PATHWAYS OF NICOTINAMIDE ADENINE DINUCLEOTIDE BIOSYNTHESIS IN NICOTINIC ACID OR NICOTINAMIDE REQUIRING MICROORGANISMS*

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Received October 27, 1967

Several microorganisms have been shown to require nicotinate or nicotinamide for growth and do not synthesize the pyridine compounds de novo of precursors to nicotinamide adenine dinucleotide (NAD). A strain of Leuconostoc mesenteroides, which grows on nicotinate but not on nicotinamide, does not possess nicotinamidase (Sundarum et al., 1960). In contrast, Lactobacillus fructosus requires nicotinamide but does not utilize nicotinate (Kodama et al., 1963). Lactobacillus casei and Lactobacillus plantarum grow on either nicotinate or nicotinamide (Snell et al., 1950). In this communication, a survey of the enzyme patterns as well as pathways of NAD biosynthesis in these microorganisms will be described.

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* This investigation has been supported in part by Public Health Service Research Grants CA-04222, from the National Cancer Institute, AM-10333, from the National Institute of Arthritis and Metabolic Diseases, and TW-00280-1, from Foreign Grants and Awards Office of International Research, National Institutes of Health; and grants from the Jane Coffin Childs Memorial Fund for Medical Research, the Squibb Institute for Medical Research, and the Scientific Research Fund of the Ministry of Education of Japan.

+ Recipient of Sigma Chemical Postgraduate Fellowship.

Table I. Apparent Enzymic Activities for NAD Biosynthesis

The enzymic activities were assayed with crude extracts. Cells were grown for 24 hours at 26° in glucose (2.0%)-polypeptone (0.5%) media supplemented with yeast extract (0.5%) and either nicotinate or nicotinamide (10 μg per ml). The extracts were made by sonicating the cells with 5 volumes of 0.05 M potassium phosphate buffer, pH 7.0 for 10 minutes at 9 kc, followed by centrifugation for 15 minutes at 12,000 X g at 0 - 4°. Nicotinate ribonucleotide pyrophosphorylase was assayed by the method of Imsande and Handler (Imsande and Handler, 1961). NMN pyrophosphorylase was assayed by measuring the formation of NMN-^{14}C and NAD-^{14}C from nicotinamide-7-^{14}C, PP-ribose-P and ATP using paper chromatography. Nicotinamidase was assayed by measuring the formation of nicotinate from nicotinamide-7-^{14}C. NAD (deamido-NAD) pyrophosphorylase was assayed by measuring the formation of either NAD-^{14}C or deamido-NAD-^{14}C from NMN-^{14}C or nicotinate-7-^{14}C ribonucleotide, respectively, and ATP using a Dowex 1-formate column. Quinolinate transphosphoribosylase was assayed as described earlier (Nakamura et al., 1963). All numbers are expressed as mμmoles of the products formed per mg of protein per hour at 37°.

Organisms	Requirement for growth	NaMN* pyrophosphorylase	NMN pyrophosphorylase	Nicotinamidase	NAD (dNAD*) pyrophosphorylase	Quinolinate transphosphoribosylase
L. mesenteroides	NA*	0.1	0	0	0.9+	0
L. fructosus	NM*	0	3.2	0	5.9	0
L. plantarum	NA or NM	0.4	0	12.3	3.6+	0
L. casei	NA or NM	0.3	0.03	0.8	1.7+	0
E. coli K 12	none					2.4

* NA: nicotinate; NM: nicotinamide; NaMN: nicotinate ribonucleotide; dNAD: deamido-NAD.

\+ The assay was carried out with nicotinate-7-^{14}C ribonucleotide as substrate.

Table I shows the activities of various enzymes responsible for the NAD biosynthesis from nicotinate, nicotinamide and quinolinate. In agreement with the results obtained by growth experiments, the extracts of L. mesenteroides catalyzes the formation of

nicotinate ribonucleotide from nicotinate, 5-phosphoribosyl-1-pyrophosphate (PP-ribose-P) and ATP (Table I). Neither nicotinamide ribonucleotide (NMN) pyrophosphorylase nor nicotinamidase is detected. The evidence indicates that L. mesenteroides synthesizes NAD only from nicotinate by way of nicotinate ribonucleotide and deamido-NAD and does not convert nicotinamide to NAD due to the lack of NMN pyrophosphorylase and nicotinamidase. This pathway will be referred to as Nicotinate Pathway (Fig. 1).

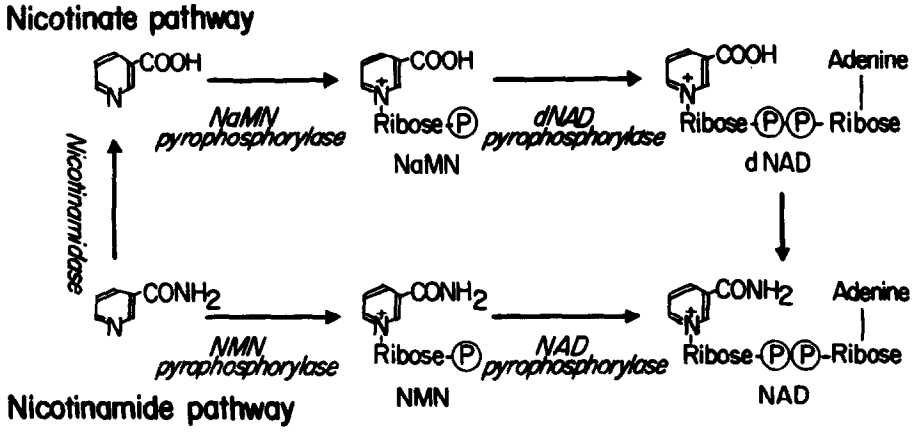

Fig. 1. Pathways of NAD biosynthesis.

In contrast, L. fructosus, which grows on nicotinamide, does not possess nicotinate ribonucleotide pyrophosphorylase nor nicotinamidase but contains NMN and NAD pyrophosphorylases. The NMN pyrophosphorylase has been purified about 60-fold from the sonic extracts by protamine treatment followed by ammonium sulfate fractionation, DEAE-Sephadex and hydroxyapatite column chromatographies. The enzyme catalyzes a stoichiometric conversion of nicotinamide to NMN in the presence of both PP-ribose-P and ATP. The product was isolated from a large scale incubation mixture by a Dowex 1-formate column and was identified as NMN in comparison with an authentic sample by paper chromatography in three different solvent systems

and by high voltage paper electrophoresis under the conditions described previously (Nishizuka and Hayaishi, 1963). Further identity of the reaction product was provided by conversion to NAD by a partially purified preparation of NAD pyrophosphorylase. The NMN pyrophosphorylase is specific for nicotinamide and does not react with nicotinate and quinolinate. The Km value for nicotinamide and PP-ribose-P are calculated to be 2×10^{-6} M and 4×10^{-5} M, respectively, and the optimal pH is found to be in the region of 6 to 7 in Tris-maleate buffer. The reaction requires ATP absolutely and the Km value for ATP is 6.7×10^{-4} M. Neither GTP, UTP nor CTP replaces ATP, but deoxy-ATP is about 25% as active as ATP[1/]. The NAD pyrophosphorylase of L. fructosus reacts with only NMN but not with nicotinate ribonucleotide. The results indicate that in L. fructosus NAD is synthesized directly from nicotinamide by way of NMN and not via Nicotinate Pathway. This pathway will be referred to as Nicotinamide Pathway.

L. plantarum grows on both nicotinate and nicotinamide, and has nicotinate ribonucleotide pyrophosphorylase and nicotinamidase but not NMN pyrophosphorylase (Table I). In order to ascertain whether the Nicotinate Pathway is a sole route of NAD biosynthesis in this microorganism, a reaction mixture (5.0 ml) containing 280 mμmoles of nicotinamide-7-^{14}C (10.7 μc/μmole), 4 μmoles of PP-ribose-P, 60 μmoles of ATP, 125 μmoles of $MgCl_2$, 250 μmoles of Tris-HCl buffer, pH 7.4, and 40 mg of the crude extracts of L. plan-

1/ A similar effect of ATP has been observed with NMN pyrophosphorylase recently isolated from rat liver (Dietrich et al., 1966), and nicotinate ribonucleotide pyrophosphorylase of beef liver (Imsande and Handler, 1961) and Bacillus subtilis (Imsande, 1964). Using a purified preparation of the NMN pyrophosphorylase from L. fructosus, one mole of ATP is shown to be expended for every mole of NMN synthesized. The detailed experimental results will be described elsewhere.

tarum was incubated for 30 minutes at 37°. After the reaction was stopped by heat treatment for one minute in a boiling water bath, the denatured protein was removed by centrifugation, and the supernatant was chromatographed on a Dowex 1-formate (X 2, 200 - 400 mesh, 0.8 cm diameter, 40 cm length) column together with various nonradioactive nicotinate and nicotinamide derivatives as carriers. The radioactive products appeared in the nicotinate, nicotinate ribonucleotide, deamido-NAD and NAD fractions with coincidence of ^{14}C-content and optical density at 260 mμ. Essentially no radioactivity was found in the NMN fraction. In addition, when NMN was added to the reaction mixture *in situ* as a co-substrate under similar conditions, the radioactivity was incorporated into NAD but not into NMN recovered from the incubation mixture (Fig. 2). These radioactive products were further identified by paper chromatography under the conditions specified earlier (Nishizuka and Hayaishi, 1963). The results indicate that *L. plantarum* possesses only *Nicotinate Pathway* and that nicotinamide is utilized as precursor to NAD after being deamidated to nicotinate.

L. casei also grows on both nicotinate and nicotinamide, and possesses both NMN and nicotinate ribonucleotide pyrophosphorylases, although the former enzyme activity is far less than the latter enzyme activity (Table I). In addition, the crude extracts rapidly hydrolyze nicotinamide to nicotinate. The results indicate that in *L. casei*, *Nicotinate Pathway* is a major, if not a sole, pathway for the NAD biosynthesis.

Quinolinate has been established to be another precursor of NAD in mammals (Nishizuka and Hayaishi, 1963) and the enzyme which catalyzes the PP-ribose-P-dependent formation of nicotinate ribonucleotide from quinolinate is widely distributed in nicotinate-autotrophic organisms including *Escherichia coli* (Nishizuka, 1964), but

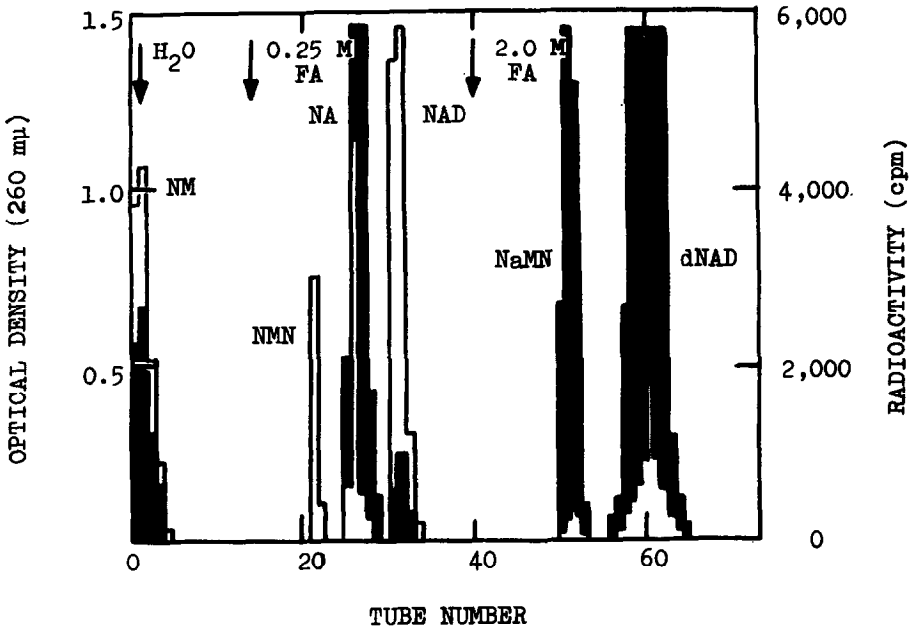

Fig. 2. Dowex 1-formate column chromatography of the reaction products. The detailed experimental conditions are described in the text. Open and solid bars represent the optical density at 260 mμ and radioactivity, respectively. The abbreviations in this figure are explained in Table I, except that FA represents formic acid.

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not in the microorganisms listed in Table I. It may be concluded therefore that nutritional requirement of the microorganisms tested correlates well to the enzyme pattern, but not to the permeability of these precursors across the cell membrane. L. fructosus is unique in the NAD biosynthesis which proceeds through a most simple pathway (Nicotinamide Pathway). A recent report by Dietrich et al., described an NMN pyrophosphorylase with a low Km value for nicotinamide occurring in rat liver, and opened a possibility of the direct utilization of nicotinamide in the NAD biosynthesis in other biological systems (Dietrich et al., 1966).

Acknowledgment - We are indebted to Drs. R. Kodama and F. Tanaka for generous gifts of the microorganisms employed in this

study. Thanks are also due to Dr. T. Suzuki, Institute for Protein Research, Osaka University, for his valuable advice and useful discussions.

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