Regulation of Pyridine Nucleotide Biosynthesis in
Escherichia coli*

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Regulation of cellular metabolism has received wide attention in recent years (1, 2). It is now well established that in a bacterium the rate of production of major small molecules, such as pyrimidines, purines, and amino acids, is controlled. The advantage for the cell in possessing such regulatory mechanisms is presumably one of economy since these compounds are precursors of most of the materials of the cell and since their production requires much of the energy expended by the cell.

In the case of enzymes, which are required only in catalytic amounts, one wonders whether it is more costly and less convenient for a cell to provide an efficient control mechanism than to produce such materials continuously in excess (3). Indeed, some vitamins (4, 5) and coenzymes (3) are produced in excess. Such evidence could indicate that the cell possesses an inefficient control over the formation of these compounds. However, little definitive information is available about the control of either vitamin or coenzyme production.

In view of the known pathway of diphosphopyridine nucleotide formation and the lack of definitive information concerning metabolic regulation of coenzyme biosynthesis, it was of interest to establish the pathway for triphosphopyridine nucleotide synthesis in bacteria and then examine the control mechanism, if any, used by the cell to regulate the intracellular levels of diphospho- and triphosphopyridine nucleotides.

The prominent role of the pyridine nucleotides, as coenzymes in biological systems, has been known for many years. Recently, the biosynthetic pathway from nicotinic acid has been established as proceeding by the following sequence of reactions (6–8):

\[ \text{Nicotinic acid} + \text{PRPP} \rightarrow \text{NAD} + \text{PPi} \]  
(1)

\[ \text{NAD} + \text{ATP} \rightarrow \text{NADH} + \text{ADP} \]  
(2)

\[ \text{NADH} + \text{H}^+ + \text{DPN} \rightarrow \text{DPNH} + \text{AMP} + \text{PPi} \]  
(3)

In addition, TPN biosynthesis in yeast (9, 10), pigeon liver (11, 12), and mammalian tissue (13) is known to proceed according to Reaction 4.

\[ \text{DPN} + \text{ATP} \rightarrow \text{TPN} + \text{ADP} \]  
(4)

However, the pathway for TPN formation in bacterial systems has not been established.

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This report will present evidence that DPN biosynthesis in Escherichia coli is controlled by a repression mechanism that seems to regulate the biosynthesis of nicotinic acid mononucleotide pyrophosphorylase, the first enzyme specific for pyridine nucleotide formation. The evidence presented also indicates that TPN formation in E. coli proceeds by Reaction 4.

EXPERIMENTAL PROCEDURE

Materials—Nicotinic acid-7-\( ^{14} \text{C} \) (specific activity, 5.88 mc per mmole) and nicotinamide-7-\( ^{14} \text{C} \) (specific activity, 5.96 mc per mmole) were obtained from New England Nuclear Corporation; ATP, PRPP; DPN, and TPN from Pabst Laboratories; protamine sulfate from Eli Lilly and Company; glucose 6-phosphate dehydrogenase from the California Corporation for Biochemical Research (Boehringer); and levigated alumina from the Aluminum Company of America (Alcoa). Unlabeled NAD was a gift from Dr. N. O. Kaplan, and nicotinic acid-\( ^{14} \text{C} \) mononucleotide, nicotinic acid-\( ^{14} \text{C} \) adenine dinucleotide, and DPN-\( ^{14} \text{C} \) were prepared by a modification of the procedure of Preiss and Handler (6). Nicotinic acid-requiring mutants of E. coli K-12 were provided by Dr. Charles Yanofsky.

Methods—Nicotinic acid mononucleotide pyrophosphorylase, nicotinic acid adenine dinucleotide pyrophosphorylase, and DPN synthetase were assayed as previously described (8). DPN kinase activity was measured spectrophotometrically (10) or by following the conversion of DPN-\( ^{14} \text{C} \) to TPN-\( ^{14} \text{C} \). Reaction mixtures for the assay of DPN kinase activity contained: MgSO_4, 2.5 \( \mu \text{mole} \); ATP, 3.0 \( \mu \text{mole} \); Tris-HCl, pH 7.5, 50 \( \mu \text{mole} \); DPN-\( ^{14} \text{C} \), 0.18 \( \mu \text{mole} \); and 0.1 ml of extract in a final volume of 0.5 ml; or when the spectrophotometric assay was employed, the DPN concentration was increased to 4.0 \( \mu \text{mole} \) and 0.2 ml of extract was added. After incubation for 1 hour at 37°, the reaction was terminated by heating in a boiling water bath for 45 seconds. Components of the incubations containing radioactive materials were separated by paper chromatography and assayed in the usual manner (8).

Bacterial growth was followed in a Beckman model DU spectrophotometer at 660 mp. Pyridine nucleotide formation in vivo was determined as previously described (8).

Bacterial Growth and Enzyme Preparation—E. coli strain K-12 was grown in a salts-glycerol medium supplemented with 2 \( \times 10^{-6} \) m thiamine (14), whereas the medium for the nicotinic acid-requiring mutants contained, in addition, 5 \( \times 10^{-6} \) m thiamine.
nicotinic acid. Cultures were grown overnight (8), and the cells were collected by centrifugation, washed once with 0.9% NaCl solution (3 ml per g of packed cells), centrifuged, and immediately disrupted by alumina grinding (2.5 g of levigated alumina per g wet weight of cells). The broken cell preparation was suspended in phosphate buffer (8), centrifuged, and the slightly turbid supernatant fluid was collected. To 10 ml of the supernatant fluid were added 3.6 ml of a 1% protamine sulfate solution. The suspension was stirred for 5 minutes, centrifuged, and the clear supernatant fluid was examined for enzyme activity.

RESULTS

Growth of Nicotinic Acid-requiring Mutants—The growth rate of a nicotinic acid auxotroph supplied with different nicotinic acid derivatives was examined to determine the relative nutritional effectiveness of such compounds. Growth of the mutant was supported by all nicotinic acid derivatives examined. However, NAD and TPN were not as effective in supporting growth as were nicotinic acid, nicotinamide, NMN, or DPN (Fig. 1). The complex nicotinic acid derivatives may require degrading before they can enter the bacteria or the differences in growth rate could reflect different rates of entry. As with other vitamin-requiring mutants (3), growth continued for several hours in the unsupplemented cultures. Internal reserves of the coenzymes probably are adequate for the needs of the organism during this time.

Pyridine Nucleotide Synthesis and Turnover—The rate of synthesis of pyridine nucleotides by a nicotinic acid-requiring E. coli mutant was investigated at two concentrations of nicotinic acid, to determine how responsive coenzyme synthesis was to this nutritional condition. The lowest concentration of nicotinic acid that supported growth at the maximal rate was 1 X 10^{-6} M. This value is in close agreement with one previously reported (15).

The rate of pyridine nucleotide formation is about 2 mmol/mg dry weight per generation when nicotinic acid is supplied in excess (5 X 10^{-6} M) of the minimal growth requirement (Table I). This rate is the same as that found for wild-type E. coli (8), and is slightly in excess of the rate required for maximal growth of the bacteria since a minor fraction of the pyridine nucleotides accumulated in the medium or was found as intermediates (NAD was detected in trace amounts only). These results suggest that the ability of E. coli to convert nicotinic acid to DPN and TPN is limited to approximately the rate of conversion of nicotinic acid to NAD.

When the mutant was grown on a suboptimal concentration of nicotinic acid (5 X 10^{-7} M), the rate of pyridine nucleotide synthesis was reduced about 6-fold (Table I). Regulatory mechanisms apparently cannot rapidly adjust the rate of synthesis to overcome this inadequate precursor supply.

If the pyridine nucleotides were degraded, then the rate of pyridine nucleotide turnover was determined for this purpose. E. coli was grown for 10 generations on C14-nicotinic acid (5 X 10^{-8} M) to label the pyridine nucleotides. A portion of this culture was centrifuged and resuspended in 12 times the original volume of fresh nonradioactive medium. At intervals thereafter, the distribution of radioactivity was determined to see whether the previously formed pyridine nucleotides were degraded. Table II shows that no appreciable loss of radioactivity from DPN or TPN was observed.

A similar experiment was performed with resting bacteria. In this case, the radioactive cells were not diluted when they were resuspended in the nonradioactive medium. Again, the loss of radioactivity from DPN and TPN was small (Table II). Approximately 2% of the pyridine nucleotides was degraded per hour in the resting culture. Thus, turnover does not seem to play a significant role in the metabolism of pyridine nucleotides in either growing or resting E. coli.

Effect of Variation in Nicotinic Acid Concentration—The large amounts of the second and third enzymes, relative to the first
(8), and also the low concentrations of the intermediates, $N\text{,}_a\text{MN}$ and $N\text{,}_a\text{AD}$ (Table I), suggest that the first enzyme limits the rate of conversion of nicotinic acid to DPN. By analogy with other pathways, a repression of this enzyme suggests itself as a possible control mechanism. To test this hypothesis, E. coli was grown on a medium that contained $1 \times 10^{-4}$ M unlabeled nicotinic acid, and then the bacteria were transferred to a medium that contained an excess of radioactive nicotinic acid ($1 \times 10^{-4}$ M). The rate of conversion of the nicotinic acid to pyridine nucleotides was determined at intervals, to measure the activity of the pathway. As shown in Fig. 2, the rate decreased, as if the high concentration of nicotinic acid prevented further enzyme synthesis; the enzyme already present was simply diluted out. These data suggest a repression of enzyme synthesis.

Repression of $N\text{,}_a\text{MN}$ Pyrophosphorylase—To determine

**Table II**

**Pyridine nucleotide turnover in E. coli**

Experimental conditions and assay procedure are described in the text.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Cells (mg dry wt/ml medium)</th>
<th>Nicotinic acid (umoles intracellular)</th>
<th>$N\text{,}_a\text{MN}$</th>
<th>DPN</th>
<th>TPN</th>
<th>DPNH$^*$</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.12</td>
<td>0.007</td>
<td>0.083</td>
<td>0.167</td>
<td>0.017</td>
<td>0.016</td>
<td>0.257</td>
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<tr>
<td>2</td>
<td>0.26</td>
<td>0.000</td>
<td>0.055</td>
<td>0.194</td>
<td>0.020</td>
<td>0.016</td>
<td>0.265</td>
</tr>
<tr>
<td>4</td>
<td>0.65</td>
<td>0.000</td>
<td>0.003</td>
<td>0.226</td>
<td>0.018</td>
<td>0.016</td>
<td>0.265</td>
</tr>
<tr>
<td>6</td>
<td>1.4</td>
<td>0.000</td>
<td>0.004</td>
<td>0.174</td>
<td>0.023</td>
<td>0.014</td>
<td>0.215</td>
</tr>
<tr>
<td>0</td>
<td>1.4</td>
<td>0.150</td>
<td>0.404</td>
<td>2.86</td>
<td>0.295</td>
<td>0.213</td>
<td>3.92</td>
</tr>
<tr>
<td>2</td>
<td>2.2</td>
<td>0.000</td>
<td>0.250</td>
<td>2.90</td>
<td>0.188</td>
<td>0.213</td>
<td>3.56</td>
</tr>
<tr>
<td>4</td>
<td>2.6</td>
<td>0.000</td>
<td>0.059</td>
<td>2.92</td>
<td>0.250</td>
<td>0.204</td>
<td>3.46</td>
</tr>
<tr>
<td>6</td>
<td>3.2</td>
<td>0.000</td>
<td>0.044</td>
<td>2.86</td>
<td>0.300</td>
<td>0.155</td>
<td>3.36</td>
</tr>
</tbody>
</table>

*DPNH and TPNH were determined as free nicotinamide since reduced forms of the pyridine nucleotide are acid-labile.

**Table III**

Repression and derepression of pyridine nucleotide-forming enzymes in E. coli

Experimental conditions and assay procedures are described in the text.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth supplement</th>
<th>Relative enzyme activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotinic acid auxotroph</td>
<td>$1 \times 10^{-4}$</td>
<td>0.05-0.10</td>
</tr>
<tr>
<td>Nicotinic acid auxotroph</td>
<td>$1 \times 10^{-6}$</td>
<td>0.5-1.0</td>
</tr>
<tr>
<td>Nicotinic acid auxotroph</td>
<td>$5 \times 10^{-7}$</td>
<td>0.21</td>
</tr>
<tr>
<td>Nicotinic acid auxotroph</td>
<td>$1 \times 10^{-4}$</td>
<td>0.03-0.10</td>
</tr>
<tr>
<td>Nicotinic acid auxotroph</td>
<td>$1 \times 10^{-4}$</td>
<td>0.10-1.0</td>
</tr>
<tr>
<td>Nicotinic acid auxotroph</td>
<td>$5 \times 10^{-7}$</td>
<td>0.24</td>
</tr>
</tbody>
</table>

*Values given for the auxotroph represent the extreme range observed since the level of the enzyme varied greatly for any given supplement concentration. Such variations depend on the final cell concentration and the time required for growth.

Fig. 2. Rate of $N\text{,}_a\text{MN}$ formation in the intact cell. Experimental conditions are described in the text. Values indicated are for E. coli (wild type) (○) and a nicotinic acid auxotroph (□); the solid line is a theoretical curve for simple enzyme dilution.

Fig. 3. Effect of ATP on TPN formation. Reaction conditions were those described under "Methods."
200 times as great in bacteria harvested from low nicotinic acid medium as in bacteria grown with excess nicotinic acid or DPN. The low activity of the latter extracts cannot be attributed to an inhibitor, since activities of mixed extracts were additive. It is concluded that N₄MN pyrophosphorylase synthesis is regulated by a repression mechanism.

**Feedback Inhibition of Enzyme Activity**—Feedback inhibition of N₄MN pyrophosphorylase activity by DPN was tested by adding $1 \times 10^{-4}$ M DPN to partly purified preparations of the enzyme. No effect of DPN on the activity of the enzyme was detected, and therefore this particular feedback does not seem to occur.

**DISCUSSION**

DPN formation seems to be regulated by the mechanism of repression. In spite of the approximately 1000-fold less rapid synthesis of this coenzyme compared to amino acids and nucleotides, major pathways have this regulatory mechanism in common with this minor one.

Although the evidence presented here provides the first case in which regulation of coenzyme synthesis has been studied at the enzyme level, other evidence is suggestive of control of these minor pathways. Thus, regulation of DPN has previously been indicated; however, the level of DPN reported (16) was 30-fold below the values reported elsewhere (8, 17). Evidence for regulation of flavin synthesis indicates that there is no feedback inhibition, but that some control of enzyme amount is likely (3). Regulation of the level of pyridine nucleotides in mammalian tissue has been suggested (18); however, the significance of such findings has not been ascertained.

Although the regulation reported here applies to the first enzyme of the pathway from nicotinic acid to DPN, it must be remembered that the biosynthesis of pyridine nucleotides actually commences with an earlier metabolite since, in bacteria, the first known intermediate in pyridine nucleotide formation. The first portion of the pathway must also be regulated since bacteria form an ample, but not excessive, amount of nicotinic acid.

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Although the regulation reported here applies to the first enzyme of the pathway from nicotinic acid to DPN, it must be remembered that the biosynthesis of pyridine nucleotides actually commences with an earlier metabolite since, in bacteria, the only function known for nicotinic acid is its role as a pyridine nucleotide precursor. Thus, nicotinic acid is merely the first known intermediate in pyridine nucleotide formation. The first portion of the pathway must also be regulated since bacteria form an ample, but not excessive, amount of nicotinic acid. Elucidation of the first steps of this pathway will be required to continue the problem of DPN regulation.

The data presented here suggest that TPN formation in *E. coli* proceeds by a mechanism analogous to that for other tissues (yeast (10), pigeon liver (12), and mammalian tissue (13)). DPN kinase is present in *E. coli* in large amounts, the rate of the enzyme-catalyzed reaction under optimal conditions being at least 100-fold greater than the rate at which it functions in the intact cell. The affinity of the enzyme for DPN is unusually low ($K_m$ is $1 \times 10^{-3}$ M). Therefore, the enzyme is highly responsive to free DPN concentration, which must be less than $5 \times 10^{-4}$ M (8). Presumably, TPN formation is dependent on the DPN concentration and it appears to be regulated by the kinetics of DPN kinase rather than by repression. In addition, the enzyme is inhibited by high concentrations of ATP (Fig. 3). Increasing concentrations of Mg²⁺ did not appreciably alter the optimal concentration of ATP ($4 \times 10^{-3}$ M). Inhibition by ATP was previously noted with DPN kinase from pigeon liver (12).

**SUMMARY**

Pyridine nucleotide formation in *Escherichia coli* is controlled, at least in part, by a repression-depression mechanism that occurs to regulate the biosynthesis of nicotinic acid mononucleotide pyrophosphorylase, the rate limiting enzyme in diposphopyridine nucleotide synthesis from nicotinic acid. The specific activity of the enzyme varies over at least a 200-fold range depending upon growth conditions.

Triphosphopyridine nucleotide formation in *E. coli* occurs by phosphorylation of diposphopyridine nucleotide. The activity of the responsible enzyme, diposphopyridine nucleotide kinase, is markedly inhibited by excess adenosine triphosphate.

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Regulation of Pyridine Nucleotide Biosynthesis in *Escherichia coli*
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