tor X. Recently, Ratnoff has also tested electrophoretically isolated thrombokinase and concluded (personal communication, 1964) that it is a rich source of Stuart factor, as determined by tests with plasma from a patient congenitally deficient in Stuart factor. Thus, correction of the Stuart defect is a fifth measurable effect of thrombokinase preparations. Are they all due to a single molecular species?

Summary. Bovine thrombokinase, incompletely purified, was subjected to gel filtration on Sephadex G-200. Almost all of the protein emerged from the column in the position corresponding to the third wave of serum proteins. The thrombokinase subfractions had the capacity to activate prothrombin in the presence of oxalate, to cause prothrombin activation in a system containing calcium, phospholipid and accelerator (factor V), and to activate chymotrypsinogen. No separation of these activities was evident. This makes it desirable to enquire further whether these activities, as well as the TAMe esterase and the power to correct the Stuart defect, are all properties of the thrombokinase molecule. Accumulating evidence continues to favor the view that thrombokinase can, under proper circumstances, activate prothrombin without the help of presently known forms of accelerator (factor V).

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Nicotinamide Adenine Dinucleotide Synthesis by *Mycobacterium tuberculosis* var. bovis.* (30307)

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Scientists have long studied the various species and strains of *Mycobacterium tuberculosis* in the hope of obtaining simple methods for distinguishing virulent from avirulent strains, and of elucidating the nature of their virulence. Most of these investigations were attempts to ascertain the nutritional requirements of the microbe or to discover morphological and biochemical differences between strains. Only in recent years have studies been undertaken to delineate basic metabolic differences between strains. Pope and Smith (1) observed that both the human H37 and bovine Ravanel strains of *M. tuberculosis,* when grown on synthetic media devoid of preformed vitamins, were capable of *de novo* synthesis of nicotinic acid. Moreover, they demonstrated that the culture filtrate of the

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human strain contained approximately 50-fold more nicotinic acid than did the Ravanel strain. Bird(2) corroborated these findings and subsequently Konno and coworkers(3-6) showed that niacin production by a wide variety of human strains of *M. tuberculosis* is greater than nicotinic acid production by bovine or avian strains.

In the past decade, investigations of niacin production by *Mycobacteria* have been more or less limited to the perfection of techniques for detection of nicotinic acid in culture filtrates(7-9). A few attempts have been made to determine the metabolic pathway of niacin synthesis(10-12), but the role of the vitamin in the metabolism of the bacterium has not been investigated.

Present knowledge of the cellular importance of nicotinic acid results from Preiss and Handler's discovery that human erythrocytes convert nicotinic acid to nicotinamide adenine dinucleotide (NAD) *via* nicotinic acid mononucleotide (NaMN) and nicotinic acid adenine dinucleotide (NaAD)(13,14). Moreover, niacin appears to be a precursor of NAD not only in mammalian systems but in several microorganisms as well. Yeast autolysates synthesize NAD from nicotinic acid but not from nicotinamide(14). *Escherichia coli* synthesizes NAD from niacin, and NaMN and NaAD have been isolated as intermediates (15).

It was the purpose of the present study to: A) determine whether NAD could be synthesized from niacin by a bovine strain of *M. tuberculosis;* B) demonstrate that both NaMN and NaAD were intermediates of the pathway; C) delineate the requirements for coenzyme synthesis.

Materials. Cell-free extracts of *Mycobacterium tuberculosis* var. bovis strain BCG Tice† were employed in this study. Stock cultures of the organism were maintained on Lowenstein egg medium. For experimental purposes the *Mycobacteria* were transferred to Hirsch charcoal agar(16) and incubated for 3 weeks at 37°C. The cells were harvested from the surface of the agar in cold distilled water and washed in a coarse sintered glass filter. They were then resuspended in cold 0.05 M potassium phosphate buffer, pH 7.4, containing 10^{-4} M ethylenediaminetetraacetic acid and 10^{-4} M glutathione, and the suspension sonicated for 1 hour in a Raytheon 10KC sonic oscillator in which the water bathing the vibrating shaft was chilled by passage through a copper coil submerged in crushed ice. Cell wall material was removed by centrifugation for 1 hour in a refrigerated centrifuge at 12,000 rpm. Nucleic acids were precipitated with cold 1% protamine sulfate (18 ml 1% protamine sulfate/50 ml of solution) and removed by centrifugation. The supernatant was brought to 60% saturation with ammonium sulfate [$(NH_4)_2SO_4$] and the protein collected by centrifugation. The protein was redissolved in cold 0.05 M buffer and dialyzed overnight. Following dialysis, the protein concentration of the solution was determined turbidimetrically(17). This buffered protein solution was employed in all experiments.

Nicotinic acid-7-C^{14}, NAD, and adenosine triphosphate (ATP) were obtained from the California Biochemical Corp. The 5-phosphoribosyl-1-pyrophosphate (PRPP) was purchased from Pabst Laboratories. Unlabeled NaMN was kindly supplied by Dr. Robert W. Wheat(18). Radioactive NaMN and NaAD were prepared enzymatically using acetone powders of human erythrocytes(19).

Methods. Enzymatic synthesis of NAD from niacin-7-C^{14}. 306-310 mg tubercle bacillus protein, 75 μM ATP, 150 μM $MgCl_2$, 14.1 μM PRPP, 15 μM glutamine, 15 μM asparagine and 6 μM niacin-7-C^{14} in a total volume of 30 ml were incubated at 37°C for 20 hours. The reaction was stopped in the cold by addition of sufficient 70% perchloric acid to bring the final acid concentration to 10%. Precipitated protein was removed by centrifugation and the supernatant adjusted to pH 6.8 with saturated potassium hydroxide. Potassium perchlorate was removed by centrifugation; the solution was lyophilized to half its original volume and applied to an 0.8 × 55 cm Dowex-1-formate, AG X 8, column. The column was washed with 500 ml of water and developed with a gradient of 0 to 4 N formic acid. Seven to 10 ml frac-

† Obtained through the courtesy of Dr. Sol Rosenthal, Tice Laboratories, Chicago, Ill.

tions were collected and the optical density of each was measured at 265 mμ. Each 265 mμ peak was combined, lyophilized to dryness, redissolved in 0.5 ml of distilled water, and tested for the presence of radioactive products.

Control experiments using niacin-7-C^{14} as the enzymatic substrate. 1 ml reaction mixtures, which contained in addition to 13-18 mg of tubercle bacillus protein, proportionally the same concentration of ingredients as the large reaction mixtures but from which each reactant was individually omitted, were incubated at 37°C for 20 hours. The reactions were stopped by immersing the reaction vessel in a boiling water bath for 1 minute and the solution was frozen overnight, thawed and centrifuged to remove precipitated protein. The zero time controls consisted of reaction mixtures containing all reactants but tubercle bacillus protein. The latter was added after incubation and the reaction stopped with heat. Enzymatic products in these experiments were not separated on a Dowex column but were identified directly.

Enzymatic synthesis of NAD from NaMN-C^{14}. 132 mg tubercle bacillus protein, 25 μM ATP, 50 μM $MgCl_2$, 5.0 μM glutamine and NaMN-C^{14} (750,000 cpm) in a total volume of 10 ml were incubated for 10 hours at 37°C. The reaction was stopped and deproteinized as described above and the resulting solution applied to a 1 × 17.5 cm Dowex-1-formate column. The column was washed and developed as outlined above.

Identification of enzymatic products. Enzymatic products were initially identified by descending paper chromatography using Whatman No. 1 filter paper. Ethanol: 1 M ammonium acetate (7:3, pH 5.0), isobutyric acid : ammonia : water (66:1:33) and 0.1 M sodium phosphate buffer, pH 6.8:$(NH_4)_2SO_4$: n-propanol (100:60:2) were used as solvents. The chromatographic spots were detected under an ultraviolet light. Radioactivity was measured on a Vanguard strip counter or in a Packard tri-carb liquid scintillation counter.

Nicotinamide nucleotides were further identified by the development of UV fluorescent spots after exposing the chromatograms to the vapors of methyl ethyl ketone and ammonia (1:1/v:v) in a closed glass cylinder for 1 hour(20). Nicotinic acid was further identified on paper by the development of yellow-orange spots when the chromatogram was exposed to cyanogen bromide (CNBr) vapors in a closed glass tank for 1 hour and sprayed with 2% p-aminobenzoic acid in alcoholic hydrochloric acid(20).

When present in sufficient quantities, the radioactive nucleotides were eluted from the chromatography paper in water and their UV spectra measured in a Bausch and Lomb recording spectrophotometer. Each was hydrolyzed in 1 N sodium hydroxide, neutralized, and tested for release of nicotinamide or niacin with 10% CNBr and 4% aniline in 95% methanol(21).

Results. Formation of NaMN-C^{14}, NaAD-C^{14}, and NAD-C^{14} from niacin-C^{14}. In these experiments 3 nucleotide peaks eluted from the Dowex-1-formate column and each contained at least one radioactive compound. Peak I appeared from paper chromatography to be a mixture of NAD-C^{14} and niacin-C^{14}. The radioactive NAD spot fluoresced after treatment with methyl ethyl ketone and ammonia. Upon elution from the paper, the nucleotide was examined spectrophotometrically. In acid it exhibited an absorption maximum at 260 mμ and, after 3 minutes incubation in 1 M KCN, a second maximum developed at 325 mμ. The nucleotide did not react with CNBr until hydrolyzed in 1 M NaOH. At this time it formed a yellow-orange complex characteristic of niacin or niacinamide. The radioactive niacin spot did not react with methyl ethyl ketone nor with KCN but reacted readily with CNBr.

Peak II was composed of 2 compounds: an unlabeled nucleotide, which migrated in all solvents as AMP, and a radioactive material which moved as NaMN. The latter failed to fluoresce following treatment with methyl ethyl ketone. The NaMN spot when eluted from paper exhibited an absorption maximum at 265 mμ at pH 1-7. When incubated for 20 minutes with 1 M KCN, the 265 mμ maximum was markedly diminished and a new absorption maximum developed at 315 mμ. After hydrolysis in 1 M NaOH, the NaMN spot reacted with CNBr to yield a

positive yellow-orange color.

Peak III was also composed of 2 compounds: a large quantity of what was believed to be an adenine-containing degradation product of ATP and a radioactive compound which migrated chromatographically as NaAD. Insufficient quantities of NaAD were formed to permit chemical and spectral examination of this material.

In order to quantitate the NAD, NaMN and NaAD formed in these experiments, reaction mixtures were prepared to which 2 μM of unlabeled NAD were added immediately prior to application of the sample onto the Dowex-1-formate column. Unfortunately, unlabeled NaMN and NaAD were not available to be used as carriers for their radioactive counterparts. Each radioactive peak was chromatographed in ethanol: 1M ammonium acetate, pH 5.0, and its radioactivity measured on the strip counter. The amount of radioactivity in each peak was then compared with a strip counter measurement of a known niacin sample and the specific activity (counts/min/mg protein) of each enzymatic product was calculated (Table I). In these studies NAD accumulated. Twice as much NAD as NaMN was detected in the reaction mixtures but little NaAD was found.

TABLE I. Specific Activity of Enzymatic Products Formed from Niacin-7-C^{14}.

Compound	Specific activity (cpm)
NaMN	615
NaAD	120
NAD	1242

TABLE II. Enzymatic Product When One or More Ingredients Were Omitted from 1 ml Reaction Mixtures.

Omission	Enzymatic product
Zero time control	None
PRPP	"
ATP	"
$MgCl_2$	"
Asparagine & glutamine	NaMN
Asparagine	NAD
Glutamine	"

TABLE III. Optimal Nitrogen Donor for NAD Synthesis.

Nitrogen donor	Specific activity (cpm)
Glutamine	1463
Asparagine	1058

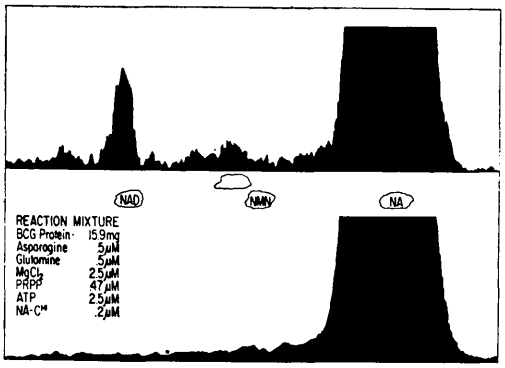

FIG. 1. Distribution of radioactivity in products of a complete 1 ml reaction mixture containing nicotinic acid-C^{14} and BCG protein. 100 μl applied to chromatogram and read at 1,000 cpm after development in ethanol:ammonium acetate. Lower part of figure shows zero time control.

Formation of radioactive enzymatic products in 1 ml control experiments. These studies were undertaken to prove that each compound other than niacin which had been added to the reaction mixtures was requisite for NAD biosynthesis (Table II). No enzymatic products were detected in the zero time controls (Fig. 1). Moreover, if PRPP, ATP or $MgCl_2$ were omitted from the reaction mixture, no enzymatic products were formed. When both glutamine and asparagine were omitted, a radioactive compound was detected which migrated chromatographically as NaMN. Omission of either glutamine or asparagine individually permitted synthesis of NAD while substitution of 0.16 μM $(NH_4)_2SO_4$ for the amino acids prevented coenzyme synthesis. Glutamine appeared to be the preferred nitrogen donor as determined by the number of counts in the NAD spot (Table III). On the basis of these experiments, only glutamine was used as the nitrogen donor in the subsequent studies.

Formation of NaAD-C^{14} and NAD-C^{14} from NaMN-C^{14}. Three nucleotide peaks were eluted from the Dowex-1-formate column in this experiment and, of the 750,000 cpm of NaMN-C^{14} in the original reaction mixture, 541,295 cpm were recovered from the column. Peak I contained 2 UV-absorbent spots, both of which were radioactive.

TABLE IV. Specific Activity of Enzymatic Products Formed from NaMN-C^{14}.

Compound	Specific activity (cpm)
Niacin	768
NaMN	1124
NaAD	2046
NAD	163

One migrated as nicotinic acid and the other as NAD. The former reacted on paper with CNBr to give a yellow-orange spot while the latter fluoresced after treatment with methyl ethyl ketone and ammonia but did not react with CNBr until hydrolyzed in 1 M NaOH. Fluorescence of the NAD spot could not be attributed entirely to freshly synthesized NAD as unlabeled NAD had been put on the column. However, since the NAD spot was also radioactive, it can be assumed that the enzymatically synthesized radioactive nucleotide was NAD. Two compounds comprised Peak II: unlabeled AMP and labeled NaMN. Peak III was composed of a single UV-absorbent and radioactive spot which migrated chromatographically just behind NAD or as NaAD. Table IV illustrates the specific activity (counts/min/mg protein) of each radioactive intermediate. Under the conditions of these experiments only small amounts of NAD-C^{14} were found and the bulk of the label was detected as NaAD-C^{14} and as nicotinic acid-C^{14}. Zero time controls were found to contain niacin-C^{14}, in addition to the NaMN-C^{14}. In fact, the specific activity of the niacin-C^{14} in the control was greater than that of the full reaction mixture.

Discussion. The studies described herein illustrate that NAD can be synthesized from niacin by a bovine strain of *M. tuberculosis* and that the nicotinic acid mono- and dinucleotides are intermediates on the pathway. In no case were NMN or other nicotinamide nucleotides detected among the enzymatic products. Enzymatically synthesized NaMN, NaAD and NAD were identified by a variety of chromatographic, chemical and spectrophotometric techniques. Each eluted from the Dowex-1-formate columns in the position described by Preiss and Handler(13). Upon paper each migrated as their unlabeled counterparts and all behaved predictably when subjected to chemical and spectrophotometric tests.

Further proof of the pathway of coenzyme biosynthesis in *Mycobacteria* was afforded by experiments in which NaMN replaced niacin as the radioactive substrate. Moreover, these studies confirmed the hypothesis that NaMN is utilized directly for NAD biosynthesis without prior conversion to niacin and that it is thus an obligatory intermediate of coenzyme synthesis. Both NAD-C^{14} and NaAD-C^{14} were formed enzymatically from the radioactive mononucleotide. In contrast to the experiments in which nicotinic acid-C^{14} was employed as the radioactive substrate, the bulk of the label was found as the dinucleotide. Large amounts of niacin-C^{14} were also detected. The vitamin appeared, however, to accumulate as the result of heat degradation of NaMN during the prolonged incubation at 37°C rather than as a consequence of enzymatic decomposition. It was the only radioactive material other than NaMN found in the control reaction mixtures and, in fact, was present in greater quantities in the controls than in the full reaction mixtures. Were NaMN not directly on the pathway of NAD synthesis or were it enzymatically degraded to niacin prior to incorporation into the coenzyme, greater concentrations of the vitamin would have been detected in the full reaction mixtures than in the controls. In addition, since PRPP was omitted from these reaction mixtures, no carbohydrate was available to combine with nicotinic acid to form NAD by an alternate pathway which would bypass NaMN as an intermediate.

The control experiments demonstrated that the requirements for NAD biosynthesis in *M. tuberculosis* parallel those previously described for human erythrocytes and *E. coli* in that PRPP, ATP, and $MgCl_2$ are requisite for formation of NaMN, and that a source of amide nitrogen is necessary for conversion of NaAD to the coenzyme. However, minor points of variation involving the last two steps of NAD synthesis did exist. To accumulate NaAD with red blood cells, Preiss and Handler(13) omitted the nitrogen source from the reaction mixture. When comparable experi-

ments were performed with tubercle bacillus protein, NaMN rather than NaAD accumulated. Yeast autolysates and *E. coli* employ either ammonium ions or glutamine as the source of amide nitrogen(14,15). The former uses glutamine preferentially and cannot utilize either asparagine, aspartic acid or glutamic acid. The latter employs ammonium ions as the optimal nitrogen donor; asparagine was not tested. The BCG strain of *M. tuberculosis* was able to use both glutamine and asparagine but not $(NH_4)_2SO_4$ as nitrogen donors. Imsande(15) has suggested that the preferential use of glutamine as the amide donor by yeast *vs* the use of ammonia by *E. coli* may "represent evolutionary changes in metabolic patterns." It is curious to note that in respect to the optimal nitrogen donor, as in so many other ways, *Mycobacteria* appear more closely related to the fungi than they are to other bacteria.

Summary. The pathway of NAD biosynthesis in *Mycobacteria* was studied with cell-free extracts of *M. tuberculosis* var. bovis strain BCG Tice. The organism converted niacin to NAD with NaMN and NaAD as intermediates. PRPP, ATP, and $MgCl_2$ were shown to be requisite for NaMN formation while glutamine or asparagine were shown to be necessary for conversion of NaAD to NAD. The pathway was further elucidated by the demonstration that NaMN was itself utilized directly for coenzyme synthesis without prior decomposition to niacin.

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Effect of 2,4-Dinitrophenol on Magnesium Metabolism.* (30308)

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Mg^{28} administered parenterally in a tracer dose is rapidly concentrated in tissues(1). This tissue uptake of Mg^{28} is facilitated by simultaneous administration of insulin and glucose(2), and thyroxine also accelerates the uptake of this isotope in certain tissues(3). This effect of thyroxine may be due to the uncoupling of oxidative phosphorylation. The present experiments were performed in an attempt to determine whether the uncoupling of oxidative phosphorylation by 2,4-dinitrophenol could be correlated with alterations in magnesium metabolism.

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