in H_2O and in 40% aqueous methanol by measuring E and calculating $\log a_H$ from the molar lyonium ion concentration and solvent dielectric constant. The values of E_0 in H_2O and aqueous methanol differed by about 2 mV, corresponding to approximately 0.03 pH units.

NMR Spectroscopy. The home-built 270-MHz spectrometer employed in these studies has been described previously.[1] It is capable of pulsed Fourier operation in nondeuterated solvents without the necessity of presaturating the strong solvent resonance. This is achieved by using a long (0.6 ms) observation pulse whose power spectrum has a null at the solvent frequency, and by analogue filtering to eliminate any residual solvent signal. Although the spectrometer is not designed to null simultaneously at two solvent frequencies, e.g., H_2O and MeOH, the analogue filter cutoff is sufficiently steep that we can easily obtain spectra downfield of H_2O in the presence of 40% MeOH, with the power null at H_2O. For work upfield of H_2O, deuterated methanol is required.

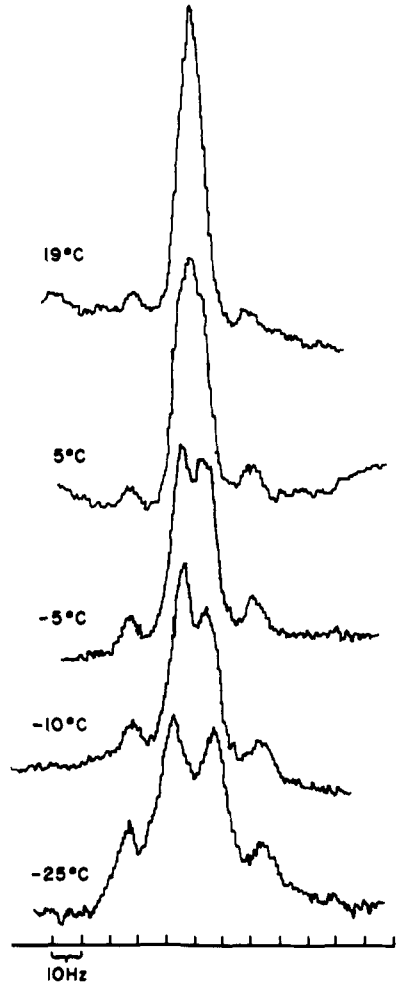


Figure 2. The dihydronicotinamide C_4-H resonance in 40% methanol-d_4 at various temperatures. The horizontal scale is shifted as in Figure 1.

For double irradiation (two pulse) experiments, the observation pulse was preceded by a long (500 ms) low-power preirradiation pulse to selectively saturate a given resonance, followed by a short (1 ms) delay. Double irradiation difference spectra (hereinafter "difference spectra") were obtained in an alternating two-cycle experiment. In the first cycle, the preirradiation pulse was set at a particular frequency of interest inside the NADH spectrum. In the second cycle, it was set several kilohertz outside the NADH spectrum. The free induction decay from the first cycle was subtracted from that of the second, and the difference was Fourier transformed.

Temperature control was achieved by standard methods and stability was typically within 0.1 °C; the estimated accuracy was ±1.0 °C as measured with copper-constantan thermocouples.

Results and Discussion

Effects of Methanol and Temperature on Conformation. The conformation of NADH has been extensively studied in aqueous solution.[4] It is generally accepted that NADH adopts a folded conformation with the adenine and dihydronicotinamide rings stacked upon each other. Strong support for this view comes from the observation that the dihydronicotinamide C_4 protons resonate as an AB quartet;[5] their magnetic inequivalence is presumably due to the stacking.

Figure 1 shows the dihydronicotinamide C_4 proton resonance at various concentrations of deuterated (d_4) methanol, near room temperature. The two central peaks gradually coalesce, while the outer peaks lose intensity, as methanol is added. Figure 2 shows the C_4 proton resonance in 40% methanol as a function of temperature. The splitting of the center

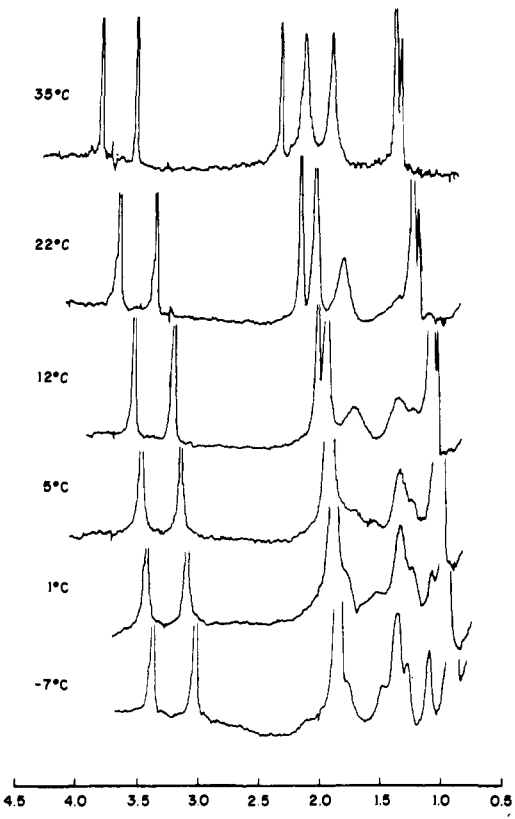


Figure 3. The spectrum of NADH in 40% methanol, downfield of H_2O, at various temperatures. The horizontal scale is parts per million from H_2O.

peaks is reestablished and the outer lines gain intensity as temperature is lowered. This behavior is approximately the reverse of that shown in Figure 1.

All spectra in Figure 1 display the characteristic AB pattern, and the splittings and intensities are accounted for by an *AB* Hamiltonian[6] of the form

$$\omega_0(I_{1z} + I_{2z}) + (\delta/2)(I_{1z} - I_{2z}) + J\mathbf{I}\cdot\mathbf{I}_2 \qquad (1)$$

where the coupling J is approximately constant, but δ is a function of methanol concentration and temperature. This suggests that NADH unstacks upon addition of methanol at 20 °C, and restacks as temperature is lowered. Since the quartet structure presumably arises from the magnetic inequivalence due to stacking, destacking the rings should cause the protons to resonate as a single line. If exchange between conformations is fast on the NMR time scale, the observed splittings should be given by an averaged Hamiltonian of the form (1) with $\delta = P\delta_0$ where P is the fraction of NADH in the stacked conformation, δ_0 is the chemical shift difference between the C_4 protons in the stacked form, and we have assumed that the coupling constant J is the same in both conformations. Although direct measurement of δ_0 has not proven feasible, several workers have obtained estimates for the fraction of stacked NADH molecules in aqueous solution. Various experimental methods lead to estimates of 24,[7] 35,[2] and 50%[8] stacking near 20 °C. Employing each of these values in turn, and assuming that δ_0 is unchanged by addition of methanol, we obtain respectively the following estimates of δ_0 and the stacking enthalpy: δ_0 0.48 ppm, $\Delta H = -3.5$ kcal/mol; δ_0 0.33 ppm, $\Delta H = -3.64$ kcal/mol; δ_0 0.23 ppm, $\Delta H = -4.2$ kcal/mol. In an earlier NMR study, the stacking enthalpy of NADH in D_2O was estimated to be -3.1 to -4.6 kcal/mol from the temperature dependence of the dihydronicotinamide C_4-H and C_2-H chemical shifts.[7]

Assignment of Exchangeable Proton Resonances. Figure 3 shows the spectrum of NADH downfield of H_2O in 40% methanol at various temperatures. At 22 °C we observe three peaks at 2.01, 1.80, and 1.36 ppm from H_2O, which are absent in D_2O solvent. The well-resolved peak at 2.01 ppm and the broad, indistinct peak at about 1.36 ppm both disappear in double-irradiation experiments where H_2O is saturated, indicating that exchange with solvent protons is rapid compared with magnetic relaxation. The peak at 1.80 ppm does not saturate with H_2O, indicating slower exchange. The peak at 2.01 ppm has an integrated intensity of two protons, and is assigned to the adenine amino protons on the basis of its resonance position and its broadening at higher temperature.[9] Note that the sharp dihydronicotinamide peak (2.14 ppm at 22 °C) moves into the amino peak at low temperatures. The peak at 1.80 ppm also corresponds to two protons, and is assigned to the amide protons. The behavior of the line shape as temperature is varied is typical of a hindered rotation. The line broadens as temperature is lowered from 40 to 10 °C, and splits in two at about 7 °C. At 5 °C the two components of the split peak are barely visible at 1.55 and 1.67 ppm. At lower temperatures the study of the amide line shape is complicated by overlap with other peaks; we postpone its discussion until later.

The broad peak at 1.36 ppm is just discernible at 22 °C. It disappears as temperature is raised to 35 °C, and moves downfield, sharpens, and splits as temperature is lowered from 22 °C. At −7 °C there are four resolved peaks between 1.0 and 1.45 ppm from H_2O. The peak at 1.45 ppm is due to the amide (see below); the remaining peaks at 1.30, 1.22, and 1.05 ppm have a total integrated intensity of four protons. We identify these as the four ribose hydroxyl protons of NADH. Although sugar hydroxyl protons might be expected to exchange very rapidly with water, there is at least one precedent for their observation by NMR in aqueous solution.[10]

These peaks are not due to low-temperature splitting of the amide or amino resonances, since double-irradiation experiments with preirradiation of the putative hydroxyl peaks causes no kinetic transfer of saturation to either the amide or amino peaks at any temperature, and vice versa. Also the hydroxyl resonances are not observed in 40% MeOH in the presence of 30 mM sodium phosphate buffer at an apparent pH of 7.0, whereas the amide and amino peaks are unaffected by this buffer. This last observation can be explained using Eigen's theory[11] for the rate of buffer-catalyzed proton exchange with solvent. We calculate pseudo-first-order rate constants of 10^3/s and <1/s for the ribose hydroxyl and amino protons respectively, in 40% M MeOH, 30 mM sodium phosphate buffer, apparent pH 7.0. These values are consistent with our observations, since a specific rate of 10^3/s would broaden the hydroxyl protons beyond detection. The calculation is subject to the following assumptions: (1) Exchange proceeds with extraction of the exchangeable proton by HPO_4^{2-}. (2) The second pK of phosphoric acid in 40% MeOH is approximately 8.0.[12] (3) The pK of any sugar hydroxyl proton[11] is about 12; the pK of an amino proton[13] is 15. We have no estimate for the pK of the amide proton; presumably it is greater than that of an adenine amino proton.

Hindered Rotation of the Amide. As noted above, the study of the amide line is complicated by the proximity of other peaks in the spectrum. Above 10 °C, the line is clearly visible, but accurate measurements of the line width are difficult due to overlap with the amino resonance. In the neighborhood of 7 °C, the amide line is broadened almost beyond detection. At lower temperatures, e.g., −7 °C, it appears to have split into two peaks, but here the situation is ambiguous. Apparently, the low-field component of the line is the shoulder on the amino resonance at about 1.73 ppm, while the upfield component resonates at 1.45 ppm but cannot be distinguished from the ribose hydroxyl protons. These difficulties can be overcome by difference spectroscopy.

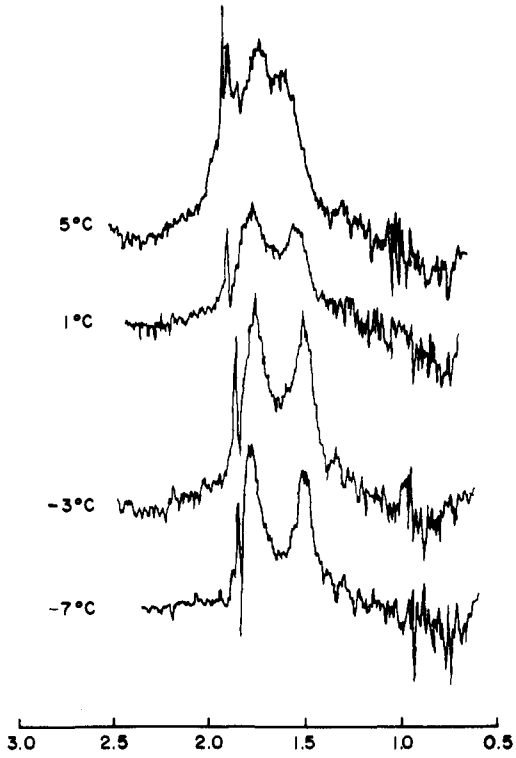


Figure 4. Difference spectra of the amide resonance at various temperatures, in 40% methanol. Refer to the text for explanation. The horizontal scale is parts per million from H_2O.

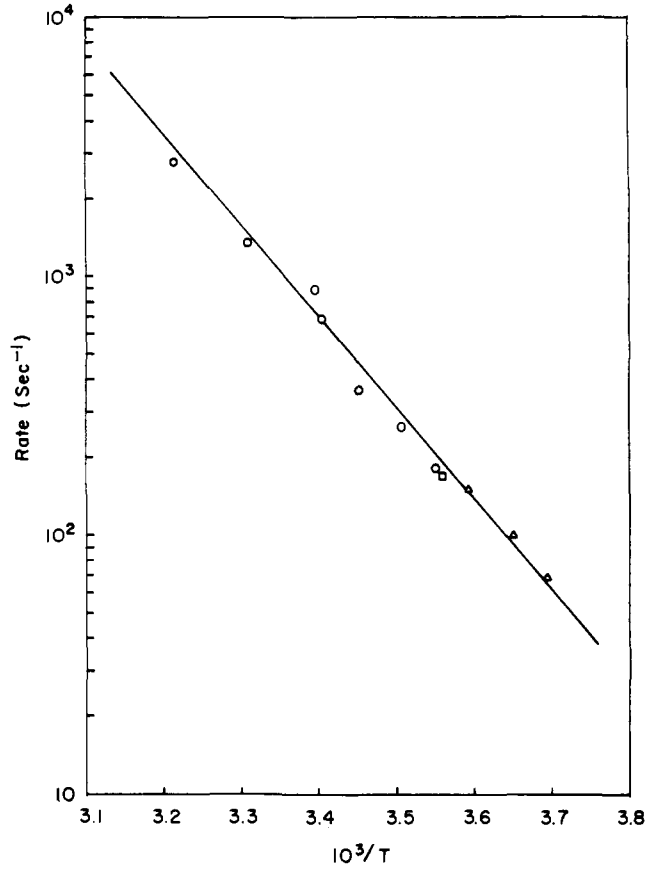


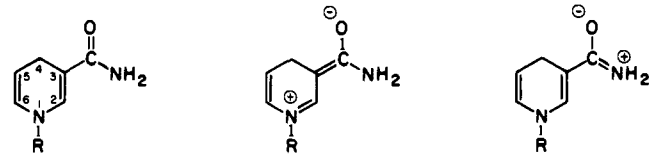
Figure 5. Arrhenius plot for the amide resonance. Circles are points from line-width data, triangles are points from splitting data, and the square is from the coalescence point. Splitting data were analyzed according to ref 16.

At temperatures above the point at which the amide peaks coalesce, we take the difference spectrum: (normal spectrum with no preirradiation pulse) minus (spectrum immediately following preirradiation pulse to saturate the amide resonance). Clearly, this difference consists of just the amide line, except for small ghosts of other peaks due either to power spillover from the preirradiation pulse or to nuclear Overhauser effects. At temperatures below the coalescence point, we take the difference spectrum: (normal spectrum with no preirradiation pulse) minus (spectrum immediately following preirradiation pulse of the presumed upfield component of the amide doublet). If both the duration of the preirradiation pulse (500 ms) and the spin-lattice relaxation time are long compared to the inverse rate constant for the hindered rotation, then saturation of the upfield component will be kinetically transferred to the downfield component,[14] and the difference spectrum will consist of the amide doublet and small ghost peaks. Figure 4 shows these spectra with preirradiation of the presumed upfield peak, at several temperatures. These spectra permit positive identification of the amide peaks, despite the interfering presence of hydroxyl and amino resonances. The difference spectra allow us to measure the amide peak separation as a function of temperature. The peak separation reaches its maximum value, 0.28 ppm, at −7 °C.

The behavior of the amide line shape indicates a hindered rotation; we estimate an activation barrier of 15 kcal/mol from the line width and splitting data.[15,16] Figure 5 shows the Arrhenius plot.

Two peculiar features of the NADH amide resonance emerge from the line-shape study. (1) Despite a moderately large activation barrier, the rotation rate at 20 °C (680/s) is unusually fast. Typical rate data for other amides at 20 °C under various solution conditions follow: formamide[17,18] (in acetone), 0.5/s, E_{act} = 18 kcal/mol; *N,N*-dimethylacetamide[19] (neat liquid), 0.7/s, E_{act} = 10.6 kcal/mol; *N,N*-dimethylbenzamide[19] (in CH_2Br_2), 27/s, E_{act} = 7.7 kcal/mol; propionamide[20] (in H_2O, pH 5), 5/s; NAD[20] (in H_2O, pH 3.5), 2/s; benzamide[20] (in H_2O, pH 5), 10/s. (The rates in H_2O are estimated from saturation transfer data at the pH minimum of the solvent exchange rate for the amide protons.) (2) The maximum peak separation at low temperature (0.28 ppm) is much smaller than that observed for a variety of amides in H_2O at 20 °C. Typical values follow:[20] acetamide, 0.75 ppm; propionamide, 0.71 ppm; NAD, 0.96 ppm; acrylamide, 0.63 ppm; benzamide, 0.86 ppm.

A possible explanation for these anomalies of NADH can be found in the resonance structures of the dihydronicotinamide group. Inspection of structures II and III shows that



I II III

two hindered rotations are possible; (1) rotation about the C–N bond and (2) rotation of the entire carboxamide group about the ring C_3–carboxyl bond. There is no experimental evidence for simultaneous hindrance of both rotations, which would lead to four amide lines at low temperature.

"Competition" between structures analogous to II and III has been invoked[19] to explain the low activation barriers (approximately 7 kcal/mol) in *N,N*-dimethylacrylamide and *N,N*-dimethylbenzamide.[19] The type II structure should be more favorable in NADH than in acrylamide or benzamide derivatives, since in NADH the formal positive charge resides on nitrogen rather than on carbon. Thus one could argue that

structure II dominates in NADH and that the observed hindered rotation is that of the entire carboxamide group.

This argument does not lead to predictions of splitting and rate behavior, not does it suggest a conformational preference for the carboxamide. However, the behavior of NADH is clearly atypical, and, since the typical amides are all presumably characterized by the C-N hindered rotation, it is not unreasonable to propose an alternative for the atypical case.

Taking an entirely different tack, one might argue that the anomalous behavior of NADH is only apparent, and that the variation of peak separation with temperature is not due to rotational coalescence, but arises instead from specific chemical shift effects due to destacking. According to this argument, peak separation should continue to increase with stacking. However, peak separation is complete at −7 °C, whereas stacking is still incomplete at −25 °C, to judge from the dihydronicotinamide C_4-H resonance. Also, this argument does not explain the linearity of the Arrhenius plot. Finally, in NAD, which is estimated to be 15-20% stacked at 20 °C with a stacking enthalpy of −5 kcal/mol, the amide peak separation is virtually unchanged between 0 and 20 °C.[21]

In conclusion, the nature of the hindered rotation in NADH is an open question whose resolution will probably require further study. Even for "typical" amides, the origin of the chemical shift difference between cis and trans protons is poorly understood.[22]

Acknowledgments. This work was supported by U.S. Public Health Service Grant GM20168 and by the Research Corporation. J.T. is a recipient of a National Institutes of Health postdoctoral fellowship (GM05542).

References and Notes

(1) Redfield, A. G. "NMR, Basic Principles and Progress", Vol. 13; Pintar, N. M., Ed.; Springer-Verlag: West Berlin, 1976; pp 137-152.
(2) Oppenheimer, N. J.; Arnold, L. J.; Kaplan, N. O. *Biochemistry* **1978**, *17*, 2613-2619.
(3) Bacarella, A. L.; Grunwald, E.; Marshall, H. P.; Purlee, B. L. *J. Org. Chem.* **1955**, *20*, 747-762.
(4) Sarma, R. H.; Mynott, R. J. "Conformation of Biological Molecules and Polymers, The Jerusalem Symposium on Quantum Chemistry and Biochemistry", Bergmann, E., Pullman, B., Eds.; Israel Academy of Arts and Sciences: Jerusalem, 1973; pp 591-623.
(5) Sarma, R. H.; Kaplan, N. O. *J. Biol. Chem.* **1969**, *244*, 771-774.
(6) Abragam, A. "The Principles of Nuclear Magnetism"; Oxford University Press: London, 1961; p 484.
(7) McDonald, G.; Brown, B.; Hollis, D.; Walter, C. *Biochemistry* **1972**, *11*, 1920-1930.
(8) Cross, D. G.; Fisher, H. F. *Biochemistry* **1969**, *8*, 1147-1155.
(9) McConnell, B.; Seawell, P. C. *Biochemistry* **1972**, *11*, 4382-4392.
(10) Harvey, J. M.; Symons, M. C. R.; Naftalin, R. J. *Nature (London)* **1976**, *261*, 435-436.
(11) Eigen, M. *Angew. Chem., Int. Ed. Engl.* **1968**, *3*, 1-23.
(12) Douzou, P.; Hui Bon Hoa, G.; Maurel, P.; Travers, F. "Handbook of Biochemistry and Molecular Biology", Fasman, G. D., Ed.; CRC Press: Cleveland, **1976**; pp 520-539.
(13) Teitelbaum, H.; Englander, S. W. *J. Mol. Biol.* **1975**, *92*, 79-91.
(14) Gupta, R. K.; Redfield, A. G. *Biochem. Biophys. Res. Commun.* **1970**, *41*, 273-281.
(15) Pople, J. A.; Scheider, W. G.; Bernstein, H. J. "High Resolution Nuclear Magnetic Resonance"; McGraw-Hill: New York, 1959; pp 218-230.
(16) Gutowsky, H. S.; Holm, C. H. *J. Chem. Phys.* **1956**, *25*, 1228-1235.
(17) Sumners, B.; Piette, L. H.; Schieider, W. G. *Can. J. Chem.* **1960**, *38*, 681-688.
(18) Leffler, J. E.; Grunwald, E. "Rates and Equilibria of Organic Reactions"; Wiley: New York, 1963; p 113.
(19) Rugers, M. T.; Woodbrey, J. C. *J. Phys. Chem.*, **1962**, *66*, 540-546.
(20) Redfield, A. G.; Waelder, S. W. *J. Am. Chem. Soc.*, **1979**, *101*, 6151-6162.
(21) Raszka, M.; Kaplan, N. *O. Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 4546-4550.
(22) Bovey, F. A. "Nuclear Magnetic Resonance Spectroscopy"; Academic Press: New York, 1969; p 76.

^{1}H NMR Spectroscopic Study of Cyclic Proton Exchange between Acetic Acid and Methanol in Tetrahydrofuran-d_8

Hans-Heinrich Limbach* and Wolfgang Seiffert

Contribution from the Institut für Physikalische Chemie der Universität Freiburg i. Br., D-7800 Freiburg i. Br., West Germany. *Received April 3, 1979*

Abstract: The kinetics of the intermolecular proton exchange between methanol and acetic acid dissolved in tetrahydrofuran-d_8 have been studied by ^{1}H NMR line shape analysis. In the slow exchange range the line shapes of the carboxyl singlet and the methanol multiplets provide information about the proton-exchange rates between methanol and acetic acid and between molecules of methanol. In very carefully prepared samples of high purity no methanol self-exchange is observed. The proton-exchange rates between methanol and acetic acid were found to be proportional to the total concentrations of the reactants. The temperature dependence of the observed bimolecular rate constants is given by $k_{obsd} = \exp(18.6 \pm 0.7) \exp(-27 \pm 1.3 \text{ kJ mol}^{-1}/RT)$, $196 \leq T \leq 258$ K. The absence of methanol self-exchange and the kinetic law prove that the exchange takes place during one encounter of the reactants in a hydrogen-bonded intermediate which contains one molecule of acetic acid and one molecule of methanol. The rate-limiting step of the proton exchange is the proton transfer in this intermediate. The observed rate constants depend on the equilibrium constants of the formation of the active 1:1 complex from the dominating quasi-monomers which form a hydrogen bond with the solvent. The values of the activation parameters provide evidence that the proton exchange takes place in a cyclic 1:1 intermediate along a reaction pathway which does not involve a solvated ion pair as intermediate.

I. Introduction

The kinetics of intermolecular proton exchange in buffered mixtures of carboxylic acids and alcohols have been studied by Grunwald et al.[1-5] using dynamic ^{1}H NMR spectroscopy. In protic media ionic exchange mechanisms dominate which consist of successive proton-transfer reactions involving free solvated ions. However, side reactions involving only neutral molecules were also observed. There is evidence that this type of exchange takes place in cyclic hydrogen-bonded intermediates as shown, for example, in Scheme I. The number of participating alcohol molecules was found to vary between one and three. The question whether the exchange proceeds via an intimate ion pair or whether there is a concerted proton motion has been discussed in several papers,[2-7] and a stepwise mechanism was favored.[5,6]

0002-7863/80/1502-0538$01.00/0