Isolation and Characterization of the Fluorescent Alkali Product from Diphosphopyridine Nucleotide*

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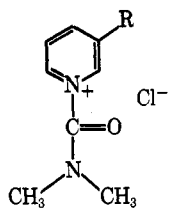
ABSTRACT: Diphosphopyridine nucleotide undergoes a ring-opening reaction in strongly alkaline solutions. This has been shown by isolation of the same final fluorescent alkaline product from both DPN and 1-(*N*,*N*-dimethylcarbamoyl)nicotinamide chloride, which is known to undergo ring-opening at pH above 7. The final alkaline products from DPN and 1-(*N*,*N*-dimethycarbamoyl)nicotinamide chloride have been shown to be identical with one another and to have the same structure as synthetic 2-hydroxynicotinaldehyde by comparison of their infrared, mass, nuclear magnetic resonance, ultraviolet, and fluorescence spectra. The yield of 2-hydroxynicotinaldehyde from DPN increases with increasing hydroxide ion concentration and reaches a maximal value of 80% at 7.5 N NaOH.

The action of strong base on DPN produces a transient 370-mμ-absorbing intermediate which rapidly decomposes to a 340-mμ-absorbing species (Johnson and Morrison, 1970a). This 340-mμ absorbing intermediate decays to a final product absorbing at 360 mμ in its basic fluorescent form and at 340 mμ in its acidic nonfluorescent form (Kaplan *et al.*, 1951). A pK_a of 9.6 has been measured both spectrally (Johnson and Morrison, 1970b) and by acid quenching of fluorescence (Kaplan *et al.*, 1951) for this 360-mμ-absorbing material. Production of fluorescence has been developed as an analytical method for DPN (Lowry *et al.*, 1957). A ring-opening reaction of 1-(*N*,*N*-dimethylcarbamoyl)nicotinamide chloride, I, where R = $CONH_2$, at pH values above 7 produces a product with the identical spectral, fluorescent, chromatographic, and pK_a properties as the 360-mμ-absorbing product from DPN (Johnson and Morrison, 1970b). The isolation and characterization of these two products are the subject of this paper.



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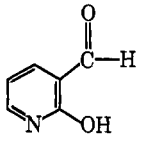

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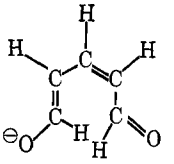

III

Experimental Section

Materials and Methods. DPN was a product of Sigma Biochemicals. Carbonate-free sodium hydroxide solutions from Fisher Scientific were used in the synthesis procedures and in the incubation studies. Bio-Rad anion-exchange resin AG 1-X-2 (200–400 mesh, Cl^- form) was obtained from Calbiochemicals and prepared by washing six to seven times with 3 N HCl until the washings were free of 260-mμ-absorbing material, and then washing with deionized, degassed water until the washings were at pH 6. Nicotinamide was obtained from K & K Chemicals and dimethylcarbamoyl chloride from Aldrich. Thin-layer chromatography was performed using Eastman Chromatogram sheets, type 6065 (cellulose with fluorescent indicator), obtained from Fisher Scientific. Distilled, deionized water was used throughout.

Solvents used for chromatography were (A) 0.05 M sodium carbonate buffer (pH 9.8) and (B) *tert*-butyl alcohol–formic acid–water (3:2:1, v/v). Compounds were located by their fluorescence under long-wave ultraviolet light for A or by examination under long-wave ultraviolet light after spraying with 0.01 N NaOH with B.

Elemental analyses were performed by the Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.

Infrared spectra were obtained from KBr disks on a Perkin-Elmer Model 457 spectrophotometer.

Fluorescence spectra were performed on an Aminco-Bowman spectrophotofluorimeter.

A Cary Model 14 recording spectrophotometer was used for ultraviolet spectral determinations.

1-(*N*,*N*-Dimethylcarbamoyl)nicotinamide chloride was prepared from nicotinamide and dimethylcarbamoyl chloride as previously described (Johnson and Morrison, 1970b).

2-Aminonicotinaldehyde was synthesized by the procedure of Albert and Reich (1960).

Mass spectra were obtained with an LKB Model 9000 mass spectrometer with an ionizing voltage of 70 eV and an ionizing current of 60 μA. The sample was introduced directly into the ion source.

Proton magnetic resonance spectra were recorded as dimethyl sulfoxide-d_6 solutions at 60 MHz on a Varian Model A60 spectrometer. Chemical shifts were determined relative to *tert*-butyl alcohol used as an internal reference.

Ionization constants for 2-hydroxynicotinaldehyde and the two 360-mμ-absorbing materials were determined both by ultraviolet spectroscopy and fluorimetrically at 26 ± 1.0° in 0.01 M potassium carbonate buffers. The final concentration at each pH for all three compounds was 1×10^{-5} and 1×10^{-4} M, respectively, for the fluorimetric and spectral pK_a determinations. The pK_a value is equal to pH + log $[(d_i - d)/(d - d_u)]$ at 370 mμ, where d_i refers to the optical density of the

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† National Institutes of Health trainee. This paper represents a portion of the work to be submitted by C. G. G. in partial fulfillment of the requirements for the Ph.D. degree at the University of Pittsburgh.

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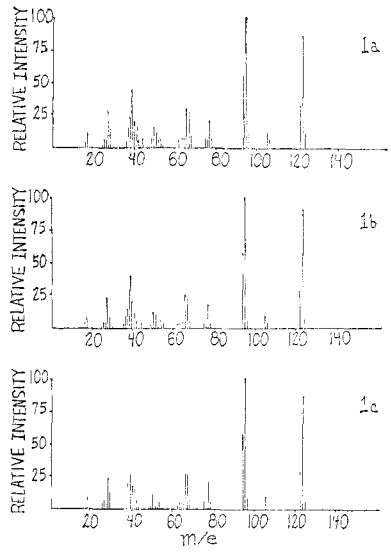


FIGURE 1: Mass spectra of 360-mμ-absorbing materials from 1-(*N,N*-dimethylcarbamoyl)nicotinamide chloride (1a), from DPN (1b), and 2-hydroxynicotinaldehyde (1c) measured at 70 eV.

completely ionized molecule measured at high pH values, where d refers to the optical density at intermediate pH values (pH 9–10), and d_u refers to the optical density of the completely un-ionized molecule measured at low pH values. A similar equation can be written for pK_a measurements based on fluorescence measured at 460 mμ (when excited at 360 mμ) by substitution of the maximal fluorescence intensity of the completely ionized molecule at high pH values for d_i, the fluorescence measured at intermediate pH values for d, and by substitution of the minimal fluorescence of the completely un-ionized molecule at low pH values for d_u. Six determinations were carried out by ultraviolet spectroscopy at 370 mμ and fluorimetrically at 460 mμ to give a value of 9.84 ± 0.05 for the pK_a of all three compounds.

The yield of 2-hydroxynicotinaldehyde from DPN as a function of NaOH concentration was determined by measurement of the absorbance at 360 mμ using a Cary 14 spectrophotometer after incubating 0.957×10^{-4} M DPN in 3.0 ml of the appropriate NaOH solution until no further change in the ultraviolet spectrum occurred. The per cent yield was calculated using a value of 8000 M^{-1} cm^{-1} for the molar absorptivity at 360 mμ. The value for ε remains constant over the entire range of NaOH concentrations.

Isolation of 360-mμ-Absorbing Material from 1-(N,N-Dimethylcarbamoyl)nicotinamide Chloride. 1-(*N,N*-Dimethylcarbamoyl)nicotinamide chloride (1.25 g) was added to 20 ml of 1 N NaOH with stirring. The solution was allowed to react for 2 hr at ambient temperature. At the end of this time the solution was adjusted to pH 11 with 1 N HCl and the total volume was brought to 75 ml. This solution was applied to a washed 15 × 50 cm AG-1-X-2 column (chloride form). The column was discontinuously eluted with 100 ml of deionized, degassed water, 100 ml of 0.001 N HCl, and 300 ml of 0.01 N HCl; 10-ml fractions were collected. Those fractions yielding a blue fluorescence when an aliquot was spotted on filter paper, treated with a drop of 5 N NaOH, and viewed under a long-wave ultraviolet source were combined and lyophilized to dryness. The residue was dissolved in 20 ml of hot ethanol and filtered. The filtrate was taken to dryness and the residue crystallized from water; 0.297 g was obtained, mp 222–223°.

Isolation of the 360-mμ-Absorbing Material from DPN. With stirring 3.75 g of β-DPN was dissolved in 20 ml of 6 N NaOH and incubated at room temperature for 1 hr. The reaction mixture was then cooled in an ice–salt bath and 9 ml of concentrated HCl was added. The solution was adjusted to pH 11 with 1 N HCl and diluted to a final volume of 100 ml. The same isolation procedure was followed for this solution as for the 360-mμ-absorbing material from 1-(*N,N*-dimethylcarbamoyl)nicotinamide chloride. Aliquots of those fractions giving a blue fluorescence on treatment with 5 N NaOH were scanned on a Cary 14 spectrophotometer and those fractions containing absorbance only at 341 and 245 mμ were combined and lyophilized to dryness. The residue was taken up in 20 ml of hot ethanol, filtered, and the filtrate taken to dryness. The residue was crystallized from water with a yield of 44.5 mg, mp 222–223°.

Synthesis of 2-Hydroxynicotinaldehyde. In small portions 0.915 g of $NaNO_2$ was added with stirring to 0.615 g of 2-aminonicotinaldehyde in 30 ml of 2.0 N HCl. The solution was allowed to stand 1 hr and then was heated to 90° for 15 min. The reaction solution was adjusted to pH 11 with 1 N NaOH and diluted to a total volume of 100 ml with deionized degassed water. 2-Hydroxynicotinaldehyde was isolated by the same ion-exchange method used for the isolation of the 360-mμ-absorbing material from 1-(*N,N*-dimethylcarbamoyl)-nicotinamide chloride. The lyophilized residue from those fractions yielding a blue fluorescence were shaken with 100 ml of hot ethanol and filtered. The filtrate was taken to dryness *in vacuo* and the residue crystallized from water. Colorless needles (0.309 g, 48% yield, mp 222–223°) were obtained: $\lambda_{max}^{0.1\ N\ NaOH}$ 361 mμ (ε 8000) and 249 mμ (ε 6300).

Anal. Calcd for $C_6H_5NO_2$: C, 58.54; H, 4.06; N, 11.38. Found: C, 58.38; H, 4.03; N, 11.52.

Results and Discussion

The 360-mμ-absorbing materials from DPN and from I, R = $CONH_2$, migrated as single spots and had identical migrations in solvents A (R_F 0.90) and in B (R_F 0.83). The infrared spectra were superimposible and had major bands at 3120 (s), 2860 (w), 1690 (s), 1670 (s), 1630 (s), 1580 (s), 1540 (s), and 1470 (m) cm^{-1}. 2-Pyridones are characterized by a strong band in the 1710- to 1640-cm^{-1} region due to C=O stretching (Spinner and White, 1966); furthermore, the four strong C=O, C=C, and C=N stretching bands in the region 1680–1500 cm^{-1} are very similar to those seen in the spectra of 2-pyridone and 2-hydroxynicotinic acid. The presence of an aldehyde group in the two 360-mμ-absorbing materials is indicated by the absorbance at 2860 cm^{-1} which is typical of an aromatic aldehyde. The two compounds also give positive Tollen's tests. The ultraviolet spectra were identical with maxima at 361 and 249 mμ in 0.1 N NaOH and maxima at 341 and 245 mμ at pH 7.

Mass spectra of the two compounds also are very similar (Figure 1a, 1b) and show a parent peak at *m/e* 123. This molecular weight was confirmed by measurement at 15 eV. The base peak at *m/e* 95 (M-28) probably arises from loss of carbon monoxide from the parent molecule. This fragment probably is 2-pyridone since the remainder of the spectrum resembles the spectrum obtained from that compound (Spiteller and Spiteller-Friedmann, 1963). The base peak of 2-pyridone is at *m/e* 94 which would also account for the intensity (57%) of this peak at *m/e* 94 in the mass spectra of these two compounds. The strong molecular ion peak (88%) is typical of substituted 2-pyridones (Kaiser, 1968; Duffield

et al., 1966). The mass spectra has intense peaks at M, M − 1, M − 29, M − 28, and a substantial intensity (10%) at *m/e* 105 (M − 18) which resembles the spectrum of salicaldehyde (Stoll *et al.*, 1967). From consideration of the above data the structure of the 360-mμ-absorbing material was postulated as 2-hydroxynicotinaldehyde (II) and this compound was synthesized as described under Methods and Materials.

Authentic 2-hydroxynicotinaldehyde gave an infrared spectrum identical with those of the 360-mμ-absorbing materials from DPN and I, R = $CONH_2$. The mass spectrum was very similar to those of the two isolated products (Figure 1c). Proton magnetic resonance spectra of all three compounds were identical and contained four signals: a triplet at −366 cps (J = 7) from the interal *tert*-butyl alcohol standard, a set of five lines from −447 to −466 cps centered at −456 cps (J = 7, ~2 cps), a singlet at −590 cps, and a broad singlet at −727 cps. The relative areas of the four groups of signals are 1:2:1:1. 2-Hydroxynicotinaldehyde was indistinguishable from the products from DPN and I, R = $CONH_2$, by comparison of ultraviolet spectra, migration in solvents A and B, and by fluorescence excitation and emission spectra. A mixture melting point of all three compounds showed no depression.

The two maxima, one at low wavelengths and the other about 100 mμ higher, in the ultraviolet spectra of 2-hydroxynicotinaldehyde are seen in many pyridone compounds (Bridges *et al.*, 1966; Bonsall and Hill, 1967; Spinner and White, 1966; Schofield, 1967). For example, 4,6-dimethyl-3-acetyl-2-pyridone has peaks at 253 mμ (log ε 3.54) and 325 mμ (log ε 3.90) in its un-ionized form (Bonsall and Hill, 1967). Upon ionization to the anionic form, pyridones undergo a bathochromic shift of approximately 5 mμ at the lower wavelength maxima and a shift of approximately 20 mμ at the higher maxima. The log ε values of 3.78 and 3.90 for the 249- and 361-mμ absorbances of 2-hydroxynicotinaldehyde in its anionic form are typical values for pyridones (Schofield, 1967).

Presence of the electron-withdrawing 3-formyl group would be expected to lower the ionization constant of 2-hydroxynicotinaldehyde compared to that of 2-pyridone. 3-Chloro-2-pyridone has a pK_a of 10.4 (Spinner and White, 1966) compared to 11.6 for 2-pyridone (Bridges *et al.*, 1966). Comparison of the pK_a's of 3-hydroxypyridine to that of 3-hydroxy-4-formylpyridine (pK_a = 8.72 and 6.77, respectively) shows a similar reduction in the ionization constant (Martell, 1963). The ionization constant of 2-hydroxynicotinaldehyde (pK_a = 9.84) is lower than expected by almost 1 pK_a unit, but this may be due to the possibility of hydrogen bonding between the 2-hydroxy group and the carbonyl group of the 3-formyl substituent such as seen in 4,6-dimethyl-3-acetyl-2-pyridone (Bonsall and Hill, 1967). This would make the preferred reference compound the 2-hydroxypyridine tautomer rather than the stable 2-pyridone structure. 2-Hydroxypyridine would be expected to be more acidic than 2-pyridone and could thus account for the abnormal lowering of the ionization constant.

If the δ value for the internal standard *tert*-butyl alcohol used in the nuclear magnetic resonance spectra is taken as 1.27 relative to tetramethylsilane (Jackman and Sternhell, 1969), the following δ values are obtained: 6.57 (triplet, J = 7 cps), 8.07 (multiplet, J = 7, ~2 cps), 10.3 (singlet), and 11.32 (broad singlet). The broad singlet at 11.32 disappeared when shaken with D_2O and is assigned to the 2-hydroxy proton. Typical values for intramolecular hydrogen-bonded phenols range from 10.5 to 16 (Jackman and Sternhell, 1969). The formyl proton is assigned to the singlet at 10.3. Nicotinaldehyde in dimethyl sulfoxide-d_6 solution has a chemical shift of 10.14 relative to internal tetramethylsilane for its formyl group

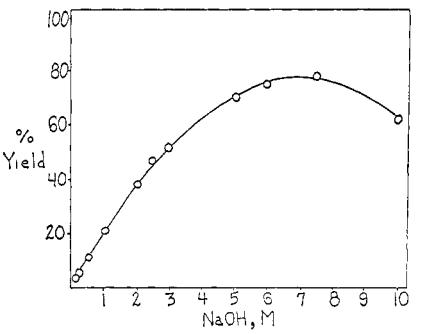


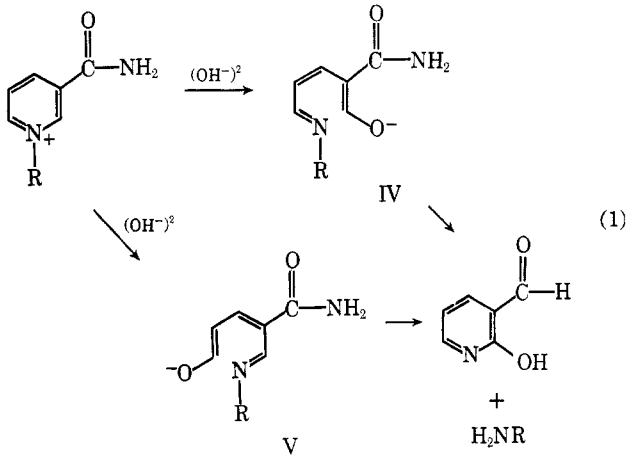
FIGURE 2: Per cent yield of 2-hydroxynicotinaldehyde from 0.957 × 10^{-4} M DPN as a function of NaOH concentration at 25°, measured at 360 mμ.

(Brügel, 1962). Aromatic formyl groups with ortho-substituents have chemical shifts of 10.2–10.5 (Jackman and Sternhell, 1969). The triplet at 6.57 is assigned to the 5-H proton of the ring on consideration of the relative area of the peak, its splitting pattern, and by comparison to the nuclear magnetic resonance spectra of 2-pyridone and 3-methyl-2-pyridone. The 5-H protons of these two compounds are split into a triplet by the 4-H and 6-H protons and are less deshielded than any of the other ring protons, having δ values (determined in dimethyl sulfoxide-d_6 relative to tetramethylsilane) of 6.43 and 6.20, respectively (Brügel, 1962). The multiplet at 8.03 is assigned to the 4-H and 6-H protons of the ring on the basis of the relative area of 2 contained by the multiplet and by comparison to the nuclear magnetic resonance spectrum of 2-pyridone. In 2-pyridone the 4-H and 6-H protons have a complex multiplet centered at 7.73 which is very similar in appearance to that of 2-hydroxynicotinaldehyde (Brügel, 1962). The shift downfield of these protons in 2-hydroxynicotinaldehyde compared to that of the 2-pyridone is of the magnitude expected for the presence of the formyl substituent. The J values of 7 for interproton coupling of the 5-H proton and the 4-H and 6-H protons and the coupling constant of J ~ 2 observed in the multiplet at 8.03 are typical for 3-substituted pyridine compounds (Brügel, 1962). For example, 2-pyridone has values of $J_{4,5}$ and $J_{5,6}$ = ~7 cps and $J_{4,6}$ = ~2 cps.

The yield of 2-hydroxynicotinaldehyde from the ring opening of DPN as a function of NaOH concentration is shown in Figure 2. There is a linear dependence on NaOH concentration up to 2 N NaOH with a leveling off at 6 N NaOH and a decline at 10 N NaOH. This parallels the leveling off and decline in yield of the 342-mμ-absorbing material from DPN which occurs at 5 N NaOH and at 10 N NaOH (Johnson and Morrison, 1970a). The maximal yield of 80% at 7.5 N NaOH for 2-hydroxynicotinaldehyde compares well to the maximal yield of glutacondialdehyde anion, III, from the ring-opening reaction of 1-(*N*,*N*-dimethylcarbamoyl)pyridinium chloride, I, R = H) at pH values above 15 (Johnson and Rumon, 1970). At 6 N NaOH which is the condition used for the analytical determination of DPN (Lowry *et al.*, 1957) the yield is 70%. The yield of 2-hydroxynicotinaldehyde from I, R = $CONH_2$, could not be quantitated because of the difficulty of obtaining pure starting material.

On the basis of the above criteria the 360-mμ-absorbing material resulting from the action of strong base on DPN has been identified as 2-hydroxynicotinaldehyde. Since this same compound is isolated as the final product from the known ring-opening reaction of 1-(*N*,*N*-dimethylcarbamoyl)nicotin-

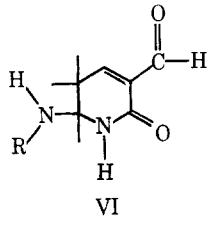
amide chloride (Johnson and Morrison, 1970b), this is strong evidence for the earlier suggestion of an analogous ring-opening reaction for DPN in strong base. Ring opening would result from attack of two hydroxide ions at either the 2 or 6 position of the nicotinamide ring, according to eq 1, where R = $CON(CH_3)_2$ or ADPR for 1-(*N*,*N*-dimethylcarbamoyl)-nicotinamide chloride or DPN, respectively.



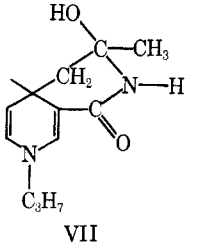
(1)

2-Hydroxynicotinaldehyde is probably produced by an internal cyclization of the open-chain compounds IV or V, resulting from 2 or 6 attack of hydroxide, respectively, where R = $CON(CH_3)_2$ or ADPR (see eq 1) or by cyclization of a hydrolysis product. The amide group of IV could undergo either a "trans-Schiffization" with the C=N group or condense with a carbonyl group produced by hydrolysis of the Schiff base. Intramolecular condensation of the amide group of V with its aldehyde group, followed by hydrolysis of the Schiff base, could also produce 2-hydroxynicotinaldehyde. Analogies for the trans-Schiffization reaction are seen in the ring-chain tautomerization of γ-imino amides which cyclize to form 2-amino-5-pyrrolidone structures in aqueous solutions (Watanabe *et al.*, 1969).

Cyclization of IV would give an aminopyridone structure, VI, which could lose the substituted amino group to form the pyridone structure, II.



VI



VII

γ-Keto amides also undergo ring-chain tautomerization to form 2-hydroxy-5-pyrrolidone structures (Flitsch, 1970). Intermediates similar to VI, where an OH group is substituted for the NHR group, could be formed by cyclization of the δ-aldehydoamide produced by hydrolysis of IV or V. The pyridone structure could then result from loss of water.

Other examples of the condensation of amides with carbonyl groups include the reaction of acetone with 1-(n-propyl)nicotinamide iodide which forms the structure, VII (Ludowieg *et al.*, 1964). Sodium formylacetone condenses with cyanoacetamide in aqueous basic solutions to form 3-cyano-6-methyl-2-pyridone (Mariella, 1963).

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