Metabolism of the Pyridine Nucleotides Involved in Nicotinamide Adenine Dinucleotide Biosynthesis by Clostridium butylicum

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In order to elucidate the mechanism of the accumulation of considerable amounts of free nicotinic acid (NA) in the culture medium of Clostridium butylicum, this organism was investigated with regard to its ability to metabolize nicotinamide adenine dinucleotide (NAD) and its immediate biosynthetic precursors, nicotinic acid mononucleotide (NAMN) and nicotinic acid adenine dinucleotide (deamido-NAD). Cell-free extracts of C. butylicum were found to degrade NAMN and deamido-NAD to NA. NAMN, in the presence of adenosine triphosphate (ATP), was converted to deamido-NAD, but only at high concentrations of ATP (20 mM) was significant synthetic activity observed in competition with NAMN degradation. Degradation of both NAMN and deamido-NAD was activated by ATP at concentrations of 5 and 10 mM. Anaerobiosis markedly enhanced the degradation of the nucleotides. The data indicate that the synthesis of NAMN and deamido-NAD prevails over their degradation only in the presence of high concentrations of ATP. NAD was degraded to nicotinamide mononucleotide (NMN) by a pyrophosphatase. Phosphate markedly inhibited both the deamido-NAD and NAD pyrophosphatases. Under anaerobic conditions there was practically no further degradation of NMN to NA, whereas barely measurable amounts of NA were formed under aerobic conditions. All of these observations suggest that, under the given conditions of anaerobiosis and physiological phosphate concentrations, there is very little degradation of NAD to NMN and practically no degradation to NA by C. butylicum. Thus, NAD represents an insignificant source of the NA accumulated in the culture medium. The intermediates in the biosynthetic pathway (NAMN and deamido-NAD) have been shown to be the major source of the NA which is accumulated by C. butylicum.

Growing cultures of Clostridium butylicum accumulate significant amounts of nicotinic acid (NA) in the culture medium (10, 26). Metabolically, free NA may be derived from the degradation of nicotinamide adenine dinucleotide (NAD) or its immediate biosynthetic precursors, nicotinic acid mononucleotide (NAMN) and nicotinic acid adenine dinucleotide (deamido-NAD). We have previously reported that C. butylicum synthesizes NAD de novo via the intermediates quinolinic acid (QA), NAMN, and deamido-NAD. NA cannot be utilized by this organism for the synthesis of NAD because it lacks the necessary pyrophosphorylase (10). The presence of the specific enzymes required for the degradative reactions has not been investigated. Mycobacterium tuberculosis var. hominis also accumulates NA in the culture medium (14, 15, 16, 20). The NA accumulated by human strains of M. tuberculosis has been shown to be derived solely from NAD (13). Evidence now will be presented that C. butylicum, in contrast to M. tuberculosis, degrades NAMN and deamido-NAD to NA, and that these nucleotides are the main source of the NA which accumulates in growing cultures of this organism.

MATERIALS AND METHODS

Chemicals. NA-carboxyl-\(^{14}\)C, specific activity of 59.1 mCi/mmol; nicotinamide-carboxyl-\(^{14}\)C, specific activity of 60 mCi/mmol; nicotinamide-carbonyl-\(^{14}\)C; NAD, specific activity of 44 mCi/mmol; and quinolinic acid-\(^{6}\)\(^{14}\)C, specific activity of 43.7 mCi/mmol,
were obtained from Amensham/Searle Corp., Des Plaines, Ill. Unlabeled NAD Chromatopure, diagnostic grade, was obtained from Pabst Laboratories, Milwaukee, Wis. Adenosine triphosphate (ATP) was purchased from Calbiochem, Los Angeles, Calif. Phosphoribose-1-pyrophosphate (PRPP) tetrasodium salt, was a product of Sigma Chemical Co., St. Louis, Mo. PRPP dimagnesium dibydrate B grade, was obtained from Calbiochem. Both forms were tested for activity with extracts of Saccharomyces cerevisiae, which actively converted NA to NAMN and were found to be active.

Preparation of 14C-labeled substrates. 14C-NA ribonucleotide and 14C-deamido-NAD were prepared according to the method of Preiss and Handler (21) by incubation of 14C-NA, ATP, and ribose-5-phosphate with an extract of acetone-dried human erythrocytes. Part of the 14C-NA ribonucleotide was used for preparation of 14C-NA ribonucleoside. The procedure was the same as described by Kaplan and Stolzenbach (12) for the preparation of nicotinamide riboside, except that orthophosphoric monoester phosphohydrolase (Boeringer Manheim Corp., New York, N.Y.) from potatoes was used instead of a prosthetic orthophosphoric monoester phosphohydrolase. The labeled substrates were isolated by Dowex-1-formate column chromatography (21). Radiochemical purity of the nucleotides was 98% and of the nucleoside 99%, as judged by paper chromatography (21).

Organism used. C. butylicum ATCC 14823 was obtained from the American Type Culture Collection.

Medium. The medium used contained the following ingredients in amounts per liter: glucose, 10.0 g; KH2PO4, 0.5 g; K2HPO4, 0.5 g; Casamino Acids (vitamin-free), 5.0 g; MgSO4, 200 mg; L-cysteine-HCl hydrate, 200 mg; DL-aspartic acid, 0.5 g; NaCl, 10.0 mg; FeSO4, 10.0 mg; MnCl2, 10.0 mg; and biotin, 1.0 μg. The pH of the medium was adjusted to 7.2 before autoclaving for 15 min at 121°C.

Growth conditions. C. butylicum was grown anaerobically by filling a carboy to the neck in order to exclude air. The medium was incubated statically for 22 h at 37°C. Cells were harvested by continuous-flow centrifugation at 29,000 × g. The resultant cell pellet was washed twice with cold, distilled water, washed once with 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.5), and frozen until used for the preparation of cell-free extracts.

Preparation of cell-free extracts. Cell-free enzyme preparations were obtained by disruption of the cells in a Biro X-Press (Nacka, Sweden). The cells were used not later than 72 h from the time of initial harvesting. The disrupted cells were extracted with 0.05 M Tris-hydrochloride buffer (pH 7.4) by using 1 mL of buffer per 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, after dialysis over night against 0.015 M Tris-hydrochloride buffer (pH 7.5), containing 5.0 mM 2-mercaptoethanol, was used as the enzyme preparation.

Determination of enzyme activities. Enzyme activities were assayed as previously described (13). Because Brown and Stadtman (3) found that NAD-

pyrophosphatases isolated from C. propionicum were markedly inhibited by orthophosphate, all reactions were conducted in both phosphate and Tris-hydrochloride buffers.

All enzyme assays were performed in test tubes (10 by 75 mm) either in air or in a H2 atmosphere. Because C. butylicum is an anaerobe, all experiments were conducted under both aerobic and anaerobic conditions. A comparison of reactions conducted under both conditions showed striking differences which will be presented under Results. For H2 atmosphere, each tube was thoroughly flushed with H2 for 3 min, stoppered immediately, and incubated at 37°C. Incubation times varied and are given with the individual results. 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 chromatography analysis.

Chromatographic analysis. The reaction products were isolated by paper chromatography. Chromatographic analysis and measurement of radioactivity were carried out as previously described (13).

Analytical methods. The protein content of the enzyme preparation was determined by the method of Lowry et al. (17) by using crystalline bovine serum albumin as a standard.

Calculations. The amount of radioactivity added to unlabeled substrate was calculated to be sufficient for detection of the products of degradation or synthesis at the level of at least 0.5% conversion of the substrate. In all cases where utilization of the substrate could not be detected at this level, the appropriate enzyme activity was considered to be absent. In all experiments, virtually all of the radioactivity added to the reaction mixture as 14C-labeled substrate was accounted for as unmetabolized substrate and reaction products. For this reason, it was possible to equate the total radioactivity recovered (in counts per minute) to 100% in order to arrive at the molar values for each compound. Molar values of products formed are given for the incubation time indicated.

RESULTS

With 14C-QA as substrate, and in the absence of ATP, crude enzyme preparations of C. butylicum synthesized NAMN, but free NA was also found in the reaction mixture. Addition of ATP resulted in synthesis of both NAMN and deamido-NAD, but again a considerable amount of NA was present (Table 1). This indicated that the synthesized nucleotides were degraded to NA and that the mechanism of NA accumulation by C. butylicum differed from that observed in M. tuberculosis (13), which was found to be incapable of degrading the intermediates on the pathway from QA to NAD.
To elucidate the metabolism of the intermediates in the biosynthesis of NAD from QA by C. butylicum, experiments were performed with \(^{14}\)C-NAMN and \(^{14}\)C-deamido-NAD as substrates.

**Metabolism of NAMN.** In the absence of ATP, \(^{14}\)C-NAMN was degraded to NA riboside (NAR) and NA (Table 2). The degradation of NAMN was considerably higher when the reaction was conducted under anaerobic conditions (Fig. 1). Activity of this enzyme was not markedly different in phosphate or Tris-hydrochloride buffers, indicating that NAMNase activity was unaffected by inorganic phosphate. In the presence of ATP (Fig. 2), conversion of NAMN to deamido-NAD was observed. A considerable amount of NA was also formed in the presence of ATP. This indicated that degradation of NAMN, and possibly deamido-NAD was occurring concurrently with the synthesis of deamido-NAD. Under anaerobic conditions, the amount of deamido-NAD formed increased only slightly at 5 mM ATP (Fig. 2). At either 10 or 20 mM ATP, considerably greater amounts of deamido-NAD were observed. The amount of NA formed under anaerobic conditions in the presence of 5 mM ATP was even slightly increased over that observed in the absence of ATP. Repeated experiments always revealed this slight increase in NA formation at 5 mM ATP. At 10 mM ATP the amount of NA was still equivalent to that formed in the absence of ATP. Considerably less NA was formed at 20 mM ATP, but the amount was still greater than the amount of deamido-NAD synthesized. Aerobically, at a lower concentration of ATP, there was increased synthesis of deamido-NAD and less degradation of NA than was observed under anaerobic conditions. Only at 20 mM ATP did the differences in the amount of deamido-NAD synthesized or in the amount of NA formed

<table>
<thead>
<tr>
<th>ATP added</th>
<th>QA remaining (nmol)</th>
<th>Products observed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Deamido-NAD (nmol)</td>
<td>NAMN (nmol)</td>
</tr>
<tr>
<td>-</td>
<td>274</td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td>116</td>
<td>152</td>
</tr>
</tbody>
</table>

*The standard incubation mixture contained: \(^{14}\)C-quinolinic acid (QA), 1.0 \(\mu\)mol; phosphoribose-1-pyrophosphate (PRPP), 2.5 \(\mu\)mol; MgCl\(_2\), 10 \(\mu\)mol; potassium phosphate buffer, pH 7.4, 40 \(\mu\)mol; enzyme preparation, 10 mg of protein; and, where indicated, adenosine triphosphate (ATP), 5.0 \(\mu\)mol. A total volume of 0.6 ml was incubated in a \(\text{H}_2\) atmosphere for 2 h at 37°C. Deamido-NAD, Nicotinic acid adenine dinucleotide; NAMN, nicotinic acid mononucleotide; NAR, nicotinic acid riboside; NA, nicotinic acid.

**Table 2. Utilization of nicotinic acid mononucleotide**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>NAMN* degraded (nmol)</th>
<th>NAR (nmol)</th>
<th>NA (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aerobic</td>
<td>175</td>
<td>11</td>
<td>164</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>265</td>
<td>16</td>
<td>249</td>
</tr>
<tr>
<td>Tris-hydrochloride buffer:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aerobic</td>
<td>165</td>
<td>45</td>
<td>120</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>302</td>
<td>60</td>
<td>242</td>
</tr>
</tbody>
</table>

* A 400-nmol amount of \(^{14}\)C-NAMN (2.4 \(\times\) 10\(^4\) counts/min) was added to the initial reaction mixture. The values given are after 2 h of incubation at 37°C. NAR, Nicotinic acid riboside; NA, nicotinic acid.

**Fig. 1. Degradation of nicotinic acid mononucleotide in the absence of adenosine triphosphate.** O, Phosphate buffer under aerobic conditions; ●, phosphate buffer under anaerobic conditions; △, Tris-hydrochloride buffer under aerobic conditions; and ▲, Tris-hydrochloride under anaerobic conditions. The reaction mixture contained: \(^{14}\)C-NAMN (2.4 \(\times\) 10\(^4\) counts/min), 0.4 \(\mu\)mol; potassium phosphate or Tris-hydrochloride buffer, 25 \(\mu\)mol (pH 7.4); and enzyme preparation, 10 mg of protein in a total volume of 0.5 ml. Incubation temperature was 37°C.
under the two conditions become insignificant. Conversion of NAR to NAMN in the presence of ATP could not be detected. (Table 3).

**Metabolism of deamido-NAD.** In the absence of ATP, deamido-NAD was degraded to NA via NAMN (Table 4). The reaction was markedly influenced by inorganic phosphate and by the imposition of aerobic conditions (Fig. 3). Inorganic phosphate inhibited the degradation of deamido-NAD by 50 to 75% under either aerobic or anaerobic conditions. In either buffer system, the reaction was considerably greater under anaerobic conditions.

Degradation of deamido-NAD was found to be markedly affected by the ATP concentration in the reaction mixture. At 5 and 10 mM ATP concentrations there was considerably more degradation than in the absence of ATP. Only in the presence of relatively high concentrations (20 mM) of ATP was a decrease in degradation observed (Fig. 4). Under these conditions, it appears that reconversion of NAMN to deamido-NAD prevails over degradation. The amount of deamido-NAD degraded was not significantly different under aerobic or anaerobic conditions except in the absence of ATP, in which case there was a marked decrease in the amount of deamido-NAD degraded under aerobic conditions.

**Metabolism of NAD.** Under all of the conditions examined, 14C-nicotinamide mononucleotide (NMN) was the only major product formed from 14C-NAD. Anaerobically, in Tris-hydrochloride buffer, substantial amounts of NMN were observed as a product. Under aerobic conditions or in the presence of phosphate buffer, or both, considerably lower amounts of NMN were produced (Fig. 5). Only under

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Deamido-NAD degraded (nmol)</th>
<th>Products formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aerobic</td>
<td>67</td>
<td>8</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>160</td>
<td>11</td>
</tr>
<tr>
<td>Tris-hydrochloride buffer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aerobic</td>
<td>258</td>
<td>23</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>374</td>
<td>17</td>
</tr>
</tbody>
</table>

* A 400-nmol amount of 14C-deamido-NAD (2.1 x 10^4 counts/min) was added to the initial reaction mixture. The values given are after 2 h of incubation at 37 C. NAMN, Nicotinic acid mononucleotide; NAR, nicotinic acid riboside; NA, nicotinic acid.
aerobic conditions; ride (deamido-NAD) cleotide mixture was under anaerobic triphosphate. 0, bation temperature deamido-NAD, significant under buffer described ports product as activity cohydrolase addition cyanide NAD glycohydrolase in detectable amounts condition, the total degradation to 1.5% experiments in (Fig. 5), because previous experiments ing (Fig. 5)t the 0.4 bacteria. X0 0.3 + in sonic extracts (2), but degradation of NAD to NMN (Fig. 5) suggests that the degradation of NAD to NMN in C. butylicum is catalyzed by a pyrophosphatase and is not due to the presence of a NAD-specific deoxyribonucleic acid (DNA) li-

![Graph](https://via.placeholder.com/150)

**FIG. 3.** Degradation of nicotinic acid adenine dinucleotide (deamido-NAD) in the absence of adenosine triphosphate. O, potassium phosphate buffer under aerobic conditions; ●, potassium phosphate buffer under anaerobic conditions; △, Tris-hydrochloride buffer under aerobic conditions; ▲, Tris-hydrochloride buffer under anaerobic conditions. The reaction mixture was the same as for Fig. 1, except ¹⁴C-deamido-NAD, (0.4 µmol, 2.1 x 10⁶ counts/min was added instead of nicotinic acid mononucleotide. Incubation temperature was 37 C.

![Graph](https://via.placeholder.com/150)

**FIG. 4.** Effect of adenosine triphosphate (ATP) on the degradation of nicotinic acid adenine dinucleotide (deamido-NAD). O, NA formed under aerobic conditions; ●, NA formed under anaerobic conditions; △, deamido-NAD degraded under aerobic conditions; and ▲, deamido-NAD degraded under anaerobic conditions. The reaction mixture contained: ¹⁴C-deamido-NAD, 0.4 µmol (2.5 x 10⁶ counts/min); potassium phosphate buffer, 25 µmol (pH 7.4); MgCI₂, and ATP in the amounts indicated; enzyme preparation, 10 mg of protein in a total volume of 0.5 ml. Incubation time was 3 h at 37 C.
gase as was found in E. coli (19, 28), because the DNA ligase was reported to be markedly stimulated by inorganic phosphate (28).

Absence of recycling of NA. In experiments with $^{14}$C-NA as substrate in the presence of ATP and PRPP, no conversion of NA to NAMN or deamido-NAD could be demonstrated. The only radioactive product found was the $^{14}$C-NA added initially.

**DISCUSSION**

Our results show that crude cell-free extracts of C. butylicum readily degraded NAMN and deamido-NAD, but not NAD, to NA. The reactions involved were found to be markedly affected by inorganic phosphate, the concentration of ATP, and the presence or absence of an anaerobic environment.

The inhibitory effect of phosphate on the pyrophosphatases of C. butylicum was not unexpected. Most enzymes which act upon substrates containing phosphate groups will be inhibited to varying degrees by phosphate, because an enzyme cationic group will usually be present at the active center for interaction with the phosphate of the substrate (27). Brown and Stadtman (3) reported that the Fe$^{3+}$-dependent NAD pyrophosphatases isolated from C. propionicum are markedly inhibited by orthophosphate. However, the mechanism of this inhibition remains unclarified because of the possibility that the inhibition resulted from the precipitation of Fe$^{3+}$ as Fe$_3$(PO$_4$)$_2$ from the reaction mixture, rather than from a direct effect on the enzyme.

ATP exerted an unexpected effect on the degradation of NAMN and deamido-NAD in that it appeared to activate the degradation of these intermediates. The degradation of NAMN and deamido-NAD was greater in the presence of 5 to 10 mM ATP than in its absence. The activities of the nucleotidases degrading NAMN and deamido-NAD were substantially reduced only in the presence of a higher concentration of ATP (20 mM). At this concentration of ATP, NAMN was converted to deamido-NAD in significant amounts, but degradation still prevailed over synthesis. In comparable experiments with S. cerevisiae, an organism known to possess all of the enzymes of the pyridine nucleotide cycle, we have observed that QA, NAMN, and deamido-NAD were almost completely reduced to degradative products in the absence of ATP. Even in the presence of relatively low concentrations of ATP, there was a definite shift of all reactions in the direction of deamido-NAD synthesis. The degradation of deamido-NAD, which proceeded via NAMN, was almost completely inhibited by ATP, obviously because of reconversion of NAMN to deamido-NAD (unpublished data). These observations are in accord with those of Nakamura, Nishizuka, and Hayashi (18), who observed that the degradation of NAMN by crude rat liver extracts was completely inhibited by ATP.

All attempts to demonstrate recycling of NA via the synthesis of NAMN in extracts of C. butylicum were unsuccessful. In experiments in which radioactively labeled QA or NAMN were utilized as substrates, all of the intermediates of synthetic as well as degradative reactions were detectable (Tables 1 and 2). Identical experiments with $^{14}$C-NA as substrate revealed $^{14}$C-NA to be the only detectable compound at the end of the incubation. If NA was being converted to NAMN, then radioactively labeled NAMN, deamido-NAD, or NAR, should have been observed in detectable amounts. All of these observations support previous investigations which revealed that C. butylicum lacks NAMN pyrophosphorylase (10).
C. butylicum was found to possess NAMNase activity, but it was unable to phosphorylate NAR in the presence of ATP. In fact, ATP appeared to activate the degradation of the riboside (Table 3).

As a result of the absence of NAMN pyrophosphatase, the incapability of phosphorylating NAR to form NAMN and the presence of very active degradation of NAMN and deamido-NAD, NA accumulates in the culture medium. ATP stimulates the degradation of NAMN, deamido-NAD, and NAR, contributing to the accumulation of NA. On the other hand, ATP is a substrate in the conversion of NAMN to deamido-NAD and in the amidation of deamido-NAD to NAD. Concentrations of 20 mM ATP were required to suppress the degradative activity on NAMN and to permit the synthesis of deamido-NAD from NAMN in a significant amount. This dual function of ATP complicates the understanding of its role in the biosynthesis of NAD in C. butylicum. The mechanism of this effect of ATP is the subject of continuing investigations.

NAD was degraded to NMN by a pyrophosphatase. Under anaerobic conditions there was practically no further degradation of NMN. Only under aerobic conditions were small amounts of NA formed from NAD. This organism was found to possess an active nicotinamide deamidase; therefore, it is obvious that NMN was not degraded further to nicotinamide to any significant extent. Because it was also shown that phosphate strongly inhibited NAD pyrophosphatase activity, it is clear that under the conditions of anaerobiosis and physiological phosphate concentrations there is very little degradation of NAD to NMN or further degradation of NMN to NA by C. butylicum.

All of the information which has been presented suggests that the intermediates on the pathway of NAD biosynthesis represent the source of the NA which accumulates in the culture medium during growth of C. butylicum. In comparison, NAD was found to be the only source of NA accumulated in growing cultures of M. tuberculosis, for NAMN and deamido-NAD were not degraded by this organism (13).

It appears unusual for an organism to irreversibly degrade such an important cofactor as NAD or its metabolic precursors. Most organisms which have been investigated to date appear to be able to recycle the products of the degradation of NAD or its biosynthetic intermediates (1, 4, 5, 6, 9, 11, 18, 22, 23, 24). C. butylicum and M. tuberculosis var. hominis appear to be unique exceptions in this regard. These organisms may represent individual species in which the more common regulatory mechanisms of feedback and repression are absent. The irreversible degradation of NAD or its precursors may represent a crude substitute for metabolic control over the intracellular concentration of this cofactor.

ACKNOWLEDGMENT

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