Substituent Effects on Pyridinium Ring-Opening Reactions. Facile Ring Opening of 1-(*N*,*N*-Dimethylcarbamoyl)nicotinamide Cation*

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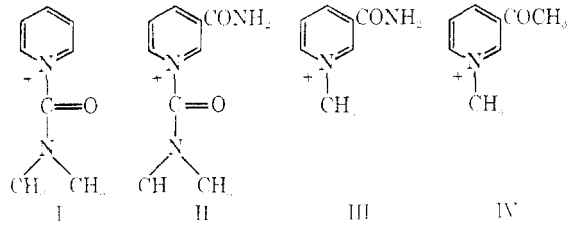
ABSTRACT: 1-(*N*,*N*-Dimethylcarbamoyl)nicotinamide cation reacts very readily with hydroxide ion in a reaction which is second order in hydroxide, to produce a ring-opened product having a pK of 9.8, and absorbing maximally at 3930 Å in its anionic form and at 3600 Å in its neutral form. The ring-opening process competes effectively at pH values above neutrality with the nucleophilic displacement of nicotinamide, a process which is first order in hydroxide. The effect of the 3-carboxamide moiety on the pyridine ring of 1-(*N*,*N*-dimethylcarbamoyl)nicotinamide cation as compared with the unsubstituted pyridinium analog is to increase by a factor of 10^5 the rate constant of the ring-opening process and by 14 the nucleophilic hydroxide ion reaction The ring-opened product decays to a material which has ultraviolet spectroscopic and fluorescent properties identical with those of the final alkaline diphosphopyridine nucleotide product. *N*-Methylnicotinamide cation and *N*-methyl-3-acetylpyridinium ion undergo reversible alkaline reactions, interpreted here as ring-opening reactions, to form products absorbing maximally at 3190–3250 and 3260–3300 Å, respectively.

Two apparent acid dissociation constants were calculated for *N*-methyl-3-acetylpyridinium ion: $K_1' = 5.0 \times 10^{-14}$ M and $K_2' = 1.8 \times 10^{-14}$ M.

The nature of alkaline pyridinium ion ring destruction in the case of 1-(*N*,*N*-dimethylcarbamoyl)pyridinium ion, I, has been described previously (Johnson and Rumon, 1970). Intermediates and final products from this reaction have been described and identified. This ring destruction process has a second-order rate dependence on hydroxide ion concentration and competes effectively with processes which are first order in hydroxide ion, such as nucleophilic displacement of the intact pyridine ring. Therefore, the ring destruction reaction becomes increasingly prominent at higher pH values. The intermediate and final products of the ring destruction reaction absorb in the visible or near-ultraviolet region. These characteristics of the ring destruction of I are strikingly similar to the destruction reaction of DPN in sodium hydroxide (Kaplan *et al.*, 1951). An even more strikingly similar phenomenon to be described here is the ring destruction reaction of the nicotinamide analog 1-(*N*,*N*-dimethylcarbamoyl)nicotinamide cation, II, which produces final fluorescent products having ultraviolet absorption and fluorescence characteristics identical with those of the DPN-alkaline product.

N-Methylnicotinamide cation, III, investigated by Martin and Hull (1964), undergoes reversible increases of absorbance at 3200 Å at higher alkalinities (>1 M NaOH) as well as at 2800 Å at lower alkalinities. The rate of disappearance of the 2800-Å band increases with hydroxide ion concentration at lower alkalinities, but loses its dependence on hydroxide concentration at 0.8 M NaOH and higher. These workers calculated an ionization constant for III from the initial spectral measurements and postulated that the equilibrium and kinetic behavior is due to ionization of the amide NH. Amide anions are known to be not nearly as susceptible as the amide itself to hydrolysis (Schowen and Zuorick, 1966). Also observed by Martin and Hull (1964) was the reversible increased absorbance of *N*-methyl-3-acetylpyridinium ion at 2800 and 3200 Å in alkaline solutions.



The alkaline destruction of II and the alkaline modifications of III and IV are the subjects of this paper. The following objectives are in mind: (1) To quantitatively compare the reactivity of nucleophiles of II with I in order to determine the electronic effect of the 3-carboxamide substituent on ring-opening and displacement reactions. (2) To determine the effect of 1-N substituents on the reversibility of pyridine ring-opening reactions.

Experimental Section

Materials. 1-(*N*,*N*-Dimethylcarbamoyl)nicotinamide chloride, II, was prepared by dissolving an excess of *N*,*N*-dimethylcarbamoyl chloride (Aldrich) with nicotinamide (Calbiochem) in acetonitrile. The resulting product, mp 120–123°, $\nu_{C=O}$ 1757 cm^{-1}, was used without further purification as

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it cannot be sublimed or recrystallized as can I due to a very facile disproportionation reaction under these conditions (Johnson and Rumon, 1964). II has a λ_{max} at 2660 Å, ϵ 3550 M^{-1} cm^{-1}. DPN was a Calbiochem product. 1-Methylnicotinamide iodide, III, was prepared by mixing benzene solutions of methyl iodide and nicotinamide. After recrystallization twice from ethanol, the product has mp 164–165° (uncor), λ_{max} 2650 Å (ϵ 1250 M^{-1} cm^{-1}). 1-Methyl-3-acetylpyridinium iodide, IV, was prepared by mixing benzene solutions of methyl iodide and 3-acetylpyridine at room temperature. Recrystallization twice from ethanol yields a product with a melting point of 208–209° (uncor).

Kinetic Studies of II. The decay of II was followed at 2660 Å, and the formation of ring-opened products was followed at 3600 or 3930 Å, depending upon the pH. Optical density values were obtained with a Beckman DU-2 spectrophotometer, thermostated at 25.0 ± 0.01° with a cell compartment of special design. Rate constants were obtained graphically from semilog $(D_t - D_\infty)$ or $(D_\infty - D_t)$ *vs.* time plots. Stock buffers of known composition were made for various pH values. Successive dilution of these buffers were prepared, the ionic strength being maintained with KCl at a constant value. The pH was measured at the completion of the reaction with a Radiometer TTT-1 pH meter. Extrapolation to zero buffer concentration with a k_{obsd} *vs.* buffer dilution plot gives a buffer-independent rate constant at the pH of the buffer series. From the slopes of such plots buffer terms can be obtained. Aliquots (usually 10 μl) of II, 0.01 M dissolved in 10^{-3} M HCl, were used to initiate kinetic experiments involving either II, or the ring-opened product of II at pH values above 9, by adding to 3 ml of buffer in a cuvet. If known quantities of ring-opened product V are needed at pH values lower than 9, known quantities of II are placed in a 0.05 M sodium hydroxide solution, where the half-life of the ring-opened product is maximal (2 hr). Aliquots of this solution are then immediately used in kinetic experiments or in pK_a determinations.

pK_a of V. The pK_a of the ring-opened product V was determined by adding identical amounts of a stock solution of V to identical amounts of buffer solutions of various pH and determining the initial optical density at 3920 Å by extrapolating to zero time on an optical density–time plot. The pH of the solution was subsequently determined. The pK_a of V was calculated from eq 1 where D_u is the initial

$$pK_a = pH + \log\frac{D_u - D}{D - D_i} \quad (1)$$

optical density of the un-ionized form of V determined at pH 7.4, D_i is the initial optical density of the completely ionized form of V determined at pH 12, and D is the initial optical density of V in the buffer solution with a pH value near the pK_a value of V. Four determinations at pH 9.5–9.8 yield a pK_a value of 9.80 ± 0.03.

Competition and Yield Studies of V. The final yield of V from the alkaline reaction of II was determined by adding known amounts of II to known amounts of stock buffer solutions and their dilutions, and determining the initial optical density at 3600 or 3830 Å by extrapolation to zero time at those pH values where V is immediately formed, or by determining the maximal optical density at those pH values where V is formed at a measurable rate and decomposes very slowly. In all cases V is formed at least ten times more rapidly than it decomposes so that the maximal optical density value is a valid measurement of the amount of V formed. The per cent of V formed is calculated from the above optical density data and the known extinction coefficients and pK_a of V. These experiments were carried out with a single batch of II which on the day of the determination was added to a 0.001 M HCl solution. Aliquots of this solution were added to the buffers.

Extinction Coefficient of V. The extinction coefficient of V was determined by placing aliquots of stock solutions of V, which had been prepared from II in 0.05 M NaOH, in a pH 7 buffer to determine the absorption characteristics of the acid form and in a 0.5 M NaOH solution to determine the absorption characteristics of the basic form: ionized V, at 3930 Å, ϵ 1.92×10^4 M^{-1} cm^{-1}; at 3600 Å, ϵ 1.17×10^4 M^{-1} cm^{-1}; neutral form of V, at 3930 Å, ϵ 0.40×10^4 M^{-1} cm^{-1}; at 3600 Å, ϵ 1.87×10^4 M^{-1} cm^{-1}.

Spectrophotometry of III and IV. Known quantities of III or IV were added to 3.00 ml of the NaOH solution and the spectra scanned repeatedly with a Cary 14 spectrophotometer at 26°. Kinetic determinations were carried out as for II. The reversibility of the alkaline reaction of III was tested in the following manner: III, 3.33×10^{-4} M, was placed in 3.0 ml of 10 M NaOH with stirring. Immediately thereafter 3.0 ml of glacial acetic acid was added and the entire mixture was cooled. For a control 1.67×10^{-4} M III was placed in a mixture of 3.0 ml of 10 M NaOH and 3.0 ml of glacial acetic acid. The identity of the spectra in the acid quenching and control experiments from 2400 to 4000 Å marks the reversal reaction. A mixture of equal amounts of the NaOH and acetic acid solutions serves as a blank. Similar experiments were carried out for IV, using 1 M NaOH and 2 M acetic acid.

Other Physical Studies. The fluorescent properties of the alkaline decomposition products of II, III, and DPN were determined with an Aminco Bowman spectrofluorometer. Ammonia determinations of decomposing solutions of III and II were made by the pyridine–pyrazolone method (Kruse and Mellon, 1953). The uv properties as a function of pH, of the final decomposition products of II, III, and DPN in alkali were determined by placing identical volumes of the completely reacted solutions in buffers of various pH values and their spectra were taken. A Varian Model A-60 spectrometer was used for the nuclear magnetic resonance analyses. A Durrum stopped-flow spectrophotometer equipped with a 18-μl cuvet was used for the rapid kinetic studies. Semilog plots of absorbance *vs.* time were made, from which the rate constant was calculated as $0.693/t_{1/2}$.

Results

Rate of Decomposition of II. II, when placed in buffer solutions of pH less than 6, decomposes to nicotinamide as is evidenced by the characteristic uv spectra in the completed reaction of nicotinamide in the 2600-Å region. The rate of displacement of nicotinamide follows rate eq 2,[1]

[1] k^f_{obsd} refers to the observed rate constant for the formation of V and k^d_{obsd} refers to the observed rate of decomposition of V.

TABLE I: Rate Constants for Nucleophilic Reactions of 1-(*N*,*N*-Dimethylcarbamoyl)nicotinamide Cation at 25.0° in Water.

Nucleophile	pK_a	Composition of Stock Buffer,[a] M Basic Form/M Acidic Form (μ)	pH (± 0.02)	k_n, M^{-1} min^{-1}
Formate	3.75	0.060/0.060, (0.60)	3.46	0.027
Acetate	4.73	0.030/0.030, (0.60)	4.55	0.038
Acetate	4.73	0.60/0.060, (0.60)	5.60	0.036
Phosphate	6.80	0.10/0.06, (0.60)	6.52	0.16
Imidazole	7.0	0.05/0.15, (0.60)	6.64	3.2
Imadazole	7.0	0.05/0.034, (0.60)	7.32	3.2
Tris(hydroxymethyl)-aminomethane	8.2	0.10/0.40, (0.40)	7.65	1.1
Triethanolamine	7.8	0.40/0.20, (0.20)	8.41	2.6
Triethanolamine	8.1	0.10/0.10, (0.60)	8.09	0.71
Triethanolamine	8.1	0.10/0.20, (0.60)	7.73	0.70
Triethanolamine	8.1	0.10/0.40, (0.60)	7.40	0.41
Water[b]	−1.8			$1.5 \pm 0.14 \times 10^{-5}$
Hydroxide[b]	15.6			$1.36 \pm 0.065 \times 10^{5}$

[a] The stock buffer and 0.8, 0.6, 0.4, and 0.2 dilutions maintained at constant ionic strength, μ, with KCl, were used in these experiments. [b] From intercept data of the above kinetic experiments. The first-order water term is converted into a second-order term by division by 55.5, the molarity of water.

where k_w is the water term equal to 8.3×10^{-4} min^{-1}, k_n is the nucleophilic term, N is a nucleophile, and k_{OH} is the hydroxide ion term. Extrapolation to zero buffer concentration at constant ionic strength of 0.60, gives the sum of $k_w + k_{OH}(OH^-)$ terms. A plot of the intercept values *vs.* hydroxide concentration, yields k_w as the intercept and k_{OH} as the slope.

$$k^f_{obsd} = k_w + k_n(N) + k_{OH}(OH^-) \quad (2)$$

In Table I are listed the kinetic terms from eq 2. Figure 1 is the pH–rate profile for the decomposition of II obtained from the extrapolated zero buffer concentration values. The

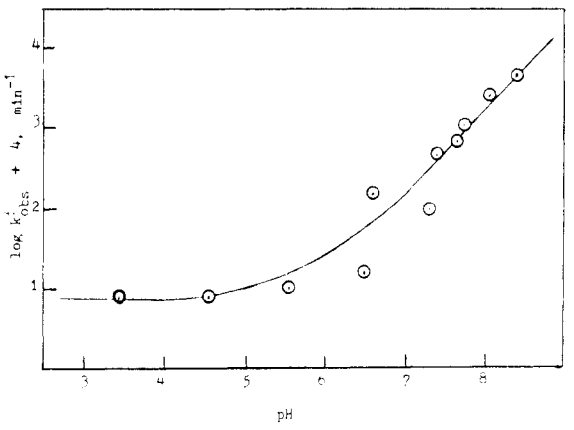


FIGURE 1: pH–rate profile for the decomposition of 1-(*N*,*N*-dimethylcarbamoyl)nicotinamide cation at 25.0° in water. The circled points obtained as intercepts, k_{int}, from rate constants *vs.* buffer concentration plots. The solid line is calculated from the equation: $k = [8.3 \times 10^{-4} + 1.4 \times 10^{5}(OH^-)]$ min^{-1}.

large negative deviations near neutrality are due to the long extrapolations necessary to make with the strongly catalyzing imidazole buffers. A plot of log k_n for the nucleophilic reaction of II with a particular nucleophile *vs.* log k_n for the corresponding reaction with *p*-nitrophenyl acetate with the same nucleophile gives a linear dependence with a slope of 1.1. Water and hydroxide as well as the other nucleophiles studied here behave toward II as they do to other ester derivatives (Johnson, 1967).

Yield of V as a Function of pH. In buffers of pH greater than 7, II undergoes in addition to nucleophilic displacement reactions a transformation to a product, V, which absorbs maximally at 3600 Å (or 3930 Å in its basic form, pK_a = 9.8). The rate of disappearance of II as measured at 2660 Å is identical with the rate of appearance of the 3600-Å-absorbing material. Increasing amounts of V are formed with increasing pH. Thus, the formation of the chromophoric product must be kinetically higher order than first in hydroxide to compete effectively with the first-order hydroxide nucleophilic displacement reaction at higher pH values. Above pH 10 no further amounts of the chromophoric product are formed indicating that above this pH complete conversion to the chromophoric material occurs. From this knowledge of the complete formation of V from II at pH 12.5 where V is relatively stable (half-life of 2 hr), the optical properties of V in its basic form were determined, and upon placing a known amount of V in a neutral solution the optical properties of its acid form were determined. The extinction coefficients resulting from this study are given in the Experimental Section.

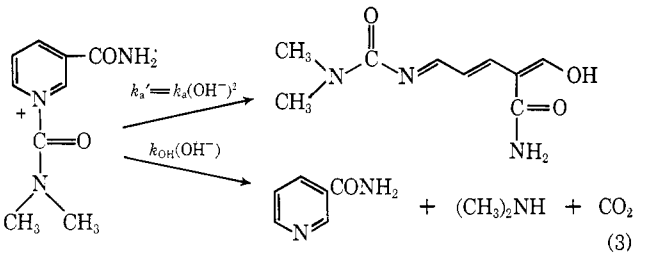
Assuming that the production of V is a process second order in hydroxide ion which competes with the first-order hydroxide nucleophilic displacement reaction as in the case of I (Johnson and Rumon, 1970), eq 3, the yield of V is

TABLE II: Yield of V from 0.33×10^{-4} M II as a Function of pH.

Buffer[a]	pH	(H^+), M $\times 10^9$	λ (Å)	A_{obsd}	V Formed,[b] M $\times 10^{-4}$	% V Formed
Tris	7.70	5.88	3600	0.108	0.058	17
Triethanolamine	8.45	1.75	3600	0.350	0.19	57
Carbonate	8.98	1.23	3600	0.478	0.27	81
Carbonate	10.7	0.02	3930	0.70	0.40	120
NaOH	11.7	0.002	3930	0.72	0.36	109
NaOH	12.7	0.0002	3930	0.76	0.37	112
NaOH	13.7	0.00002	3930		0.40	120

[a] There is a marked trend to buffer catalysis of V formation. Therefore the yield of V is extrapolated to zero buffer concentration. [b] Calculated from eq 4, using the extinction coefficients of V described in the Experimental Section.

related to the ratio of rate constants according to eq 4, where K_w is the dissociation constant of water. The percentage of V formed can be determined from the amount



(3)

$$\frac{k_a'}{k_{OH}} = \frac{\%\,V}{(100 - \%\,V)} \times \frac{(H^+)}{K_w} \qquad (4)$$

of absorption resulting when II is placed in a solution of a given pH as follows. The absorbance of a solution of V is given by eq 5 where ϵ_V and ϵ_{VH} refer to the extinction coefficients of anionic and neutral V, respectively, at a given wavelength, and K_a is the acid dissociation constant of V which

$$A_{obsd} = \epsilon_V(V) + \epsilon_{VH}(VH) = \left[\epsilon_V\left(\frac{K_a}{(H^+) + K_a}\right) + \epsilon_{VH}\left(\frac{(H^+)}{(H^+) + K_a}\right)\right]V_{total} \qquad (5)$$

is equal to $10^{-9.8}$ M. The percentage yield of V is then given by eq 6, where c_0 is the initial concentration of II added to the solution. The yields of V at various pH values calculated according to eq 6 are given in Table II. There is significant catalysis in the formation of II so that extrapolation to zero buffer concentration is necessary. Rearranging eq 4

$$\%\,V = \frac{100\,(V\ formed)}{c_0} = \frac{100\,A_{obsd}}{c_0}\left(\frac{(H^+) + K_a}{\epsilon_V K_a + \epsilon_{VH}(H^+)}\right) \qquad (6)$$

results in eq 7 which describes the dependence of the yield of V on hydrogen ion concentration. A plot of $100/\%$ V

$$100/\%\,V = 1 + (H^+)k_{OH}/K_w k_a' \qquad (7)$$

vs. (H^+) yields a straight line from whose slope the value of k_a/k_{OH} can be calculated. The value of k_{OH} is already known from the experiments summarized in Figure 1 and Table I, therefore, the absolute value of k_a is calculated to be $3.9 \pm 1.9 \times 10^{10}$ M^{-2} min^{-1}. This value is a factor of 10^5 greater than the corresponding ring-opening coefficient of I (Johnson and Rumon, 1970).

Rate of Decomposition of V. The rate of decomposition of V at various pH values is shown in Figure 2. The similarity between the pH–rate profile for the decomposition of V when compared with that observed for the ring-opened product for I (Johnson and Rumon, 1970) is most striking. The rate constants represented in Figure 2 are intercept values from plots of k^d_{obsd} *vs.* buffer concentration. Strong buffer catalysis is observable in the carbonate, Tris, and triethanolamine buffers which were used in the pH range 7.6–9.6. Two buffer series of carbonate were used: pH 8.92 and 10.61. No catalysis is seen in the latter buffer, or in a triphosphate buffer at pH 11.2. It can be concluded that no general catalysis is observable on the alkaline side of the pH–rate maximum at pH 9.8. The catalytic coefficients obtained, assuming general base catalysis on the acid side of the pH–rate maximum are as follows: carbonate, 2.0 M^{-1} min^{-1}; Tris, 0.19 M^{-1} min^{-1}; triethanolamine, 0.095 M^{-1} min^{-1}, as determined in 1:10 carbonate–bicarbonate, 1:4 Tris–TrisH, and 2:1 triethanolamine–triethanolamine·H buffers, respectively. In each case the kinetics in five buffer

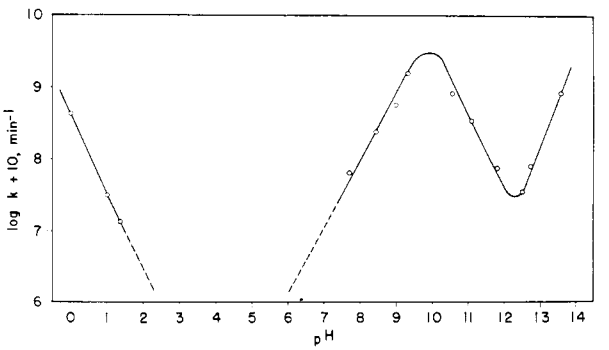


FIGURE 2: pH–rate profile for the decomposition of V.

TABLE III: pH–λ_{max} Behavior of VI and of the Alkaline DPN Product.

Buffer pH	VI[a] λ_{max} (Å)	DPN-alkali Product[b] λ_{max} (Å)
3.0	3410	
4.0	3410	
5.0	3410	
6.0	3410	
7.0	3410	3400
8.0	3415	3400
9.0	3420	3410
10.0	3510	3510
11.0	3520	
12.0	3610	3605
12.7	3610	3610
13.8	3610	3610
5 M NaOH	3610	

[a] A 2×10^{-4} M solution of II in pH 10 buffer for 25 min, then placed in various buffers. [b] A 10^{-3} M solution of DPN in 10 M NaOH for 2 hr, then adjusted with HCl to pH 9, then placed in various buffers.

TABLE IV: Spectrophotometric Properties of III in Alkaline Solutions.

NaOH, M	III, M $\times 10^4$	Long-Wavelength Absorption λ_{max} (Å)	Long-Wavelength Absorption Optical Density[a]	Low-Wavelength Absorption λ_{max} (Å)	Low-Wavelength Absorption Optical Density[a]
None	1.00			2650	0.417
1.00	3.33	None	0.090[b]	2645	1.316
2.00	3.33	None	0.136[b]	2640	1.392
2.00	13.16	3190 (shoulder)	0.551[b]	[c]	
3.00	13.16	3190 (shoulder)	0.818[b]	[c]	
3.00	3.33	None	0.208[b]	2640	1.461
4.00	3.33	3190	0.294	2640	1.535
5.00	3.33	3220	0.461	2645	1.630
7.50	1.67	3230	0.592	2645	1.043
10.0	1.67	3250	0.867	2645	1.204
15	1.67	3250	1.116	2645	1.400
19	1.67	3250	0.967	2625	1.214

[a] The optical density given here is the optical density of III in its solution minus the optical density of the solution, in 1.00-cm cells. [b] The optical density value for 3190 Å. [c] Too concentrated for observation.

dilutions of each buffer were determined. Linear plots of k^{d}_{obsd} *vs.* buffer dilutions were obtained with two- to fivefold increases in rate from the most dilute to the most concentrated buffer.

Product Studies. During the decomposition of V a highly fluorescent product, VI, which absorbs maximally in alkaline solutions at 3610 Å and also at 2500 Å is formed from pH 9.7–14. The rate of appearance of VI is equal to the rate of disappearance of V. The absorption maxima of this material shifts to 3410 Å below pH 8. The 3610-Å band can be regenerated by making the neutral solution strongly alkaline. Hence an acid–base behavior is apparent. The pH–λ_{max} behavior of VI and also of the alkaline DPN product is given in Table III. There are two apparent pK values because sharp changes in λ_{max} occur in the pH 9.6 region and also in the pH 12 region. The first pK value is in agreement with that of 9.6 found by Kaplan *et al.* (1951) for the alkaline DPN product, by fluorescence analysis. Simple pK values are not easily extractable from the optical density data. The DPN alkaline product and VI fluoresce maximally at 4550 Å, and are maximally excited at 3600 Å. Furthermore, the fluorescence behavior of VI and the alkaline DPN product are qualitatively similar as a function of pH. The fluorescence of VI is about half-maximal at pH 9.5. Below this pH the fluorescence diminishes to nothing. The fluorescence of a solution which has been acid quenched can be regenerated by making the solution alkaline. A further similarity between VI and the alkaline DPN product is their identical chromatographic behavior (Guilbert, 1969, unpublished work) using thin-layer cellulose with a carbonate buffer.

The yield of VI produced from V does not seem to vary greatly with pH from 9 to 14, although it is possible that a mixture of products which absorb similarly is produced. Ammonia analysis of reacting solutions of V by the pyridine–pyrazolone method (which we ascertained to be insensitive to amines) shows that less than 10% ammonia is produced during the decomposition of II at pH 9.6, and that ammonia is produced only very slowly at pH 13.7. In the latter experiment the appearance of VI occurs with a half-life of 6 min, but during this time only 11% of the amide nitrogen of II is released; in 25 min only 25% amide nitrogen is released.

Product analysis of decomposing solutions of V by nuclear magnetic resonance spectrometry at pH 13 shows that 1,1-dimethylurea is the main methyl-containing product from II. The resulting nuclear magnetic resonance spectrum at low field indicates that little niacin or nicotinamide is formed under these conditions.

That VI does not result from a hydrolytic reaction at the amide bond is demonstrated by the fact that 1-(*N*,*N*-dimethylcarbamoyl)nicotinic acid, when placed in 10 M NaOH does not produce VI. Instead a nonfluorescent product is formed. This material absorbs maximally at 3675 Å in strong alkali and at 3580 and 3100 Å at pH 6.

Behavior of III in NaOH Solutions. In solutions more basic than 2 M NaOH, a new ultraviolet band at 3190 Å and an increase in intensity of the 2650-Å band are produced from III. At higher NaOH concentrations increased intensity of both bands and a small wavelength shift occur. The intensities of the 2650- and 3190–3250-Å bands increase in a parallel manner with increasing NaOH concentrations. These results are presented in Table IV. The new absorbances form very rapidly with a half-life of less than 1 sec.

The new absorption at 3190–3250 Å is unstable at "lower" NaOH concentrations, producing eventually new absorption at 3610–3620 Å. The disappearance of the 3190–3250-Å

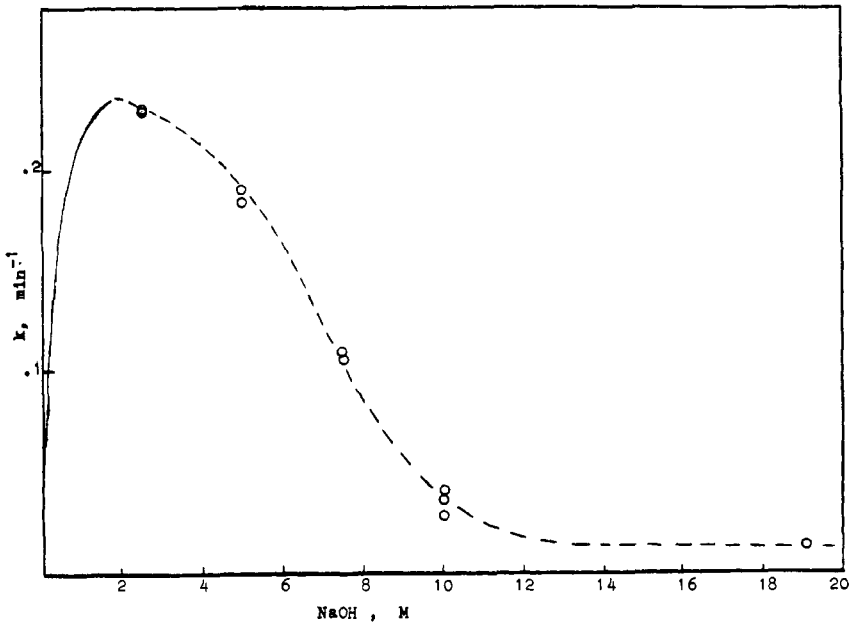


FIGURE 3: Rate constants for the decomposition of *N*-methylnicotinamide cation: (····) alkaline modification at 3250 Å or at 2800 Å as a function of sodium hydroxide concentration at 25°; (—) as a function of NaOH concentration, calculated from the data of Brooke and Guttman (1968) extrapolated to 25°.

band follows accurately first-order kinetics to greater than 90% reaction. The rate constants decrease with increasing alkalinity, as shown in Figure 3. Identical rate constants are obtained when the decrease in absorbance is measured at 2800 or at 3190–3250 Å. After the 3250-Å-absorbing material has disappeared a material absorbing maximally at 3610 Å is formed at a much slower rate. The final product is slightly fluorescent: maximal excitation occurs at 3600 and maximal emission occurs at 4550 Å.

The alkaline modification of III is a reversible reaction. Acid quenching of III in 10 M NaOH results in a 93 ± 5% recovery of III based upon the absorption at 2640 Å, the λ_{max} of III. The band in the 3250-Å region disappears and the resulting final spectrum from 2500 to 4000 Å is that of III, with the exception of a small amount of absorption at 3680 Å, the apparent extinction coefficient of which is 9.0% of that for III at 2640 Å. The total spectrum is independent of quenching time from 2 to 60 sec.

Properties of IV in NaOH Solution. IV, when added to 0.01 M NaOH produces a new band of low intensity at 3260 Å. This band gradually shifts to 3330 Å and increases in intensity as the NaOH concentration increases to 0.3 M. A further increase in intensity of the 3330 Å occurs up to 1 M NaOH as shown in Figure 4. No further increase in intensity or shift in λ_{max} occurs up to 5 M NaOH. With increasing basicity from 0.01 to 1 M NaOH concomitant increases in optical density also occur in the 2675-Å region. The alkaline modification of IV is stable for 20 min. During this time acidification results in a 100 ± 2% regeneration of IV based upon the absorbance at the λ_{max} of IV at 2660 Å. The spectra from 2400 to 4000 Å are identical in the quenching and control experiments. Because of the production of 3260-Å-absorbing materials at lower basicities and 3330-Å material at higher basicities, IV may be treated as a dibasic acid, which produces the neutral species XII and the ionic species XIII. The spectroscopic titration data are analyzed as follows

$$A_{obsd} = \epsilon_{XIII}(XIII) + \epsilon_{XII}(XII) \qquad (8)$$

where A_{obsd} is the observed absorbance of IV at a specified wavelength (3330 Å here) and ϵ is an extinction coefficient. Defining K_1 and K_2 as (XII)/(IV) (OH^-) and (XIII)/(XII) (OH^-), eq 8 becomes

$$A_{obsd} = \frac{c_0(\epsilon_{XII}K_1(OH^-) + \epsilon_{XIII}K_2K_1(OH^-)^2)}{(1 + K_1(OH^-) + K_1K_2(OH^-)^2)} \qquad (9)$$

where c_0 is the concentration of IV added to the solution. Equation 9 contains the four unknown quantities ϵ_{XII}, ϵ_{XIII}, K_1, and K_2. Equation 9 was evaluated at four different data points, giving rise to four simultaneous equations from which the unknowns were calculated. The values of these are:

$$\epsilon_{XII} = 6390\ M^{-1}\ cm^{-1}$$

$$\epsilon_{XIII} = 13{,}800\ M^{-1}\ cm^{-1}$$

$$K_1 = 5.0\ M^{-1}$$

$$K_2 = 1.8\ M^{-1}$$

In Figure 4 is shown a plot of eq 9 shown as the solid line for alkaline solutions containing 0.332×10^{-4} M IV. The data points are shown as circles.

The rate of formation of chromophoric products from IV was measured at 3330 Å by stopped-flow kinetics. At low alkalinities (<0.125 M NaOH) the rate constant is "low" and nearly independent of sodium hydroxide concentration as shown in Table V. Above 0.1 M NaOH the rate constant abruptly becomes much larger, suggesting a dependence on hydroxide which is higher than first order. At 0.15 M NaOH two relaxations are observable, a slow one and a fast one.

The alkaline modification of IV can easily be prepared as a tacky yellow material by mixing equal quantities of 1 M NaOH and 1 M IV with vigorous stirring. This material

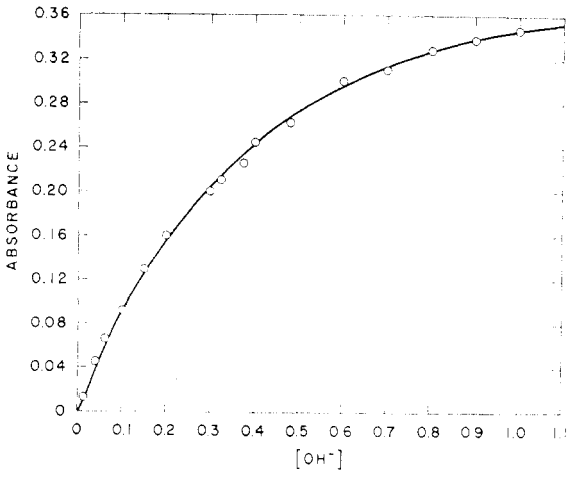


FIGURE 4: Absorbance at 3330 Å of a 0.3322×10^{-4} M solution of 1-methyl-3-acetylpyridinium ion as a function of sodium hydroxide concentration: (○) actual data points; (—) calculated from eq 9 using ϵ_{XII} and ϵ_{XIII} 6390 and 13800 M^{-1} cm^{-1}, and K_1 and K_2 = 5.0 and 1.8 M^{-1}.

TABLE VI: Polarography of 1-Methyl-3-acetylpyridinium Iodide.[a]

pH or NaOH, KOH, M	First Wave Reduction, $E_{1/2}$ (V) *vs.* sce[b]	Limiting Current (μA)
pH 7	−0.819	22.1
pH 8	−0.832	28.3
pH 9	−0.832	28.3
pH 10	−0.847	28.6
pH 11	−0.853	28.0
0.0091 KOH	−0.872	30.0
0.0167 NaOH	−0.94, −0.99	26.2
0.0364 KOH	−0.92, −0.97	19.4
0.083 NaOH	−0.955	12.0
0.83 NaOH	−1.06	9.83
4.17 NaOH	−1.12	4.1

[a] Concentration of 1-methyl-3-acetylpyridinium iodide is 9.09×10^{-3} M. [b] Standard calomel electrode, sce.

has all the ultraviolet properties of the alkaline product prepared *in situ* and can regenerate IV when treated with acid. It has a carbonyl stretching frequency of 1630 cm^{-1} which replaces the 1710-cm^{-1} band of IV. Other prominent infrared bands of this product occur at 1530, 1400, 1180, and 1100 cm^{-1}. This material is also characterized by a polarographic reduction at a dropping mercury electrode with half-wave potentials occurring at −0.95, −1.06, and −1.12 V *vs.* sce in 0.083, 0.83, and 4.17 M NaOH, respectively. The corresponding limiting currents decrease with increasing alkalinity, as shown in Table VI.

Spectroscopic Properties of Known Substances in NaOH. Because of the possibility of spectral shifts in the spectra of III and IV, in NaOH the spectra of two known substances which undergo no change in structure in base were measured as a function of alkalinity. The λ_{max} of glutacondialdehyde remains at 3625 Å in solutions ranging from 0.02 to 10 M NaOH. The λ_{max} of *p*-nitrophenol is independent of NaOH concentrations from 0.02 to 2 M where the λ_{max} remains at 4000 Å. However, a large spectral shift is apparent at 5 and 10 M NaOH where the λ_{max} occurs at 4050 and 4140 Å, respectively.

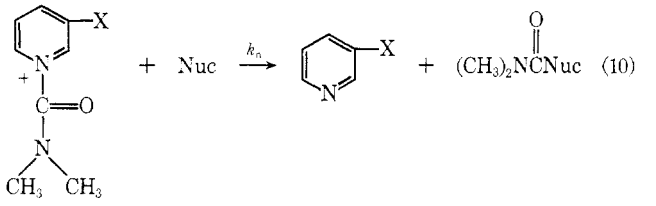
TABLE V: Rate of Formation of Chromophoric Products from IV at 30°.

NaOH, M	IV_e, %[a]	k, min^{-1}[b]
0.010	95	11.9 ± 2.9
0.025	88	8.22 ± 0.96
0.050	79	5.20 ± 0.40
0.125	57	4.65 ± 0.75
0.150	51	693 ± 70, 3.47 ± 0.42
0.240	37	924 ± 92
0.250	36	842 ± 82

[a] Calculated from $\% IV_e = 100/(1 + K_1[OH^-] + K_1K_2[OH^-]^2)$. [b] Each rate constant is the average of at least five determinations.

Discussion

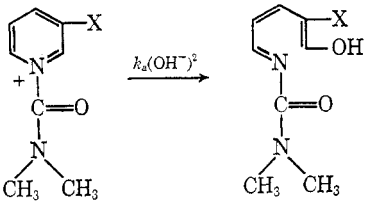
The Behavior of Dimethylcarbamoylnicotinamide Cation in Basic Solutions. The electronic effect of the 3-carboxamide group of II is very small when the reaction under consideration is the direct displacement at the heterocyclic N atom, eq 10. For the nucleophiles water, acetate, dianionic phosphate, imidazole, Tris, and hydroxide, the ratio of the rate coefficients of eq 10 with X as carboxamide compared with X as hydrogen,



(10)

k^{CONH_2}/k^H, is 4.2, 5.0, 5.3, 3.2, 12, and 14, respectively. Thus, the influence of the 3-carboxamide group is to increase the rate of direct displacement reactions by about sevenfold.

The effect of the 3-carboxamide group on the rate of the ring-opening reaction shown in eq 11 as measured by the rate coefficient k_a as compared with the unsubstituted compound is an increase by a factor of 100,000. This means



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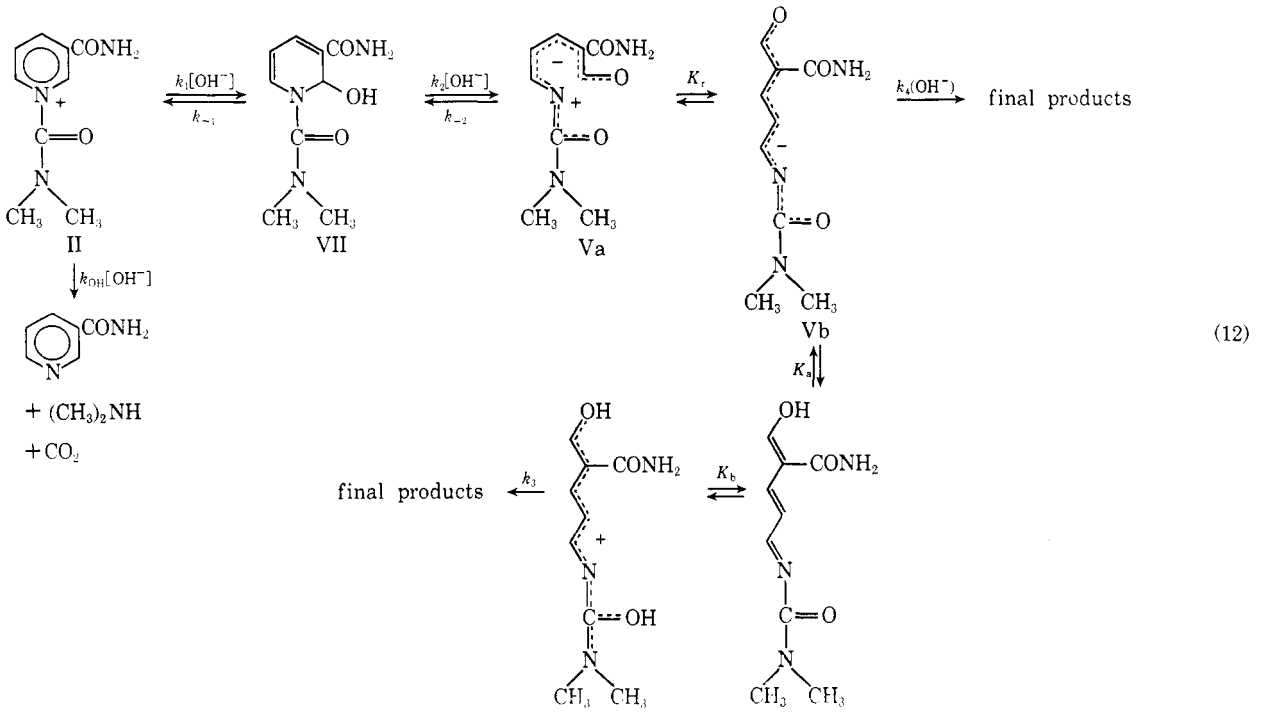
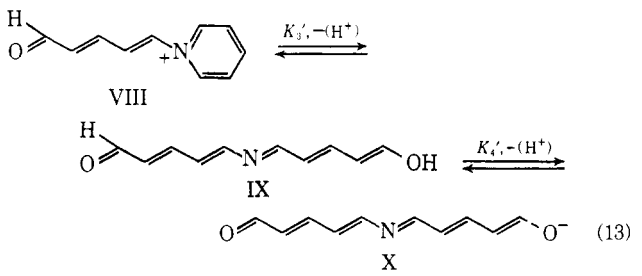


(12)

that the ring-opening reaction which is second order in hydroxide will compete more effectively with displacement reactions with nicotinamide derivatives compared with pyridine derivatives. Thus, such ring-opening reactions can occur at much lower pH values. In the case of II *vs.* I, the rate of direct displacement reaction is about 10 times faster for the nicotinamide derivative but the ring-opening reaction is 10^5 times faster, at any given pH. Therefore the pH value at which ring opening of II will be important is 5 − 1 or 4 pH units lower than the pH at which ring opening becomes important with I. Ring opening becomes important for I at pH 11 and for II at pH 7.

An analysis of the pH–rate profile for the decomposition of V can be carried out similarly to the previous analysis for the ring-opened product of I (Johnson and Rumon, 1970). We are assuming here that the striking similarity of the pH–rate profiles for V and for the ring-opened product of I suggest that the ring-opened products are reacting identically. The similarities are: (1) the pH–rate maximum at intermediate pH values with the rate maximum occurring at a pH identical with the pK of the ring-opened product, (2) hydrogen ion dependence at low pH, and (3) hydroxide ion dependence at high pH. The reaction path is given in eq 12. In Table VII are listed the calculated rate and equilibrium constants and the ratios of these constants for the 3-carboxamide-substituted derivatives and the unsubstituted derivatives, assuming that the pH–rate maximum at pH 9.8 is due to the reversal of V to II. The reason for this assumption lies in the known reversibility of 5-pyridinium-2,4-pentadienal, VIII, as shown in eq 13 (Schwarzenbach, 1943) and in the reversibility of the alkaline reactions of III and IV to be discussed later in this paper.



(13)

The value of $K_r k_2 k_1/k_{-2}k_{-1}$, which is the equilibrium constant of the ring-opening reaction of II, is given as 2×10^9 M^{-2}, a factor of 6000 greater than the ring-opening equilibrium of I. This means that V and II are present at equal concentrations at pH 8.6, whereas I and its ring-opened product are present at equal concentrations at pH 10.6. This is a remarkable effect for a single carboxamide substituent. It must be pointed out, however, that the true equilibrium situation is never achieved under the conditions of our experiments because of the slow rate of return of the ring-opened products to the ring-closed form and the subsequent secondary reactions of the ring-closed forms. However, if

TABLE VII: Rate and Equilibrium Constants for Reactions of II.

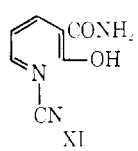
Constant	Value	Value Ratio (II/I)
K_a	1.59×10^{-10} M	1.59×10^3
$k_1k_2/k_{-1} = k_a$	3.30×10^{10} M^{-2} min^{-1}	0.84×10^5
k_{OH}	8.3×10^4 M^{-1} min^{-1}	1.4×10
k_{-1}/k_2	5.0×10^{-3} M	2.3×10^{-2}
k_1	1.6×10^6 M^{-1} min^{-1}	10^3
k_{-2}/K_r	16.8 min^{-1}	15
$K_rk_2k_1/k_{-2}k_{-1}$	2.0×10^9 M^{-2}	5.7×10^3
k_3/K_b	4.28×10^{-2} M^{-1} min^{-1}	7.1×10^{-1}
k_4	2.30×10^{-15} M^{-1} min^{-1}	1.53×10

DPN is stored in a ring-opened form on the enzyme surface, as discussed by Johnson and Rumon (1970), an equilibrium situation would undoubtedly prevail because most probably the approach to equilibrium would be catalyzed by the enzyme. Furthermore, the enzyme-stored open form of DPN could have a more favorable equilibrium constant of formation which is quite different from that obtained in aqueous solution because of special effects on the enzyme surface such as its hydrophobic nature.

The rate-determining step for the ring-closure reaction on the acid side of the pH–rate maximum is the k_{-2} step which involves the formation of the cyclic carbinolamine VII. On the basic side of the pH–rate maximum the k_{-1} step, which is the elimination of water from the carbinolamine, is the rate-controlling step for the ring-opened product of I; on the other hand, the k_{OH} step, which is the basic hydrolysis of II, is the rate-controlling step of the ring closure of V. The reason for this lies in the relative values of k_{OH} and k_1.[2] For I, $k_{OH} > k_1$; for II, $k_{OH} < k_1$.

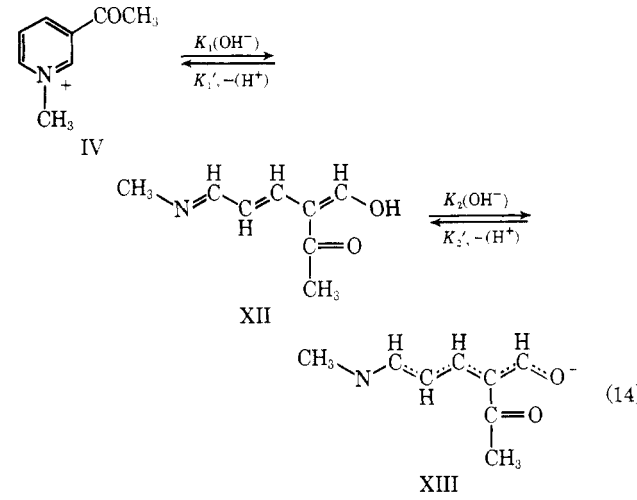
The reason for the large substituent effect due to the 3-carboxamide group in II on the rate constant for the ring-opening reaction, the specific rate constant of which is k_1k_2/k_{-1}, is largely due to the 1000-fold increase in the k_1 term due to the inductive effect of the electron-withdrawing carboxamide moiety. Well known is the fact that equilibrium additions of nucleophiles do not occur with unsubstituted *N*-alkylpyridinium ions, whereas pyridinium ions negatively substituted at the 3 or 5 positions undergo such additions with ease (Gauthier, 1966; Lamborg *et al.*, 1957). This is the first time that we know of, that the ring-substituent effect of the *rate* of this process has been measured, although Lindquist and Cordes (1968) have measured substituent effects due to *N*-alkyl substituents in cyanide addition reactions.

The nature of the final fluorescent product, VI, is not yet known. It is probably a rearranged product or internal cyclization product of V or a hydrolysis product of V. Not present in VI is the original heterocyclic nitrogen substituent present in the starting materials II, III, or DPN, because these materials each give rise to VI. VI is definitely not 5-hydroxy-2,4-pentadienal-2-carboxamide or the corresponding carboxylic acid because such compounds, instead of having a pK value greater than 9, would be expected to have pK values of 5.75 or lower in analogy with the pK value of 5.75 for 5-hydroxy-2,4-pentadienal (Schwarzenbach and Lutz, 1940). There is a known analogy for the rearrangement of V: the BrCN product of nicotinamide, XI, which is formed under slightly acid conditions, rearranges in alkaline solution to yield a fluorescent product absorbing maximally at 3500 Å at pH 13 (Douzou and LeClerk, 1955).



XI

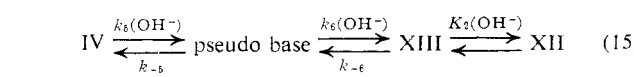
The Behavior of N-Methyl-3-acetylpyridinium Ion in Basic Solutions. We propose that IV is acting as a dibasic acid according to eq 14[3] with the ring-opened product XII absorbing maximally at 3260 Å, and the ionized ring-opened product XIII absorbing maximally at 3330 Å. This type of ring-opening behavior has an analogy with 5-pyridinium-2,4-pentadienal, VIII, which behaves as a readily reversible dibasic acid in alkaline solutions (Schwarzenbach, 1943), providing IX and its anion X as shown in eq 13. The second acid dissociation constant is larger than the first so that no IX is actually ever seen except under certain quenching conditions. Since K_4' is greater than K_3' in eq 13 only the geometric mean or $(K_3'K_4')^{1/2}$ is experimentally known. This quantity is equal to 2×10^{-13} M.



(14)

The polarographic behavior of IV in basic solutions is in agreement with the structures in eq 14. At pH values where no new ultraviolet bands are seen IV behaves as a typical *N*-alkylpyridinium ion in that from pH 7 to 11 there is a nearly pH-independent reduction potential (Kolthoff and Lingane, 1952). Above pH 11 where ultraviolet changes take place the polarographic behavior changes concomitantly. The nature of the high pH polarographic behavior can be accounted for if XII–XIII are the species present. XII is a pentadienal derivative and would be expected to behave as a conjugated trienal. It has nearly the same $E_{1/2}$ values as 2,4,6,8-decatetraenal has, and also the same $E_{1/2}$ – pH dependence (Fields and Blout, 1948). Also the limiting current decreases with increasing alkalinity above pH 13 due to the conversion of the aldehyde into its less reducible anionic form.

The kinetic data for the rate of formation of XII and XIII given in Table V can be interpreted according to the same mechanism for the ring-opening reaction of I and II, with the intermediacy of a pseudo base (carbinolamine) according to eq 15. Assuming a steady-state concentration of the pseudo



(15)

[2] For more details refer to eq 20 of Johnson and Rumon (1970).

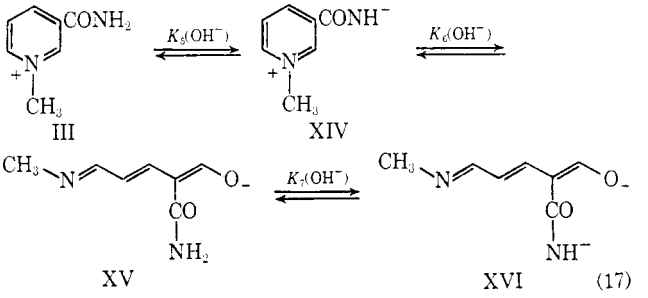
[3] K_n refers to the equilibrium constants with hydroxide; for example, K_1 is equal to (XII)/(IV)(OH$^-$). K_n' refers to the acid dissociation constant, for example, K_1' is equal to (H$^+$)(IV)/(XII) = $K_1 \times K_w$, where K_w is the dissociation constant of water.

base intermediate the rate of approach to equilibrium is given by

$$k_{obsd} = \frac{k_5k_6(OH^-)^2 + k_{-5}k_{-6}}{k_{-5} + k_6(OH^-)} \quad (16)$$

At low alkalinities the numerator and denominator hydroxide terms should be smaller than the constant terms, giving rise to k_{-6} as the rate constant. At higher alkalinities the hydroxide terms in the numerator or denominator will independently become important, giving rise to a complex rate dependence. At high alkalinity both the numerator and denominator hydroxide terms become important, giving rise to a rate constant equal to $k_5(OH^-)$. We have not attempted to analyze in detail the rate data because of its complexity. It is important to note that at lower alkalinities a nearly pH-independent rate constant obtains, as predicted by eq 16. At higher alkalinities, where $k_{-6}k_{-5} < k_5k_6(OH^-)^2$, as calculated from the equilibrium constants K_1 and K_2 in terms of the percentage of IV present at equilibrium, IV_e (the remainder being present as XII and XIII), the rate constant increases sharply, indicating that the numerator becomes hydroxide dependent. This happens at those alkalinities where IV_e nears 50% as seen in Table V. The occurrence of both a slow and a fast rate process at 0.150 M NaOH suggests that an intermediate (pseudo base) is on the reaction path between IV and its alkaline modification.

The Behavior of N-Methylnicotinamide Cation in Basic Solutions. The reversible near-ultraviolet absorption of III in alkaline solutions and its 60-Å red λ_{max} shift with alkalinity can be interpreted as due to ring opening and ionization of the ring-opened product, similar to that of IV. The shift in λ_{max} is also consistent to the well-known red shift of polar species in solvents of increasing polarity or polarizability (Basu, 1964). That such a shift is possible is suggested by the 140-Å red shift of *p*-nitrophenol in 2–10 M NaOH. However, the less polar glutacondialdehyde undergoes no shift at all under these same conditions. Because of these results it is presently not possible to conclude from the spectrophotometric data in Table IV that two ring-opened species in ionic equilibrium are observed in alkaline solutions. The 3190–3250-Å form of III must involve *at least* two hydroxide ions (perhaps three) rather than one hydroxide ion (or two at higher alkalinity) as in the case of IV. This is because III undergoes an ionization of its amide hydrogen at lower alkalinity. The K_5 value, defined as (amide anion)/(OH^-)(III) is 6.8 ± 1.0 M^{-1} at 25° as determined spectrophotometrically at 2800 Å (Martin and Hull, 1964), or 3.5 ± 0.2 M^{-1} as determined kinetically by Brooke and Guttman (1968) and extrapolated at 25°. If the chromophoric alkaline modification of III involves only one hydroxide ion, then the concentration of this material would exist independently of the concentration of the amide anion as a function of hydroxide ion concentration, *i.e.*, increasing alkalinity would not change the ratio existing between these two species. Since this is not the case, and also because the 3190–3250-Å product forms only at alkalinities higher than those where III exists mostly in the form of its amide anion (according to the K_5 values of 3–7), then the 3190–3250-Å product most probably can be represented as XV (and possibly XVI) in eq 17. Because of the multiplicity of products possible due to the involvement of two or three hydroxide ions, all neutral species can be grouped together as involving one hydroxide ion, all monoanionic species as involving two hydroxide ions, etc., XV in eq 17 is represented as the most probable species in its class only for convenience.



(17)

The rate of disappearance of XV and its instability as compared with XII can be explained in terms of the hydrolysis of III and the equilibrium between III and XV. The rate of disappearance of XV and III above 2 M NaOH as seen in Figure 3 meets with the rate of disappearance of III from 0.02 to 0.10 M NaOH as calculated from the data of Brooke and Guttman (1968) extrapolated to 25°. Because XV and III disappear at the same rate (as measured at 3250 and 2800 Å) and because of the decrease in ther ate of disappearance of XV at higher alkalinities these data are consistent with the reversible formation of hydrolytically more stable ring-opened products of III at higher alkalinities. This would act to reversibly reduce the concentration of the reactive form III. Kinetically this is accomplished by substituting the quantity $[1 + K_5(OH^-)]$ by $[1 + K_5(OH^-) + K_5K_6(OH^-)^2]$ in the denominator of the kinetic equations of Brooke and Guttman (1968). The rate will not level off above 1 M NaOH as predicted by Brooke and Guttman, but will instead decrease at increasing alkalinity, as observed in Figure 3.

Consistent with ring opening of III is the production of a small amount of fluorescent material which has absorption and fluorescent characteristics identical with the final alkaline product of II and DPN. This material is probably produced by hydrolysis or rearrangement of XV.

Conclusions. The rate of the ring-opening process of pyridinium ions is greatly increased by electron-withdrawing substituents. This is seen in the 100,000-fold increase in the third-order ring-opening coefficient for II *vs.* I. The equilibrium constants of ring opening are greatly affected by substituents: the corresponding values for I, II, and IV are 3.5×10^5, 2×10^9, and 9 M^{-2}, respectively. Comparing I with II it is apparent that electron-withdrawing groups greatly increase the extent of ring opening. Comparing II with IV and assuming that the 3-carboxamide and 3-acetyl moieties have similar inductive effects it is apparent that the substitution of a dimethylcarbamoyl moiety on the hetero nitrogen with a methyl group remarkably decreases the extent of ring opening. The hetero nitrogen substituent exerts a much greater effect than ring substituents. The nature of the heterocyclic nitrogen substituent also greatly affects the ease of reversibility of the ring-opening reaction. Electron-withdrawing groups greatly decrease the reversibility as in I and II, whereas electron donating groups greatly increase the ease of reversibility as is III and IV. The reversibility is felt mostly in the ring-closing reaction, therefore, it is reasonable to presume that electron-donating

groups on the nitrogen atom of the ring-opened forms increase the rate of pseudo base formation in the ring-closure reaction.

The substituent effects of both the third-order ring-opening reaction and the overall ring-opening equilibrium constant are the cumulative result of two separate reactions with the unstable pseudo base as the intermediate. Because the substituent effects are in the same direction for these two reactions the total substituent effect is large.

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In Vitro Synthesis of Lignoceric and Nervonic Acids in Mammalian Liver and Brain*

Stephen C. Boone† and Salih J. Wakil

ABSTRACT: A fatty acid elongation system has been identified in 21-day-old rat liver and brain mitochondria. This fatty acid system will elongate a multitude of saturated and unsaturated acyl coenzymes (acyl-CoAs) ranging in chain length from C_{12} to C_{22}. The components necessary for this elongation have been found to be a precursor acyl-CoA, acetyl coenzyme A reduced nicotinamide–adenine dinucleotide and reduced nicotinamide–adenine dinucleotide phosphate. Acetyl-CoA and not malonyl-CoA has been found to be the immediate precursor of the two-carbon addition unit. It was this mitochondrial system which was found to elongate behenyl-CoA to lignoceric acid and erucyl-CoA to nervonic acid. The synthesized radioactive lignoceric and nervonic acids were identified by thin-layer and gas chromotography. Their response to hydrogenation was further proof of their identity. Decarboxylation of the [^{14}C]ligonceric and nervonic acids revealed they had both been synthesized by an elongation process.

Studies *in vivo* have shown that the brain is capable of synthesizing lignoceric ($C_{24:0}$) and nervonic acids ($C_{24:1}$ Δ^{15}). In these studies radioactive lignoceric and nervonic acids were isolated from the brains of animals which had previously been injected with [1-^{14}C]acetate. These acids were then decarboxylated in an effort to determine the mechanism by which they had been synthesized. Fulco and Mead (1961) found an even distribution of radioactivity in the isolated lignoceric acid and therefore postulated that it has been synthesized by a *de nono* process. Bernhard *et al.* (1962), Hajra and Radin (1963a,b) and Kishimoto and Radin (1963a) felt however that lignoceric acid had been synthesized by an elongation process because they observed a preferential location of radioactivity at the carboxyl end of the fatty acid. Fulco and Mead (1961), Hajra and Radin (1963b), and Kishimoto and Radin (1963b) agreed that the decarboxylation data indicated an elongation process was responsible for the synthesis of nervonic acid.

Evidence will be presented in this paper that the enzyme system used for elongation is intramitochondrial. The components necessary for fatty acyl elongation will be documented using a system which elongates palmityl-CoA to stearic acid.

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