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Relationship of Structure to Properties of Diphosphopyridine Nucleotide and Other Pyridinium Compounds[1]

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A number of 3- or 4-substituted 1-methylpyridinium compounds were synthesized as model systems for studying the chemical reactions of diphosphopyridine nucleotide.[3] These compounds were classified according to their ability to undergo the following reactions: (1) reaction with aqueous potassium cyanide, (2) reaction with alcoholic potassium cyanide, (3) chemical reduction by sodium hydrosulfite and (4) oxidation by purified rabbit liver aldehyde oxidase. It was shown that the cyanide addition reaction was, in several cases, strongly dependent upon the dielectric constant of the solvent, suggesting a bimolecular nucleophilic addition reaction. Spectrophotometric evidence based on the cyanide addition reaction is presented to indicate a physical and chemical function for the ARPPR moiety of DPN.

Introduction

1-Alkyl pyridinium compounds have been extensively employed as models for the study of the chemical properties of DPN. Karrer, *et al.*,[4,5a] and Rafter and Colowick[5b] studied the reduction of some of these compounds by sodium hydrosulfite. Meyerhof, *et al.*,[6] Colowick, *et al.*,[7] and San Pietro[8] analyzed the potassium cyanide addition reaction and Knox[9] and Hurwitz[10] have investigated the oxidation of 1-methyl-3-carbamoylpyridinium iodide (1-methylnicotinamide iodide) by an aldehyde oxidase obtained from rabbit liver. This paper presents data with 1-methylpyridinium compounds, DPN and DPN derivatives in the three type reactions, which permits some evaluation of the physical and chemical effects of the carbamoyl and ARPPR groups in the DPN molecule.

Experimental

Materials.—Various pyridine derivatives were purchased from Distillation Products Industries, Aldrich Chemical Co. and Nutritional Biochemicals Corp. 1-Methylnicotinic acid (trigonelline) was purchased from Nutritional Biochemicals Corp. Methyl iodide was purchased from Fischer Scientific Co. and redistilled prior to use.

Aldehyde oxidase was prepared according to the method of Hurwitz.[10] Twice recrystallized yeast alcohol dehydrogenase was obtained from Worthington Biochemical Corp. This was diluted $^1/_{25}$ (v./v.) with potassium phosphate buffer (0.1 *M*; *p*H 7.5). APDPN was prepared according to the method of Kaplan and Ciotti.[11]

Methods.—All 1-methylpyridinium iodide compounds studied were synthesized in the following general manner: 0.02 mole of the pyridine compound was dissolved in 5 to 10 ml. of methanol (alternatively benzene), 0.03 mole of methyl iodide was added and the mixture was refluxed for 5–10 hours. Recrystallization was effected from hot methanol after treatment with animal charcoal.

Aqueous cyanide addition reactions were carried out according to the method of Colowick, *et al.*[7] Alcoholic cyanide addition reactions were carried out in the same manner except that methanol replaced water as solvents. The methanol was saturated with potassium cyanide (approx. 0.7 *M* at 25°).

Reductions of the pyridinium compounds were carried out in sodium carbonate solution with sodium dithionite (hydrosulfite) as described by Karrer and Blumer[12] except that the extracting solvent, chloroform, was not removed prior to recording the spectrum of these compounds. This precaution was taken since it was found that one of these compounds, trigonelline (1-methylnicotinic acid), was particularly labile, yielding a product with an absorption maximum at 290 mμ.

Oxidation of the pyridinium compounds by the aldehyde oxidase was measured by the reduction of 2,6-dichloroindophenol at 610 mμ. The oxidations were carried out in air. Corrections were made for non-enzymatic chemical oxidation of the dye.

All spectrophotometric measurements were made with the models DU or DK-2 Beckman spectrophotometers using 3-ml. quartz cells with a light path of 1 cm. The Beckman model G *p*H meter was used for all *p*H measurements.

Results and Discussion

A compilation of the 1-methylpyridinium iodide compounds studied is listed in Table II. It can be seen from the data that most of these compounds fit in one of three general categories. I. Compounds which are capable of aqueous potassium cyanide addition reactions, sodium hydrosulfite reduction and oxidation by aldehyde oxidase. II. Compounds which will not give an aqueous cyanide addition product but will form an addition product with alcoholic potassium cyanide. This group of compounds can in general be reduced and they are poorly oxidized.[13] III. Compounds which will not give a cyanide addition reaction, cannot be reduced by the method employed and are poorly oxidized.

1-Methylnicotinamide iodide and the monosubstituted amides undergo only partial aqueous cyanide addition. As expected from the inductive effect of the alkyl group the substituted amides yielded less addition product at equilibrium than 1-

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(3) The following abbreviations are used: DPN, diphosphopyridine nucleotide; ARPPR, the non-nicotinamide moiety of DPN, adenosinediphosphate ribosyl; NR, nicotinamide riboside; NMN, nicotinamide mononucleotide; APDPN, the 3-acetylpyridine analog of DPN.

(4) (a) P. Karrer and O. Warburg, *Biochem. Z.*, **285**, 297 (1936); (b) P. Karrer, G. Schwarzenbach, F. Benz and U. Solmssen, *Helv. Chim. Acta*, **19**, 811 (1936).

(5) (a) P. Karrer and F. J. Stare, *ibid.*, **20**, 418 (1937); (b) G. W. Rafter and S. P. Colowick, *J. Biol. Chem.*, **209**, 773 (1954).

(6) O. Meyerhof, P. Ohlmeyer and W. Mohle, *Biochem. Z.*, **297**, 113 (1938).

(7) S. P. Colowick, N. O. Kaplan and M. M. Ciotti, *J. Biol. Chem.*, **191**, 447 (1951).

(8) A. San Pietro, *ibid.*, **217**, 579 (1955).

(9) W. E. Knox, *ibid.*, **163**, 699 (1946).

(10) J. Hurwitz, *ibid.*, **212**, 757 (1955).

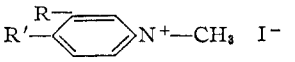
(11) N. O. Kaplan and M. M. Ciotti, *ibid.*, **221**, 823 (1956).

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

(13) 1-Methyl-3-butoxycarbonylpyridinium iodide is an unexplained exception to this generalization.

TABLE I

ANALYTICAL DATA



R	R′	M.p. (uncorr.) (°C.)	Formula	Analyses, % Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found	Nitrogen Calcd.	Nitrogen Found	Ref.
$-CONHCH_3$	.....	169.6	$C_8H_{11}ON_2I$	34.56	34.66	3.96	3.93	10.08	9.85	..
$-CON(CH_3)_2$	.....	137.0	$C_9H_{13}ON_2I$	37.01	37.63	4.45	4.60	9.59	9.36	..
$-COOC_2H_5$	.....	93.8–94.2	$C_9H_{12}O_2NI$	36.89	36.86	4.10	4.11	4.18	4.55	..
$-CONHC_2H_5$	.....	79.5–82.0	$C_9H_{13}ON_2I$	37.01	36.92	4.45	4.44	9.59	9.77	..
-CHO	.....	164.5–166.0	C_7H_6ONI	33.76	33.37	3.21	3.16	5.63	5.29	[a]
$-COOC_4H_9$	.....	96.5–97.5	$C_{11}H_{16}O_2NI$	41.15	41.04	4.98	4.97	4.36	4.58	..
.....	$-CONH_2$	258.6	$C_7H_9ON_2I$	31.84	31.73	3.41	3.47	10.61	10.37	[b]
$-COCH_3$	.....	154.5–155.2	C_8H_7ONI	36.53	36.60	3.80	3.85	5.33	5.06	[a]
$-CON(C_2H_5)_2$	.....	148.2–148.5	$C_{11}H_{17}ON_2I$	41.28	41.42	5.31	5.34	8.75	8.75	[c,d]
-CN	.....	194.5–195.5	$C_7H_7N_2I$	34.17	34.48	2.85	2.94	11.39	11.29	..
.....	.....	110.5–111.5	C_6H_5NI	32.61	32.47	3.62	3.60	6.34	6.23	[e]
$-COOCH_3$	.....	127.5–128.5	$C_8H_{10}O_2NI$	34.44	34.52	3.58	3.64	5.02	4.74	[f]
$-CONH_2$	.....	202.7–203.4	$C_7H_9ON_2I$	31.84	31.68	3.41	3.39	10.61	10.40	[g]
.....	$-CONHNH_2$	205.2–206.8	$C_7H_{10}ON_3I$	30.13	30.38	3.58	3.68	15.06	15.15	..
$-CH_3$	.....	91.8–92.0	$C_7H_{10}NI$	35.77	35.48	4.30	4.11	5.96	5.64	..

[a] Sara Ginsburg and E. W. Wilson, THIS JOURNAL, **79**, 481 (1957). (1-Methyl-3-formylpyridinium iodide, m.p. 173°) (1-methyl-3-acetylpyridinium iodide, m.p. 163–164°). [b] P. Karrer, F. W. Kahnt, R. Epstein, W. Jaffee and T. Ishii, *Helv. Chim. Acta*, **21**, 236 (1938); m.p. 255°. [c] E. Gryszkiewicz-Trochimowski, *Arch. Chem. Farm.*, **3**, 211 (1937); m.p. 157–158°. [d] G. Machek, *Monatsh.*, **72**, 77 (1938), m.p. 186–188°. [e] A. Hantzsch, *Ber.*, **42**, 68 (1909), m.p. 116°. [f] Y. Hukusima, *J. Chem. Soc. Japan*, **61**, 121 (1940), m.p. 130°. [g] P. Karrer, G. Schwarzenbach, F. Benz and U. Solmssen, *Helv. Chim. Acta*, **19**, 811 (1936).

methylnicotinamide. The partial cyanide addition of 1-methylnicotinamide iodide can be seen by comparing the spectra of this compound in water, aqueous cyanide and alcoholic cyanide with those of 1-methyl-3-acetylpyridinium iodide as shown in Fig. 1. In the former the cyanide addition reaction is markedly increased if the solvent is changed from water to methanol. This is not the case with the latter compound. Since the absorption at 260 mμ of 1-methylnicotinamide is due to the pyridinium ring system and is absent in the dihydropyridine derivative, the difference in absorption at 260 mμ of 1-methylnicotinamide iodide in the presence and absence of potassium cyanide can be used to calculate the extent of addition. In aqueous cyanide only 18% of the addition compound is formed,

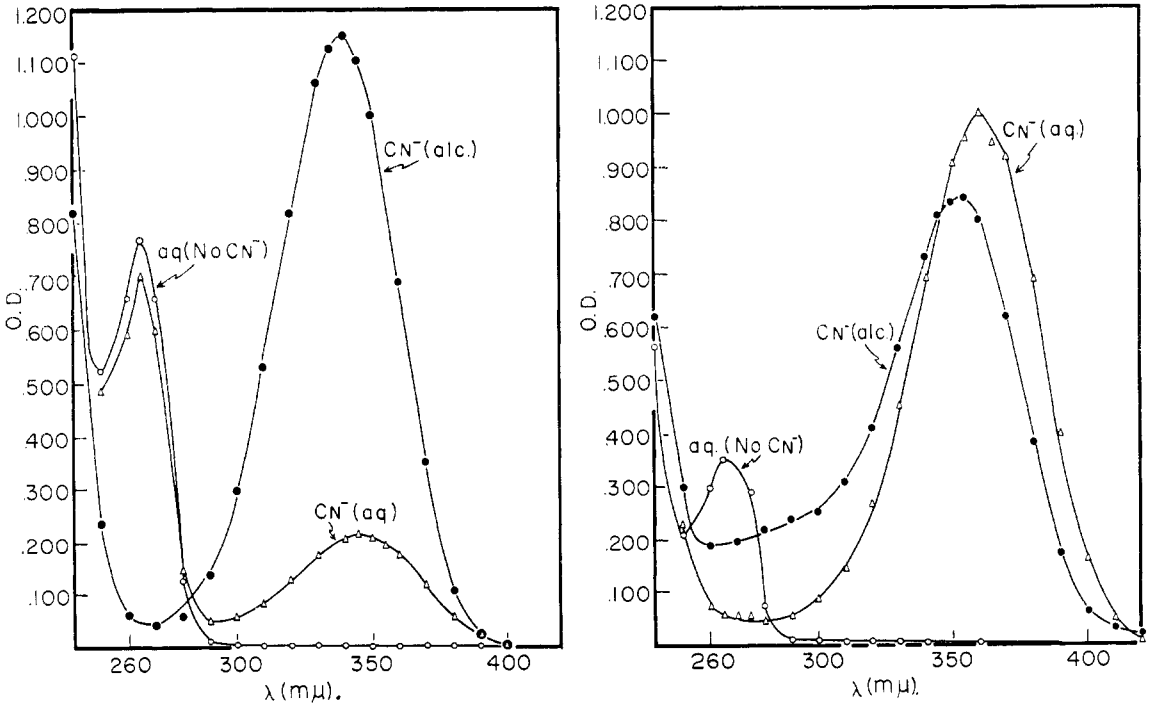
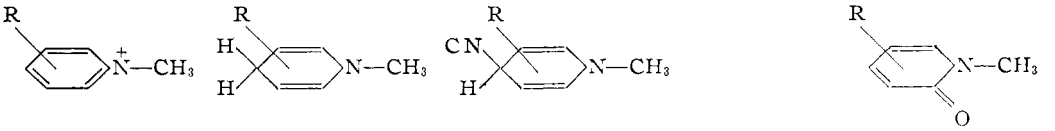


Fig. 1.—The aqueous and methanolic potassium cyanide addition reactions: 0.19 μmole of 1-methyl nicotinamide iodide (or 1-methyl-3-acetylpyridinium iodide) was treated with molar potassium cyanide (aq.) or saturated potassium cyanide (methanol) (final volume 3.0 ml.; 25°).

TABLE II

SPECTROPHOTOMETRIC OXIDATION, REDUCTION AND CYANIDE ADDITION REACTIONS OF SOME 1-METHYLPYRIDINIUM IODIDE COMPOUNDS



R-group	Oxidized form λ_{max} (mμ)	Oxidized form $a_m \times 10^{-3}$	Reduced form λ_{max} (mμ)	Aq. cyanide addn. λ_{max} (mμ)	Aq. cyanide addn. $a_m \times 10^{-3}$	Alcoholic cyanide addn. λ_{max} (mμ)	Alcoholic cyanide addn. $a_m \times 10^{-3}$	Aldehyde oxidase %[a]
3-CN	270 265[c]	3.9	365	335–340	6.2			91.8
3-CHO	260 265[c]	4.1	360	340[f]	0.53	340[f]	3.5	57.0
3-$COCH_3$	265	3.5	370	360[f]	10	355[f]	8.4	112
3-$CONH_2$	265	4.1	365	345[b]	1.1	340	6.1	100
3-$CONHCH_3$	265	4.6	355	325[b]		340	6.2	
3-$CONHC_2H_5$	265	4.7	352.5	335[b]		340	6.1	
3-COO^-	265	9.3	355	No addition		330	6.8	12.4
3-$COOCH_3$	260 270[c]	3.2	360–365	No addition		340	8.4	
3-$COOC_2H_5$	260	6.3	365	No addition		340	10	5.9
3-$COOC_4H_9$	265	5.7		No addition		340	9.0	80.0
3-$CON(CH_3)_2$	265	4.7	345	No addition		330	4.6	
3-$CON(C_2H_5)_2$	265	4.1	No reduction	No addition		330	6.0	21.8
3-H	260	4.3	No reduction	No addition		No addition		15.9
3-CH_3	265	4.1	370	No addition		No addition		12.7
4-$CONH_2$	270 260[c]	5.0	No reduction	No addition		No addition		18.8
4-$CONHNH_2$	270 262[c]	11	No reduction	No addition		No addition		9.42

[a] Approximately 4 μmoles of the pyridinium compound was incubated in 0.02 *M* potassium phosphate buffer (*p*H 7.1) containing 69γ of 2,6-dichloroindophenol. The reaction was started with aldehyde oxidase (final volume = 3.0 ml.) and the change in optical density at 610 mμ was recorded. (Corrections were made for non-enzymatic, chemical oxidation reactions.) The percentage recorded in this table was calculated as that change in optical density in three minutes as compared with the same three minute change of an equimolar quantity of 1-methylnicotinamide iodide. The reactions were carried out aerobically. [b] Only partial addition see Fig. 1. [c] Spectra were taken in water, 0.17 *N* HCl and 0.17 *N* NaOH (approximately 0.3 μmole of pyridinium compound in a total volume of 3 ml.). The alternatively listed wave length maxima represent a shift in spectra in the basic solution. [d] Blank spaces indicate no data obtained for this reaction. [e] a_m is defined as molar absorbancy index = As/c where c = concentration in moles per liter and $As = -\log_{10}$ transmission. [f] When 3-acetylpyridine and pyridine-3-aldehyde were mixed with molar aqueous potassium cyanide (25°) no change in absorption was noted in their spectra when compared with the control (no cyanide) in the region 260 to 400 mμ. Employing these conditions, 1-methyl-3-acetylpyridinium iodide and 1-methyl-3-formylpyridinium iodide showed maxima at 360 and 340 mμ, respectively, with a complete loss of the 265 mμ peak which is characteristic for pyridinium compounds undergoing addition. While cyanohydrin formation cannot be ruled out as a possibility, the shift in absorption from 265 mμ to the 350 mμ region is more characteristic of the pyridinium ring reacting to form the cyanide addition product.

while in methanolic cyanide 97% of the compound undergoes addition. The same type of calculation can be applied to the reaction of cyanide with DPN, nicotinamide riboside and nicotinamide mononucleotide. The data listed in Table III show that in the case of nicotinamide riboside there is little change in the percentage of cyanide addition when the solvent system is changed. This is also true for nicotinamide mononucleotide and DPN. 1-Methylnicotinamide, on the other hand, exhibits a change of addition product of 79% on changing the solvent. We feel that the increase in ability of the riboside and ribotides to yield an aqueous addition product is due to a direct electronic effect, influencing the polarization of the pyridinium ring *via* the carbamoyl grouping. Another possible explanation for the results of Table III can be that of a transmission effect acting through the N-1 position of the pyridinium ring. Clarification of this important point in the mechanism of action of DPN must await infrared and dipole moment analysis.

TABLE III

CYANIDE ADDITION REACTIONS OF RELATED PYRIDINIUM COMPOUNDS[a]

Compound	% Addn. with CN^- (aq.)	% Addn. with CN^- (alc.)
1-Methyl nicotinamide iodide	18.2	96.7
Nicotinamide riboside	98[b]	100[b]
Nicotinamide mononucleotide	101.1[b]	100[b]
Diphosphopyridine nucleotide	91.4	98.6

[a] Conditions as described in Fig. 1. [b] Corrected for 20–25% 260 mμ absorbing, non-reactive, material.

Function of Dielectric Constant in Potassium Cyanide Addition.—1-Methyl-3-ethoxycarbonylpyridinium iodide gave no apparent addition reaction with aqueous molar cyanide (*p*H 11.0–11.5). If the aqueous cyanide reaction is carried out at *p*H 10.7 an absorption maximum at 340 mμ is observed followed by a rapid loss of this peak. Coincident with the loss in absorption at 340 mμ, a corresponding increase in absorption at 260 mμ

is noted. This reaction is shown in Fig. 2 (measurements at 340 mμ). The addition of alcoholic (methanol) cyanide at the completion of the reaction initiated the reappearance of the peak but the maximum absorption was shifted from 340 to 332.5 mμ. After recrystallization from hot ethanol

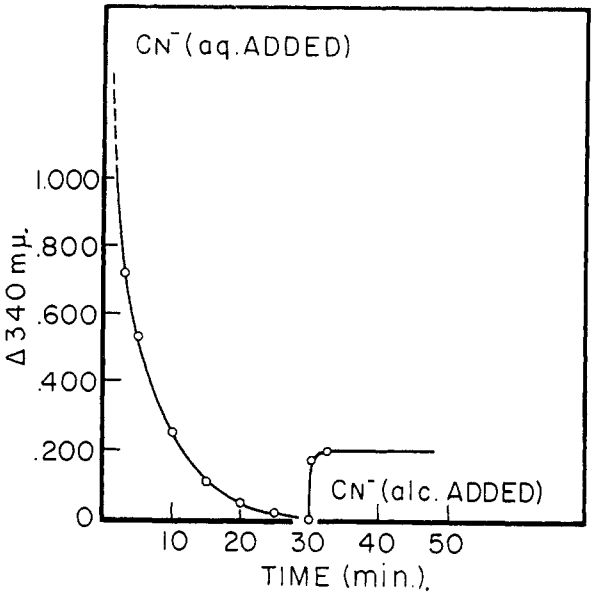


Fig. 2.—Hydrolysis of 1-methyl-3-ethoxycarbonyl pyridinium iodide: 1.0 μmole of 1-methyl-3-ethoxycarbonyl pyridinium iodide was treated with potassium cyanide (0.3 *M* final concentration) in a solution buffered with 0.5 *M* potassium phosphate (final volume 3.0 ml.; final *p*H 10.7; 25°). After 30 minutes 5 ml. of saturated methanol-potassium cyanide was added to the mixture (no correction made for the dilution factor). A 3-ml. aliquot was extracted and the change in optical density with time was measured at 330 mμ. Concentration of 1-methyl-3-ethoxycarbonylpyridinium iodide 0.2 μ*M*.

the white material was identified as trigonelline by its melting point, ultraviolet absorption spectrum and electrophoretic mobility. The conversion of the ester to the acid was duplicated with sodium hydroxide utilizing the same conditions (*p*H 10.7 22°). Confirmation of the hydrolysis reaction was obtained by measuring ethanol liberation from 1-methyl-3-ethoxycarbonylpyridinium iodide by a specific enzymatic assay.[14] The unmethylated parent compound 3-ethoxycarbonylpyridine (ethyl nicotinate) is not hydrolyzed under these circumstances.

Figures 3a and 3b show the influence of dielectric constant on the cyanide addition reaction. Figure 3a shows that the cyanide addition product increases as the dielectric constant decreases. That is, the equilibrium of the addition reaction is displaced toward the formation of the addition product as the dielectric constant is lowered. If one plots the data of Fig. 3a in terms of apparent equi-

(14) Ethanol can be assayed quantitatively with yeast alcohol dehydrogenase if one substitutes APDPN for DPN. This is in part due to differences in potential of the two coenzymes (APDPN–APDPNH = −0.248 v.; DPN–DPNH = −0.320 v.). For details concerning the assay see N. O. Kaplan and M. M. Ciotti in "Methods in Enzymology," Vol. III, S. P. Colowick and N. O. Kaplan, eds., Academic Press, Inc., New York, N. Y., 1956, p. 253.

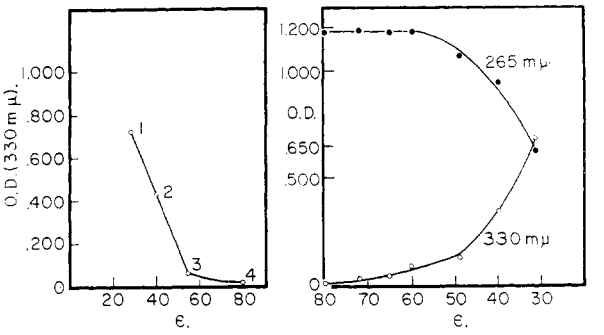


Fig. 3a.—Potassium cyanide addition as a function of dielectric constant: solvents of various dielectric constant. 0.37 μmole of trigonelline was treated with molar potassium cyanide. The following solvents are employed: water (ε = 80.1; point 4), methanol-water (ε = 40.4; point 2), ethanol-water (ε = 55.0; point 3) and a dioxane-water mixture (ε = 29.2; point 1).

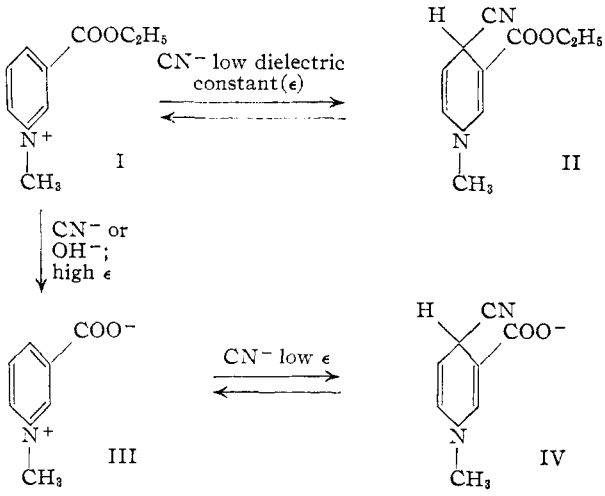
Fig. 3b.—Potassium cyanide addition as a function of dielectric constant, mixtures of methanol and water: 0.16 μmole of trigonelline was mixed with appropriate amounts of *M* KCN (aq.) and saturated KCN (methanol) to give the desired dielectric constant indicated as the abscissa[15] (final volume 3.0 ml.; 25°). The absorption of the various mixtures was recorded at 330 mμ (O) and 265 mμ (●).

librium constant *versus* reciprocal of dielectric constant a straight line relationship is obtained. The dielectric constant can also be varied using mixtures of the same solvents (in this case methanol and water). As can be seen from Fig. 3b as the 330 mμ absorption increases there is a corresponding decrease in the 265 mμ absorption which represents material that has not reacted. The possibility of re-esterification as a part of the reaction sequence can be ruled out for two reasons. First, the maximum absorption for the esters (methyl and ethyl) occurs at 340 mμ while that of the 1-methylnicotinic acid is at 330 mμ. When hydrolysis of the esters is complete and methanolic cyanide is added the new maximum is shifted to 332.5 mμ. The product as discussed previously is trigonelline and the discrepancy in wave length is probably due to small contamination of the original ester. Secondly, re-esterification could not take place in the dioxane–water solvent system (Fig. 3a). A possible sequence for the reaction of the ethyl ester in low and high dielectric constants is shown.

The scheme takes account of the following experimental facts: (a) 1-methyl-3-ethoxycarbonylpyridinium iodide can undergo reversible cyanide addition mediated by a solvent of lower dielectric constant than that of water (I ⇄ II). The addition product has an absorption maximum of 340 mμ. (b) In aqueous cyanide (or in alkali of the same *p*H) 1-methyl-3-ethoxycarbonylpyridinium iodide is irreversibly hydrolyzed to trigonelline (I → III). (c) Trigonelline can undergo reversible dielectric constant dependent cyanide addition,

(15) The dielectric constants for mixtures of methanol–water and dioxane–water were obtained from H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolytic Solutions," ACS Monograph Series, 2nd edtn., Reinhold Publ. Corp., New York, N. Y., 1950, pp. 118, 544.

yielding a product whose absorption is at 330 mμ (III ⇄ IV).

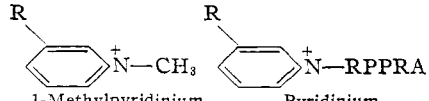


The effect of solvent on this reaction is not unexpected if one assumes the mechanism is that of nucleophilic addition. Kinetic studies of this reaction series have not been made.

Structure of DPN: Chemical and Physical Action of the ARPPR Moiety.—Spectral comparisons of the cyanide addition reaction of the 1-methylpyridinium iodide model compounds with the corresponding dinucleotide analogs of DPN show that in every case the dinucleotide–cyanide absorption maximum is shifted 10 to 20 mμ toward the ultraviolet region. This information is presented in Table IV.

TABLE IV

SPECTRAL COMPARISON OF THE CYANIDE ADDITION PRODUCT OF SOME 1-METHYLPYRIDINIUM COMPOUNDS WITH THEIR CORRESPONDING DINUCLEOTIDES (DPN ANALOGS)[a]



R	1-Methylpyridinium compd. λ_{max} (mμ)	Pyridinium dinucleotide λ_{max} (mμ)
$-CONH_2$	345	325
$-COO^-$	330[c]	315[b,c]
$-COOC_2H_5$	340[c]	325[14]
$-COCH_3$	360	340[11]
$-CHO$	340	330[16]

[a] Conditions employed were the same as in Fig. 1. [b] Unpublished data. [c] Measured in alcoholic cyanide.

As previously observed from the data presented in Table II, some of the 1-methylpyridinium iodide compounds were incapable of aqueous potassium cyanide addition but would undergo alcoholic cyanide addition. These compounds must have a decreased ability to potentiate nucleophilic addition at the 4 (*para*) position compared to those which are capable of aqueous cyanide addition. It was found that the pyridine dinucleotide analogs of some of these 1-methyl compounds would in fact yield an addition product in an aqueous solvent despite the fact that the 1-methylpyridinium counterpart (model) would not. A few of the model compounds in this class are the 1-methyl-4-hydrazide pyridinium iodide, trigonelline, 1-methyl-3-ethoxycarbonylpyridinium iodide and 1-methyl-3-(N-methylcarbamoyl)-pyridinium iodide. This shows that the electronic structure of the pyridinium compound is markedly affected by the adenosine diphosphate ribosyl moiety. Three reasons can be advanced which may possibly help to explain this difference in behavior. First, the electronic inductive effects of the 1-methyl and 1-ribosyl groups are quite different. Secondly, we have shown that the 1-methyl ester compound undergoes an aqueous potassium cyanide addition followed by a rapid hydrolysis. The ester analog of DPN may be protected from the hydrolytic reaction by a steric effect of ARPPR portion of DPN. Finally, associated with the steric effect, a direct electronic effect might exert its influence upon the polarization of the pyridinium ring by hydrogen bonding with the substituent at the 3-position and a phosphate moiety in the case of the nucleotides or the 5′-hydroxyl grouping in the case of the riboside.

It has been known for some time that the pyridinium ring is the site of oxidation–reduction and addition reactions for DPN. The ARPPR portion of the molecule must play an important part in preserving the functional activity of the coenzyme and in stabilizing its structural integrity.

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(16) N. O. Kaplan and M. M. Ciotti, *J. Biol. Chem.* **221**, 833 (1956).