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Metabolism of Nicotinamide Adenine Dinucleotide in Human and Bovine Strains of *Mycobacterium tuberculosis*

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A marked difference was found to exist between the nicotinamide adenine dinucleotide (NAD) glycohydrolase activity of human strains of *Mycobacterium tuberculosis* as compared with bovine strains. Human strains had from 6- to 20-fold higher NAD glycohydrolase activity than bovine strains. This finding explains the accumulation of free nicotinic acid in the culture medium by human strains and not by bovine strains. The biosynthetic intermediates nicotinic acid mononucleotide and deamido-NAD were not degraded by either human or bovine strains of *M. tuberculosis;* hence these nucleotides do not represent a source of the nicotinic acid accumulated by the human strains.

The major differences between the human and bovine varieties of *Mycobacterium tuberculosis* are based primarily upon cultural characteristics and pathogenicity. There are relatively few biochemical differences between the human and bovine strains. Pope and Smith (15) reported a marked quantitative difference in nicotinic acid production between a human strain of *M. tuberculosis* ($H_{37}R_v$) and a bovine strain (Ravenel). The human strain produced approximately 50-fold more nicotinic acid in culture filtrates than the bovine strain. Furthermore, Konno et al. (8-10) showed that human strains produced from 13.5 to 32.0 μg of nicotinic acid per ml of culture medium, whereas only minimal amounts (1.0-1.5 μg/ml) were produced by bovine strains. Konno's test for differentiating between human and bovine strains of *M. tuberculosis* is based upon the marked difference in nicotinic acid production by human strains as compared with bovine strains (11, 12).

The purpose of the present investigation was to determine the differences in the metabolic processes between the human and bovine strains of *M. tuberculosis* which result in the accumulation of nicotinic acid only by the human strains. It will be shown that the source of nicotinic acid which accumulates in the culture medium is nicotinamide adenine dinucleotide (NAD) and that a major difference between the two varieties of *M. tuberculosis* is the relatively high activity of NAD glycohydrolase in human strains as compared with negligible activity of this enzyme in bovine strains.

MATERIALS AND METHODS

Chemicals. Nicotinic acid-*carboxyl*-^{14}C, nicotinamide-*carbonyl*-^{14}C, NAD-*carbonyl*-^{14}C, and quinolinic acid-6-^{14}C were obtained from Amersham/Searle Corp., Des Plaines, Ill. Unlabeled NAD, chromatopure, diagnostic grade, was obtained from Pabst Laboratories, Milwaukee, Wisc. Adenosine triphosphate (ATP) was purchased from Calbiochem, Los Angeles, Calif. Phosphoribose-1-pyrophosphate (PRPP) was a product of Sigma Chemical Co., St. Louis, Mo.

Preparation of ^{14}C-labeled substrates. ^{14}C-nicotinic acid ribonucleotide and ^{14}C-nicotinic acid adenine dinucleotide (deamido-NAD) were prepared by the method of Preiss and Handler (16) by incubation of ^{14}C-nicotinic acid, ATP, and ribose-5-phosphate with an extract of acetone-dried human erythrocytes. The labeled substrates were isolated by Dowex-1-formate column chromatography.

Organisms. The following strains of *M. tubercu losis* were employed in these investigations. *M. tuberculosis* var. *bovis* strain BCG and *M. tuberculosis* var. *hominis* $H_{37}R_a$ and 71-72 were obtained from the collection of the Henry Phipps Institute through the courtesy of Harry Morton, University of Pennsylvania. *M. tuberculosis* var. *bovis*, strains TMC 405 (4228-4) and TMC 409 (Vallee-Pasteur) and *M. tuberculosis* var. *hominis* strain TMC 206 (R_1R_a) were provided by the U.S.-Japan Cooperative Medical Science Program.

Cultural procedures. The organisms were grown at 37 C, as surface cultures, in the chemically de-

fined medium of Sauton as modified by Konno et al. (10). After 28 to 35 days of incubation at 37 C, the cells were harvested by filtration with Whatman no. 1 filter paper, washed twice with cold 0.9% KCl, then with cold distilled water, and frozen at −20 C until used.

Preparation of cell-free extracts. Cell-free enzyme preparations were obtained by disruption of the cells in a Biox X-Press, Nacka, Sweden. The cells were used not later than 72 hr after the time of initial harvest. The disrupted cells were extracted with 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, *p*H 7.4, with 1 ml of buffer/g (wet weight) of cells. The cell debris and unbroken cells were removed by centrifugation at 31,000 × *g* in a Sorvall refrigerated centrifuge. The resulting clear supernatant fluid was used as the enzyme preparation.

Determination of enzyme activities. Nicotinamidase activity was assayed by measuring the formation of ^{14}C-nicotinic acid from ^{14}C-nicotinamide. The reaction mixture contained ^{14}C-nicotinamide, 1 μmole; 0.5 M potassium phosphate buffer, *p*H 7.4, 40 μmoles; and 0.25 ml of enzyme preparation in a total volume of 0.4 ml. The incubation time was 1 hr.

Nicotinic acid adenine dinucleotide pyrophosphorylase activity was assayed by following the conversion of ^{14}C-nicotinic acid mononucleotide to ^{14}C-deamido-NAD. The reaction mixture contained ^{14}C-nicotinic acid mononucleotide, 0.4 μmole; ATP, 2.5 μmoles; $MgCl_2$, 5 μmoles; 0.5 M potassium phosphate buffer, *p*H 7.4, 20 μmoles; and 0.25 ml of enzyme preparation in a final volume of 0.5 ml. The incubation time was 0.5, 1, 2, and 3 hr.

NAD glycohydrolase activity was determined by the method of Gopinathan et al. (4) with heat-activated crude enzyme preparation. An amount (4 ml) of the crude cell-free preparation in a tube (10 by 100 mm) was heated in a water bath at 85 C for 1 min and immediately cooled in an ice bath. The precipitated proteins were removed by centrifugation, and the clear supernatant fluid was used as the enzyme preparation. Before heat activation, the protein content of the crude enzyme preparation was adjusted to 12 mg/ml for all strains. The glycohydrolase activity was followed by measuring the increase in ^{14}C-nicotinamide and ^{14}C-nicotinic acid concentration and the concomitant decrease in ^{14}C-NAD concentration. The experimental mixture contained ^{14}C-NAD, 1 μmole; 40 μmoles of 0.5 M Tris-hydrochloride buffer, *p*H 6.5 (4); and 0.3 ml of the enzyme preparation in a final volume of 0.4 ml. Incubation time was 3 hr.

Nicotinic acid mononucleotide pyrophosphorylase activity was assayed by following the formation of ^{14}C-nicotinic acid mononucleotide and ^{14}C-deamido-NAD from ^{14}C-nicotinic acid. The reaction mixture contained ^{14}C-nicotinic acid, 1 μmole; 5-phosphoribose-1-pyrophosphate, 2.5 μmoles; ATP, 6 μmoles; $MgCl_2$, 12 μmoles; 0.5 M potassium phosphate buffer, *p*H 7.4, 40 μmoles; and 0.30 ml of the crude enzyme preparation in a final volume of 0.5 ml. The incubation time was 12 hr.

All of the assays were conducted in test tubes (10 by 75 mm). The incubation temperature was 37 C. A control for nonenzymatic degradation of the substrate accompanied each assay. After cooling in an ice bath, the reaction was stopped by the addition of cold 30% perchloric acid, and the precipitated proteins were removed by centrifugation in a Sorvall GLC-1 refrigerated centrifuge at 1,240 × *g*. The supernatant fluid was neutralized with 20% KOH. An 80-μliter sample of the clear, deproteinized solution was used for paper chromatographic analysis.

Chromatographic analysis. The products of the enzymatic reactions were identified by paper chromatography. The following solvent systems were employed: (i) 70% ethanol-1.0 M ammonium acetate (7:3) adjusted to *p*H 5.0 with concentrated HCl; (ii) 1-butanol-acetic acid-water (4:1:2); and (iii) 1-propanol-water (4:1).

After drying in air, the products were located under ultraviolet light. Nicotinamide nucleotides and nucleosides were distinguished from the nicotinic acid analogues by development of fluorescent spots by the method of Kodicek and Reddi (7). Nicotinamide and nicotinic acid were located by exposure of the dry chromatogram to cyanogen bromide vapors and spraying with 2% *p*-aminobenzoic acid (7).

Measurement of radioactivity. The chromatograms were cut into segments along the direction of solvent flow, and samples to be counted were transferred to stainless-steel planchets. Radioactivity was determined with a Baird-Atomic low-beta counter system. All samples were counted to a degree of statistical validity.

Analytical methods. The protein content of the enzyme preparation was determined by the method of Lowry et al. (14) with crystalline bovine serum albumin as a standard. NAD was measured by the alcohol dehydrogenase assay (17).

RESULTS

NAD glycohydrolase activity. Figure 1 demonstrates the marked difference in the specific activity of the NAD glycohydrolase in the enzyme preparation of the bovine strains as compared with the human strains of *M. tuberculosis*. The activity of the NAD glycohydrolase of the human strains was from 6- to 20-fold higher than that of the bovine strains. The bovine strains appeared to have a very low capability to degrade NAD.

Prior to heat treatment, the enzyme preparations failed to reveal any NAD-cleaving activity.

Degradation of nicotinamide. Nicotinamidase activity was observed to be very high in the human strains and very weak in the bovine strains as shown in Table 1.

Absence of recycling of nicotinic acid. Dudley and Willett (2) reported that *M. tuberculosis* var. *bovis* strain BCG incorporated ^{14}C-nicotinic acid into NAD. In our hands, the

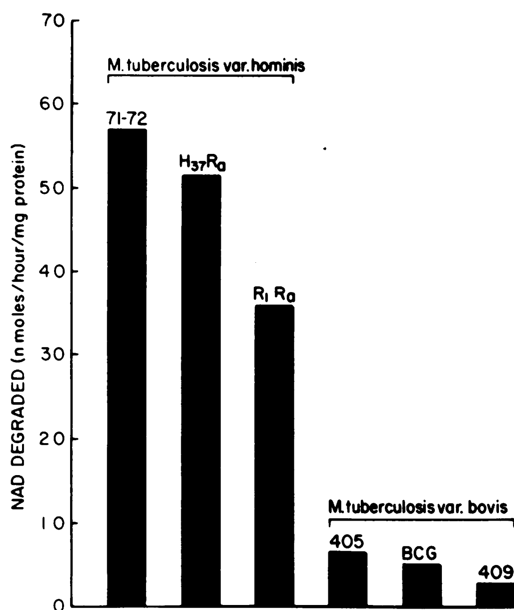

FIG. 1. *Comparison of NAD glycohydrolase activity in human and bovine strains of Mycobacterium tuberculosis.*

TABLE 1. *Comparison of nicotinamidase activity of human and bovine strains of Mycobacterium tuberculosis*

Mycobacterium tuberculosis strain	Nicotinamide degraded (nmoles per min per mg of protein)
Human strain $H_{37}R_a$	4.28
Human strain R_1R_a	3.50
Human strain 71-72	3.17
Bovine strain BCG	0.15
Bovine strain TMC 405	0.11
Bovine strain TMC 409	0.11

enzyme preparations from either human or bovine strains failed to yield more than traces of NAD and the intermediates when ^{14}C-nicotinic acid was employed as substrate. The enzyme preparations, however, showed high activity with regard to the enzymes of the biosynthetic pathway when ^{14}C-quinolinic acid or nicotinic acid mononucleotide was used as substrate instead of nicotinic acid. Thus, with quinolinic acid as substrate, in the presence of PRPP, nicotinic acid mononucleotide was synthesized. When ATP was added to the experimental mixture, the nicotinic acid mononucleotide was converted to deamido-NAD. Upon addition of glutamine and ATP, NAD was synthesized (Table 2). This finding confirmed that nicotinic acid mononucleotide and deamido-NAD are intermediates in the pathway of NAD biosynthesis in *M. tuberculosis* as shown previously by Dudley and Willett (2).

Activity of M. tuberculosis on nicotinic acid mononucleotide and deamido-NAD. Extracts of bovine and human strains of *M. tuberculosis* converted nicotinic acid mononucleotide to deamido-NAD in the presence of ATP. Upon addition of glutamine and ATP, deamido-NAD was converted to NAD. Neither the bovine nor human strains displayed detectable degradative activity towards nicotinic acid mononucleotide or deamido-NAD.

DISCUSSION

Metabolically, free nicotinic acid may be derived from the degradation of NAD or of nicotinic acid mononucleotide and deamido-NAD which are precursors on the pathway of NAD biosynthesis. It is apparent that the marked quantitative difference which exists between the human and bovine strains of *M. tuberculosis* with respect to the accumulation of nicotinic acid in the culture medium is the result of difference in the degradation of these nucleotides. Our results show that there is no degradation of nicotinic acid mononucleotide or deamido-NAD by either the bovine or human strains of *M. tuberculosis*. Thus, the degradation of NAD is the only process which

TABLE 2. *Synthesis of NAD and intermediates with ^{14}C-quinolinic acid as substrate by Mycobacterium tuberculosis var. bovis strain BCG*[a]

Additions[b]	Products formed[c]		
	NAMN	Deamido-NAD	NAD
PRPP	0.69		
PRPP + ATP	0.18	0.49	
PRPP + ATP + glutamine	0.19	0.35	0.12

[a] Analogous results were obtained with enzyme preparations from the human and bovine strains. Abbreviations: NAMN, nicotinic acid mononucleotide; NAD, nicotinamide adenine dinucleotide; deamido-NAD, nicotinic acid adenine dinucleotide; PRPP, phosphoribose-1-pyrophosphate; ATP, adenosine triphosphate.

[b] The standard incubation mixture contained (μmoles): ^{14}C-quinolinic acid, 1; $MgCl_2$, 12; phosphate buffer, *p*H 7.4, 40; enzyme preparation, 0.3 ml; and where indicated, PRPP, 2.5; ATP, 6; and glutamine, 4. A total volume of 0.6 ml was incubated for 6 hr at 37 C.

[c] Nanomoles per minute per milligram of protein.

leads to the production of free nicotinic acid by mycobacteria.

Two enzymes are involved in the degradation of NAD by *M. tuberculosis:* a NAD glycohydrolase and a nicotinamidase. The first enzyme degrades NAD to nicotinamide and adenosine diphosphate ribose. The second catalyzes the hydrolysis of nicotinamide to nicotinic acid. It is known that a great difference exists in nicotinamidase activity between the bovine and human strains of *M. tuberculosis* (1, 5). The nicotinamidase activity of bovine strains is less than one-thirtieth that of human strains (13). We have confirmed this difference in nicotinamidase activity, and we have also shown that a marked difference exists between the NAD glycohydrolase activity of human strains as compared with bovine strains. Bovine strains were found to have a negligible capacity to degrade NAD. The NAD glycohydrolase activity of human strains of *M. tuberculosis* has been shown to be from 6- to 20-fold higher than that of bovine strains. As a result of the higher activity of both enzymes in human strains and the inability to recycle nicotinic acid, they accumulate nicotinic acid in the culture medium, whereas bovine strains do not.

Our results with regard to the lack of recycling of nicotinic acid can be reconciled with those of Dudley and Willett (2) if it is considered that the very low activity which they observed after 20 hr of incubation of the experimental mixture is well below the level of physiological significance.

Gopinathan et al. (3) reported the presence of NAD glycohydrolase in an inhibited state in cell-free extracts of *M. tuberculosis*. The enzyme could be activated by heat treatment for 1 to 2 min at 85 C. The basis for heat activation was considered to be due to the destruction of a heat-labile protein inhibitor bound to the heat-stabile enzyme. The fact that growing cultures of human strains of *M. tuberculosis* accumulate nicotinic acid suggests that, under physiological conditions, the NAD glycohydrolase is activated.

Another microorganism which accumulates nicotinic acid in the culture medium is *Clostridium butylicum* (6). However, the mechanism of accumulation of nicotinic acid by this organism is entirely different from that observed in *M. tuberculosis*. In *M. tuberculosis* nicotinic acid is a product of NAD degradation by NAD glycohydrolase. In *C. butylicum* nicotinic acid is predominantly a product of the degradation of the intermediates in the biosynthetic pathway (*unpublished data*, presented in part at the 10th International Congress for Microbiology, Mexico, 1970).

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