Bull. Chem. Soc. Jpn., **55**, *1531—1537 (1982)*

Enzymatic Synthesis of Pyridine Nucleotides. Structural Property of Some New NAD-analogs, and Base Conditions Available for the Analog Formation[1]

Shuichi TONO-OKA
Institute of Immunological Science, Hokkaido University, Kita 15-jo, Nishi 7-chome,
Kita-ku, Sapporo 060

(Received August 3, 1981)

Some new β-NAD-analogs were prepared by a base-exchange reaction catalyzed by pig-brain NADase. The analogs produced from methyl 4-(methylamino)- and 4-(dimethylamino)nicotinates were proved to be 1,4-dihydropyridine form. On the contrary, the analog obtained from 4-(acetylamino)nicotinamide was shown to be a quaternary pyridinium form. Moreover, an unexpected analog of 1,4-dihydro-4-pyridinone form was given by 4-methoxynicotinamide. It was shown to be indispensable for the formation of NAD-analog that the ring nitrogen of pyridine bases concerned should at least be free from protonation around pH 7. Interestingly nicotinic acids with 4-amino, methylamino, and dimethylamino groups were found to be much more basic for the ring nitrogen atom than their respective methyl esters.

Previously we have reported[2,3] the enzymatic synthesis of new pyridine nucleosides utilizing the corresponding NAD-analogs (**2** and **3**) as intermediates, which were prepared from β-NAD (**1**) and 4-amino derivative of methyl nicotinate or nicotinamide by a NADase-catalyzed base-exchange reaction (Scheme 1). These analogs were proved to take a 1,4-dihydropyridine form instead of a quaternary pyridinium one.

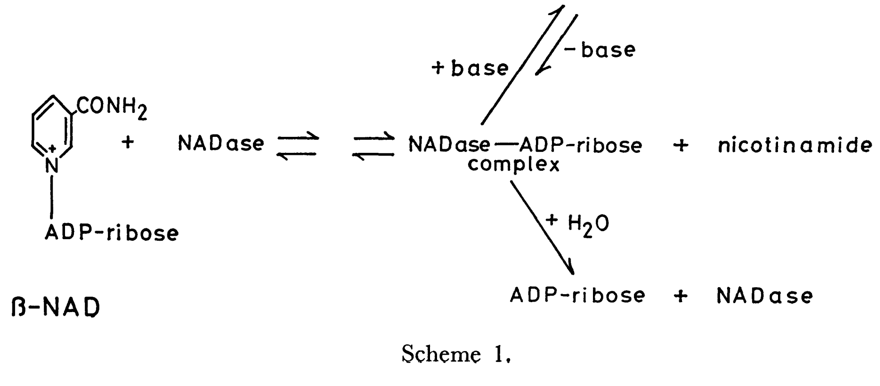
Now our attention has been extended to investigate the structural property of pyridine moiety of NAD-analogs which will be produced from other *N*-substituted 4-amino derivatives (methylamino, dimethylamino, *etc.*) of methyl nicotinate or nicotinamide. Moreover, these analogs are also of interest in connection with their possible efficiency as competitive inhibitors[4] for NAD. On the other hand, it comes to be an important problem whether the pyridine bases concerned bring about the corresponding NAD-analog or not, when this method is utilized for the synthesis of pyridine nucleosides. As previously reported,[3] nicotinic acids with 4-amino, methylamino, and dimethylamino functions produced no detectable amount of analog unless the 3-carboxyl group was pre-transformed into a methoxycarbonyl or carbamoyl one. In view of these facts, the spectroscopic behaviors of pyridine bases were examined with alterations in pH to clarify the relationship between their reactivity and chemical property.

The present paper describes the structural properties of newly prepared NAD-analogs and necessary conditions of pyridine bases for the analog formation in the base-exchange reaction.

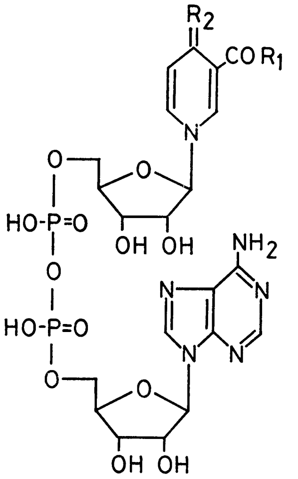
Results and Discussion

Preparation and Structural Property of NAD-analogs. On the basis of the results described in the previous paper,[3] various NAD-analogs were prepared under the following conditions: Base concentration was more than 25 mM (1 M=1 mol dm^{-3}) if possible, and the base/NAD molar ratio was about 4:1. The yield and some spectral data of the analogs obtained were summarized in Table 1.

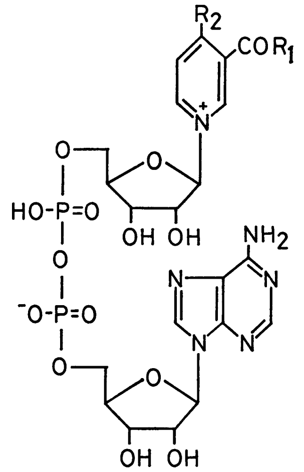
In the UV spectra (pH 7), although the replacing base, methyl 4-(methylamino)nicotinate and ADP-ribose have an absorption at 260 (log ε 4.08) and 259 nm (log ε 4.20), respectively, methylamino analog (**4**) obtained showed its maximum at 268 nm (log ε 4.27) with a bathochromic shift, suggesting the structural change of pyridine chromophore. In the ^{1}H-NMR spectra, a methyl group adjacent to the nitrogen atom of the replacing base showed a doublet in DMSO-d_6 with the vicinal coupling of $J_{\mathrm{NH,CH_3}}$=5.2 Hz, which collapsed by the addition of deuterium oxide. In contrast, compound **4** showed a singlet for the methyl group in the same solvent. From these observations, the 4-nitrogen atom of pyridine moiety in **4** proved to bear no proton, indicating 1,4-dihydropyridine structure of **4**.



Scheme 1.



2: $R_1=OCH_3$ $R_2=NH$
3: $R_1=NH_2$ $R_2=NH$
4: $R_1=OCH_3$ $R_2=NCH_3$
5: $R_1=OCH_3$ $R_2=\overset{+}{N}(CH_3)_2$
8: $R_1=OCH_3$ $R_2=O$
9: $R_1=NH_2$ $R_2=O$



1: $R_1=NH_2$ $R_2=H$
6: $R_1=NH_2$
$R_2=NHCOCH_3$
7: $R_1=OH$
$R_2=NHCOCH_3$
10: $R_1=NH_2$ $R_2=CH_3$
11: $R_1=OH$ $R_2=CH_3$

Table 1. Yields and spectral properties of analogs obtained from various pyridine bases

Compd No.	Yield [a)] %	^{1}H-NMR[b)] C_1' (δ)	^{1}H-NMR[b)] $J_{5,6}$/Hz	UV$_{max}$ (λ/nm)[c)] Bases	UV$_{max}$ (λ/nm)[c)] Analogs[d)]
4	76	5.63	8.0	260	268
5	58	5.62	8.2	273	296
6	72	6.00	6.0	272	268
7	50	6.01	6.0	268	266
8	64	5.60	8.0	233	265
9	73	5.60	8.0	234	266
10	68	6.00	6.0	261	260
11	48	5.98	6.0	261	260

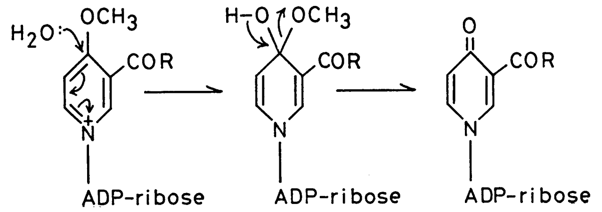
a) Analog/(analog+ADP-ribose)×100. b) Measured in deuterium oxide. c) Measured in water. d) All the compounds showed the ε-value of more than 1.6×10^4.

Dimethylamino analog (**5**), produced from methyl 4-(dimethylamino)nicotinate which bears no proton at the nitrogen atom to participate in tautomerism, was anticipated to take a pyridinium form instead of a 1,4-dihydropyridine one. However, compound **5** was shown to be the dihydropyridine structure of 4-dimethyliminium type from the following findings. The ^{1}H-NMR spectrum of **5** exhibited a six-proton singlet at δ 3.11 due to the two methyl groups, and the anomeric proton of pyridine side appeared at δ 5.62 accompanying 0.4 ppm higher field resonance compared with that of adenine side. Moreover, the $H_{5,6}$ coupling of pyridine moiety was estimated to be 8.2 Hz, indicating the olefinic character of C_5–C_6 bond. Its UV spectral behavior was also compatible with the structure of **5** (Table 1).

Acetylamino analogs (**6** and **7**) were remarkably different from **4** and **5** in some respects in the ^{1}H-NMR spectra. Both the two anomeric protons appeared as a broad two-proton doublet at δ 6.0, and the $H_{5,6}$ coupling of pyridine ring was 6.0 Hz, the value of which was distinctly smaller than for **4** and **5**. In addition, its pyridine protons showed the lower field shift of 0.4—0.6 ppm as a whole compared with those of the acetylamino bases alone. The UV spectrum of these analogs nearly corresponded with the sum absorptions of acetylamino base and ADP-ribose. These observations indicated the quaternary pyridinium form of **6** and **7**.

It has thus been shown that even if the amino group was methylated, its lone pair electrons contributed to the 1,4-dihydropyridine structure, and that when it was acetylated, its resonance effect on pyridine nucleus was prevented by the electron-withdrawing acetyl group in spite of possessing a proton at the nitrogen atom.

In connection with dimethylamino base in which an amino group is methylated, it seems of deep significance to investigate methyl 4-methoxynicotinate and 4-methoxynicotinamide in which a hydroxyl group is methylated. If they bring about a similar type of analog as described for **5**, the formation of the system $-N-C=C-C=\overset{+}{O}CH_3$ must be expected. However, the oxonium form is generally unstable compared with the ammonium or iminium one,[5)] whereby the above system with methoxonium ion can hardly be considered. The analogs formed were thus anticipated to have the alternative pyridinium system $-\overset{+}{N}=C-C=C-OCH_3$ instead. They, however, exhibited an unexpected UV absorption at 266 nm (log ε 4.24), accompanying a large bathochromic shift of more than 30 nm from that of the methoxy bases alone. The ^{1}H-NMR spectra of these analogs (**8** and **9**) showed the absence of a three-proton singlet due to a methoxyl methyl group. The anomeric proton in question exhibited a similar behavior to that of **4** and **5**, and the $H_{5,6}$ coupling was observed to be 8.0 Hz. These facts demonstrate the 1,4-dihydro-4-pyridinone form of **8** and **9**, which would probably be brought about from once formed 4-methoxypyridinium compound by demethoxylation subsequent to undergoing the nucleophilic attack of water at the 4-position (Scheme 2).



Scheme 2.

Table 2. Susceptibility of NAD-analogs to the hydrolase action of NADase

Compd No.	Hydrolysis rate (%)		
	3 h	6 h	18 h
1 (NAD)	76	100	—
2	0	5	9
3	0	0	5
4	0	4	8
5	0	5	10
6	25	42	68
7	24	36	52
8	0	4	6
9	0	0	4
10	34	56	74
11	26	45	60
12[a]	58	80	100

a) NAD-analog of INH.

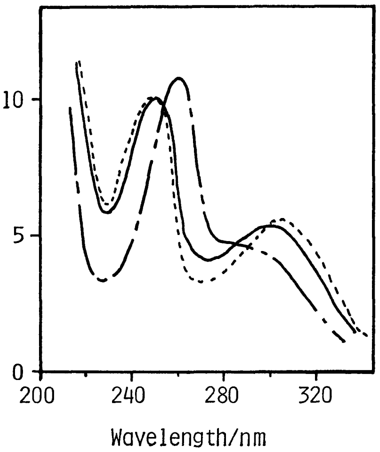


Fig. 1. Absorption spectra of methyl 4-aminonicotinate (**13**) at some values of pH.
———: pH 2, ——: pH 7, ----: pH 12.

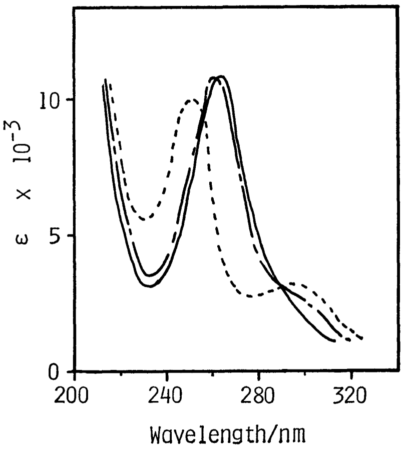


Fig. 2. Absorption spectra of 4-aminonicotinic acid (**16**) at some values of pH.
———: pH 2, ——: pH 7, ----: pH 12.

By using 4-methylnicotinamide as a replacing base, which has an UV maximum at 261 nm (log ε 3.60), 4-methyl analog (**10**)$^{6)}$ was obtained which showed the absorption at 260 nm (log ε 4.26). In the ^{1}H-NMR spectrum of **10**, the methyl protons were clearly observed at δ 2.62 as a three-proton singlet and the $H_{5,6}$ coupling was 6.0 Hz, indicating a quaternary pyridinium form of **10** rather than 1,4-dihydro-4-methylenepyridine one. Such was also found to be the case of the analog (**11**) obtained from 4-methylnicotinic acid.

As previously suggested,$^{2)}$ the susceptibility of NAD-analogs to the hydrolase action of NADase might be employed as an indication of their pyridinium ion character. In order to clarify this assumption more precisely, the above obtained analogs were incubated with NADase, and the amount of ADP-ribose liberated was examined with elapse of time on TLC in comparison with NAD itself and its isonicotinic acid hydrazide (INH) analog (**12**),$^{7)}$ which is a typical NAD-analog of pyridinium form (Table 2). All the analogs (**6**, **7**, and **10**—**12**) which exist in pyridinium form underwent hydrolysis more or less, though not so fast as NAD. On the other hand, the analogs (**2**—**5**, **8**, and **9**) which exist in dihydropyridine form were little susceptible to the enzyme, indicating that their glycosyl bonds were much more stable than those of the former analogs. These results strongly supported the validity of the assumption.

Necessary Conditions of Bases for the Analog Formation. This base-exchange reaction can be assumed to proceed through the nucleophilic attack of pyridine base on an enzyme–ADP-ribose complex. From this point of view, the reactivity of pyridine base was considered to be notably affected by the nucleophilicity of ring nitrogen. As previously described, nicotinic acids with 4-amino group inclusive of methyl- and dimethylamino ones were found to produce no NAD-analog, although nicotinic acid itself and its 4-methyl derivative produced successfully the corresponding analogs. In order to infer the chemical property of analog-productive pyridine bases and non productive ones around pH 7 wherein the NADase-catalyzed reaction proceeds, their UV spectra were firstly examined at some different pHs.

In the case of methyl 4-aminonicotinate (**13**) which gives rise to a NAD-analog in good yield, its absorption maximum at pH 7 approximated closely to that at pH 12 (Fig. 1). A similar spectral pattern was also observed for 4-aminonicotinamide. On the other hand, in the case of 4-aminonicotinic acid (**16**) which gives rise to no NAD-analog, its absorption maximum approximated closely to that at pH 2 (Fig. 2). The same spectral correlations were also recognized in a pair of corresponding 4-methylamino compounds (**14** and **17**) and 4-dimethylamino ones (**15** and **18**). The structure of now described compounds (**13**—**18**) are summarized in Table 3.

Brown and Plasz$^{8)}$ have previously reported that the first protonation of 4-aminopyridine occurred at the ring nitrogen and not at the substituent amino group. Since the primary amino function is a very weak base, the second protonation occurs only in more strongly acidic solutions (pH$\lesssim-3$) like 2 M sulfuric acid.$^{9)}$ In the acidic solution of pH 2, therefore, the

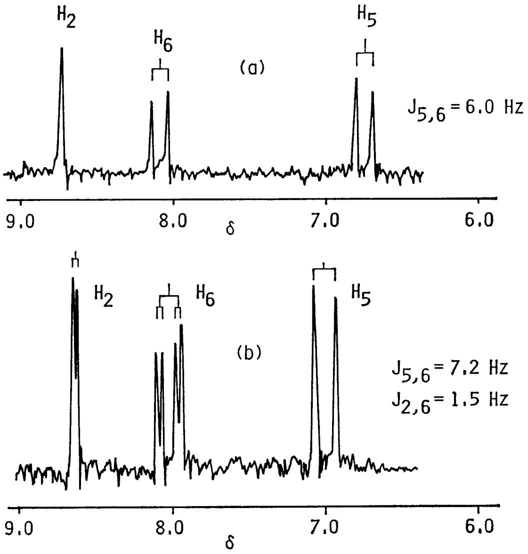


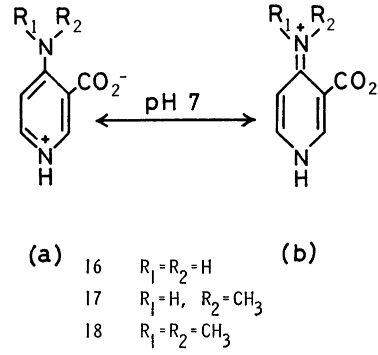
Fig. 3. ^{1}H-NMR spectral patterns of **13** (a) and **16** (b) at pH 7.

pyridine bases dealt with here should be protonated at the ring nitrogen. In the alkaline solution, on the contrary, the nitrogen should be completely free from protonation. It is accordingly suggested that the ester bases (**13**—**15**) would mainly exist in a free form, whereas the carboxyl bases (**16**—**18**) suffer a protonation in the neutral solution.

When ^{1}H-NMR spectra were measured for these two kinds of bases, their spectroscopic differences were revealed more clearly. The signal pattern of **13** in the neutral solution was substantially identical with that of pH 12, and the $H_{5,6}$ coupling was 6.0 Hz (Fig. 3a). In contrast, **16** showed a different signal pattern almost identical with that of pH 2; the $H_{5,6}$ coupling became larger ($J_{5,6}=7.2$ Hz) and the $H_{2,6}$ long range coupling appeared clearly ($J_{2,6}=1.5$ Hz) (Fig. 3b). These spectroscopic behaviors were also observed for the couples of **14** and **17** as well as **15** and **18**.

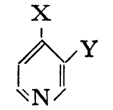
Craig *et al.*[10] have previously found in the structural studies of 4-aminoquinoline derivatives that a coupling constant between H_2 and H_3 of the quinoline ring could be an indication of the extent of aromaticity. This concept was well applicable to the 3,4-disubstituted pyridine compounds now dealt with. If the ring nitrogen was protonated, the cation formed could be stabilized by resonance, such as that between the cannonical forms (**a**) and (**b**) (Scheme 3). The C_5–C_6 bond should then come to have an olefinic character due to the contribution of the structure **b**. It is well known that the vicinal coupling of olefines is usually larger than that of aromatic environment, and the $H_{2,6}$ coupling is increased by *ca.* 1 Hz on undergoing a protonation.[11]

Thus, the ring nitrogen of ester bases were proved to be essentially free around pH 7, whereas that of carboxyl bases suffered a protonation under the same conditions. On acetylation of **16**, however, its spectral properties came to be similar to those of **13**, and the acetyl compound (**19**) certainly provided a NAD-analog in 50% yield (Table 1). Apart from this, other analog-productive 4-methyl- and 4-methoxynicotinic acids were also found to be free from protonation at pH 7.



Scheme 3.

Table 3. pK_a values for pyridine bases in aqueous solution at 25 °C

Base No.	 X	Y	pK_a (proton gained)
	X	Y	
13	NH_2	CO_2CH_3	6.6
14	$NHCH_3$	CO_2CH_3	6.6
15	$N(CH_3)_2$	CO_2CH_3	6.9
16	NH_2	CO_2H	9.1
17	$NHCH_3$	CO_2H	9.2
18	$N(CH_3)_2$	CO_2H	9.4
19	$NHCOCH_3$	CO_2H	6.2
20	H	CO_2CH_3	3.1[a]
21	H	CO_2H	2.1[a]
22	NH_2	H	9.2[a]
23	$NHCH_3$	H	9.4[b]
24	$N(CH_3)_2$	H	9.6[b]

a) A. Albert, "Physical Methods in Heterocyclic Chemistry," ed by A. R. Katritzky, Academic Press, New York (1963), Vol. I, p. 72. b) A. Albert, *ibid.*, (1971), Vol. IV, p. 9.

These observations demonstrated that the mostly free state of ring nitrogen in the pyridine bases concerned is at least necessary for the formation of NAD analog.

Basicity of Pyridine Bases. To undergo an easy protonation implies the high basicity of ring nitrogen. Strangely, there has so far been no report on the basicity of pyridine bases here dealt with. Therefore, their pK_a values were determined by the convenient spectrometric method of Albert *et al.*[12] Contrary to our intuitional expectation, carboxyl bases (**16**—**18**) (p$K_a \gtrsim 9$) proved much more basic than corresponding ester bases (**13**—**15**) (p$K_a \lesssim 7$) and showed no significant difference in basicity compared with 4-aminopyridines (**22**—**24**) (Table 3). This fact indicates that the presence of 3-carboxyl group has no special influence on the basicity of ring nitrogen, whereas 3-methoxycarbonyl group has an effect to reduce it, unlike the correlation of nicotinic acid (**21**) and its methyl ester (**20**). The lower basicity of **19** compared to **16** should be considered due to the inhibitory effect of electron-withdrawing acetyl group on the dispersal of lone pair electrons of amino function into the ring nitrogen. In view of this explanation, the unexpected decrease of basicity by esterification of carboxyl group seems possibly dependent on an intermolecular non-bonded interaction between the amino nitrogen and the carbonyl carbon of ester group. On that account, the lone pair electrons of amino function can hardly contribute to the resonance effect, resulting in the reduced basicity of ring nitrogen. On the other hand, there would occur no appreciable interaction in the case of carboxyl bases, presumably due to the deficiency of carbonium ion character in the carboxyl group.

In conclusion, the basicity of pyridine bases is surely an important factor for the formation of NAD-analogs. However, since the enzymatic reaction must be carried out around pH 7, highly basic pyridine bases can no longer function as nucleophiles because of suffering a protonation. Thus, the suitable basicity ($pK_a \lesssim 7$) was shown to be one of the necessary conditions for enough nucleophilicity of replacing bases.

The biological activity of here prepared pyridine nucleotides will be reported elsewhere.[13)]

Experimental

All the melting points are uncorrected. The UV spectra were measured on a Hitachi 200-20 spectrophotometer, and the ^{1}H-NMR spectra were determined on a Hitachi R-20B (60 MHz) spectrometer in deuterium oxide with sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal standard unless otherwise stated. The abbreviations "s, d, dd, br, bs, and bd" denote "singlet, doublet, double doublet, broad, broad singlet, and broad doublet," respectively. Optical rotations were measured with a Union PM 101 digital polarimeter. Ionic strength and pH measurements were made on an Emuesu CD-35M II conductivity meter and a Radiometer pHM-26 pH meter equipped with a G-202 glass electrode, respectively. Thin layer chromatographies (TLC) were carried out on silica gel 60 F_{254} HPTLC plates (Merck, 10 cm × 10 cm) using 2-propanol–0.2% aqueous ammonia (7 : 3, v/v) as the developing system. The analysis of chromatograms were performed by a Shimadzu CS-910 scanner equipped with a chromatopack C-R1A. Column chromatographies[14)] were carried out on DEAE-Sephadex (A-25, 3.5 mequiv./g) connected to LKB Uvicord II (254 nm). β-NAD (Grade III) was purchased from Sigma Chemical Co.

Pig-brain NADase (EC 3.2.2.5). The crude particulate enzyme was prepared by the method of Zatman *et al.*[7a)] The colloidal supernatant fluid containing *ca.* 0.4 U/ml[15)] of NADase activity was used without further purification.

4-(Methylamino)nicotinic Acid. Prepared from 4-chloronicotinic acid 1-oxide[16)] and methylamine in two steps, and recrystallized from aqueous methanol: mp 277—279 °C (decomp) [lit,[17)] 274—276 °C (decomp)].

Methyl 4-(Methylamino)nicotinate. Prepared by esterification of the above compound, and recrystallized from dichloromethane–petroleum benzine: mp 71—73 °C. Found: C, 57.86; H, 6.04; N, 16.76%. Calcd for $C_8H_{10}N_2O_2$: C, 57.82; H, 6.07; N, 16.86%.

4-(Dimethylamino)nicotinic Acid. Prepared from 4-chloronicotinic acid 1-oxide[16)] and dimethylamine in two steps, and recrystallized from aqueous ethanol: mp 223—225 °C (decomp). Found: C, 57.74; H, 6.21; N, 16.65%. Calcd for $C_8H_{10}N_2O_2$: C, 57.82; H, 6.07; N, 16.86%.

Methyl 4-(Dimethylamino)nicotinate. Prepared by the esterification of the above compound, and recrystallized from dichloromethane-petroleum benzine: mp 75—77 °C. Found: C, 60.07; H, 6.61; N, 15.55%. Calcd for $C_9H_{12}N_2O_2$: C, 59.99; H, 6.71; N, 15.55%.

4-(Acetylamino)nicotinic Acid. Prepared by the acetylation of 4-aminonicotinic acid[18)] with acetic anhydride, and recrystallized from water: mp 252—254 °C (decomp). Found: C, 53.45; H, 4.37; N, 15.82%. Calcd for $C_8H_8N_2O_3$: C, 53.32; H, 4.43; N, 15.55%.

4-(Acetylamino)nicotinamide. Prepared by the acetylation of 4-aminonicotinamide,[19)] and recrystallized from ethanol: mp 223—225 °C (decomp). Found: C, 53.76; H, 4.98; N, 23.71%. Calcd for $C_8H_9N_3O_2$: C, 53.63; H, 5.03; N, 23.47%.

Methyl 4-Methoxynicotinate. Prepared from 4-nitronicotinic acid 1-oxide[18)] in two steps, and recrystallized from benzene: mp 85—87 °C (lit,[20)] 83—84 °C).

4-Methoxynicotinamide. Prepared by ammonolysis of the above compound, and recrystallized from benzene: mp 155—157 °C (lit,[20)] 153—154 °C).

4-Methylnicotinic Acid. Prepared from 4-methylquinoline in two steps, and recrystallized from water: mp 215—217 °C (decomp) (lit,[21)] 215—217 °C).

4-Methylnicotinamide. Prepared by the ammonolysis of the above compound, and recrystallized from methanol: mp 167—169 °C (lit,[19)] 165—167 °C).

*Methyl 1,4-Dihydro-4-(methylimino)nicotinate Adenine Dinucleotide (**4**).* Methyl 4-(methylamino)nicotinate (1.6 mmol, 265 mg) and NAD (0.4 mmol, 280 mg, neutralized with 2M NaOH to pH 7) were incubated with pig-brain NADase (6 U, 15 ml) in 0.1 M Tris–HCl (pH 7.2, 30 ml) at 37 °C for 5 h. The incubation mixture was treated with 70% trichloroacetic acid (1 ml) and the precipitated protein was removed by centrifugation. To the clear supernatant thus obtained, cold acetone (300 ml) was added to give yellowish hygroscopic powder (235 mg) as the crude product. It was dissolved in water (20 ml) and then applied to a column of DEAE-Sephadex (HCO_3^--form) (column size; 2.5 cm × 45 cm). The column was washed with 0.02 M NH_4HCO_3 (250 ml) to remove the included free bases and then eluted with 300 ml portions of 0.2, and 0.5 M NH_4HCO_3 (pH 7.8), successively. The first major component showing UV-absorbing peak at 268 nm was eluted with the 0.2 M solution. The eluate (50 ml) was immediately collected and lyophilized repeatedly to give **4** (186 mg) as the ammonium salt. An analytical sample of **4** was obtained by rechromatography and drying over P_2O_5 *in vacuo* for 12 h at 40 °C: $[\alpha]_D^{20}$ −60.9° (*c* 0.92, H_2O); UV (H_2O), λ_{max} 268 nm (log ε 4.26); ^{1}H-NMR (δ, D_2O), 3.05 (3H, s, $NC\underline{H}_3$), 3.98 (3H, s, $CO_2C\underline{H}_3$), 4.2—4.6 (10H, br, riboses), 5.63 (1H, bd, J=5 Hz, py-$C_1'\underline{H}$), 6.01 (1H, d, J=5.5 Hz, ad-$C_1'\underline{H}$), 6.87 (1H, d, J=8.0 Hz, py-$\underline{H}_5$), 8.03 (1H, s, ad-$\underline{H}_2$), 8.15 (1H, dd, J=1.5, 8.0 Hz, py-$\underline{H}_6$), 8.28 (1H, s, ad-$\underline{H}_8$), 8.67 (1H, d, J=1.5 Hz, py-$\underline{H}_2$). Found: C, 34.05; H, 5.71; N, 15.79%. Calcd for $C_{23}H_{31}N_7O_{15}P_2 \cdot 2NH_3 \cdot 4H_2O$: C, 33.95; H, 5.53; N, 15.50%.

*Methyl 1,4-Dihydro-4-(dimethyliminio)nicotinate Adenine Dinucleotide (**5**).* Methyl 4-(dimethylamino)nicotinate (1.5 mmol, 270 mg) and NAD (0.4 mmol, 275 mg) were incubated with NADase (6 U, 15 ml) in 0.1 M Tris–HCl (pH 7.2, 30 ml) at 37 °C for 5 h. The incubation mixture was treated in a similar manner to that described above to give the crude product (228 mg), which was dissolved in water (20 ml) and applied to a column (HCO_3^--form). The column was eluted with aqueous NH_4HCO_3 similarly as described above. The first major component was eluted with the 0.2 M solution. The eluate (40 ml) showing the UV maximum at 266 and 296 nm was immediately collected and lyophilized repeatedly to give **5** (113 mg) as the ammonium salt: $[\alpha]_D^{20}$ −46.3° (*c* 0.76, H_2O); UV (H_2O), λ_{max} 266 nm (log ε 4.23), 296 nm (log ε 4.24); ^{1}H-NMR (δ, D_2O), 3.11 (6H, s), 3.96 (3H, s), 4.2—4.6 (10H, br), 5.62 (1H, bd, J=5 Hz), 6.00 (1H, d, J=5.5 Hz), 7.02 (1H, d, J=8.2 Hz), 8.04 (1H, s), 8.12 (1H, dd, J=1.5, 8.2 Hz), 8.31 (1H, s), 8.44 (1H, d, J=1.5 Hz). Found: C, 35.34; H, 5.52; N, 14.18%. Calcd for $C_{24}H_{33}N_7O_{15}P_2\cdot NH_3\cdot 4H_2O$: C, 35.52; H, 5.42; N, 13.81%.

*4-(Acetylamino)nicotinamide Adenine Dinucleotide (**6**).* A mixture of 4-(acetylamino)nicotinamide (2 mmol, 355 mg) and NAD (0.6 mmol, 390 mg) was incubated with NADase (6 U, 15 ml) in 0.1 M Tris–HCl (40 ml) for 7 h. The incubation mixture was treated as described for **4** to give the crude hydroscopic product (408 mg), which was purified by the chromatographic procedures using aqueous HCO_2NH_4 (pH 6.8) as eluent in the manner as described for **4** to provide **6** (332 mg) as the hygroscopic ammonium salt: $[\alpha]_D^{20}$ −12.4° (*c* 0.64, H_2O); UV (H_2O), λ_{max} 268 nm (log ε 4.07), 340 nm (log ε 3.77); ^{1}H-NMR (δ, D_2O), 2.35 (3H, s), 4.25—4.65 (10H, br), 6.00 (2H, bd), 7.43 (1H, d, J=6.0 Hz), 7.95 (1H, s), 8.21 (1H, s), 8.62 (1H, dd, J=1.5, 6.0 Hz), 9.15 (1H, d, J=1.5 Hz). Found: C, 35.15; H, 5.15; N, 18.12%. Calcd for $C_{23}H_{30}N_8O_{15}P_2\cdot 2NH_3\cdot H_2O$: C, 35.70; H, 4.92; N, 18.11%.

*4-(Acetylamino)nicotinic Acid Adenine Dinucleotide (**7**).* A mixture of 4-(acetylamino)nicotinic acid (2.9 mmol, 523 mg) and NAD (1.3 mmol, 935 mg) was incubated with NADase (8 U, 20 ml) in 0.1 M Tris–HCl (70 ml) for 12 h. The crude product (776 mg) obtained was purified by the chromatographic procedures to give **7** (372 mg): $[\alpha]_D^{20}$ −44.5° (*c* 1.1, H_2O); UV (H_2O), λ_{max} 266 nm (log ε 4.18), 283 nm (log ε 4.18); ^{1}H-NMR (δ, D_2O), 2.39 (3H, s), 4.3—4.7 (10H, br), 6.01 (2H, bd), 8.15 (1H, s), 8.35 (1H, s), 8.63 (2H, bs), 9.05 (1H, bs). Found: C, 35.39; H, 4.88; N, 14.73%. Calcd for $C_{23}H_{29}N_7O_{16}P_2\cdot NH_3\cdot 2H_2O$: C, 35.66; H, 4.65; N, 14.47%.

*Methyl 1,4-Dihydro-4-oxonicotiate Adenine Dinucleotide (**8**).* Methyl 4-methoxynicotinate (6.5 mmol, 1080 mg) and NAD (1.6 mmol, 1050 mg) were incubated with NADase (12 U, 30 ml) in 0.1 M Tris–HCl (120 ml) for 6 h. The crude product (852 mg) obtained by treating the reaction mixture as described for **4** was purified by column chromatography on DEAE-Sephadex (HCO_2^--form) as described for **6** to give **8** (546 mg): $[\alpha]_D^{20}$ −29.6° (*c* 1.05, H_2O); UV (H_2O), λ_{max} 265 nm (log ε 4.26); ^{1}H-NMR (δ, D_2O), 3.90 (3H, s), 4.2—4.6 (10H, br), 5.60 (1H, bd, J=5 Hz), 5.97 (1H, d, J=5.5 Hz), 6.85 (1H, d, J=8.0 Hz), 7.95 (1H, s), 8.02 (1H, dd, J=1.5, 8.0 Hz), 8.24 (1H, s), 8.68 (1H, d, J=1.5 Hz). Found: C, 34.28; H, 5.11; N, 11.24%. Calcd for $C_{22}H_{28}N_6O_{16}P_2\cdot 2NH_3\cdot 2H_2O$: C, 34.55; H, 4.97; N, 10.99%.

*1,4-Dihydro-4-oxonicotinamide Adenine Dinucleotide (**9**).* In 0.1 M Tris–HCl (80 ml), 4-methoxynicotinamide (6.6 mmol, 998 mg) and NAD (1.7 mmol, 1140 mg) were incubated with NADase (12 U, 30 ml) for 6 h. The reaction mixture was treated in a similar manner as described for **4** to give **9** (770 mg): $[\alpha]_D^{20}$ −37.9° (*c* 0.69, H_2O); UV (H_2O), λ_{max} 266 nm (log ε 4.24); ^{1}H-NMR (δ, D_2O), 4.25—4.65 (10H, br), 5.60 (1H, bd, J=5 Hz), 6.04 (1H, d, J=5.5 Hz), 6.90 (1H, d, J=8.0 Hz), 8.12 (1H, dd, J=1.5, 8.0 Hz), 8.15 (1H, s), 8.44 (1H, s), 8.60 (1H, d, J=1.5 Hz). Found: C, 34.14; H, 4.47; N, 17.41%. Calcd for $C_{21}H_{27}N_7O_{15}P_2\cdot 2NH_3\cdot H_2O$: C, 34.44; H, 4.78; N, 17.24%.

*4-Methylnicotinamide Adenine Dinucleotide (**10**).*6) A mixture of 4-methylnicotinamide (2.8 mmol, 380 mg) and NAD (0.7 mmol, 475 mg) was incubated with NADase (8 U, 20 ml) in 0.1 M Tris–HCl (60 ml) for 6 h. The reaction mixture was treated as described for **4** to give **10** (324 mg): $[\alpha]_D^{20}$ −24.8° (*c* 0.85, H_2O); UV (H_2O), λ_{max} 260 nm (log ε 4.27); ^{1}H-NMR (δ, D_2O), 2.66 (3H, s), 4.1—4.6 (10H, br), 6.0 (2H, bd), 7.65 (1H, d, J=6.0 Hz), 8.05 (1H, s), 8.40 (1H, s), 8.70 (1H, dd, J=1.5, 6.0 Hz), 8.82 (1H, d, J=1.5 Hz). Found: C, 34.86; H, 5.04; N, 15.22%. Calcd for $C_{22}H_{29}N_7O_{14}P_2\cdot NH_3\cdot 3H_2O$: C, 35.29; H, 5.08; N, 14.97%.

*4-Methylnicotinic Acid Adenine Dinucleotide (**11**).* A mixture of 4-methylnicotinic acid (2.4 mmol, 330 mg) and NAD (0.6 mmol, 404 mg) was incubated with NADase (8 U, 20 ml) in Tris–HCl (50 ml) for 5 h. The reaction mixture was treated as described for **4** to give **11** (196 mg): $[\alpha]_D^{20}$ −36.1° (*c* 0.90, H_2O); UV (H_2O), λ_{max} 260 nm (log ε 4.23); ^{1}H-NMR (δ, D_2O), 2.62 (3H, s), 4.1—4.6 (10H, br), 5.98 (2H, bd), 7.78 (1H, d, J=6.0 Hz), 8.02 (1H, s), 8.33 (1H, s), 8.63 (1H, dd, J=1.5, 6.0 Hz), 8.75 (1H, d, J=1.5 Hz). Found: C, 35.48; H, 5.16; N, 12.94%. Calcd for $C_{22}H_{28}N_6O_{15}P_2\cdot NH_3\cdot 3H_2O$: C, 35.24; H, 4.94; N, 13.08%.

Susceptibility of NAD-analogs to the Hydrolase Action of NADase. A series of mixtures (0.6 ml) containing 30 mM NAD-analog (pH 7, 0.2 ml), NADase (0.12 U, 0.3 ml), and 0.5 M Tris–HCl (pH 7.2, 0.1 ml) was incubated at 37 °C for 18 h. In the course of incubation, the aliquot (1 μl) of the resulting mixture was analyzed by TLC using a thin layer scanner with a chromatopack. Based on the chromatograms thus obtained the amount of ADP-ribose produced was estimated semi-quantitatively. The data in Table 2 show the averages of three measurements.

pK_a Values of Pyridine Bases. The values were determined by the spectrometric method of Albert and Phillips12) using the following series of 0.01 M buffers; glycine-hydrochloric acid (pH 2.00), acetate (pH 5.00), phosphate (pH 7.00), borate (pH 9.00), and glycine–sodium hydroxide (pH 12.00).

The author wishes to thank Prof. T. Masamune (Department of Chemistry, Faculty of Science) and Prof. I. Azuma (Institute of Immunological Science) for their encouragement and useful suggestions during this work.

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