tained as an extremely hygroscopic yellow powder in a yield of 20–25 mg.

References

Albert, A., and Serjeant, E. P. (1962), Ionization Constants of Acids and Bases, London, Methuen, pp 51–56.
Brockmann, H., and Lackner, H. (1960), *Naturwissenschaften 47*, 230.
Chinard, F. P. (1952), *J. Biol. Chem. 199*, 91.
Cornforth, J. W., Cornforth, R. H., Popják, G., and Gore, I. Y. (1958), *Biochem. J. 69*, 146.
Diekmann, H., and Zähner, H. (1968), *Helv. Chim. Acta* (in press).
Emery, T. (1965), *Biochemistry 4*, 1410.
Emery, T. (1966), *Biochemistry 5*, 3694.
Emery, T., and Neilands, J. B. (1961), *J. Am. Chem. Soc. 83*, 1626.
Jencks, W. P., (1958), *J. Am. Chem. Soc. 80*, 4581.
Longley, R. I., Jr., Emerson, W. S., and Shafer, T. C. (1952), *J. Am. Chem. Soc. 74*, 2012.
Neilands, J. B. (1966), *Struct. Bonding (Berlin) 1*, 59.
Prelog, V., and Walser, A. (1962), *Helv. Chim. Acta 45*, 631.
Schwarzenbach, G., and Schwarzenbach, K. (1963), *Helv. Chim. Acta 46, 1390.*
Staab, H. A. (1959), *Angew. Chem. 71*, 194.
Synge, R. L. M. (1948), *Biochem. J. 42*, 99.

Spontaneous Reactions of 1,3-Substituted 1,4-Dihydropyridines with Acids in Water at Neutrality. II. Nuclear Magnetic Resonance Studies*

Kyoung S. Choi† and Spyridon G. A. Alivisatos

ABSTRACT: Spin–spin coupling assignments for the ring protons in 1-methyl-1,4-dihydronicotinamide were studied with the aid of double-irradiation decoupling techniques in a 100-Mcycle nuclear magnetic resonance spectrometer. The mechanism and kinetics of the primary protonation reactions of 1-methyl-, 1-propyl-, and 1-benzyldihydronicotinamides were also studied with the aid of nuclear magnetic resonance spectroscopy. It was shown that all signals due to ring protons decay simultaneously, following second-order kinetics. At the same time, new signals appear in the nuclear magnetic resonance spectra of these reaction mixtures. Assignments of chemical shifts for the new signals were achieved with the aid of 2-, 4-, and 6-monodeuterio derivatives of the above compounds and by means of kinetic considerations. The structure of the primary protonation product of these dihydropyridine derivatives, as emerges from this study, is more or less consistent with concepts based on spectrophotometric studies.

The spontaneous reactions of reduced nicotinamide–adenine dinucleotide (NADH)[1] with orthophosphates and other acidic anions in neutral watery milieu were previously followed by ultraviolet spectroscopy and the results were analyzed kinetically (Alivisatos *et al.*, 1964, 1965). These spontaneous reaction sequences may be related, as models, to biological interactions occurring during the first step (references in Lehninger and Wadkins, 1962) of oxidative phosphorylation. In this context, it was of interest to explore the applicability of nulcear magnetic resonance spectroscopy to our studies. In the present communication we report results obtained by interaction of model 1-substituted 1,4-dihydronicotinamides (1-R-DHN) with phosphate and monochloroacetate. It is shown that the kinetics and the mechanism of these processes may be substantially clarified by this new approach.

Materials and Methods

1-Methylnicotinamide iodide was prepared according to Karrer *et al.* (1936). It was reduced with sodium dithionite to 1-methyl-1,4-dihydronicotinamide by the

* From the Division of Enzymology, The Chicago Medical School, University of Health Sciences, Chicago, Illinois 60612. *Received August 14, 1967.* This investigation was supported in part by grants from the National Science Foundation (NSF GB 4645), the U. S. Public Health Service (CA 10143), and the American Cancer Society, Inc. (P-424). A preliminary report of this work was presented at the 154th Meeting of the American Chemical Society (Choi and Alivisatos, 1967). Inquiries should be made to Dr. S. G. A. A.

† Postdoctoral trainee.

[1] Abbreviations: NADH, reduced nicotinamide–adenine dinucleotide; 1-R-DHN, variously substituted (at position 1) 1,4-dihydronicotinamides.

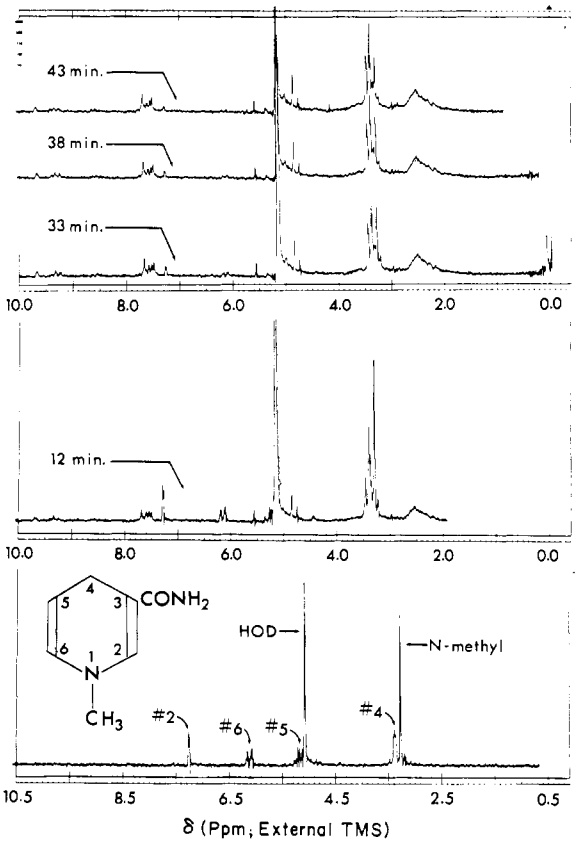


FIGURE 1: Proton magnetic resonance spectra of 1-methyldihydronicotinamide (0.5 M in D_2O at 32.5°) in the absence (lower) and in the presence of potassium phosphate (0.5 M, pH 7.0). Spectra after the addition of phosphate were taken at various intervals and a few are shown in this figure. Sweep rates were 4 min.

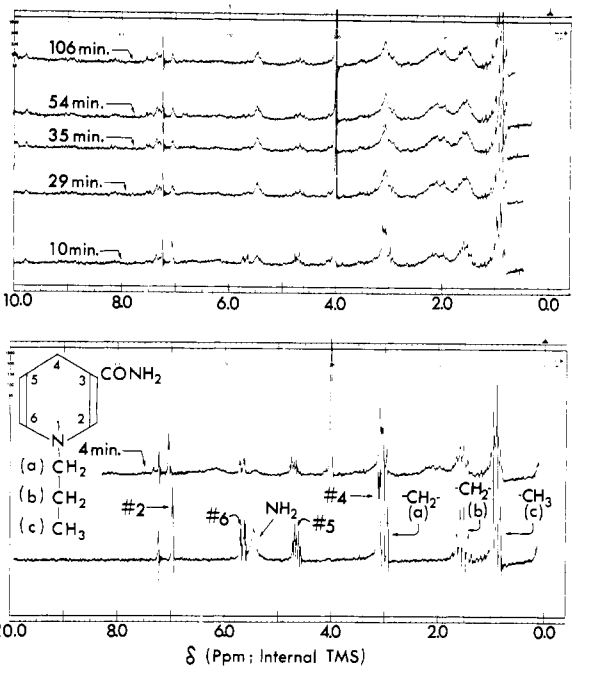


FIGURE 2: Proton magnetic resonance spectra of 1-propyldihydronicotinamide (0.3 M in $CDCl_3$ at 32.5°) in the absence (lower) and in the presence of $CH_2ClCOOH$ (0.3 M). Spectra after the addition of monochloroacetic acid were taken at various intervals and a few are shown in this figure. Sweep rates were 4 min.

method of Karrer and Blumer (1947) as modified by Hutton and Westheimer (1958). 1-Propylnicotinamide iodide and 1-benzylnicotinamide chloride, and their 1,4-dihydro derivatives, were prepared according to Karrer and Stare (1937).

2,3-Pyridinedicarboxylic acid (quinolinic acid), 2,5-pyridinedicarboxylic acid (isocinchomeronic acid), and tetramethylsilane were purchased from Aldrich Chemical Co., Inc. Deuterium oxide (99.75%) and deuteriochloroform (99%) were E. Merck, AG, Darmstadt (Germany), products. Monochloroacetic acid was a Fisher reagent.

Deuterated Derivatives. 2-Deuterionicotinamide and 6-deuterionicotinamide were prepared as described by Mauzerall and Westheimer (1955). The 1-methiodides of these compounds were reduced with dithionite in H_2O, as above, to the 1,4-dihydro derivatives (Hutton and Westheimer, 1958). Nicotinamide methiodide, reduced with dithionite in D_2O, resulted in a 4-monodeuterio-1,4-dihydro derivative (Hutton and Westheimer, 1958). Inorganic potassium orthophosphate solutions (pH 7.0) were prepared from the mono- and dipotassium salts. Whenever the deuterated buffer was required, such solutions were freeze dried and redissolved in D_2O four times.

Nuclear magnetic resonance spectra were recorded on a high-resolution Varian Model HR-100 100-Mcycle spectrometer equipped with a Varian-4343 temperature-control unit. Most studies were performed at 32.5 ± 0.5°. Chemical shifts were measured in δ (parts per million) from either internal or external tetramethylsilane. In the solvents used in this study, chemical shifts measured from external tetramethylsilane are generally found at δ 0.45–0.49 downfield as compared to internal tetramethylsilane. However, since the nature of our studies required only relative localization of shifts within a given spectrum, in most cases no attempt was made to correct for bulk susceptibility, etc. (references in Jardetzky and Jardetzky, 1962). Decoupling was achieved by double irradiation, using a Hewlett-Packard 200 AB audiooscillator and a V4354A frequency sweep model.

Results and Discussion

The 100-Mcycle proton resonance spectra of 1-methyl-DHN (in D_2O, external tetramethylsilane) and of 1-propyl-DHN and of 1-benzyl-DHN (both in $CDCl_3$, internal tetramethylsilane) are shown in the lower section of Figures 1, 2, and 3, respectively.

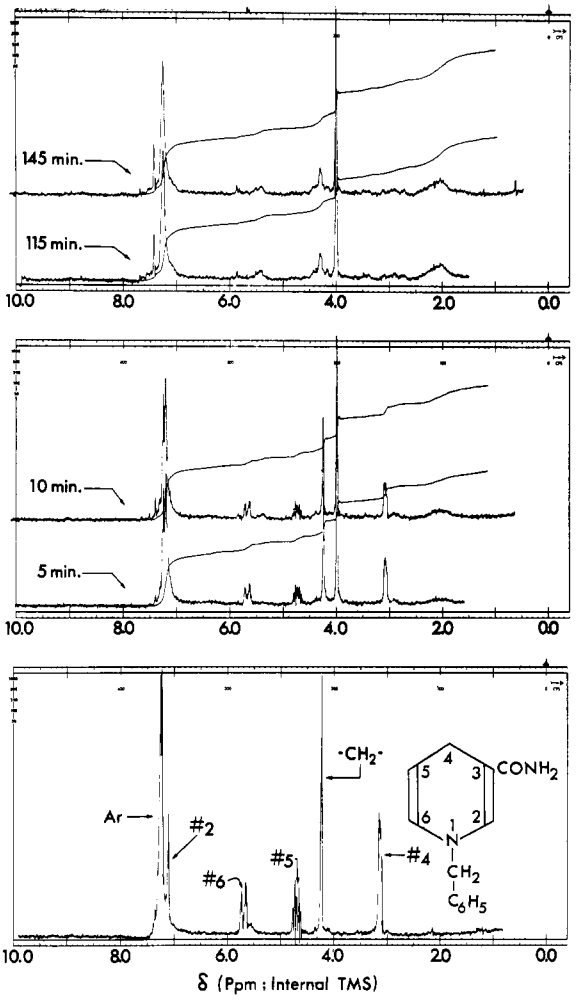


FIGURE 3: Proton magnetic resonance spectra of 1-benzyldihydronicotinamide (0.3 M in $CDCl_3$ at 32.5°) in the absence (lower) and in the presence of $CH_2ClCOOH$ (0.3 M). Spectra after the addition of monochloroacetic acid were taken at various intervals and a few are shown in this figure. Sweep rates were 4 min.

Chemical shifts and spin-coupling constants obtained from these spectra are gathered in Table I. Values for 1-benzyl-DHN are practically identical with those reported by Diekmann *et al.* (1964); they are also in excellent agreement with shifts in $CDCl_3$ reported by Meyer *et al.* (1962) with reference to external cyclohexane. Similarly, chemical shifts for 1-propyl-DHN observed in our laboratory are practically identical with those reported by Ludowieg *et al.* (1964). The downfield shift of the signal of NH_2 protons in compounds reported here is approximately 0.5 ppm smaller than shifts of signals of the same protons reported by other investigators mentioned above, indicating differences in experimental conditions. Most probably, this is due to lower concentrations of the compounds used in our studies. Shifts originally assigned by Hutton and Westheimer (1957) to signals

TABLE I: Chemical Shifts and Coupling Constants of 1-Substituted 1,4-Dihydronicotinamides.[a]

Substituents (M)	Chemical Shifts[b]									Coupling Constants[f]				Solvent
	H_2	H_4	H_5	H_6	NH_2	$>NCH_3$	$>NCH_2$	CH_2	CH_3	C_6H_5	$J_{5,6}$	$J_{4,5}$	$J_{2,6}$	$J_{4,6}$	
Methyl (0.5)[e]	7.25	3.39	*e*	6.13		3.30					8	*e*	1.7	1.7	D_2O
Methyl (0.3)[c]	6.67	2.78	4.41	5.54	5.95	2.71					8	3.4	1.7	1.7	Acetone-d_6
Benzyl (0.3)[d]	6.97	3.05	4.66	5.64	5.42		2.98	1.54	0.89		8	3.4	1.7	1.7	$CDCl_3$
Propyl (0.3)[d]	7.08	3.10	4.66	5.68	5.56		4.21			7.22	8	3.4	1.7	1.7	$CDCl_3$

[a] All spectra were registered at 32.5 ± 0.5°. [b] In parts per million (δ) downfield from the reference. [c] Reference external tetramethylsilane. [d] Reference internal tetramethylsilane. [e] 5-proton signal (sextet) is partly overlapping with HDO peak (~5.10 ppm). [f] Cycles per second.

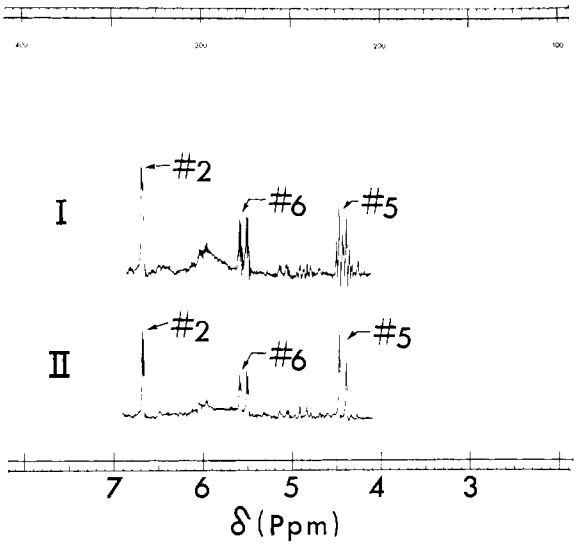


FIGURE 4: A part of the nuclear magnetic resonance spectrum of 1-methyl-DHN in acetone-d_6 recorded (I) in the absence and (II) during irradiation at the region of the H_4 proton (δ 2.90 downfield from external tetramethylsilane; amplitude 600 mv). All δ values are in parts per million.

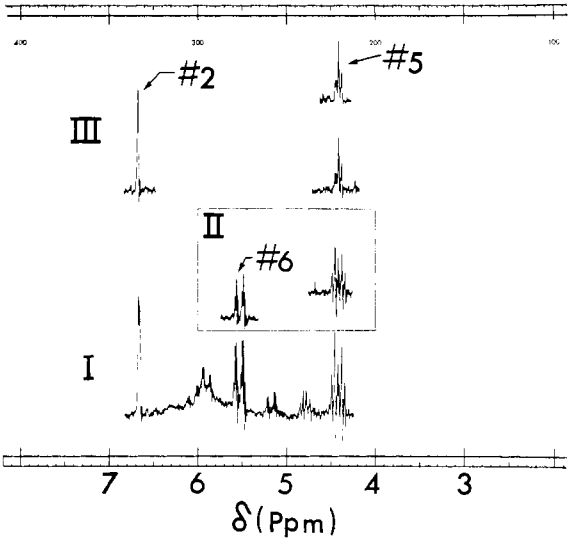


FIGURE 5: Portions of the nuclear magnetic resonance spectrum of 1-methyl-DHN in acetone-d_6 recorded (I) in the absence, (II) during irradiation at the region of the H_2 proton signal (δ 6.69 downfield from external tetramethylsilane; amplitude 700 mv), and (III) during irradiation at the region of the H_6 proton (δ 5.59 downfield from external tetramethylsilane; amplitude 700 mv).

from protons of 1-methyl-DHN in deuterioacetone with reference to external cyclohexane are also in very close agreement with our data obtained under similar conditions (not included in Table I). They appear at 2.04 ppm upfield as compared to the signals of the same protons in D_2O with reference to external tetramethylsilane.

Spin–spin coupling assignments for the protons of 1-methyl-DHN have not been studied systematically in the past. In the course of the present work, 1-methyl-DHN in acetone-d_6 was used in double-irradiation experiments. As shown in Figure 4, irradiation at the region of the H_4 signal produced modifications of signals due to H_5 and H_6 protons. Corresponding modifications of signals due to various protons were also observed when the regions due to H_2 and H_6 protons were irradiated (Figure 5). These modifications provided necessary and sufficient data for unambiguous coupling assignments of all ring protons. The actual values of coupling constants (Table I) were determined from expanded spectra. These coupling constants are identical with constants reported for the benzyl (Meyer *et al.*, 1962; Diekmann *et al.*, 1964) and the propyl (Ludoweig *et al.*, 1964) derivatives. They are also in good agreement with a value for $J_{2,6}$ of the methyl derivative, first reported by Hutton and Westheimer (1958). The latter coupling (*i.e.*, $J_{2,6}$ across the C–N–C bridge) may be used as an additional argument in favor of the planarity of the ring in 1-substituted 1,4-dihydronicotinamides (Meyer *et al.*, 1962). As Kosower (1962) pointed out, a nonplanar model of such compounds would require a tetrahedral nitrogen atom involving four sp^3 bonds, one containing the lone electron pair, whereas the planar structure would require a trigonal nitrogen atom (*i.e.*, three sp^2 bonds, one p orbital for the lone pair). Obviously, the latter arrangement would be much more favorable for a coupling between H_2 and H_6.

Time-dependent changes of chemical shifts due to protons of 1-substituted 1,4-dihydronicotinamides in the presence of acidic anions (in D_2O) or acids (in $CDCl_3$) were followed by recording nuclear magnetic resonance spectra at frequent intervals after admixture of the reactants. Spectra included in Figures 1–3 are representative examples. In general, as the reactions progressed, areas under the signals due to protons H_2, H_4, H_5, and H_6 diminished simultaneously and finally disappeared. This was taken as an indication of completion of reaction 3 (primary protonation; see Alivisatos *et al.*, 1965).

Simultaneously with the decay of previously well-defined signals, new peaks appeared in the nuclear magnetic resonance spectra of reaction mixtures. These new signals were, as a rule, wider and more diffuse (see Figures 1–3). Long after completion of reaction 3, the set of newly formed signals showed changes, as expected from previous spectrophotometric studies (Alivisatos *et al.*, 1965). However, in the present work only changes corresponding to reaction 3 (*i.e.*, the primary protonation) were studied in detail.

Signals appearing during interaction of 1-methyl-DHN with orthophosphate are listed in Table II.

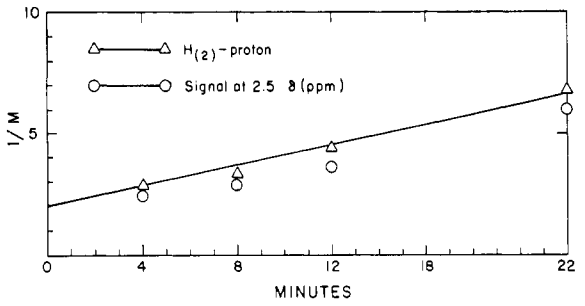


FIGURE 6: Time dependence of the changes of the areas under the signal due to H_2 (one proton) and under the wide peak at $\delta \sim 2.5$ (four protons) in the nuclear magnetic resonance spectra of reaction mixtures of 1-methyl-DHN and $D_2PO_4^-$ in D_2O at 32.5 ± 0.5°.

Assignments for these signals are based on a combination of studies with deuterio derivatives and kinetic analysis, as follows. The multiplet at δ 7.4–7.8 is missing when the 2-deuterio derivative is serving as the reactant (Table II). On this basis, it was assumed that the decaying signal due to H_2 (δ 7.26) is actually shifted downfield in its new (multiplet) form. Consequently, the addition product of areas under the decaying signal of H_2 and the growing signal at δ 7.4–7.8 should remain constant (0.5 M) during the initial phase of the sequence (reaction 3). This and the (known) ratios of the areas of the two peaks permitted computation of the molarity of intact 1-methyl-DHN at any time (t), while reaction 3 was in progress. Assuming that a second-order process was in progress (*i.e.*, one molecule of the DHN interacting with one molecule of phosphate; see Alivisatos *et al.*, 1965) and considering that initially the two reactants were present at equimolar concentrations, it was expected (Frost and Pearson, 1961) that at the initial stages of reaction 3, the integrated equation (eq 1) would be valid.[2] In this equation

$$\frac{1}{(c - x)} - \frac{1}{c} = k_1 t \qquad (1)$$

[2] In previous studies (Alivisatos *et al.*, 1965) the concentration of the proton donor (*i.e.*, $H_2PO_4^-$) was several orders of magnitude higher than that of the dihydronicotinamide derivative (NADH). This ensured practically constant pH throughout the duration of the reaction and permitted a treatment of the primary protonation of NADH in terms of pseudo-first-order kinetics. In the present study, the two reactants were used in equimolar concentrations. The obvious experimental disadvantage of this setup in a watery milieu (D_2O) is that as the reaction progresses the concentration of $H_2PO_4^-$ will be different (higher) than that predicted from eq 1 due to interaction of one of the products (HPO_4^{2-}) with water (see also Johnston *et al.*, 1963). Thus, under our conditions (initial pH 7.0), at a time when the concentration of 1-methyl-DHN is less than one-third of its initial value, eq 1 is no more applicable. Although a more suitable kinetic treatment accounting for this "catalytic" role of phosphates as proton donors in watery milieu could be worked out, we preferred in the present contribution to describe the initial stages of the process in terms of strictly second-order kinetics (see Figure 6), thus stressing the analogy with the similar reactions of the benzyl and propyl derivatives in organic solvents.

TABLE II: Chemical Shifts of Signals from Mixtures[a] of Potassium Phosphate with 1-Methyl-1,4-dihydronicotinamide and Its Deuterio Derivatives at Times of Completion of Reaction 1.

		Derivatives[c]			
No.	Chemical Shifts[b]	R	R-2d	R-4d	R-6d
1	Wide peak at ~2.5	3	3	2	3
2	Complex at 3.2–3.6	3	3	3	3
3	Sharp singlet at ~4.86	+	+	+	+
4	Multiplet at 7.4–7.8	1	–	1	1
5	Small peak at 8.2–10.0	+	+	+	+

[a] Reaction mixtures contained equimolar quantities (0.5 M) of potassium orthophosphate (pH 7.0) and of the desired 1-methyl-1,4-dihydronicotinamide derivative in D_2O at 32.5 ± 0.5°. [b] Values represent δ (parts per million) downfield from external tetramethylsilane. [c] R = methyl. Values represent number of protons corresponding to the signal.

c stands for the initial concentration of the reactants ($c - x$) is the concentration of 1-methyl-DHN at time t, and k_1 is the rate constant for reaction 3 (*i.e.*, the primary protonation). As shown in Figure 6, this expectation was fulfilled until approximately two-thirds of the initial quantity of 1-methyl-DHN had been consumed. The rate constant determined from this plot was $\sim 2 \times 10^{-1}\ M^{-1}\ min^{-1}$.

Our next attempt was to determine the origin of the wide peak appearing at δ 2.5 (Figure 1 and Table II). Using as a yardstick the combined areas under the decaying signal due to the H_2 proton and under the growing multiplet at δ 7.4–7.8 we established that this growing signal at δ 2.5 was due to three protons. Thus, using the expression

$$\frac{1}{0.5\ M\left[1 - \dfrac{\text{area under signal at } \delta\ 2.5}{3(\text{area under } \delta\ 7.26 + \text{area under } \delta\ 7.4\text{–}7.8)}\right]} \qquad (2)$$

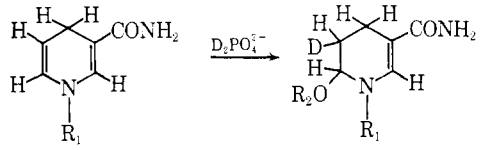
we obtained for various times (t) the values plotted as circles in Figure 6. This relation (1:3) was also maintained when the 2- and 6-deuterio derivatives were used as the reactants with phosphate in D_2O. When, however, the 4-deuterio derivative was present in the mixtures, the area under the δ 2.5 signal corresponded to approximately two protons. Considering that the corresponding signals in reaction mixtures containing either 1-benzyl-DHN or 1-propyl-DHN

TABLE III: Chemical Shifts of Signals from Mixtures of Monochloroacetic Acid with 1-R-1,4-Dihydronicotinamides and Their Deuterio Derivatives at Times of Completion of Reaction 1.[a]

No.	Chemical Shifts[b]	Derivatives[c]					
		R_1	R_1-2d	R_1-4d	R_1-6d	R_2	R_2-6d
1	Wide signal at ~1.5–2.5	4	4	3	4		
1a	Wide signal at ~1.8–2.5[d]					(4)	(4)
2	Signal at ~5.40	1	1	1	–	1	–
2a	Signal at ~4.70					+	+
3	Signal at ~5.82	+	+	+	+		
4	Complex at 6.8–7.8	6	5	6	6	1	1
5	Small peaks at 8.2–10.0	+	+	+	+	+	+

[a] Reaction mixtures contained equimolar quantities (0.3 M) of the acid and the desired dihydronicotinamide derivative in $CDCl_3$ at 32.5 ± 0.5°. [b] Values represent δ (parts per million) downfield from internal tetramethylsilane. [c] R_1 = benzyl; R_2 = propyl. Values represent number of protons corresponding to the signal. [d] The upfield portion of this signal is covered by the β-CH_2 of propyl; see Figure 2. Parentheses indicate uncertainties in the evaluation of this area.

corresponded to four protons (see below) it was concluded that the signal at δ 2.5 is due to protons of two methylene pairs of the product (reaction 3): the protons of the methylene at position 4 and the two protons of a newly formed methylene group at position 5.



(3)

In reaction 3, R_1 stands for methyl, benzyl, or propyl, and R_2, in watery milieu, is a hydrogen atom (Anderson and Berkelhammer, 1958).

The upfield migration of signals stemming from these two groups of protons lends further support to the mechanism depicted in reaction 3. Loss of $\Delta_{5,6}$ results in a more shielded environment for protons in positions 4 and 5 of the product. The downfield migration of the signal due to H_2 indicates more extensive deshielding occurring simultaneously with the loss of a cross-conjugated[3] two-double-bond system.

The complex at δ 3.2–3.6 (Figure 1) originally corresponds to the two H_4 protons and the three 1-methyl protons. During the course of the reaction the shape of this complex and the area under the signals change drastically. At completion of the primary protonation step (reaction 3), this area corresponds to three protons (Table II) and it is probably solely due to the three methyl protons. Changes in the shape of this signal (multiplet instead of the expected singlet) are also observed in the corresponding signal stemming from the protons of the C_6H_5 group of the benzyl derivative[4] and, less clearly, in the diffuse signal stemming from the protons of the α-methylene group of the propyl derivative. These apparent multiplet structures of signals due to the H_2 (sextet?) and the methyl group protons (octet?), clearly observed after completion of the primary protonation, cannot be solely due to changes of the configuration of the molecule (*i.e.*, deviations from planarity) and are difficult to reconcile with present concepts of the mechanism of this reaction (presence of isomeric forms?). They are now under careful scrutiny in this laboratory.

The sharp signal at δ ~4.86 (Figure 1) did not vary its position at different spinning speeds of the sample tube in the instrument. Consequently, it is not a side band of the neighboring HDO signal. This signal should be considered together with small signals developing in the range of 8–10 ppm downfield (Figure 1). As a group, they are reminiscent of the spectrum of nicotinamide methiodide (ring protons resonating downfield and methyl protons at δ 4.86). It is evident that a rather small percentage of 1-methyl-DHN is oxidized to 1-methylnicotinamide during the reaction period.

The presence of the intense HOD band and changes of the signal due to the methyl protons (see above) prevented unequivocal conclusions regarding the fate of H_6 during the primary protonation step of 1-methyl-

[3] For evidence pointing toward overlap between the orbital containing the nonbonding electrons on nitrogen and the π orbitals of the double bonds see Kosower (1962).

[4] A glance at the spectra of Figure 3 gives the impression that the signal due to H_2 shifts during the reaction from the upfield side to the downfield side of the signal due to the C_6H_5 protons. This impression, however, is not substantiated by studies with the 2-deuterio derivative. In such studies, the general area under the curve at δ 6.8–7.8 corresponds to five (instead of six) protons. Despite this, the signal downfield of the large band of C_6H_5 forms gradually as occurs with the nondeuterated derivative. It appears that the signal from the C_6H_5 protons changes to a complex of peaks (or a multiplet), as happens with the signal of the methyl protons in the 1-methyl derivative (see text).

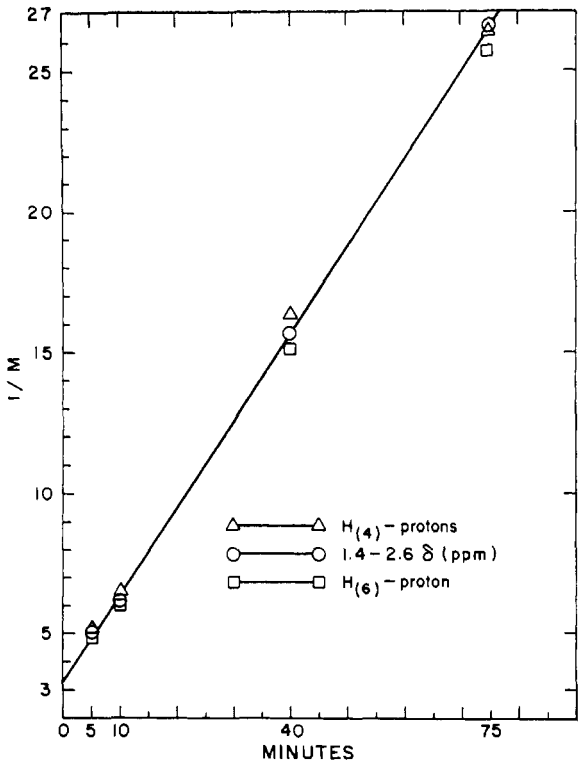


FIGURE 7: Time dependence of the changes of the areas under the signals due to H_4 (two protons) and H_6 (one proton) and of the wide peak at 1.4–2.6 (four protons) in the nuclear magnetic resonance spectra of reaction mixtures of 1-benzyl-DHN and $CH_2ClCOOH$ in $CDCl_3$ at 32.5 ± 0.5°.

DHN. The benzyl and propyl derivatives proved more suitable for such studies. Reactions were carried out in $CDCl_3$. The solubility of monochloroacetic acid in this solvent and the sharp singlet of its two methylene protons (δ 4.02 downfield from internal tetramethylsilane) were the reasons for choosing this moderately strong acid ($pK' = \sim 2.85$) as a proton donor in our systems.

Signals appearing during interactions of monochloroacetic acid with either 1-benzyl-DHN or 1-propyl-DHN are listed in Table III. Assignments for the nuclear magnetic resonance signals due to protons in reaction mixtures of the benzyl derivative were based on the following observations and kinetic considerations. The area of signals between δ 6.8 and 7.8 corresponds, at $t = 0$, to six protons, *i.e.*, the five protons of C_6H_5 and the H_2 (Figure 3). It is assumed that as in the reactions of the methyl and propyl derivatives, the signal due to H_2 moves upfield during this reaction.[4] It remains, however, in the same general region (δ 6.8–7.8) and this renders the area under the complex constant (*i.e.*, corresponding to six protons) throughout the reaction period. An additional reference is provided by the constant area under the signal at δ 4.02 (*i.e.*, due to the two protons of monochloroacetic acid). Since changes of the nuclear magnetic resonance signals of all protons in the dihydronicotinamide moiety occur concomitantly, relative changes of the areas under any of these signals with respect to the constant areas cited above may be used for the computation of the molarity of intact 1-benzyl-DHN remaining at time t in the mixture. In Figure 7 the rates of decay of the signals due to the H_4 and H_6 protons were used, separately, in computing $1/(c - x)$ values for the dihydronicotinamide moiety in 1-benzyl-DHN. Additional values were obtained from the growing area under the signal at δ 1.5–2.5. The latter was assumed to correspond to four methylene protons (see above) and a modified version of expression 2 was used for this computation. As shown in Figure 7, values from all three parameters defined closely the same straight line when the reciprocals of the molarity were plotted *vs.* time. The k_1, computed from the slope of this curve, was $\sim 3 \times 10^{-2}$ M^{-1} min^{-1}.

As mentioned previously,[4] the 2-deuterio derivative of 1-benzyl-DHN was helpful in the clarification of the upfield migration of the signal of H_2 during protonation of this compound. In a similar manner, the 6-deuterio derivative served in the elucidation of the fate of the signal due to this proton. As shown in Table III, the growing signal at δ 5.40 was missing from such spectra (see below).

We cannot offer, at present, an interpretation for the signal at δ 5.82 (see below). The small peaks at δ 8.2–10.0 are due to the oxidized form of the benzyl derivative.

Similar considerations were applied in the interpretation of nuclear magnetic resonance spectra of reaction mixtures containing the 1-propyl derivative (Figure 2 and Table III). The areas of the methyl proton in the side chain and the methyl proton of $CH_2ClCOOH$ were used as yardsticks of comparison. As in the 1-methyl derivative, the signal due to H_2 migrated downfield, where it appeared as a multiplet (probably sextet). As in the 1-benzyl derivative, the signal due to H_6 migrated upfield (from δ 564 to 540). Following the pattern set by both the methyl and benzyl derivatives, the methylene protons at positions 4 and 5 moved upfield. Signals due to the α and β CH_2 protons in the side chain became rather diffuse indicating similar changes as those observed in the 1 substituents of the other two derivatives (see above). The rate constant for the propyl derivative was estimated to be of the order of 5.5×10^{-1} M^{-1} min^{-1}.

In spectra of reaction mixtures containing propyl or benzyl derivatives it was desirable to exclude the possibility that signals in the general area between δ 5 and 6 were due to NH_2 protons. Thus, temperature elevation (from 33 to 50°) and deuterium exchange (shaking of the organic phase with D_2O) did not alter the signals in this area. In the latter experiments the spectrum of material extracted in D_2O exhibited mostly signals of the oxidized forms of the substituted dihydronicotinamide.

In conclusion, primary protonation of 1-methyl-DHN by $D_2PO_4^{2-}$ in D_2O exhibits almost *identical changes* of the nuclear magnetic resonance spectra with

those observed during primary protonation of 1-benzyl-DHN or 1-propyl-DHN by $CH_2ClCOOH$ in $CDCl_3$. The salient points of such changes and the interpretations given at present are as follows. (1) Signals due to protons H_2, H_4, H_5, and H_6, originally present in the spectra, decay concomitantly. This indicates general structural changes, involving readjustment of all bonds in the molecule. The rate of this decay may be described in terms of second-order kinetics, indicating a bimolecular reaction. (2) The signal due to H_2 migrates downfield by approximately δ 0.18. This migration is indicative of more extensive deshielding and it occurs concomitantly with the loss of a cross-conjugated two-double-bond system in the molecule. This same change is probably responsible for the increased ultraviolet absorption in the range of 290–300 mμ. The shape of the nuclear magnetic resonance signal due to the H_2 proton also changes during this migration (from a doublet to a multiplet, probably a sextet). The origin of the multiplicity is now under investigation. (3) The signal due to the H_4 methylene protons moves upfield by approximately δ 0.5. This, together with the movement of two additional protons from position 5 to the same general area (broad signal at approximately 2.5 ppm downfield from internal TMS) reflects the loss of $\Delta_{5,6}$ during primary protonation. (4) In reaction mixtures in $CDCl_3$ (*i.e.*, benzyl and propyl derivatives) the signal due to H_6 moves upfield by approximately δ 0.25. The analogous upfield movement of the signal due to H_6 in the reaction of 1-methyl-DHN with $D_2PO_4^-$ in D_2O is concealed by the intense signal due to HDO. Considering that the original signal due to H_6 in the methyl derivative (*i.e.*, before protonation) was separated by $\delta > 1.0$ from the HDO band, it follows that this signal migrates to a much larger extent upfield[5] as compared to the upfield migrations of its counterparts in $CDCl_3$. Now, signals due to methyne protons adjacent to OC(=O) R appear, in general, at lower fields ($\sim$1.2 ppm) as compared to methyne protons adjacent to OH. The above-mentioned differences are taken as indication of an addition of the anion (CH_2ClCOO^-) to the carbon at position 6. In D_2O, any association of the phosphate anion is subject to hydrolysis, thus giving rise to the hydroxylated product (Anderson and Berkelhammer, 1958). It would be of interest to study the possibility of such a transient state (see also footnote 3 in Alivisatos *et al.*, 1965) with the aid of a phosphorus resonance spectrometer. (5) Signals due to protons of the 1 substituent (*i.e.*, methyl, benzyl, or propyl) change shape during protonation (singlets become multiplets and existing multiplets acquire rather diffuse shape). The source of these changes is presently unknown, but it is believed that they will prove useful, in the future, in the clarification of the mechanism of the reaction and a better understanding of the structure and *shape* of the product.

[5] With approximate corrections (0.45–0.50 ppm) for external–internal tetramethylsilane, the original position of the signal due to H_6 (before reaction) is practically the same in all derivatives (see Table I).

Acknowledgments

We wish to thank Drs. C. L. Bell, R. H. Bible, L. K. Keefer, and C. S. Mahajanshetti for their advice in the interpretations of nuclear magnetic resonance spectrograms, and Mr. James L. Loo, Division of Oncology of the Chicago Medical School, for his help in the operation of the nuclear magnetic resonance spectroscope. We are indebted to Miss Nina Permutt and Miss Andrea Dietrich for diligent technical assistance, especially at the last stages of this work.

References

Alivisatos, S. G. A., Ungar, F., and Abraham, G. (1964), *Nature 203*, 973.

Alivisatos, S. G. A., Ungar, F., and Abraham, G. (1965), *Biochemistry 4*, 2616.

Anderson, A. G., Jr., and Berkelhammer, G. (1958), *J. Am. Chem. Soc. 80*, 992.

Choi, K. S., and Alivisatos, S. G. A. (1967), 154th National Meeting of the American Chemical Society, Chicago, Ill., Sept, Abstract C52.

Diekmann, H., Englert, G., and Wallenfels, K. (1964), *Tetrahedron 20*, 281.

Frost, A. A., and Pearson, R. G. (1961), Kinetics and Mechanism, 2nd ed, New York, N. Y., Wiley, p 19.

Hutton, R. F., and Westheimer, F. H. (1958), *Tetrahedron 3*, 73.

Jardetzky, O., and Jardetzky, C. D. (1962), *Methods Biochem. Anal. 9*, 235.

Johnston, C. C., Gardner, J. L., Suelter, C. H., and Metzler, D. E. (1963), *Biochemistry 2*, 689.

Karrer, P., and Blumer, F. (1947), *Helv. Chim. Acta 30*, 1157.

Karrer, P., Schwarzenbach, G., Benz, F., and Solmssen, U. (1936), *Helv. Chim. Acta 19*, 811.

Karrer, P., and Stare, F. J. (1937), *Helv. Chim. Acta 20*, 418.

Kosower, E. M. (1962), Molecular Biochemistry, New York, N. Y., McGraw-Hill, p 198.

Lehninger, A. L., and Wadkins, C. L. (1962), *Ann. Rev. Biochem. 31*, 47.

Ludowieg, J., Bhacca, N., and Levy, A. (1964), *Biochem. Biophys. Res. Commun. 14*, 431.

Mauzerall, D., and Westheimer, F. H. (1955) *J. Am. Chem. Soc. 77*, 2261.

Meyer, W. L., Mahler, H. R., and Baker, R. H., Jr., (1962), *Biochim. Biophys. Acta 64*, 353.