mean that the attack of the dihydroxyacetone anion on the pyridinium ring was rate-limiting.

The reaction of acetone with N-substituted pyridinium derivatives has been reported from several laboratories (Huff, 1947; Kaplan *et al.*, 1951). Since progesterone is a substituted acetone derivative, similar reactivity would be anticipated, but it was not observed. This may be due to the low rate of enolization of progesterone, and the low concentration of steroid used.

The nature of the linkage in the present case is clearly different from that described by Munck *et al.* (1957), who reported the formation of complexes between steroids and pyridine nucleotides in which the attractive forces between the components were of the London–Van der Waals type. It is not known at present whether the reactions described in this paper may contribute to an explanation of the mode of action of certain of the corticosteroids. However, it may be significant that, of the various steroids examined, this model reaction was restricted to steroids with a 17-hydroxy- or 17-deoxyketol side chain.

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Acid-Catalyzed Addition of Water to 1,4-Dihydronicotinamide Derivatives*

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The acid modification reactions of reduced diphosphopyridine nucleotide and of *N*-propyl-1,4-dihydronicotinamide have been studied spectrophotometrically. The primary reaction is a general acid-catalyzed transformation. Catalysis constants for a series of acids have been measured: phosphate is especially effective in the neutral *p*H range. The primary acid modification product from *N*-propyl-1,4-dihydronicotinamide exhibits a strong absorption band with a peak at 290 mμ, which shifts reversibly, with a pK_a of 2.3, to a low peak at 304 mμ in acid solutions. The product from diphosphopyridine nucleotide behaves similarly except that the peak in acid solution is at 300 mμ and very high and the pK_a is 0.6. The rate of the secondary modification reaction of *N*-propyl-1,4-dihydronicotinamide is *p*H dependent, the protonated and unprotonated forms of the primary product reacting with different velocities.

Reduced diphosphopyridine nucleotide (DPNH)[1] and other 1,4-dihydronicotinamide derivatives (I) undergo a rapid and apparently irreversible alteration in acidic solutions resulting in a shift of the characteristic ultraviolet absorption band in the 340–360 mμ region downward to around 290 mμ (Fig. 1). This primary acid modification product undergoes a slower secondary reaction to form less strongly absorbing compounds. In most studies of the reaction no products have been isolated, but from the acid decomposition of 1-benzyl-3-acetyl-1,4-dihydropyridine (II) Anderson and Berkelhammer (1958) have prepared two crystalline products. The major product was identified as the hydrated compound, III (equation 1), and the second one as a dimer. It appears likely that similar products are formed from the 1,4-dihydronicotinamides, and Segal and Stein (1960) have isolated a crystalline hydrate from the action of acid on 1-benzyl-1,4-dihydronicotinamide. Most of the earlier literature has been reviewed by Anderson and Berkel-

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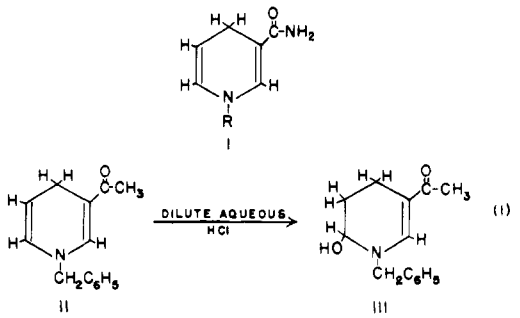
[1] Abbreviations used in this paper are as follows: DPNH-reduced diphosphopyridine nucleotide; NPrNH, *N*-propyl, 1,4-dihydronicotinamide; DPN, oxidized diphosphopyridine nucleotide.



I II III

hammer (1958) and by Wallenfels and Schüly (1957). More recent experiments have been reported by Wallenfels *et al.* (1959) and by Stock *et al.* (1961).

Our interest in this reaction was aroused by the observation that whereas reduced DPN does not undergo the acid modification at an easily noticeable rate at 25°, *p*H 7 in tris(hydroxymethyl)aminomethane or triethanolamine buffers, it does react slowly in phosphate buffer (Suelter, 1959).[2] Any reaction of reduced DPN which proceeds spontaneously under such mild conditions may be of biochemical importance. The catalysis by phosphate was especially intriguing since it might suggest possible mechanisms of adenosine triphosphate synthesis, which is known to be coupled *in vivo* to oxidation of reduced DPN.

In order to study the apparent catalysis, we chose to study principally a simpler compound than reduced DPN, namely, the crystalline *N*-propyl-1,4-dihydronicotinamide (NPrNH), which reacts in acidic solutions even more rapidly than does DPNH. The results obtained suggest that a general acid-catalyzed hydration occurs, presumably at the 5,6-position of the dihydropyridine ring.

Experimental

Materials and Solutions.—1-Propyl-1,4-dihydronicotinamide (NPrNH) was prepared in crystalline form (Suelter and Metzler, 1960). Fresh 1.4–3.6 $\times 10^{-3}$ M stock solutions were prepared by dissolving 6–15 mg of the compound in 25 ml of distilled water containing one-half drop of approximately 0.001 N KOH to stabilize the solution. Reduced diphosphopyridine nucleotide (DPNH) tetrahydrate, disodium salt, was purchased from Sigma Chemical Company. Two lots were stated to be 93% and 98% pure, respectively. Stock solutions in distilled water were 1.0–3.1 $\times 10^{-3}$ M.

Stock buffers were prepared, all having ionic strengths of 0.5. More dilute buffers were prepared by dilution with 0.5 M sodium chloride.

Absorption Spectra and Spectrophotometric Titrations.—A Cary Model 14 spectrophotometer and a Beckman Model DU spectrophotometer were employed. The spectrophotometric titration curve of the primary acid modification product of NPrNH (Fig. 3, curve A) was determined as follows: 0.1 ml of a 1.4 $\times 10^{-3}$ M stock solution of NPrNH was rapidly mixed with 1.0 ml of 0.1 N HCl containing 0.4 M NaCl (added to give an ionic strength of 0.5). After 60 seconds (a time which previously had been determined to be sufficient for the practical completion of the primary acid reaction, but insufficient for extensive secondary reaction), 2.0 ml of buffer (ionic strength = 0.5, except for 1 N HCl solution) was added. A series of buffers of different *p*H values was used. Since the secondary acid modification reaction proceeds at a significant rate, the optical density at 290 mμ was then read at 1.0-minute intervals and the optical densities were extrapolated back to the time when the buffer was first added. Additional small corrections were applied to allow for slow changes in the stock solution occurring during the titration.

A similar procedure was used for the spectrophotometric titration of the acid modification product of DPNH at 300 mμ (Fig. 3, curve B) except that the reaction was allowed to proceed in 0.1 N HCl for 10 minutes before addition of buffer, and the final ionic strength was 1.0.

The pK_a values calculated from these curves are apparent dissociation constants based on molar concentrations and hydrogen ion activities as measured by the *p*H meter.

Kinetics of the Primary Acid Modification Reaction.—Reaction rates were measured through the use of a linear recorder (Varian Associates, Model G-10) attached to the Beckman spectrophotometer, which was equipped with a cell compartment thermostatted at 25°. In a typical experiment, 3.0 ml of the desired buffer (which was previously maintained at 25°) was pipetted into a 1-cm cuvet and placed in the spectrophotometer. To start the reaction, 0.1 ml of NPrNH or DPNH stock solution was added to the buffer with an adder-mixer (Boyer and Segal, 1954) and the per cent transmission at 360 mμ or at 340 mμ compared to a distilled water blank was recorded versus time. In a typical experiment the optical density of a 1.16 $\times 10^{-4}$ M NPrNH solution dropped from an initial value of about 0.80 to 0.032 to 0.023 at the end of the reaction. It should be noted that the expected initial optical density value for such an NPrNH solution is 1.04. It was discovered that even crystalline NPrNH when not used immediately after crystallization (or kept very carefully desiccated) had partially reacted, apparently to form some of the primary acid modification product. This did not in any way interfere with the evaluation of the rate constants, however.

When the logarithms of the observed optical densities minus the final optical density were plotted against time, a nearly linear plot was obtained indicating a first-order decay. The first-order rate constants, k (in sec^{-1}) for the decay were evaluated from the slopes. (The average deviation from the mean of duplicate determinations as plotted in Figure 5 was ± 3%in most cases.) Immediately after the rate measurement, the *p*H of the solution was determined using a Beckman Model GS *p*H meter. The final ionic strength of each of the solutions was 0.48.

The rate constant, k_0, for "spontaneous" decay of NPrNH was evaluated at *p*H 8.0 in very dilute (10^{-4} M $H_2PO_4^-$) phosphate buffer containing 0.48 M NaCl in a cuvet covered to exclude atmospheric carbon dioxide. From the observed rate of reaction and the previously determined values of the catalytic constants of H^+ and of $H_2PO_4^-$, the constant k_0 was calculated. The catalytic constant of hydrogen ion, k_{H^+}, was obtained from measurements in 10^{-3} M and 10^{-5} M HCl in the case of NPrNH and in 10^{-2} M and 10^{-3} M HCl in the case of DPNH. The ionic strength was made up to 0.48 with NaCl. Catalytic constants k_{HA} for the various buffer acids were evaluated from the observed rate constants k and equation (2). When values of $(k - k_0)/[H^+]$, obtained at a single *p*H (± 0.1 *p*H unit), were plotted against [HA], a straight line with a slope $k_{HA}/[H^+]$ and intercept k_{H^+} was obtained for most acids (e.g., Fig. 5, curve A). When data at several *p*H values were obtained, they were

[2] The effect of phosphate on DPNH stability has also been noted by Winer and Schwert (1958) and by Lowry *et al.* (1961).

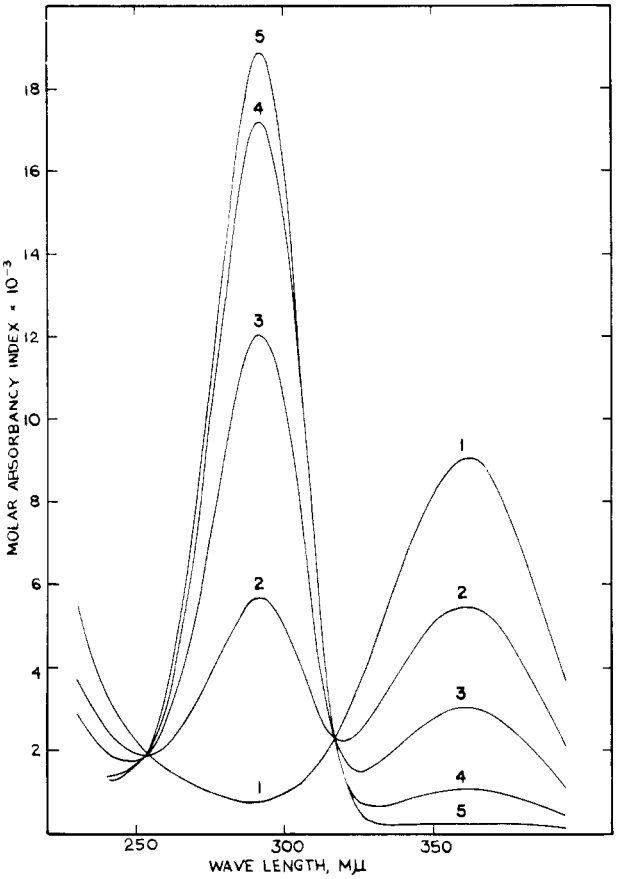


FIG. 1.—Absorption spectra of 1-propyl-1,4-dihydronicotinamide (NPrNH) and of its primary acid modification product. Curve 1 is the spectrum of freshly recrystallized NPrNH in 0.5 M NaCl solution at *p*H 8.7. Curves 2-5 were measured at various times after plunging an 0.1-ml aliquot of stock NPrNH solution into 3.0 ml of phosphate buffer of *p*H 7.1 and ionic strength 0.5. Curve 2 was obtained with a recording spectrophotometer with measurements starting at 400 mμ immediately after mixing (60 seconds or less) at a scanning rate of 150 mμ per minute. Curve 3 was begun 6 minutes after mixing and curves 4 and 5 at 18 and 50 minutes, respectively.

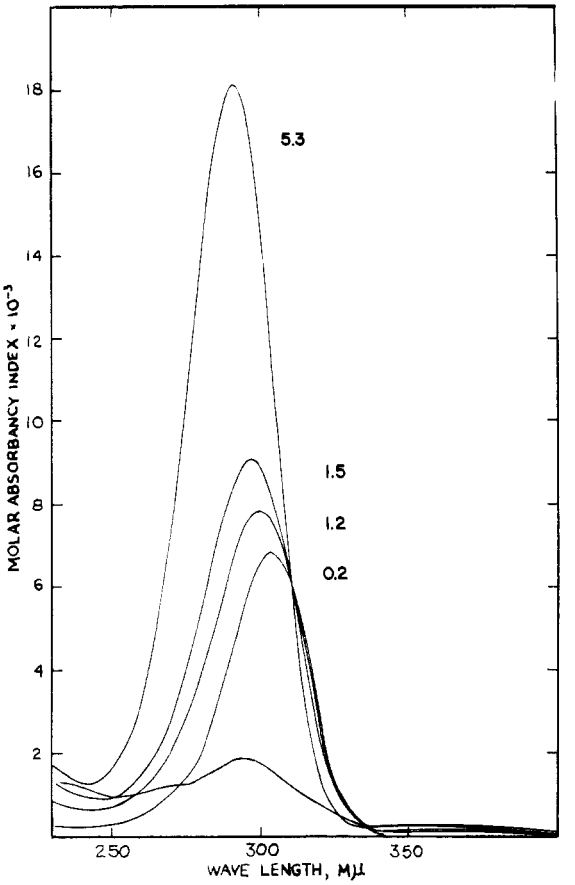


FIG. 2.—Absorption spectra of the primary acid modification product of NPrNH at various *p*H values, and the spectrum of the secondary modification product(s). An 0.1-ml aliquot of stock NPrNH solution was added to 1 ml of 0.1 N HCl and allowed to stand for 60 seconds. Then 2.0 ml of an appropriate buffer or HCl solution was added. The solution was mixed and the spectrum was measured immediately. The *p*H values are given beside the curves. Final ionic strength = 0.5. The lowest curve is the spectrum of the secondary modification product(s), which was obtained on a solution of NPrNH having a *p*H of 1.6 about 9 hours after conversion to the primary acid modification product.

sometimes plotted against the conjugate base concentration, $[A^-]$. In these plots the slope equals k_{HA}/K_{HA}, where K_{HA} is the dissociation constant of the acid. The intercept is again k_{H^+}. The values of k_{H^+} obtained from these intercepts agreed, within experimental error, with the values obtained from HCl solutions (Table II). Data in acetate buffers were obtained at *p*H 3.8, 4.6, and 5.0, and data in phosphate buffers were obtained at five *p*H values between 5.2 and 7.7.

The possible errors in k_{HA} were estimated from the graphs and represent the maximum errors which we judged to be likely.

The apparent acid dissociation constants of the catalytic acids were determined by measuring the *p*H values of carefully prepared buffer solutions of ionic strength 0.48.

Kinetics of the Secondary Modification Reaction of NPrNH.—Samples of the primary acid modification product at *p*H 1.0 were prepared in buffers of various *p*H as described under Absorption Spectra and Spectrophotometric Titrations. The rate of secondary reaction was readily followed by the decrease in absorbancy at 290 mμ. In order to calculate the first-order rate constants the final optical density ($a_M = 2.02 \times 10^3$, after eight half-lives, for a solution of *p*H 1.0) was subtracted from the observed optical densities, and the logarithms of the differences were plotted against time.

RESULTS

Characteristics of the Acid Modification Products of Dihydronicotinamide Derivatives.—Decay of *N*-propyl-1,4-dihydronicotinamide (NPrNH) in dilute (0.001 M) HCl, in acetate buffer at *p*H 5, or in phosphate buffer at *p*H 7 leads to what is apparently the same compound. In each case the product displays the same ultraviolet absorption spectrum with a maximum absorption at 292 mμ (Fig. 1). When this primary acid modification product is acidified, the absorption band is lowered and shifted to a peak at 304 mμ (Fig. 2). This spectral shift is completely reversible if studied rapidly before secondary modification reactions take place. Figure 3 shows spectrophotometric titration curves of the primary acid modification products of both NPrNH and reduced diphosphopyridine nucleotide (DPNH). The higher *p*H portion of the curve for the NPrNH modification product can be explained by the assumption that a single proton dissociates with an apparent pK_a of 2.3 ± 0.1, However, the fit of a theoretical curve to the observed data is not entirely satisfactory. The pronounced deviation at the low

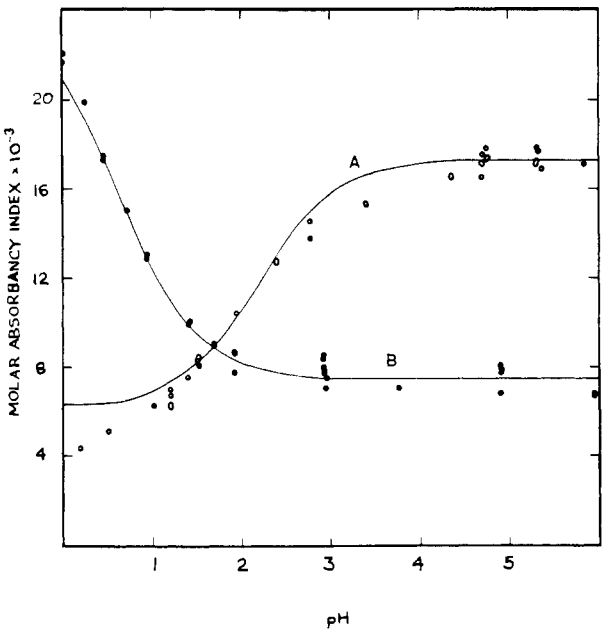


FIG. 3.—Spectrophotometric titration curves of the primary acid modification products. Curve A: product from reaction of NPrNH; absorbancy at 290 mμ. Curve B: product from reaction of DPNH; absorbancy at 300 mμ (see text for details).

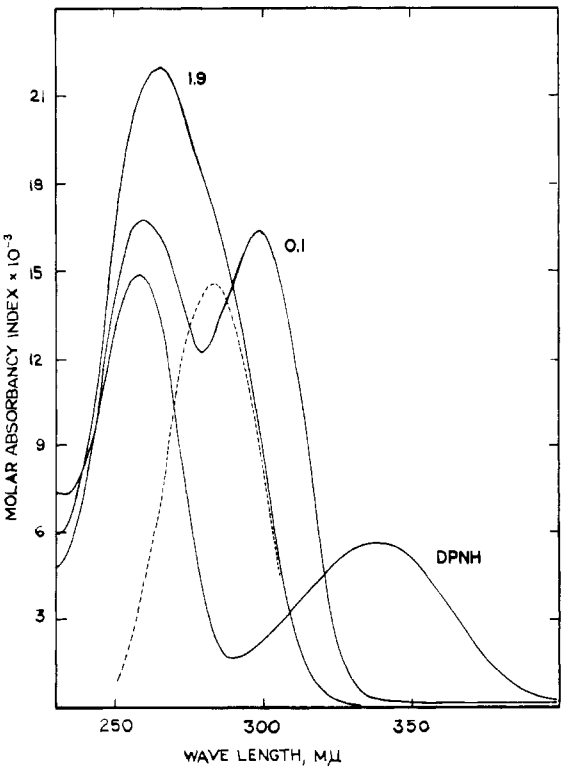


FIG. 4.—Spectrum of DPNH in sodium chloride solution, and spectrum of its primary acid modification product at *p*H 1.9 and *p*H 0.1 (ionic strength = 1.0). The dotted curve is derived from the curve at *p*H 1.9 by subtracting off the remaining absorption of the solution after approximately eight half-lives of the primary acid modification reaction.

*p*H end suggests that a second group is protonated at low *p*H, but it is not possible to establish this with certainty. The time required to add the buffer solutions to the solutions of the primary modification product of NPrNH at *p*H 1 and to mix may explain the lesser but significant discrepancy between the observed and theoretical curves at higher *p*H values.

Spectrophotometry does not reveal the existence of any intermediates between the initial compound and that absorbing at 292 mμ in the primary acid modification reaction of NPrNH. Sharp isosbestic points in a series of spectra measured at different times during the reaction at *p*H 5 or above occur at about 253 mμ and 318 mμ (Fig. 1).

In acidic solution a secondary modification reaction occurs more slowly, leading to the conversion of the primary product to a much more weakly absorbing form (Fig. 2). At *p*H 2.7, 25°, the apparent half-life for this first-order process is approximately 58 minutes. The conversion may be carried nearly to completion in 1 hour at 50°, however. Addition of hydroxylamine to the secondary decay product causes a shift of the absorption maximum to 265 mμ, as reported by Rafter (1953) for the decay product of 1-methyl-1,4-dihydronicotinamide.

The reaction of DPNH in acid, which has also recently been studied in detail by Stock *et al.* (1961), is similar in several respects to that of NPrNH. Figure 4 shows the initial spectrum of DPNH and spectra of the primary modification product at *p*H 1.9 and at *p*H 0.1. The spectrum of the primary product at *p*H 1.9 is similar to that from NPrNH when allowance is made for the additional adenylic acid chromophore. The dotted line curve in Figure 4 was derived by subtracting from the spectrum of the primary product the spectrum of the secondary modification product. Since the chromophore derived from the nicotinamide ring in the secondary product presumably absorbs very weakly as in the case of NPrNH (Fig. 2), this subtraction should give *approximately* the spectrum of the substituted tetrahydropyridine chromophore in the primary acid modification product of DPNH (Fig. 4, dotted line). Indeed this spectrum is quite similar to that of the primary modification product of NPrNH at a *p*H above 4.4. However, an interesting difference is seen in strongly acidic solutions. Protonation of the DPNH primary product gives rise to a high peak at about 300 mμ (Fig. 3, curve B; Fig. 4, *p*H 0.1) instead of the low peak at 304 mμ observed with the primary modification product from NPrNH and the pK_a is 0.6 rather than 2.3–2.4. Again the protonation is reversible.

Table I summarizes the molar absorbancy indices of the various compounds at selected wavelengths.

Kinetics of the Primary Acid Modification Reaction.— The experimentally observed first-order rate constants for the decay of NPrNH to the primary modification product at 25° can for the most part be satisfactorily described by a general acid catalysis equation (equation 2), in which k_0 (where $k_0 = k_w [H_2O]$) is the "spontaneous" reaction rate with water as the only catalytic

$$k = k_0 + k_{H^+}[H^+] + k_{HA}[HA] \qquad (2)$$

acid, and where k_w, k_{H^+}, and k_{HA} are the catalytic constants of the water, hydrogen (hydronium) ion, and buffer acid, HA, respectively. The value of k_{H^+} was obtained by measurements in dilute HCl. The value of k_0 is negligible in comparison to $k_{H^+}[H^+]$ under these conditions, and in dilute HCl the observed rate constant, k, is strictly proportional to the hydrogen ion activity (as measured by the *p*H meter).

Values of k_{HA} for a variety of acids were measured by plotting $k/[H^+]$ versus the concentration of HA for a series of buffers at constant *p*H (±0.05 *p*H unit) and ionic strength, but of different total buffer concentrations. At higher *p*H values, where k_0 is not negligi-

TABLE I

WAVELENGTHS OF ABSORPTION MAXIMA, SELECTED ABSORBANCY INDICES, AND pK_a VALUES

	λ_{max} (mμ)	Molar Absorbancy Indices ($a_M \times 10^{-3}$) At λ_{max}	290 mμ	300 mμ	pK_a
NPrNH	362	9.02	0.74 (0.75 at 292 mμ)		
Primary acid modification product from NPrNH					
In neutral solution	292	17.4	17.3		
In acidic solution	304	6.81	6.36[a]		2.3–2.4
Secondary modification product from NPrNH	294	*ca.* 2.1[b]	2.02[b]		
DPNH	338	5.66[c]		1.82	
Primary acid modification product from DPNH					
In neutral solution	284	14.6[d]		7.52[d]	
In acidic solution	300			24.1[a] (estimated)	0.6

[a] See titration curve, Fig. 3. [b] This value was obtained at *p*H 1.0 after about 2.5 hours as described in the experimental section under Kinetics of the Secondary Modification Reaction of NPrNH. It does not agree exactly with the spectrum of Figure 2 in which the sample, at *p*H 1.6, was allowed to stand 9 hours, and for which a lower absorbancy ($a_M = 1.6 \times 10^3$) was observed. This difference may, at least in part, be a *p*H effect. The variation of this spectrum with *p*H has not studied. [c] Compare with value of 6.22 (Chaykin *et al.*, 1956). [d] Corrected for absorption of the adenylic acid moiety. See text.

TABLE II

CATALYTIC CONSTANTS OF VARIOUS ACIDS IN THE PRIMARY ACID MODIFICATION REACTION AT 25° AND IONIC STRENGTH 0.48

		Catalytic Constants, liters mole^{-1} sec^{-1}					
Acid	pK_a[a]	$k_{H^+} \times 10^{-2}$	k_{HA}	p[b]	q[b]	$-\log\left(\frac{k_{HA}}{p}\right)$	$p\left(\frac{qK_a}{p}\right)$ $pK_a - \log(q/p)$
For the reaction of NPrNH							
Hydronium ion[c]	−1.74	1.3 ± 0.4	1.3 ± 0.4 × 10^{+2}	1	1	−2.11 ± 0.2	−1.74
Formic	3.46	1.2 ± 0.2	8 ± 2 × 10^{-1}	1	2	0.10 ± 0.12	3.16
Lactic	3.45	1.0 ± 0.3	7 ± 3 × 10^{-1}	1	2	0.14 ± 0.14	3.15
Acetic	4.61	2.6 ± 0.6	1.5 ± 0.1 × 10^{-1}	1	2	0.81 ± 0.02	4.31
H Malonate	5.15	1.3 ± 0.5	1.2 ± 0.2 × 10^{-1}	1	4	0.92 ± 0.1	4.55
Cacodylic	6.00	1 ± 3	7 ± 2 × 10^{-2}	1	2	1.16 ± 0.1	5.70
$H_2PO_4^-$	6.81	About 2	3.3 ± 0.3 × 10^{-2}	2	3	1.78 ± 0.04	6.63
HSO_3^-[d]	6.40		1.4 ± 1 × 10^{-1}	1	3	0.85 ± 0.2	5.92
2-Picoline·H$^+$	5.92		1.9 ± 1.6 × 10^{-2}	1	1	1.72 ± 0.8	5.92
Pyridine·H$^+$	5.38		5.5 ± 3 × 10^{-2}	1	1	1.26 ± 0.2	5.38
Imidazole·H$^+$[e]	7.15		3.6 ± 3 × 10^{-3}	2	1	2.74 ± 0.6	7.45
Hydroxylamine·H$^+$	5.67	1.2	2.6 ± 1 × 10^{-2}	1	1	1.59 ± 0.3	5.67
Triethanolamine·H$^+$[f]	7.83	1.3	2.5 ± 0.5 × 10^{-4}	1	1	3.6 ± 0.1	7.83
Water[g]	15.75		4.7 ± 0.9 × 10^{-7}	1	1	6.33 ± 0.1	15.75
For the reaction of DPNH							
Hydronium ion[c]	−1.74	7.7 ± 0.9	7.7 ± 0.9 × 10^{-1}[i]	1	1	0.11 ± 0.05	−1.74
$H_2PO_4^-$	6.81		6.5 ± 1 × 10^{-4}	2	3	3.49 ± 0.3	6.63
Imidazole·H$^+$[h]	7.15		7.5 ± 4 × 10^{-6}	2	1	5.43 ± 0.6	7.45
Water	15.75		1.79 × 10^{-7}	1	1	6.75 ± 0.1	15.75

[a] Apparent pK_a values observed under conditions of these experiments, except for those of hydronium ion and water, which were taken from Bell (1941). [b] p and q represent the numbers of dissociable protons in the acid and number of equivalent points at which a proton can be attached to the conjugate base, respectively (Bell, 1941). [c] Used synonomously with H^+, as in k_{H^+}. [d] $k' = (2.6 \pm 1) \times 10^{-1}$; $k = k_0 + k_{H^+}(H^+) + k_{HA}(HA) + k'(HA)^2$. [e] Best estimate at zero buffer concentration. Rate is slower at finite buffer concentrations. [f] Best estimate of value; lower bound overlaps K_w (H_2O). [g] In water (no salt present) the rate is slower, $k_{HA} = (1.4 \pm 0.1) \times 10^{-7}$; $pk_{HA} = 6.84 \pm 0.04$. [h] Rate is too slow to easily obtain an accurate k_{HA} at *p*H = 7.1; average of two values for k in more concentrated (0.968 M) imidazole buffer was $(4.5 \pm 0.6) \times 10^{-6}$ sec^{-1}. [i] However, Lowry *et al.* (1961) give a value equal to 6.3 liters mole^{-1} sec^{-1} at 23°.

ble, $(k - k_0)/[H^+]$ was plotted against [HA]. These plots were usually straight lines with an intercept equalling, within experimental error, the expected value, k_{H^+} (e.g., Fig. 5, curve A).

The reaction was studied in most detail in acetate buffers and in phosphate buffers. As previously mentioned, the changes in absorption spectrum with time were consistent with the assumption that only two species, the unchanged NPrNH and the primary acid modification product, were present. The catalysis in the phosphate buffers could all be accounted for through the effect of the $H_2PO_4^-$ ion as the acid. Though it doubtless occurs, catalysis by H_3PO_4 and by HPO_4^- could not be observed readily because of the very high and low acidities, respectively, of these components. The catalytic constants of acetic acid and other catalytic acids are summarized in Table II and in Figure 6. Statistical corrections were made according to Bell (1941) before the data were plotted.

For a few of the acids, namely, the pyridine, 2-picoline, and imidazole cations and bisulfite, nonlinear plots of $(k - k_0)/[H^+]$ versus [HA] were obtained

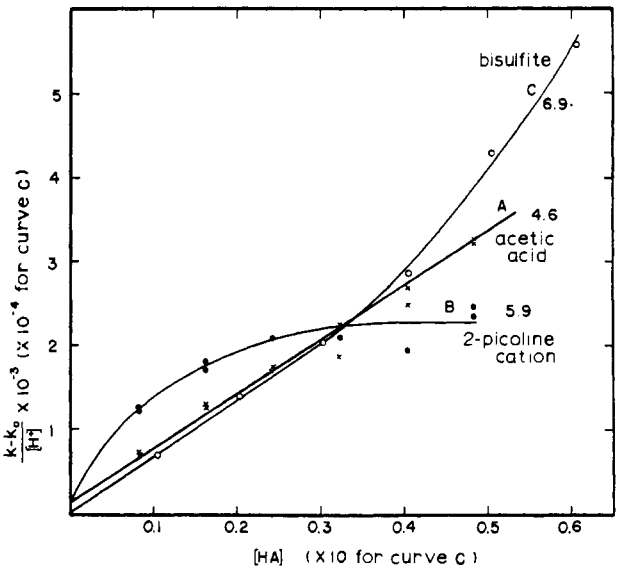


FIG. 5.—Determination of catalytic constants of various acids. Values of $(k - k_0)/[H^+]$ are plotted against the concentrations of weak acid. With most acids such as acetic acid (curve A) straight lines are obtained. With others such as protonated 2-picoline (curve B) and bisulfite anion (curve C) nonlinear relationships were observed. All curves were drawn to pass through the known value of k_{H^+} (see text) at the intercept on the vertical axis.

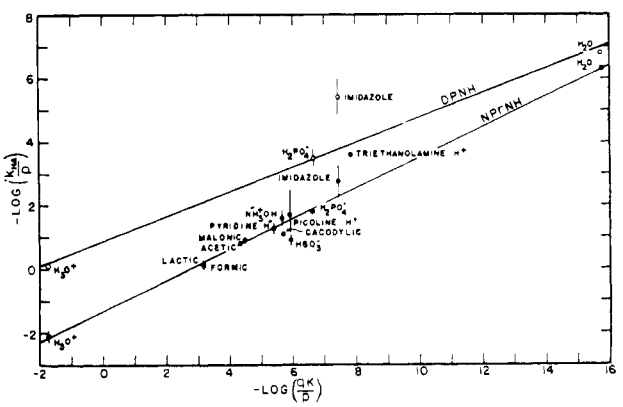


FIG. 6.—"Brönsted plot" of catalysis constants against acidity for catalytic acids. The negative logarithm of the catalysis constant, k_{HA}, divided by p, the number of equivalent dissociable protons, is plotted against the negative logarithm of the acid dissociation constant, K multiplied by the statistical correction factor q/p, where q is the number of equivalent sites in the conjugate base available for protonation (Bell, 1941). The solid lines represent the equations $k_{HA}/p = 23.2\ (qK/p)^{0.48}$ for NPrNH and $k_{HA}/p = 0.12\ (qK/p)^{0.38}$ for DPNH.

(Fig. 5). The nitrogenous acids exhibited decreased catalytic activity at high concentrations. The exact cause of this decrease was not investigated, but we estimated the k_{HA} value by drawing a tangent to the curve at zero [HA], making use of the known value of the intercept k_{H^+}. The uncertainties in these estimations are indicated in Table II and in Figure 6. A similar decrease of catalytic activity was observed with DPNH even with phosphate buffer. Bisulfite was found to be more active than expected in the reaction with NPrNH at high concentrations (Fig. 5, curve C). At three pH values the data could be fitted satisfactorily to equation (2) only by inclusion of an additional term $k'[HSO_3^-]^2$.

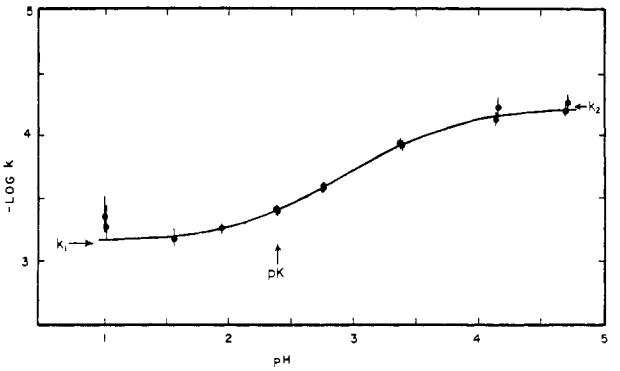


FIG. 7.—Rate constants for the secondary decay reaction of NPrNH. The negative logarithms of the apparent first-order rate constants are plotted against pH. The circles represent experimental points, the short vertical lines indicating the estimated maximum limits of error. The solid line is a theoretical curve (see text).

Kinetics of the Secondary Modification Reaction.—The secondary modification reaction of NPrNH was followed spectrophotometrically at 290 mμ. First-order kinetics were observed. The reaction is pH-dependent, the rate constants at various pH values being shown in Figure 7. Above pH 5 the rate is almost constant, but as the pH decreases, the rate increases by as much as 12-fold at around pH 1.5. There appears to be a small decrease again at yet lower pH values. The results may be explained by assuming that the protonated form of the primary acid modification product, BH^+, reacts more readily than the unprotonated form B, but that each reacts in a first-order process. Then the total rate of reaction is as shown in equation (3), where $[b] = [B] + [HB^+]$,

$$-d[b]/dt = k_1[HB^+] + k_2[B] \qquad (3)$$

the total concentration of the primary acid modification product, and k_1 and k_2 are the rate constants for reaction of HB^+ and B, respectively. It follows that the observed rate constant, k, is given by equation

$$k = k_1\Big/\left(1 + \frac{K_a}{[H^+]}\right) + k_2\Big/\left(1 + \frac{[H^+]}{K_a}\right) \qquad (4)$$

(4), where K_a is the apparent acid dissociation constant of the primary acid modification product. The solid line in Figure 7 was constructed to follow equation (4) with $pK_a = 2.4$, $k_1 = 7.2 \times 10^{-4}$, and $k_2 = 5.9 \times 10^{-5}$ sec^{-1}.

The value of $pK_a = 2.4$ for the primary acid product obtained during this curve-fitting process is in good agreement with the value of 2.3 ± 0.1 obtained by spectrophotometric titration (Fig. 3, curve A) and lends additional support to our interpretation of the change of spectrum with pH.

Hydroxylamine is reported to accelerate the disappearance of the primary acid modification product of DPNH (Burton and Kaplan; see Kaplan, 1960).

Equilibrium in the Primary Acid Modification Reaction.—After the primary modification reaction is complete a small amount (about 2.7%) of the initial absorption at 360 mμ remains. This may indicate that there is an equilibrium between the modification product and some unaltered NPrNH. The equilibrium constant may be estimated on this basis as 36. From data of Chaykin *et al.* (1956), the corresponding constant for acid modification of DPNH is estimated as 30.

The observation by Suelter (1959) that the primary acid modification product of NPrNH can be slowly

TABLE III

RATES OF REACTION FOR VARIOUS 1,4-DIHYDROPYRIDINES AT 25°

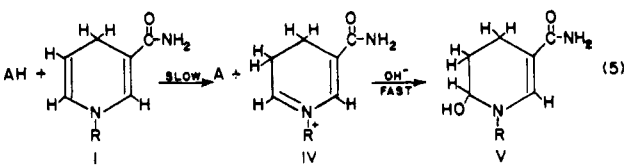
	NPrNH	DPNH	1-Benzyl-1,4-dihydronicotinamide	1-Benzyl-3-acetyl-1,4-dihydropyridine
Second order rate constant k_{H^+}, in liters mole^{-1} sec^{-1} for primary acid modification reaction				
This study	130	0.77		
Anderson and Berkelhammer[a]				
In 49% alcohol	5.3		1.5	0.043
In water			17	
Second-order rate constant for oxidation of riboflavin[b]	118	0.75	29	
Equilibrium constant for dissociation of the cyanide adduct of the oxidized dihydropyridine derivative $1/K_A \times 10^{2}$[c]	124	0.40	15.4	

[a] Calculated from first-order constants given by Anderson and Berkelhammer (1958) by dividing by HCl concentration. [b] Suelter and Metzler (1960). [c] Wallenfels and Diekman (1959).

oxidized by riboflavin to give authentic *N*-propylnicotinamide may be explained by such an equilibrium between NPrNH itself and the modification product. However, other mechanisms cannot be ruled out. Similarly, Stock *et al.* (1961) have reported the oxidation of the primary modification product of DPNH by permanganate in 30% yield.

DISCUSSION

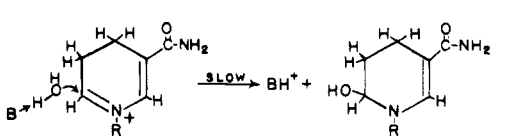
The initial acid modification reaction of 1,4-dihydropyridine derivatives is apparently a general acid-catalyzed hydration at the 5,6-position (equation 5).



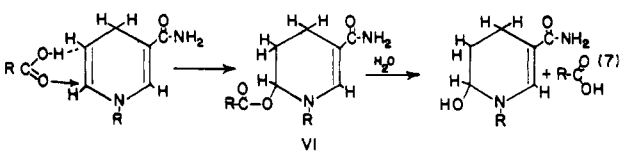
I IV V

It seems likely that the first step, the proton transfer to C-5 of the dihydropyridine ring, is the slow step. Such a protonation reaction has been established for other enamines (Leonard and Hay, 1956). As would be predicted, electron-withdrawing substituents at N-1 (benzyl, ribosyl) and at C-3 (acetyl) decrease the electron density on the ring nitrogen and slow the reaction, as shown in Table III and by the data of Stock *et al.* (1961).

An alternative interpretation, advanced by Anderson and Berkelhammer (1958), postulates a rapid, reversible protonation for the first step followed by an addition of OH^- at C-6 in the rate-determining step. Our results do not rule out this possibility, if the addition of OH^- occurs by means of a general base-catalyzed reaction with water (equation 6), in the second step. A mechanism involving general base catalysis of this step is kinetically indistinguishable from general acid catalysis of the first step followed by rapid addition of base as in equation (5). Reaction 6 would be expected to display an increase, rather than a decrease, in rate for compounds with electron-withdrawing substituents. However, the same substituents would cause a decrease in the equilibrium constant for the preliminary protonation, and it is hard to predict to what extent the two effects would cancel.



A third possibility lies in a "concerted" catalysis in which an acid such as carboxylic acid or phosphoric acid would simultaneously add both a proton and an anion as shown in equation (7).



VI

No evidence for an intermediate VI can be detected spectrophotometrically, but it is possible that the spectrum of the intermediate is too similar to that of the final product. In the case of catalysis by phosphate buffer, no phosphate-containing compounds have been detected chromatographically. We thought that indirect evidence for such addition might be obtained by measuring the catalytic constants of a series of acids. As seen in Figure 6, most oxygen-containing acids and several of the amines fall on a single Brönsted plot. If the acid modification reaction occurred by the concerted mechanism of equation (7), we would expect that pyridine hydrochloride would be a much poorer catalyst than the carboxylic acids, and since this is not so, it is probable that, in aqueous solution, anions other than the hydroxyl ion do not add at C-6. The imidazolium and triethanolammonium cations are less reactive than predicted by the Brönsted relationship. In the triethanolammonium cation steric hindrance may be a factor, but it is not clear why the imidazolium ion should be less reactive. In another example of general base catalysis, that of ester hydrolysis, the HPO_4^- ion and imidazole are equally effective (Jencks and Carriuolo, 1961).

Bisulfite appears to represent a special case, and Wallenfels and Schüly (1957) have isolated a sulfite-containing adduct. While at first Wallenfels *et al.* (1959) assigned this a structure containing the sulfite group at C-5, it is probable that the anion actually added at C-6[3] (Anderson and Berkelhammer, 1958). The enhanced catalytic activity of bisulfite may reflect the occurrence of a "concerted" addition at positions 5 and 6 with this acid. The catalytic effect at higher bisulfite concentrations (Fig. 5), which can only be

[3] K. Wallenfels, private communication.

described by inclusion of a term containing the square of the bisulfite concentration, suggests that a "concerted" addition process involving two molecules of bisulfite, one as an acid and one as a nucleophile, may occur.

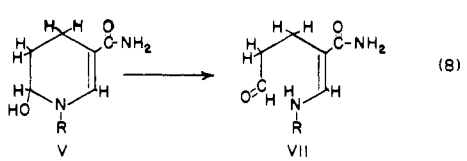
In appropriate solvents other adducts can be formed. For example, Wallenfels and co-workers (1959) have shown that thiphenols add, as does 2,4-dinitrophenylsulfenic acid, the sulfur atom of the latter attaching at C-5 and the chloride at C-6. The adduct so formed can reversibly lose HCl and the resulting compound can add acetic acid.

The addition of mercuric chloride to DPNH (Hill, 1956; Shifrin and Kaplan, 1961; Stock *et al.*, 1961) appears to be a closely related reaction.

A completely different explanation for the primary acid modification reaction has been advanced by Burton and Kaplan (see Kaplan, 1960). They suggest a protonation at N-1 followed by a ring-opening as part of the primary reaction. Stabilization of the primary modification product by bisulfite is taken to indicate a potential carbonyl group at C-6. However, this effect may simply reflect the addition of the sulfite ion as discussed above.

The reversible protonation of the primary acid modification product (a vinylogous substituted urea) may involve the carbonyl oxygen atom of the carboxamide group. The different, electron-withdrawing properties of the substituents on the nitrogen atoms of the primary acid modification products of NPrNH and DPNH, and the intramolecular and steric influences of the adenylic acid moiety of the DPNH product, may explain the remarkable differences in pK_a values and absorption spectra observed between the primary acid modification products of NPrNH and DPNH. (The dihydronicotinamide and adenine ring systems in the DPNH molecule are known to interact in solution [see Kaplan, 1960], and hydrogen bonding is possible between the oxygen atom of the carboxamide group of the dihydronicotinamide moiety and the amino group of the adenine moiety.) However the explanation of these differences is far from clear.

The nature of the secondary modification reaction is also uncertain. The simplest explanation assumes a ring-opening reaction (equation 8) to give an aldehyde, VII. This in turn might react in various ways. For



(8)

example, a different sort of ring closure could occur between the aldehyde group and the carboxamide group. Stock *et al.* (1961) offer an alternative explanation. In the case of the secondary acid modification product of DPNH, Chaykin *et al.* (1956) have reported spectrophotometric evidence for the existence of a basic group with a pK_a of about 7.

Burton and Kaplan (see Kaplan, 1960) report that strong acid (pH 1) cleaves the acid modification products of DPNH to yield adenosine diphosphate ribose and an unidentified dialdehyde.

Whether any biological function is reflected in the acid modification reactions of DPNH is completely uncertain. If oxidative phosphorylation occurs when bound DPNH is oxidized by a flavoprotein in the respiratory chain, it seems clear either that some other intermediate hydrogen carrier must be involved or that some unknown chemistry of either DPNH or riboflavin must be utilized by nature in providing a mechanism for coupling phosphorylation and oxidation. The possibility exists that the addition of an anionic group to the 6-position of DPNH is in some way involved in oxidative phosphorylation.

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