Note

Production of Nicotinamide Adenine
Dinucleotide Glycohydrolases
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Nicotinamide adenine dinucleotide glycohydrolases (NADases, EC 3.2.2.5) hydrolyze the bond between nicotinamide and ribose moiety in NAD^+ to yield nicotinamide and ADP-ribose. They have been found in higher animals and in microorganisms, purified and investigated in detail.^{1,2)} Most of mammalian NADases are associated with microsomal particles, sensitive to inhibition by nicotinamide, and able to catalyze the transfer of ADP-ribose moiety of NAD^+ to certain acceptors such as pyridine derivatives or proteins resulting the formation of NAD^+ analogues or ADP-ribosylated proteins.^{3,4)} In contrast, microbial enzymes are soluble, rather resistant to nicotinamide inhibition, and unable to catalyze ADP-ribose transfer.⁵⁾ Bacterial enzymes so far known are heat stable and usually accompanied with heat labile endogenous inhibitors, which makes them designated as "heat activated" enzymes.⁶⁾ Recently some bacterial exotoxins such as diphtheria toxin,⁷⁾ cholera enterotoxin,⁸⁾ exotoxin A of *Pseudomonas aeruginosa*,⁹⁾ and heat labile enterotoxin of *Escherichia coli*¹⁰⁾ have been demonstrated to exert their toxic effects on host cells through ADP-ribose transfer by NADases commonly present in their molecules and have attracted a great attention.

We have investigated whether nontoxic, at least toxicity is not yet known, bacteria produce non "heat activated" NADases as seen in the above toxin molecules and whether they excrete the enzymes into surrounding media.

Bacterial strains examined and their cultures were as follows: Seventy five strains of Group I were obtained from the IAM Culture Collections, the University of Tokyo, and 100 strains of Group II were new isolates from soil, which covered a wide variety of gram positive and negative bacteria. They were inocubated to a medium (pH 7.0) containing 3% Daigo polypepton and 0.5% Difco yeast extract and cultured for 2 days at 30°C with shaking. Ninety strains of Group III and 30 strains of Group IV were new isolates from sea water with Eiken trypto soy agar and Eiken thiosulfate-citrate-bile-sucrose agar, respectively. Most of the former were halophilic *Pseudomonas*

and the latter were halophilic *Vibrio*. They were cultured in the similar manner as the cases of bacteria of Groups I and II except that a mixture of sea water and deionized water (50:50) was used. One hundred and fifty three strains of Group V were actinomycetes newly isolated from soil. They were cultured in 0.05 M phosphate buffer (pH 6.0) containing 2% lactose, 0.5% Kyokuto peptone, 0.1% Difco yeast extract, and 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ for 2 days at 30°C with shaking. The resulting culture broths or their supernatant fluids obtained by removing cells with centrifugation at $8000 \times g$ for 10 min were used as samples for NADase assay. Twenty μl of culture broth or supernatant was mixed with 20 μl of 2 mM NAD^+ -0.05 M phosphate buffer, pH 7.4, and the mixture was incubated at 37°C for 2 hr. One drop of 10% $\text{CNBr-H}_2\text{O}$ and the same volume of 1.5% tridrine-95% EtOH were added to the mixture successively to detect nicotinamide or nicotinic acid liberated from NAD^+ by strong orange or red coloration.

Table I shows the results of this screening tests on the bacteria of Group I. Bacteria having an activity of liberating nicotinamide or nicotinic acid from NAD^+ were about 20% of this group. The activity seems to be distributed most frequently in enteric gram negative rods

TABLE I. LIBERATION OF NICOTINAMIDE OR
NICOTINIC ACID FROM NAD^+
BY BACTERIA OF GROUP I
(IAM CULTURE COLLECTIONS).

Group	No. tested	No. positive	
		Culture broth	Supernatant
<i>Pseudomonas</i>	10	1	1
<i>Xanthomonas</i>	2	0	0
<i>Azotobacter</i>	1	0	0
<i>Alcaligenes</i>	2	0	0
<i>Escherichia</i>	5	2	0
<i>Enterobacter</i>	3	2	0
<i>Serratia</i>	3	1	0
<i>Proteus</i>	3	1	0
<i>Erwinia</i>	2	1	0
<i>Aeromonas</i>	2	1	0
<i>Chromobacterium</i>	2	0	0
<i>Flavobacterium</i>	2	1	0
<i>Micrococcus</i>	5	0	0
<i>Staphylococcus</i>	3	0	0
<i>Bacillus</i>	8	1	1
<i>Corynebacterium</i>	2	0	0
<i>Arthrobacter</i>	2	0	0
<i>Brevibacterium</i>	5	1	0
<i>Mycobacterium</i>	2	0	0
<i>Nocardia</i>	4	1	0
Others	7	1	0
Total	75	14	2

TABLE II. LIBERATION OF NICOTINAMIDE OR NICOTINIC ACID FROM NAD⁺ BY BACTERIA OF GROUPS II TO V

Group	No. tested	No. positive ^a	
		Culture broth	Supernatant
II	100	10 (10)	3 (3)
III	90	41 (46)	4 (4)
IV	30	25 (83)	1 (3)
V	153	43 (28)	30 (20)

^a Numbers in parentheses are percentages.

and, in almost all the cases, associated with bacterial cells.

Table II shows the results of the tests of the bacteria of Groups II to V. It was clearly demonstrated that nicotinamide or nicotinic acid liberating activity was distributed in soil bacteria, but its frequency was far below that in sea water bacteria. Many of halophilic *Pseudomonas* and most of halophilic *Vibrio* decomposed NAD⁺. This fact reminded us of NADase-active toxin production by *Ps. aeruginosa*⁹⁾ and *V. cholerae*.⁸⁾ None of the bacteria examined here, however, was *Ps. aeruginosa* or *V. cholerae* (data not shown) and the activity was absent in supernatants of the cultures unlike such toxins. The activity was also found in actinomycetes and in contrast to the cases of soil and sea water bacteria, it was excreted out of cells.

Further analysis of decomposition products of NAD⁺ was performed by using thin layer chromatography. Twenty μ l of culture broth showing nicotinamide or nicotinic acid liberating activity was mixed with 20 μ l of 2 mM NAD⁺-0.05 M phosphate buffer (pH 7.4). The mixture was incubated at 37°C for 5 hr and centrifuged at 8000 $\times$ g for 10 min. Two μ l of the supernatant obtained was applied to a cellulose plastic plate (E. Merck, Darmstadt Germany) and developed with isobutyric acid-28% NH₃-water (66:1.7:33) or with 95% EtOH-1 M ammonium acetate (7:3) for 7 hr. Authentic NAD⁺, NADH, NMN⁺, nicotinamide, nicotinic acid, ADP-ribose, ADP, AMP, adenosine, and adenine were used as standards. Adenine and its derivatives were detected by UV absorption (λ , 253.6 nm). NAD⁺, NADH, and NMN⁺ were detected by light blue fluorescence ($\lambda_{\max}$, 350 nm) given when they were exposed to the vapor of methylethylketone-28% NH₃ (1:1) for an hour and then irradiated by UV light (λ , 253.6). Nicotinamide and nicotinic acid were detected by orange or yellow coloration given when they were exposed to the vapor of 5% CNBr-H₂O for an hour followed by a spray of 2% *p*-aminobenzoic acid-H₂O.

The results are shown in Table III. When ADP-ribose was detected together with nicotinamide or nicotinic acid as the reaction product, the bacterium examined was judged as an NADase producer. According to this

TABLE III. SPECTRUM OF DECOMPOSITION PRODUCTS OF NAD⁺ BY CULTURE BROTHS OF BACTERIA OF EACH GROUP ANALYZED BY THIN LAYER CHROMATOGRAPHY.^a

	Group				
	I	II	III	IV	V
No. tested	14	10	41	25	43
No. positive					
Nicotinamide	7	7	41	25	13
Nicotinic acid	8	3	30	19	41
NMN	1	3	0	0	0
ADP-ribose	2	1	0	0	29
ADP	0	0	1	0	0
Adenosine	0	0	0	7	0
Unknown compounds ^b					
X	4	6	34	18	3
Y	0	0	5	1	0
Z	0	0	0	0	12

^a Two solvent systems were used for development. The first one was isobutyric acid-28% NH₃-water (66:1.7:33) and the second one was 95% EtOH-1 M ammonium acetate (7:3).

^b These compounds were detected by UV absorption (λ , 253.6 nm). The *R_f* values for X, Y and Z in the first solvent system were 0.61, 0.53 and 0.15 respectively and in the second solvent system, 0.52, 0.54 and 0.14, respectively.

criterion, a few strains of the bacteria of Group I (*Nocardia pseudosporangefera* and *Flavobacterium ferrugineum*) and soil bacteria, none of sea water bacteria, and many of actinomycetes were designated as NADase producers. In some cases when nicotinamide or nicotinic acid was detected but ADP-ribose was not detected, NADase may probably be absent in the bacteria. NAD pyrophosphatase in combination with NMN glycohydrolase could decompose NAD⁺ to produce nicotinamide, and nicotinic acid being produced from nicotinamide deaminase. Most of sea water bacteria and many of actinomycetes could not be proved to be NADase producers. They produced ADP, adenosine, and some unidentified compounds (shown in Table III) instead of ADP-ribose.

In most cases, the major product other than ADP-ribose was nicotinic acid in NAD⁺ decomposition by culture broths of actinomycetes, but it was nicotinamide in that by their culture supernatants (shown in Tables III and IV). Nicotinic acid was also produced from nicotinamide both by culture broths and washed cells of them, but not by supernatants (data not shown), which suggests that many of actinomycetes possess nicotinamide deaminase in their cells in addition to extracellular NADase.

Heat stability of the NADases obtained here was varied, but none of their activities increased by heat treatment.

TABLE IV. SPECTRUM OF DECOMPOSITION PRODUCTS OF NAD⁺ BY CULTURE SUPERNATANTS OF BACTERIA OF EACH GROUP ANALYZED BY THIN LAYER CHROMATOGRAPHY.^a

	Group				
	I	II	III	IV	V
No. tested	2	3	4	1	30
No. positive					
Nicotinamide	2	3	4	1	29
Nicotinic acid	0	0	0	0	1
ADP-ribose	1	1	0	0	24
Unknown compound Z ^b	0	0	0	0	8

^a Solvent systems used were the same as in Table III.

^b This is the same as in Table III.

It remains to be elucidated whether the NAD⁺ decomposition by most of sea water bacteria depends on intracellular NADase, and if so, whether such enzyme can be excreted out of cells under certain conditions, and whether the properties of such enzyme resemble that of toxin-NADases produced by the related bacteria. Physiological significance of NAD⁺ decomposition by extracellular NADases of actinomycetes is also unknown, but it

may be considered to be different from that of the decomposition by intracellular enzymes of other bacteria. Investigation of actinomycete enzymes is now in progress in comparison with other bacterial enzymes.

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