Production of Nucleic Acid-Related Substances by Fermentative Processes

Part XVII. Production of NAD and Nicotinic Acid Mononucleotide with *Brevibacterium ammoniagenes*

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Received May 13, 1968

A new preparative method of NAD was provided by the finding that a large amount of NAD accumulated with AMP, ADP and ATP in the culture broth when *Brevibacterium ammoniagenes* ATCC 6872 was incubated in the medium containing adenine and nicotinic acid or nicotinamide. A large amount of nicotinic acid mononucleotide (NaMN) with a small amount of NAD accumulated when nicotinic acid or nicotinamide was singly added.

NAD was isolated from the culture broth by ion exchange chromatography, and identified by paperchromatography, ultraviolet and infrared spectra, analyses of ribose and phosphate, and reduction by alcohol dehydrogenase from yeast.

An example of the time course of the fermentation for the production of NAD was shown.

The biosynthetic pathway of NAD from nicotinic acid or nicotinamide in the organism was discussed.

The amount of NAD accumulated reached to several mg per ml.

NAD has been prepared from baker's yeast from which NAD has been extracted with hot water and purified from the extracts. The accumulation of a large amount of NAD in culture broth has not been reported except the paper by Hayano *et al.*¹⁾ which described the leakage of a small amount of NAD from yeast.

Previously, it was reported that *Brevibacterium ammoniagenes* produced large amounts of IMP, AMP, ADP, ATP, GMP, GDP, and GTP from the corresponding purine bases.^{2,3)}

Now, it was found that the same organism

produced a large amount of NAD when it was cultured in the presence of adenine and nicotinic acid or nicotinamide. The production of nicotinic acid mononucleotide (NaMN) was also noted when it was cultured with nicotinic acid or nicotinamide.

METHODS

Microorganism and cultivation. *Brevibacterium ammoniagenes* ATCC 6872 was used. The cultivation of the organism was carried out according to the methods described in the previous paper.³⁾

Analytical methods. The quantities of NAD, NaMN, AMP, ADP and ATP were determined by the optical density of the extracts of their spots on paperchromatograms. The paperchromatography was performed on Toyo Roshi No. 51A paper using the following solvent system; isobutyric acid-acetic acid-1 N ammonia (10:1:5). The *R_f* values of adenine, nicotinamide, nicotinic acid, 5'-AMP, NAD, ADP,

1) K. Hayano, I. Takebe and K. Kitahara, *Hakko to Taisha*, **9**, 37 (1964).

2) T. Nara, M. Misawa and S. Kinoshita, *Agr. Biol. Chem.*, **32**, 561 (1968).

3) H. Tanaka, Z. Sato, K. Nakayama and S. Kinoshita, *ibid.*, **32**, 721 (1968).

ATP and NaMN were 0.90, 0.90, 0.80, 0.60, 0.54, 0.48, 0.37 and 0.38, respectively. Ribose and phosphate contents in NAD and NaMN were estimated by orcinol method⁴⁾ and Allen's method,⁵⁾ respectively. Reduction of NAD by sodium hydrosulfite was carried out according to the procedure of LePage⁶⁾ modified from that of Warburg and Christian.⁷⁾ Reduction by alcohol dehydrogenase from yeast was carried out according to the procedure of Ciotti and Kaplan.⁸⁾ Alcohol dehydrogenase from yeast was purchased from Sigma Chemical Co. The last procedure could be applied to the estimation of NAD in culture broth.

RESULTS

Isolation and identification of NAD

When *B. ammoniagenes* ATCC 6872 was incubated in the medium containing adenine and nicotinic acid or nicotinamide, a ultraviolet absorbing spot besides those of AMP, ADP and ATP was noted on a paperchromatogram. The *R_f* value of the spot was 0.48 and identical with that of NAD (Fig. 1). The formation of the ultraviolet absorbing

substance was dependent on the presence of nicotinic acid or nicotinamide in the medium. After treating with charcoal, the culture filtrate was chromatographed on Dowex $-1 \times 2 (\text{Cl}^-)$ according to the method described in the previous paper.³⁾ As shown in Fig. 2 the substance was eluted with dilute hydrochloric acid following the elution of nicotin-

FIG. 2. Column Chromatography of Culture Filtrate on Dowex- $1 \times 2 (\text{Cl}^-)$ after Treatment with Charcoal.

FIG. 1. Paper Chromatography of Culture Filtrate.

Solvent: Isobutyric acid-acetic acid-1 N ammonia (10 : 1 : 5).

4) M. L. Morse and C. E. Carter, *J. Bacteriol.*, **58**, 317 (1949).

5) R. J. L. Allen, *Biochem. J.*, **34**, 858 (1940).

6) O. Warburg and W. Christian, *Biochem. Z.*, **287**, 291 (1936).

7) G. A. LePage, *J. Biol. Chem.*, **168**, 623 (1947).

8) "Methods in Enzymology," Vol. III, ed. by S. P. Colowick and N. O. Kaplan, Academic Press Inc., New York, N. Y. 1957, p. 891.

amide and adenine with water. AMP, ADP and ATP followed the substance. In the paperchromatography with 4 different solvent systems the R_f values of the substance were coincided with those of authentic NAD as shown in Table I. The molar ratio of the substance (as NAD) to ribose to phosphate was 1.0:2.1:1.9 (Table II).

The white powder (3.52 g, purity 89%) was obtained from the culture filtrate by the procedure of Shin *et al.*⁹⁾ (Fig. 3). The ultraviolet absorption spectrum of the preparation of NAD was identical with that of an authentic sample of NAD. When the preparation was reduced with sodium hydrosulfite or alcohol dehydrogenase from yeast, the characteristic absorption peak of reduced

TABLE I. PAPERCHROMATOGRAPHY OF NAD AND NaMN FROM CULTURE FILTRATE

Substances	R_f values			
	a	b	c	d
(1) NAD, authentic	0.55	0.88	0.10	0.54
(2) NAD, from culture filtrate	0.54	0.88	0.10	0.54
(3) 1+2	0.54	0.88	0.11	0.53
(4) NMN, authentic	0.52	0.82	0.30	0.46
(5) NaMN, from culture filtrate	0.38	0.87	0.32	0.34

a: isobutyric acid, acetic acid, 1 N ammonia (10:1:5).

b: 60% acetone.

c: *n*-butanol, 50% acetone (1:1).

d: isobutyric acid, water, ammonia water (100:58:2).

TABLE II. ANALYTICAL DATA FOR NAD AND NaMN CULTURE FILTRATE

Samples	Molar ratios		
		Ribose	Phosphate
NAD, authentic	(1.0)	1.9	2.1
NAD, from culture filtrate	(1.0)	2.1	1.9
NaMN, from culture filtrate	(1.0)*	0.84	0.78

* The concentration of NaMN was estimated from the molar extinction coefficient at 265 m μ ; 4400.¹³⁾

9) M. Shin, Y. Hamano and K. Okunuki, *J. Biochem.*, **48**, 579 (1960).

FIG. 3. Isolation of NAD from Culture Filtrate.

FIG. 4. UV-Absorption Spectra of NAD before and after Reduction by Sodium Hydrosulfite or Alcohol Dehydrogenase from Yeast.

---- before reduction.
— after reduction.

FIG. 5. IR-Spectra of NAD from Culture Filtrate.

---- NAD, authentic.
 — NAD, from culture filtrate.

FIG. 6. Paper Chromatography of Culture Filtrate.

Solvent: Isobutyric acid-acetic acid-1 N ammonia (10:1:5).

NAD at 340 m μ was noted as shown in Fig. 4. The infrared absorption spectrum was identical with that of an authentic sample of NAD (Fig. 5).

Identification of NaMN

With the medium containing nicotinic acid or nicotinamide, *B. ammoniagenes* produced a substance having the *R_f* value, 0.30 with a small amount of NAD (Fig. 6). The substance was separated from the culture filtrate by column chromatography on Dowex -1×2

(Cl⁻). As shown in Fig. 7 the substance was eluted just after AMP. The substance was discriminated from ATP in the ultraviolet absorption spectrum and from nicotinamide mononucleotide (NMN) in the *R_f* value (Table I). Its ultraviolet absorption spectra in alkaline, neutral and acidic solutions were identical with those of NaMN¹⁰⁾ (Fig. 8).

10) Y. Nishizuka and O. Hayaishi, *J. Biol. Chem.*, **238**, 3369 (1963).

FIG. 7. Column Chromatography of Culture Filtrate on Dowex-1 $\times$ 2 (Cl⁻) after Treatment with Charcoal.

FIG. 8. UV-Absorption Spectra of NaMN from Culture Filtrate.

— · — alkaline (pH ?).
 — neutral
 - - - acidic (pH ?).

The molar ratio of this substance (as NaMN) to ribose to phosphate was 1.00:0.84:0.78 (Table II).

Accumulation of NAD and NaMN

Accumulation of nucleotides in the cultured broth incubated with nicotinic acid or nicotinamide was compared with that in the cultured broth incubated with both adenine and nicotinic acid or nicotinamide. When either nicotinic acid or nicotinamide was

TABLE III. NAD AND NaMN ACCUMULATION IN ADDITION OF NICOTINIC ACID, NICOTINAMIDE AND ADENINE

Addition* (μ moles/ml)	Accumulation, μ moles/ml				
	NAD	NaMN	ATP	ADP	AMP
Nicotinic acid (16.2)	trace	7.0	trace	trace	trace
Nicotinamide (16.4)	1.5	6.7	trace	trace	trace
Nicotinic acid (16.2)+adenine (14.8)	2.9	trace	1.3	2.1	4.0
Nicotinamide (16.2)+adenine (14.8)	2.8	trace	1.5	2.2	3.5

* Each was added to 48 hr age culture.

added to the medium, a large amount of NaMN and a small amount of NAD accumulated. On the other hand, when adenine besides nicotinic acid or nicotinamide was added, the accumulation of NaMN reduced, while the accumulation of NAD increased accompanying the accumulation of AMP, ADP and ATP (Table III).

FIG. 9. Time Course of NAD Production.

Culture medium: glucose 10%, urea 0.6%, KH_2PO_4 1.0%, K_2HPO_4 1.0%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.0%, meat ext. 0.25%, biotin 30 $\mu\text{g/liter}$, Ca-D-pantothenate 12 mg/liter, thiamine hydrochloride 10 mg/liter. Nicotinamide and adenine were added at 48 hr.

—●— NAD, —△— AMP,
 - - -○- ATP, - - -○- growth,
 - - -□- ADP, - - -×- pH.

Time course of fermentation

An example of the time course of fermentation was shown in Fig. 9. Accumulation of NAD, AMP, ADP and ATP began just after the addition of adenine and nicotinamide to 2 days culture. Accumulation of NAD continued until 5th day when the concentration of NAD in the culture broth reached to 2.3 mg/ml.

DISCUSSION

It was found that a large amount of NAD accumulated with AMP, ADP and ATP in the culture broth when *B. ammoniagenes* ATCC 6872 was incubated in the medium containing adenine and nicotinic acid or nicotinamide. A large amount of NaMN with a small amount of NAD accumulated when nicotinic acid or nicotinamide was singly added. The amount of NAD accumulated reaches several mg per ml under a certain culture condition and this process provides a very effective method for the preparation of NAD.

Two pathways, one via NMN^{11,12)} and the other via NaMN,^{13~15)} are known in the biosynthesis of NAD from nicotinic acid or nicotinamide. The facts, that NaMN accumulated in the culture medium of *B. ammoniagenes* grown with nicotinamide and that nicotinic acid was detected on the paper-chromatogram of the culture filtrate, support the salvage biosynthetic pathway via NaMN in the organism as in *Escherichia coli*¹⁵⁾ (Fig. 10).

Imsande^{16~18)} reported that the NaMN pyrophosphorylase activity in *Bacillus subtilis* was dependent upon the presence of a nucleo-

FIG. 10. Estimated Pathway of NAD Biosynthesis from Nicotinic Acid or Nicotinamide in *Brevibacterium ammoniagenes*.

side-triphosphate,¹⁷⁾ and the repression-derepression control mechanism and feedback inhibition by NAD might not be widespread in nature, while pyridine nucleotide formation was controlled by the intracellular concentration of ATP.^{16,18)} Recently, Honjo *et al.*¹⁹⁾ reported that ATP was stoichiometrically utilized in NaMN synthesis from nicotinic acid and 5-phosphoribosyl-1-pyrophosphate as energy source of the reaction. Considering those literatures, NaMN and NAD accumulation by *B. ammoniagenes* will be reasonably explained as follows. Since *B. ammoniagenes* accumulated ATP a little without addition of adenine, NAD is synthesized utilizing this ATP in single addition of nicotinic acid or nicotinamide. Further addition of adenine besides nicotinic acid or nicotinamide led to the accumulation of a large amount of NAD. It will be speculated that the synthesis of a large amount of NaMN in single addition of nicotinic acid or nicotinamide might occurred utilizing GTP which accumulated without addition of guanine.³⁾ The replacement of ATP by GTP in NaMN synthesis was shown in *B. subtilis* by Imsande.¹⁷⁾ To prove the speculation, further experiments are required.

11) A. Kornberg, *J. Biol. Chem.*, **182**, 779 (1950).

12) J. W. Rowen and A. Kornberg, *ibid.*, **193**, 497 (1951).

13) J. Preiss and P. Handler, *ibid.*, **233**, 488 (1958).

14) J. Preiss and P. Handler, *ibid.*, **233**, 493 (1958).

15) J. Imsande, *ibid.*, **236**, 1494 (1961).

16) J. Imsande, *Biochim. Biophys. Acta*, **82**, 445 (1964).

17) J. Imsande, *ibid.*, **85**, 255 (1964).

18) J. Imsande and L. S. Prestidge, *ibid.*, **85**, 265 (1964).

19) T. Honjo, S. Nakamura, Y. Nishizuka and O. Hayaishi, *Biochem. Biophys. Res. Commun.*, **25**, 199 (1966).