Summary

A new glucuronide of sulfathiazole was isolated from human urine after administration of sulfathiazole. This glucuronide was isolated as its ammonium salt with melting point 176~178° (decomp.). It was confirmed that the conjugate is sulfatiazole-N^1-glucuronide by the data of ultraviolet and infrared spectroscopy.

(Received October 29, 1962)

UDC 612. 398. 145

122. Mikio Honjo, Yoshiyasu Furukawa, Hiroki Moriyama, and Kuniyoshi Tanaka : Synthesis of Nicotinamide Adenine Dinucleotide Analogs and their Coenzymatic Activities.*1

(*Research Laboratories, Takeda Chemical Industries, Ltd.**2)

Many NAD*3 analogs have so far been prepared enzymatically,[1~8] but the analogs synthesized chemically are only NMN-IMP,[9] the N-hydroxyethyl derivative of NAD[10] and nicotinic acid adenine dinucleotide.[11]

This paper describes the chemical synthesis of NAD analogs in which the adenosine portion is replaced by other naturally occurring nucleosides derived from RNA and DNA, and their reaction with dehydrogenases.

Among the compounds of this kind, only NMN-dAMP has so far been prepared enzymatically.[6]

In general, the phosphoramidate method is superior to the DCC method in the synthesis of nucleotide coenzymes such as FAD, UDPG, etc.[12] Therefore, the synthesis

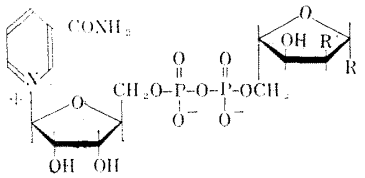
---

*1 After our publication of the short communication in this Bulletin, **10**, 73 (1962), a similar paper on the synthesis of nicotinamide adenine dinucleotide analogs and their properties was published in J. Biol. Chem., **237**, 1709 (1962) by C.P. Fawcett and N.O. Kaplan.

*2 Juso-Nishino-cho, Higashiyodogawa-ku, Osaka (本庄美喜男, 古川純康, 森山博規, 田中邦喜).

*3 Abbreviations used : NAD, Nicotinamide adenine dinucleotide; NMN-IMP, Nicotinamide hypoxanthine dinucleotide; RNA, ribonucleic acid; DNA, Deoxyribonucleic acid; NMN-dAMP, 2′-Deoxyadenosine analog of NAD; FAD, Flavin adenine dinucleotide; UDPG, Uridine diphosphate glucose; NMN, Nicotinamide mononucleotide; AMP-NH_2, Adenosine 5′-phosphoramidate; DCC, Dicyclohexylcarbodiimide; dAMP, 2′-Deoxyadenosine 5′-phosphate; GMP, Guanosine 5′-phosphate; CMP, Cytidine 5′-phosphate; UMP, Uridine 5′-phosphate; TMP, Thymidine 5′-phosphate; NMN-GMP, Nicotinamide guanine dinucleotide; NMN-CMP, Nicotinamide cytosine dinucleotide; NMN-UMP, Nicotinamide uracil dinucleotide; NMN-TMP, Nicotinamide thymine dinucleotide; NADH, Reduced NAD.

1) N.O. Kaplan, M.M. Ciotti : J. Biol. Chem., **221**, 823 (1956).
2) N.O. Kaplan, *et al.* : *Ibid.*, **221**, 833 (1956).
3) H. Klenow, B. Andersen : Biochim. et Biophys. Acta, **23**, 92 (1957).
4) L.S. Dietrich, *et al.* : J. Biol. Chem., **233**, 964 (1958).
5) S.G.A. Alivisatos, *et al.* : *Ibid.*, **235**, 1742 (1960).
6) F. Schlenk, *et al*, : Ber., **71**, 1471 (1938).
7) B.M. Anderson, N.O. Kaplan : J. Biol. Chem., **234**, 1226 (1959).
8) L.W. Cunningham Jr., *et al.* : Biochem. Biophys. Res. Commun., 8, 243 (1962).
9) N.O. Kaplan, *et al.* : J. Biol. Chem., **194**, 579 (1952).
10) H.G. Windmueller, N.O. Kaplan : *Ibid.*, **236**, 2716 (1961).
11) M.R. Atkinson, R.K. Morton : Nature, **188**, 58 (1960).
12) J.G. Moffatt, H.G. Khorana : J. Am. Chem. Soc., **80**, 3756 (1958).



NMN-dAMP :	R=adenine,	R′=H
NMN-GMP :	R=guanine,	R′=OH
NMN-CMP :	R=cytosine,	R′=OH
NMN-UMP :	R=uracil,	R′=OH
NMN-TMP :	R=thymine,	R′=H

of NAD was first attempted by condensing NMN with AMP-NH_2, but NAD was not detected in the reaction mixture by paper partition chromatography (PPC), paper electrophoresis (PEP), and enzymatic determination[13] with yeast alcohol dehydrogenase. This is probably due to the intramolecular salt formation of the tertiary amine with the phosphoryl group in the molecule of NMN. Accordingly, NAD analogs were synthesized by condensing NMN with various naturally occurring nucleotides according to the DCC method. The NMN was prepared by the degradation of NAD, which was extracted from baker's yeast, with crude potato pyrophosphatase,[14] while the phosphatase activity of the latter was inhibited with sodium arsenate. This NMN was ascertained in advance to be free of NAD and proved to be *beta*-form[15] (of α- and β-NAD's only β-form shows coenzymatic activity) by measurement of its specific optical rotation. β-NMN was allowed to react with dAMP in the presence of DCC in aqueous pyridine at 20° (or 30°) for six days. The reaction mixture was chromatographed on Dowex-1 (formate-form), the fraction eluted with 0.01*N* formic acid (Fig. 1) was adsorbed on

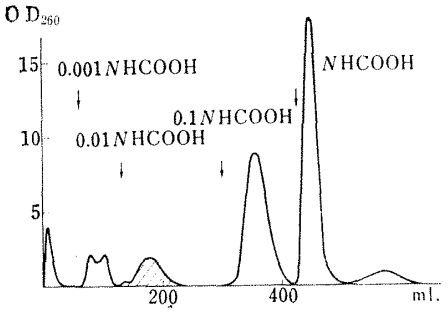


Fig. 1. NMN-dAMP

charcoal, and the solution obtained by eluting the charcoal with 50% ethanol containing 0.5% of aqueous ammonia was concentrated and the concentrate was found to contain only NMN-dAMP by the following tests. a) PEP (0.05*M* phosphate buffer, pH 8.0; all PEP's hereafter were conducted under the same conditions) gave only one ultraviolet absorbing spot showing the same migration value as that of NAD. b) PEP after reaction with potato pyrophosphatase exhibited two ultraviolet absorbing spots, the positions of which were in agreement with those of NMN and dAMP, respectively. The eluate of the spot corresponding to dAMP showed the same ultraviolet spectrum as that of this compound. c) Molar ratio between deoxyribose[16] and total phosphorus[17] was 1:2.28, the theoretical value being 1:2.00. d) No inorganic phosphate[17] was detected. The yield of NMN-dAMP based on NMN was 22 per cent. The concentration of NMN-dAMP in this solution was calculated from the content of the organic phosphorus,[17] and the solution showed $[\alpha]_D^{22}$ −20.5° (H_2O, c=1.22). The ammonium salt of NMN-dAMP was isolated as a hygroscopic white powder by addition of acetone to the concentrated

13) M.M. Ciotti, N.O. Kaplan : Methods in Enzymology, Vol. III, Academic Press, Inc., New York, 1957, p. 891.
14) A. Kornberg, W.E. Pricer, Jr. : J. Biol. Chem., **182**, 763 (1950).
15) N.O. Kaplan, *et al.* : J. Am. Chem. Soc., **77**, 815 (1955).
16) F.B. Seibert : J. Biol. Chem., **133**, 593 (1940).
17) R.J.L. Allen : Biochem. J., **34**, 858 (1940).

eluate. *Anal.* Calcd. for $C_{21}H_{26}N_7O_{13}P_2 \cdot NH_4 \cdot 8H_2O$: P, 7.67. Found : P, 7.67. With a view to preparing free NMN-dAMP from its ammonium salt, a solution of the salt was passed through a column of Dowex-50 (hydrogen-form), but it was found by PEP that the bond between adenine and deoxyribose was partially splitted, though NAD was stable in such a procedure.

β-NMN was condensed with GMP, CMP, UMP, and TMP in the presence of DCC as in the case of dAMP, the reaction mixtures were chromatographed on Dowex-1 (formate-form), and NMN-GMP, NMN-CMP, NMN-UMP, and NMN-TMP showing a single ultraviolet absorbing spot and the same migration value as NAD on PEP respectively were obtained as their ammonium salt solutions (Figs. 2~5), The structure of NMN-GMP was confirmed by splitting it with snake venom phosphodiesterase into its components, and NMN-UMP and NMN-TMP into their components with potato pyrophosphatase. Attempts were also made to synthesize NMN-dGMP and NMN-dCMP, but their poor yields prevented their isolation.

Determination of the organic phosphorus and measurement of the optical densities at 260 mμ of the five NAD analogs thus synthesized were performed, giving the following ε_{260} (pH 2) values; NMN-dAMP; 18.0×10^3, NMN-GMP; 15.0×10^3, NMN-CMP; 10.5×10^3, NMN-UMP; 13.3×10^3, NMN-TMP; 11.9×10^3.

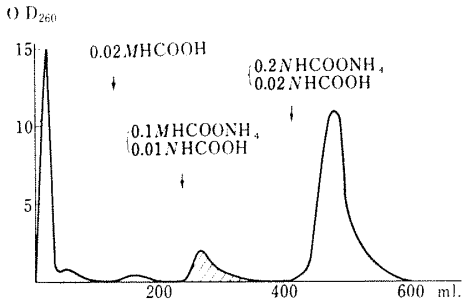


Fig. 2. NMN-GMP

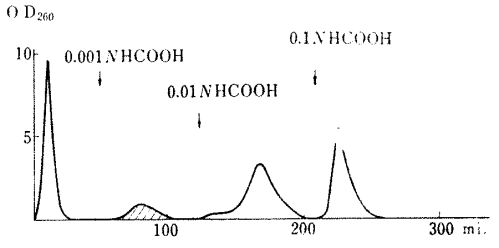


Fig. 3. NMN-CMP

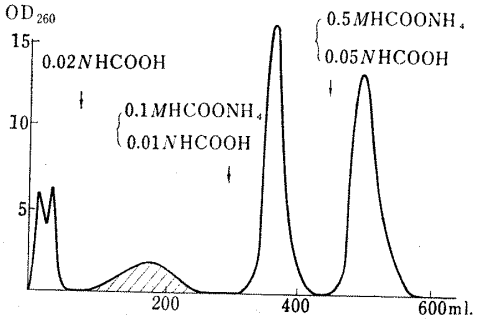


Fig. 4. NMN-UMP

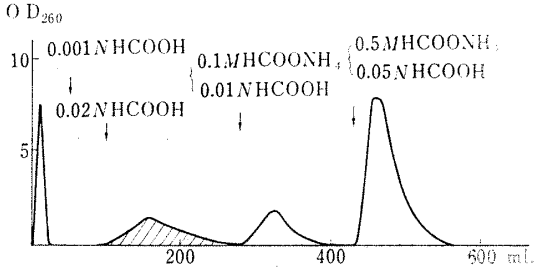


Fig. 5. NMN-TMP

Next, the coenzymatic activities of the analogs in the presence of dehydrogenases were compared with that of NAD to get further information about the functional groups of the natural coenzyme. NMN-dAMP, NMN-GMP, NMN-CMP, and NMN-TMP were reduced with the yeast alcohol dehydrogenase, respectively, when a new absorption maximum of dihydronicotinamide appeared at 340 mμ. The reaction rates of the analogs were followed by the increase in optical density at 340 mμ. In these cases, the molar concentrations of the analogs and NAD were the same and the enzyme was saturated with respect to NAD. As shown in Fig. 6, all the analogs reacted with much lower rates than NAD, especially NMN-UMP and NMN-TMP showed little

activity. Accordingly, the yeast alcohol dehydrogenase is very sensitive to the replacement of the adenosine moiety of NAD. In contrast, the analogs reacted as efficient coenzymes with the beef liver glucose dehydrogenase (Fig. 7), and it is of special interest to note that NMN-UMP reacted more rapidly than NAD. Keeping in mind the fact that the equilibrium of NAD+lactate $\rightleftharpoons$ NADH+pyruvate is markedly shifted to the NAD side,[18] NAD and the analogs were previously reduced with the beef liver glucose dehydrogenase, and the reaction rates of NADH analogs $\longrightarrow$ NAD analogs in the presence of the rabbit muscle lactic dehydrogenase were followed by the decrease in the optical density at 340 mμ (Fig. 8). With this system, too, the analogs showed considerable activity. The ratios of the reaction rates of the analogs to that of NAD are summarized in Table I.

Table I. Ratios of the Reaction Rates of the NAD Analogs to that of NAD in the Dehydrogenase Systems

Enzymes \ Analogs	NAD	NMN-dAMP	NMN-GMP	NMN-CMP	NMN-UMP	NMN-TMP
Alcohol Dehydrogenase	100	10	10	4	0	0
Glucose Dehydrogenase	100	90	95	90	125	35
Lactic Dehydrogenase	100	60	90	80	80	15

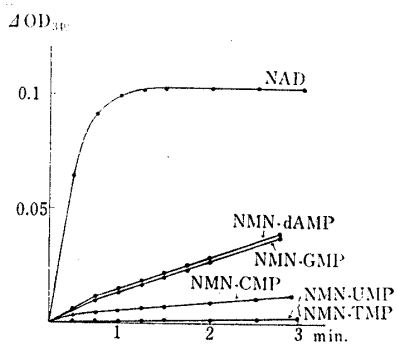


Fig. 6. Rate of Reaction with Alcohol Dehydrogenase

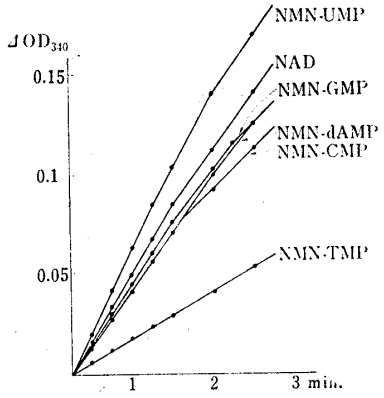


Fig. 7. Rate of Reaction with Glucose Dehydrogenase

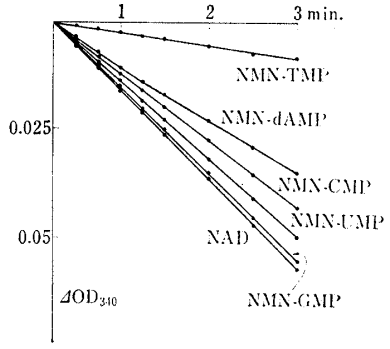


Fig. 8. Rate of Reaction with Lactic Dehydrogenase

An attempt was also made to examine the reaction rate of NAD plus each analog in the presence of the dehydrogenase. In this case, equal moles of NAD and the analog were used. With the yeast alcohol dehydrogenase, the amount of the enzyme was so small that except for NAD, every analog showed on detectable activity in the determination of their individual activities. Under these conditions, interestingly

18) A. Kornberg : Methods in Enzymology, Vol. 1, 1955, p. 441.

enough, the reaction rates were higher than that of NAD in the absence of the analogs (Fig. 9), namely, the analogs stimulated the reduction of NAD. The phenomenon may be explained like this; an electron could be transported enzymatically or non-enzymatically (depending on the oxidation-reduction potential) to the analogs *via* NADH, though it is uncertain whether the analogs are reduced or not. But obtaining a definite proof, further investigations are necessary. With the beef liver glucose dehydrogenase, NMN-dAMP or NMN-TMP did not affect the reaction rate of NAD (Fig. 10). With the rabbit muscle lactic dehydrogenase, neither reduced NMN-dAMP nor reduced NMN-TMP affected the oxidation rate of NADH (Fig. 11). Namely, the analogs that showed low activity with the dehydrogenases showed no inhibition of the reaction of NAD in the presence of the dehydrogenases.

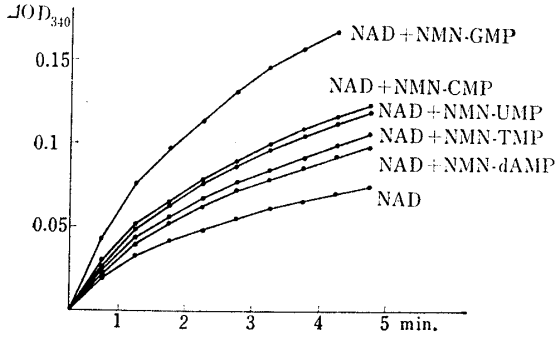


Fig. 9. Rate of Reaction of NAD Plus Analogs with Alcohol Dehydrogenase

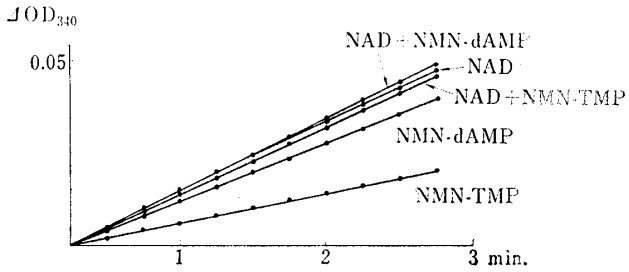


Fig. 10. Rate of Reaction of NAD Plus Analogs with Glucose Dehydrogenase

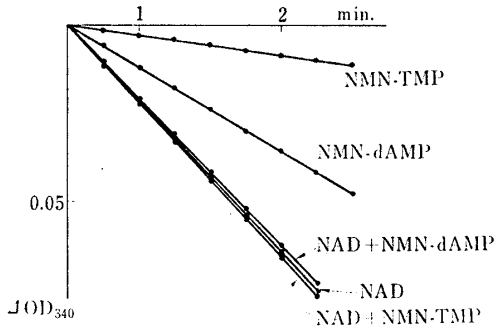


Fig. 11. Rate of Reaction of NAD Plus Analogs with Lactic Dehydrogenase

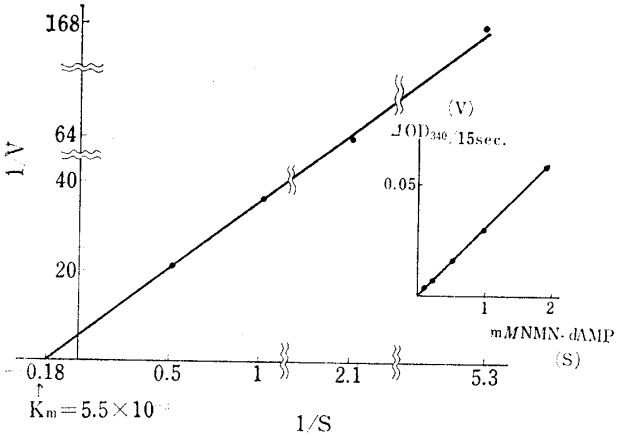


Fig. 12. Effect of NMN-dAMP Concentration on the Reaction Rate of Alcohol Dehydrogenase

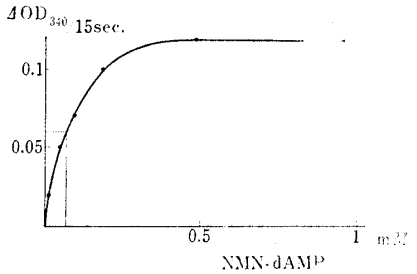


Fig. 13. Effect of NMN-dAMP Concentration on the Reaction Rate of Glucose Dehydrogenase

The Km value for NMN-dAMP with the yeast alcohol dehydrogenase was $5.5\times10^{-3}M$ (Fig. 12), that of NAD used as the control being $1.7\times10^{-4}M$. The Km value for NMN-dAMP with the beef liver glucose dehydrogenase was $6.8\times10^{-5}M$ (pH 7.6) (Fig. 13), that of NAD described in the literature[19] being below $10^{-5}M$.

Kaplan, *et al.*[2,7] have synthesized various 3-substituted pyridine analogs of NAD and reported that the amide group on the pyridine moiety of NAD is not essential for the coenzymatic function with dehydrogenases. The authors have synthesized the five analogs, in which the adenosine portion of NAD is replaced by other naturally occurring nucleosides. The investigation of the activities of these compounds in the presence of the three dehydrogenases showed many interesting facts and made it clear that the adenine base and the 2′-hydroxyl group in the adenosine moiety are not essential for NAD to exhibit the coenzymatic activity.

Experimental

NMN——A mixture of 847 mg. (1.25 m mol.) of NAD, 38 ml. of 0.5*M* potassium phosphate buffer (pH 7.7), 9.5 ml. of crude potato pyrophosphatase (Ethanol fraction II-2c by Kornberg and Pricer,[14] of which the activity was 264 units per ml., and further purification remarkably lowered its total activity, and 19 ml. of 0.5*M* Na_2HAsO_4 was made 95 ml. with H_2O. The mixture was incubated at 38° for 3 hr. Determination with yeast alcohol dehydrogenase[13] detected no measurable NAD. The reaction mixture was treated in accordance with the method by Kára and Sorm[20] to give a pure aqueous solution of NMN (858 μ moles, determined by the KCN addition reaction[13]). A 68% yield of NMN was obtained from NAD. A single UV absorbing spot was detected at the R_{AMP}*4 value of 0.64 by PEP (0.05*M* Na_2HPO_4, pH 8, 5.5 v./cm., 6 hr.) and at the value of 0.77 by PPC (BuOH-CH_3COOH-H_2O=5:2:3, by ascending method). No inorganic phosphorus[17] was detected. The nicotinamide riboside[13]/total phosphorus[17] value was 1.06, the calcd. value being 1.00. $[\alpha]_D^{24.5}$ −37.0° (c=0.74, H_2O), where the concentration was measured by the KCN addition reaction, the literature value[15] for β-NMN being $[\alpha]_D^{23}$ −38.3° (c=1, H_2O).

NMN-dAMP——14 mg. of NMN and 28 mg. of dAMP were dissolved in a mixture of 1.05 ml. of H_2O and 4.55 ml. of pyridine and to this was added 0.252 ml. of DCC. The mixture was allowed to stand overnight at 20°. An additional 0.252 ml. amount of DCC was added to the reaction mixture, which was left standing overnight at the same temperature. This procedure was repeated 5 times. The reaction mixture was poured into 20 ml. of H_2O and extracted 3 times with 4.2 ml. portions of $CHCl_3$. The aqueous layer was concentrated *in vacuo* and subjected to the column chromatography using 2 ml. of Dowex-1 X-8 (formate-form), 200~400 mesh (Fig. 1).

Table II.

Fraction	Solvent	Volume (ml.)	Total optical density (hereafter abbreviated to TOD) at 260 mμ	Components
1	H_2O	40	42	nicotinamide, small amounts of NMN and NMN-NMN[a]
2	0.001*N* HCOOH	100	73	NMN, adenine ?
3	0.01*N* HCOOH	150	90	NMN-dAMP
4	0.1*N* HCOOH	120	314	dAMP
5	1*N* HCOOH	70	315	dAMP-dAMP, unknown substances

a) NMN-NMN is the abbreviation of P^1, P^2 di-(nicotinamide riboside 5′-)pyrophosphate. Hereafter the same way of abbreviation as this was used.

255 mg. of NMN and 509 mg. of dAMP were allowed to react in aqueous pyridine at 30° for 6 days as described above. The reaction mixture was subjected to the chromatography using Dowex-1. 2L. of the fraction eluted with 0.01*N* HCOOH (TOD 2830, a 22% yield from NMN) was passed through a column of 3 g. of charcoal. The column was washed with H_2O and eluted with 120 ml. of 50% aqueous

*4 Ratio of the migration distance of a sample to that of adenosine 5′-phosphate.

19) H. J. Strecker : Methods in Enzymology, Vol. 1, 1955, p. 339.

20) J. Kára, F. Sorm : Collection Czech. Chem. Commun., **24**, 2471 (1959).

EtOH containing 0.5% of conc. NH_4OH. The eluate was concentrated to 3 ml. *in vacuo* and 340 ml. of Me_2CO was added. The mixture was left standing in a deep freezer overnight and the resulting precipitate was collected by centrifugation, dried over P_2O_5 at 0° to give 44 mg. of a hygroscopic white powder.

Degradation of NMN-dAMP with Potato Pyrophosphatase——To 0.5 ml. of an aqueous solution of the pure ammonium salt of NMN-dAMP (TOD 49) were added 0.1 ml. of 0.5*M* potassium phosphate buffer (pH 7), 0.2 ml. of the potato pyrophosphatase[14] (5.3 units) and 0.1 ml. of 0.5*M* Na_2HAsO_4. The mixture, after incubating at 37° for 1 hr., was adjusted to pH 2 with *N*HCl and passed through a column of 100 mg. of charcoal, which was eluted with 25% aqueous EtOH containing 1.5% of conc. NH_4OH. The eluate was concentrated *in vacuo* and subjected to PEP (0.05*M* phosphate buffer, pH 8, 13 v./cm., 2.5 hr.), whereby 2 spots of UV absorption corresponding to NMN and dAMP were detected. The spot corresponding to dAMP was eluted with 0.01*N* HCl. The UV absorption spectrum of the eluate was in accordance with that of dAMP.

Degradation of NMN-dAMP with Dowex-50 (hydrogen-form)——2 ml. of an aqueous solution of the pure ammonium salt of NMN-dAMP (TOD 278) was passed through a column of 0.2 ml. of Dowex-50 (hydrogen-form), which was washed with H_2O. The combined effluent and washings (7.6 ml.) were concentrated *in vacuo*. PEP (0.05*M* phosphate buffer, pH 8.0, 12 v./cm., 3 hr.) of the resulting solution detected two new UV absorbing spots at the starting point and closely near the (+) side of the starting material. The former was confirmed to be adenine in view of the fact that the eluate of Dowex-50 column with 3 ml. of 0.05*N* NH_4OH gave the substance of TOD 40 which was identical with adenine on PEP, UV absorption spectrum and its solubility in H_2O. The latter might be a substance which lacks the adenine moiety of NMN-dAMP.

NMN-GMP——21 mg. of NMN and 42 mg. of GMP were allowed to react with DCC in aqueous pyridine at 30° for 6 days as in the case of NMN-dAMP. The reaction mixture was fractionated using 3 ml. of Dowex-1 X-8 (formate-form), 200~400 mesh (Fig. 2).

Table III.

Fraction	Solvent	Volume (ml.)	TOD	Components
1	H_2O	130	283	NMN, NMN-NMN, nicotinamide
2	0.02*N* HCOOH	110	11	
3	0.01*N* HCOOH + 0.1*M* $HCOONH_4$	120	75	NMN-GMP
4	0.05*N* HCOOH + 0.5*M* $HCOONH_4$	200	630	GMP, GMP-GMP

Fraction 3 was treated with charcoal to give a solution of the ammonium salt which showed a single UV spot on PEP. An 11% yield (average) of NMN-GMP was obtained based on NMN.

Degradation of NMN-GMP with Snake Venom Phosphodiesterase——To 0.1 ml. of the pure ammonium salt solution of NMN-GMP (TOD 20) were added 1 ml. of 0.1*M* Tris buffer (pH 9.1), 0.1 ml. of 0.3*M* $MgCl_2$ and 0.1 ml. of the snake venom phosphodiesterase (20 γ protein), which was kindly supplied by Dr. S. Iwanaga, Faculty of Pharmaceutical Sciences, Kyoto University. The mixture was incubated at 37° for 30 min., adjusted to pH 2 with HCl and adsorbed on 100 mg. of charcoal. The charcoal was washed with H_2O and eluted with 50% aqueous EtOH containing 1% of conc. NH_4OH. The eluate was concentrated *in vacuo* and subjected to PEP (0.05*M* phosphate buffer, pH 8, 13 v./cm., 3 hr.), which detected two UV absorbing spots corresponding to NMN and GMP. The spectra of their eluates with 0.01*N* HCl were in accordance with those of NMN and GMP respectively, GMP-NMN being 1:0.9 (calculated from their molar absorption coefficient).

NMN-CMP——15.4 mg. of NMN and 29.4 mg. of CMP were allowed to react in aqueous pyridine with DCC at 30° for 6 days as in the case of NMN-dAMP. The reaction mixture was fractionated using 2 ml. of Dowex-1 X-8 (formate-form), 200~400 mesh (Fig. 3).

Table IV.

Fraction	Solvent	Volume (ml.)	TOD	Components
1	H_2O	30	81	NMN, NMN-NMN, nicotinamide
2	0.001*N* HCOOH	40	17	NMN-CMP
3	0.01*N* HCOOH	85	70	CMP
4	0.1*N* HCOOH	90	75	CMP-CMP

Fraction 2 was treated with charcoal to give a solution of the ammonium salt which showed a single UV absorbing spot on PEP. A 15% yield (average) of NMN-CMP was obtained based on NMN.

NMN-UMP——21 mg. of NMN and 42 mg. of UMP were allowed to react in aqueous pyridine with DCC at 30° for 6 days as in the case of NMN-dAMP. The reaction mixture was fractionated using 3 ml. of Dowex-1 X-8 (formate-form), 200~400 mesh (Fig. 4).

Table V.

Fraction	Solvent	Volume (ml.)	TOD	Components
1	H_2O	110	195	NMN, NMN-NMN, nicotinamide
2	0.02N HCOOH	280	202	NMN-UMP
3	0.01N HCOOH +0.1M $HCOONH_4$	130	507	UMP
4	0.05N HCOOH +0.5M $HCOONH_4$	150	545	UMP-UMP

Fraction 2 was treated with charcoal to give a solution of the ammonium salt which showed a single UV absorbing spot on PEP. A 37% yield (average) of NMN-UMP was obtained based on NMN.

Degradation of NMN-UMP with Potato Pyrophosphatase——A mixture of 0.5 ml. of the pure ammonium salt solution of NMN-UMP (TOD 66), 0.2 ml. of the potato pyrophosphatase (5.3 units),[14] 0.1 ml. of 0.5M Na_2HAsO_4 and 0.1 ml. of 0.5M potassium phosphate buffer (pH 7) was incubated at 37° for 1 hr., adjusted to pH 2 with N HCl and adsorbed on charcoal, which was eluted with 50% aq. EtOH containing 1% of conc. NH_4OH. The eluate was concentrated *in vacuo* and subjected to PEP (pH 8), which showed spots corresponding to NMN and UMP besides the spot of the starting material. The new spots were eluted separately with 0.01N HCl. The spectra of the eluates were in accordance with those of NMN and UMP respectively, NMN-UMP being 1:1.1 (calculated from their optical densities at 260 mμ).

NMN-TMP——10 mg. of NMN and 20 mg. of TMP were allowed to react in aqueous pyridine with DCC at 30° for 6 days as in the case of NMN-dAMP. The reaction mixture was fractionated using 2 ml. of Dowex-1 (formate-form), 200~400 mesh (Fig. 5).

Table VI.

Fraction	Solvent	Volume (ml.)	TOD	Components
1	H_2O	30	85	NMN, NMN-NMN, nicotinamide
2	0.001N HCOOH	200	None	
3	0.02N HCOOH	200	110	NMN-TMP
4	0.01N HCOOH +0.1M $HCOONH_4$	170	75	TMP
5	0.05N HCOOH +0.5M $HCOONH_4$	200	330	TMP-TMP

Fraction 3 was treated with charcoal to give a solution of the ammonium salt which showed a single UV absorbing spot on PEP. A 32% yield (average) of NMN-TMP was obtained based on NMN.

Degradation of NMN-TMP with Potato Pyrophosphatase——To 0.5 ml. of an aqueous solution of the pure ammonium salt of NMN-TMP (TOD ca. 30) were added 0.1 ml. of 0.5M potassium phosphate buffer (pH 7), 0.2 ml. of the potato pyrophosphatase[14] (5.3 units) and 0.1 ml. of 0.5M Na_2HAsO_4. The mixture was incubated at 37° for 1 hr., adjusted to pH 2 with HCl and adsorbed on charcoal, which was eluted with 50% aq. EtOH containing 1% of conc. NH_4OH. The eluate was concentrated *in vacuo* and subjected to PEP (pH 8) which detected two UV absorbing spots corresponding to NMN and TMP. The spots were respectively eluted with 0.01N HCl and the optical densities were measured at 260 mμ, TMP-NMN being 1:1.02.

Reaction of the Analogs with Yeast Alcohol Dehydrogenase[2,13]——The reaction mixture (3 ml.) contained 2.7 ml. of 0.5M EtOH in 0.1M sodium pyrophosphate (pH 10), 0.3 μmole of NAD analog and 0.1 ml. of the enzyme (purchased from Sigma Chemical Co.) solution (1000 units). The optical density change in this system at 340 mμ was measured every 15 seconds (Fig. 6).

Reaction of the Analogs with Beef Liver Glucose Dehydrogenase[21]——The reaction mixture (1.1 ml.) contained 0.7 ml. of 0.05M potassium phosphate buffer (pH 7.6), 0.1 ml. of 1M glucose, 0.2 ml. of the enzyme solution (the enzyme purified to Step 7 by Strecker's methed,[21] which was kindly supplied by Prof. Hayaishi and Dr. Nishizuka) (68 units) and 0.1 μmole of NAD analog. The optical density change in this system at 340 mμ was measured every 15 seconds (Fig. 7).

21) H. J. Strecker : Methods in Enzymology, Vol, 1, 1955, p. 335.

Reaction of the Analogs with Rabbit Muscle Lactic Dehydrogenase[18]——The reaction mixture contained 2.6 ml. of 0.05*M* potassium phosphate buffer, 0.2 ml. of 1*M* glucose, 0.1 μmole of NAD analog and 0.2 ml. of the glucose dehydrogenase (mentioned above) (68 units) in a total volume of 3.1 ml. and was allowed to react at 20° until the optical density change at 340 mμ was not measured (for about 2 hr.). The reaction mixture was heated at 100° for 1 min. to inactivate the glucose dehydrogenase. To this were added 0.1 ml. of 0.01*M* sodium pyruvate and 0.02 ml. of the lactic dehydrogenase (Sigma Chemical Co.) (0.02 unit). The optical density change in this system at 340 mμ was measured every 15 seconds (Fig. 8).

Influence of the NAD Analogs on the Reaction Rate of NAD——a) With yeast alcohol dehydrogenase : The reaction mixture contained 2.5 ml. of 0.5*M* EtOH in 0.1*M* sodium pyrophosphate (pH 10), 0.3 ml. of 1 m*M* NAD, 0.1 ml. of 3 m*M* analog and 0.1 ml. of the yeast alcohol dehydrogenase (100 units) in a total volume of 3 ml. The optical density change at 340 mμ was measured every 30 seconds. In this case, the enzyme amount used was only 100 units with which NAD was reduced, but the NAD analog was not. The ratios of the reaction rates of NAD plus the analog to that of NAD (100) were as follows : NAD+NMN-GMP 215, NAD+NMN-CMP 150, NAD+NMN-UMP 145, NAD+NMN-TMP 130, NAD+NMN-dAMP 120 (Fig. 9).

b) With beef liver glucose dehydrogenase : The reaction mixture (3.0 ml.) contained 2.2 ml. of 0.05*M* potassium phosphate buffer (pH 7.6), 0.2 ml. of 1*M* glucose, 0.2 ml. of the glucose dehydrogenase (mentioned above) (60 units), 0.3 ml. of 1 m*M* NAD and 0.1 ml. of 3 m*M* NAD analog. The optical density change in this system at 340 mμ was measured every 15 seconds (Fig. 10).

c) With rabbit muscle lactic dehydrogenase : A mixture of 2.6 ml. of 0.05*M* potassium phosphate buffer (pH 7.6), 0.2 ml. of 1*M* glucose, 0.1 ml. of 3 m*M* NAD, 0.1 ml. of 3 m*M* NAD analog and 0.1 ml. of the glucose dehydrogenase (mentioned above) was allowed to react at 20° for 2 hr. The reaction mixture was heated at 100° for 1 min. to inactivate the enzyme. To this were added 0.1 ml. of 0.01*M* sodium pyruvate and 0.01 ml. of the muscle lactic dehydrogenase (mentioned above) (0.02 unit). The optical density change at 340 mμ was measured every 15 seconds (Fig. 11).

Michaelis Constant (Km) for NMN-dAMP with Dehydrogenases——a) With yeast alcohol dehydrogenase : The reaction mixture contained 0.7 ml. of 0.5*M* EtOH in 0.1*M* sodium pyrophosphate (pH 10) 0.1 ml. of the yeast alcohol dehydrogenase (100 units) and 0.1~1.9 μmoles of NMN-dAMP in a total volume of 1.0 ml. and the Km value was calculated from the Lineweaver-Burk plott[22] (Fig. 12).

b) With beaf liver glucose dehydrogenase : The reaction mixture (1.0 ml.) contained 0.7 ml. of 0.05*M* potassium phosphate buffer (pH 7.6), 0.1 ml. of 1*M* glucose, 0.05 ml. of the enzyme (mentioned above) and 0.1~1.00 μmole of NMN-dAMP, and the Km value was given by the NMN-dAMP concentration (S) at half-maximum velocity (Fig. 13).

The authors are grateful to Dr. S. Tatsuoka, Director of the Laboratories, for his encouragement throughout the present work. Thanks are also due to Prof. O. Hayaishi and Dr. Y. Nishizuka of the Department of Medical Chemistry, Faculty of Medicine, Kyoto University, for their supply of dehydrogenases and for their advice in enzymatic measurements.

Summary

Five NAD analogs have been synthesized in which the adenosine portion of NAD was replaced by deoxyadenosine, guanosine, cytidine, uridine and thymidine, and their comparative coenzymatic activity with that of NAD was investigated with yeast alcohol dehydrogenase, beef liver glucose dehydrogenase and rabbit muscle lactic dehydrogenase. The influence of the analogs on the reaction rate of NAD was also studied.

(Received November 8, 1962)

22) H. Lineweaver, D. Burk : J. Am. Chem. Soc., **56**, 658 (1934).