De Novo Biosynthesis of Nicotinamide Adenine Dinucleotide in *Escherichia coli*: Excretion of Quinolinic Acid by Mutants Lacking Quinolinate Phosphoribosyl Transferase¹

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The excretion of quinolinic acid was studied in growing and resting cells of *Escherichia coli* K-12 *nadC*₁₃. Under optimal conditions, this organism could synthesize quinolinic acid in several-fold excess of the amount which would be required for normal growth. The excretion of quinolinic acid was controlled by the concentration of nicotinamide adenine dinucleotide (NAD) precursors available to the organism either during growth or during incubation in dense cell suspensions. These observations suggest that biosynthesis of NAD de novo is regulated by both repression and feedback inhibition. Analogues of niacin which inhibit bacterial growth also inhibited and repressed the synthesis (excretion) of quinolinic acid. The pH optimum for quinolinic acid excretion agreed favorably with the optimum observed for its synthesis in vitro. The rate of quinolinic acid excretion was strongly influenced by the concentration of ribose or glycerol in the medium.

The pathway for the de novo biosynthesis of nicotinamide adenine dinucleotide (NAD) in bacteria and plants remains to be clarified. Reports from several laboratories have indicated that quinolinic acid (pyridine-2,3-dicarboxylic acid, QA) is a key intermediate in this biosynthetic pathway in *Escherichia coli*, *Mycobacterium tuberculosis*, and higher plants (1, 5, 6, 8). Available evidence indicates that quinolinic acid is synthesized from L-aspartate and a three-carbon precursor closely related to glycerol, by at least two separate enzymes and gene products in *E. coli* K-12 (J. L. R. Chandler, R. K. Gholson, and T. S. Matney, Fed. Proc. 28:351, 1969). In this paper, experiments on the excretion of QA by *E. coli* K-12 *nadC* mutants (14) and some possible regulatory mechanisms controlling the synthesis of QA are described. The availability of mutants blocked in the *nadA* and *nadB* genes (13, 15) suggests that similar experiments might be useful for producing the postulated intermediates between L-aspartate and QA.

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MATERIALS AND METHODS

Bacterial strain. The organism used in this study is a niacin-requiring mutant of *E. coli* K-12 originally obtained from John Imsande and designated by him as E-126. This organism has subsequently been shown to lack the enzyme quinolinate phosphoribosyl transferase, which catalyzes the conversion of QA to nicotinic acid mononucleotide (10). The genetic locus specifying this enzyme has been designated *nadC* and has been mapped at approximately minute 1.5 on the *E. coli* chromosome (14). The original *nadC* mutant (E-126) is now designated as *nadC*₁₃.

Medium. The minimal salts medium of Yates and Pardee (16) was used throughout these experiments. Unless otherwise indicated, the medium was supplemented with 10⁻⁶ M nicotinic acid and 0.5% glycerol. Other additions are given in the text.

Growth of cells. For experiments with growing cells, cultures were grown at 37°C in 18-mm culture tubes containing 5 ml of medium on a reciprocating shaker. For experiments with cell suspension, 500-ml overnight cultures were harvested at 4°C by centrifugation; the cells were washed with saline and added to the minimal medium, supplemented as indicated in the tables. (The dry weight of cell mass added was routinely determined by weighing in duplicate.) These experiments were at 37°C on a reciprocating shaker for the period of time indicated.

The experiments were terminated by rapidly cooling the cultures to 0°C in an ice bath and immediately removing the cells by centrifugation. The cells were washed once with water, and the supernatant solutions were combined. Control experiments indicated that less than 10% of the QA remained in the cells under these experimental conditions. The amount of QA in the supernatant fluid was determined by chromatography and ultraviolet (UV) spectroscopy. The validity of this assay and rigorous identification of the product have previously been described (2). The purity of the QA obtained by this procedure was routinely checked by its distinctive UV spectrum, by paper chromatography, and by decarboxylation to form nicotinic acid (2).

Chemicals. Vitamin-free casein hydrolysate (Casamino Acids) was obtained from Difco. The pure, crystalline L-amino acids were obtained from Sigma Chemical Co., St. Louis, Mo. 5-Fluoronicotinamide and 5-fluoronicotinic acid were gifts from P. McGuire, Eli Lilly & Co. NAD was generously donated by D. Brusca, Enzymedic Laboratories. All other chemicals were of reagent grade.

RESULTS

Nicotinic acid-requiring mutants of *E. coli* require between 5×10^{-7} and 1×10^{-6} M nicotinic acid (or nicotinamide or NAD) in the medium for normal growth (7). This is equivalent to 2.5 to 5 nmoles per 5 ml of culture. However, *E. coli* cells are capable of the synthesis of QA in amounts in excess of 100 nmoles per 5 ml of culture, as shown by the data summarized in Table 1. After growth of the *nadC*₁₃ mutant in minimal medium containing 10^{-6} M nicotinic acid, the amount of QA detected in the supernatant fluid was small but significant. Additions of dicarboxylic acids and 0.5% vitamin-free casein hydrolysate stimulated QA synthesis during growth several-fold (Table 1, experiment I).

The results summarized in experiment II of Table 1 demonstrate that the excess production of QA is controlled by the availability of nicotinic acid. An increase in the nicotinic acid concentration from 10^{-6} M to 2×10^{-5} M reduced QA synthesis to 42% of the control value. When nicotinic acid was added to the growth medium at a 10-fold excess, i.e., 10^{-5} M, no QA was detected in the culture. This observation confirms regulation of QA biosynthesis by repression, which had been suggested by the results of in vitro enzyme assays (12).

The relationships among growth rate, the final concentrations of cells, and medium supplements complicate the interpretation of experiments with growing cells. Therefore, further experiments were conducted with heavy cell suspensions. Although it was necessary to

TABLE 1. Excretion of quinolinic acid (QA) by *E. coli nadC*₁₃ grown with various supplements

Addition ^a	Optical density ^b	QA yield (μmoles/5 ml) ^c
<i>Experiment I</i>		
None	0.96	<.10
Aspartate	0.87	.51
Malate	0.90	.35
Aspartate + pyruvate	1.05	.53
Malate + pyruvate	1.04	.31
Succinate + pyruvate	1.06	.40
Aspartate + 0.5% Casamino Acids	1.13	.68
<i>Experiment II</i>		
Nicotinic acid (1 μM)	0.85	.69
Nicotinic acid (2 μM)	0.72	.29
Nicotinic acid (10 μM)	0.70	<.05

^a The growth medium in experiment I was minimal salts (16) with 0.5% glycerol, 2 μM thiamine, 1 μM niacin, and 5 μM substrates; the incubation time was 30 hr. In experiment II, the growth medium was minimal salts (16) with 0.5% glucose as the carbon source, supplemented with 0.1% Casamino Acids and 5 μM aspartate, 2 μM thiamine, and nicotinic acid as shown; incubation time was 30 hr.

^b Optical density was determined in the growth tubes on a Coleman Junior colorimeter at 660 nm.

^c Cultures were in late exponential or early stationary phase when cells were harvested and the QA content of the medium was determined (2).

carry out these experiments in the presence of both a nitrogen (i.e., L-aspartate) and an energy source (i.e., a carbohydrate precursor of the 3-carbon source for QA), the initial cell density of the suspension would allow little growth under these experimental conditions. Furthermore, pH 8.0, at which most of the resting-cell experiments were conducted, is highly unfavorable for growth of *E. coli*.

The experiment summarized in Table 2 demonstrates that heavy suspensions of *nadC*₁₃ cells grown in the presence of 10^{-6} M niacin produce QA when incubated in the presence of a carbohydrate and organic acids. The addition of glucose, vitamin-free casein hydrolysate, or citrate stimulated QA production in this long-term experiment. During the incubation period, the pH tended to drop 0.3 to 0.8 pH units.

The effect of pH on the production of QA by suspensions of *nadC*₁₃ cells is shown in Table 3. The pH optimum was between 7.8 and 8.5, which is in good agreement with the pH optimum for the enzymatic synthesis of QA in vitro (3).

The effect of niacin vitamers, niacin ana-

TABLE 2. *Synthesis of quinolinic acid (QA) by suspensions of nadC₁₃ cells^a*

Additions to the medium			QA yields (μ moles/5 ml)
Glycerol (%)	Aspartate (mmoles)	Other	
0.5	10	—	0.98
0.5	20	—	1.07
0.5	50	—	1.16
2.0	20	—	1.09
—	20	Glucose (0.5%)	1.55
0.5	20	Casamino Acids (0.5%)	2.20
0.5	20	Citrate (20 mM)	1.39
0.5	—	Glutamate (20 mM)	0.97

^a A 500-ml culture of *nadC₁₃* was grown overnight on minimal medium supplemented with 10^{-6} M nicotinic acid. After the bacteria were harvested by centrifugation at 4 C, the cells were washed once with 0.9% NaCl and resuspended in 10 ml of water. Portions of the cell suspension (0.5 ml; 18.5 mg, dry weight) were added to test tubes (16 to 150 mm) containing 5 ml of minimal medium and the additions given in the table. The suspensions were aerated on a reciprocal shaker for 26 hr at 37 C. The cells were removed by centrifugation and washed once with water; the supernatant fluids were assayed.

logues, and nucleic acid bases on QA synthesis is shown in Table 4. The addition of nicotinic acid or nicotinamide to the cell suspension reduced the amount of QA synthesis to about 20% of the control. The growth-inhibiting analogues of niacin (6-aminonicotinamide, 6-aminonicotinic acid, 5-fluoronicotinamide, and 5-fluoronicotinic acid [9], also effectively inhibited the synthesis of QA. 6-Chloronicotinic acid, which does not inhibit the growth of *E. coli* (9), did not inhibit the synthesis of QA. Since we have previously observed the apparent feedback inhibition of QA synthesis in vitro by NAD but not by nicotinic acid or nicotinamide (3), these results suggest that the inhibiting niacin analogues are converted into the corresponding NAD analogues, which in turn are the actual feedback inhibitors in vivo. Further experiments will be required to clarify these points. Since the purines and pyrimidines did not inhibit QA synthesis, the effect appears to be specific for growth-inhibiting analogues of nicotinic acid and precursors of NAD.

In studies designed to gain insight into the nature of the three-carbon precursor of QA, a series of carbohydrates were tested for their effect on QA excretion. Surprisingly, ribose stimulated QA excretion in short-term experiments with resting-cell suspensions. The effect

of equimolar concentrations of glycerol and ribose on QA synthesis as a function of time is shown in Table 5. However, the incorporation of 14 C-ribose into QA in vitro could barely be detected (Chandler and Gholson, *unpublished data*). One possible explanation of this discrepancy could be that the in vitro experiments were conducted with radioactive substrates at 1 mM, whereas in the in vivo experiments the substrates were at 20 mM. To test this possibility, various concentrations of glycerol and ribose were incubated with a suspension of *nadC₁₃* cells (Table 6). Surprisingly, either 1 mM glycerol or ribose greatly reduced QA synthesis when compared to either *lower* or *higher*

TABLE 3. *Effect of pH on quinolinic acid (QA) synthesis by suspensions of nadC₁₃ cells^a*

pH	QA yield (μ moles/5 ml)
6.0	0.42
6.5	0.61
7.0	0.74
7.5	0.87
7.8	1.01
8.0	1.08
8.2	1.18
8.5	0.93

^a The experimental conditions are described in Table 2. The medium was supplemented with 0.02 M L-aspartate and 1% glycerol. The pH of the medium was adjusted by addition of 1 N HCl or KOH. The cell suspensions (18.5 mg, dry weight) were incubated for 26 hr.

TABLE 4. *Effect of organic bases on quinolinic acid (QA) synthesis by suspensions of nadC₁₃ cells^a*

Addition	QA yield (μ moles/5 ml)
None	0.93
5-Fluoronicotinic acid	0.13
5-Fluoronicotinamide	0.14
6-Aminonicotinic acid	0.16
6-Aminonicotinamide	0.19
6-Chloronicotinic acid	1.06
Nicotinic acid	0.18
Nicotinamide	0.19
Guanine	1.03
Adenine	1.03
Cytosine	1.05
Uracil	1.04

^a The experimental conditions are described in Table 2. The cell suspension (0.5 ml; 22 mg, dry weight) was added to pH 8.0 minimal medium supplemented with 0.02 M L-aspartate, 0.02 M ribose and either 5 μ moles of the pyridine derivatives or 2.5 μ moles of the pyrimidine or purine derivatives.

concentrations of the substrates. This experiment was repeated twice with essentially similar results.

In addition to mutant *nadC*₁₃, four other *nadC* mutants (14) were observed to produce QA when incubated with L-aspartate and glycerol. Therefore, the excretion of QA appears to be a general property of *nadC* mutants.

As previously mentioned, vitamin-free casein hydrolysate stimulated the synthesis of QA in growing cells. It has also been observed that 1% vitamin-free casein hydrolysate will replace the niacin requirement of a significant percentage of the *nad* mutants isolated. These two facts suggest that alternative regulatory mechanisms may control QA synthesis *in vivo*. To test this possibility, cells were grown on minimal medium and minimal medium supplemented with 1% vitamin-free casein hydrolysate. The ability of the cells to synthesize QA was then measured in the presence of three different carbohydrates and three niacin vitamins. No major differences were observed between the two cultures. Hence, it appears that the biochemical basis of the amino acid effects on QA synthesis must be further explored at the molecular level.

DISCUSSION

This series of experiments was undertaken to determine whether *E. coli nadC* mutants which lack quinolinate phosphoribosyl transferase would excrete significant quantities of one of the intermediates of the *de novo* pathway of NAD biosynthesis into the medium. Clearly, these *E. coli* strains lacking quinolinate phosphoribosyl transferase produce

TABLE 5. Time course of quinolinic acid (QA) synthesis with suspensions of *nadC*₁₃ cells in the presence of glycerol or ribose^a

Incubation time (min)	QA yield (μmoles/5 ml)	
	Glycerol	Ribose
0	<0.05	<0.05
30	0.22	0.29
60	0.34	0.37
120	0.53	0.62
240	0.76	0.81

^aThe experimental conditions are described in Table 2. Mutant cells (24.5 mg, dry weight) were incubated in minimal medium at pH 8.0 supplemented with 0.02 M L-aspartate and either 0.02 M glycerol or 0.02 M ribose on a reciprocal shaker at 37°C. After the reaction had proceeded for the described time, the cells were removed by centrifugation and the QA in the medium was determined.

TABLE 6. Effect of glycerol and ribose concentrations on quinolinic acid (QA) synthesis in suspensions of *nadC*₁₃ cells^a

Addition	Concn (mM)	QA yield (μmoles/5 ml)
None	—	.07
Glycerol	0.20	.16
Glycerol	1.00	<.05
Glycerol	5.00	.62
Glycerol	100.00	.51
Ribose	0.50	.49
Ribose	1.00	.12
Ribose	2.00	.15
Ribose	5.00	.65

^aThe experimental conditions are described in Table 2. The cell suspension (21 mg, dry weight) was added to pH 8.0 minimal medium supplemented with 0.02 M L-aspartate and incubated for 4 hr.

excessive quantities of quinolinate when grown or incubated in niacin-deficient medium. The amount of QA production by these mutants can exceed the amount of NAD synthesis required under normal growth conditions (7) by a factor of 5 to 100. Thus, cells of *E. coli* K-12 must contain regulatory mechanisms which govern the production of NAD under normal growth conditions.

These *in vivo* experiments provide suggestive evidence that three separate regulatory mechanisms are operating. At low precursor concentrations, very little QA is excreted into the medium, which suggests that the enzymes which synthesize QA are operating far below their maximal potential velocity. Under normal growth conditions on minimal medium, no QA was found in the presence of excess nicotinic acid (Table 1) or when heavy suspensions of cells grown on excess nicotinic acid were incubated with L-aspartate and a carbohydrate (data not shown). This is consistent with previous data from *in vitro* experiments (12) and suggests repression of enzymes which synthesize QA (12). Addition of nicotinic acid, nicotinamide, NAD, or growth-inhibitory analogues of nicotinic acid effectively reduced the ability of derepressed cells to synthesize QA. This suggests that the pathway is also subject to regulation by feedback inhibition. Superficially, then, it appears that the regulation of the biosynthesis of the coenzyme NAD in *E. coli* is similar to that observed in major pathways such as those of amino acids or nucleic acids. Naturally, experiments reported here do not exclude the presence of other regulatory mechanisms nor do they define the molecular nature of these regulatory

mechanisms. The unusual inhibition of QA synthesis by intermediate levels of carbohydrates (Table 6) and the stimulation of QA synthesis and the stimulation of growth of certain *nadB* mutants by vitamin-free casein hydrolysate make it tempting to speculate that further regulatory mechanisms will be discovered.

Our experiments on the production of QA in *nadC* mutants of *E. coli* are in general agreement with the exploratory experiments of Ortega and Brown (11) on the synthesis of nicotinic acid in suspensions of *E. coli* cells.

Optimal conditions for the production of quinolinate, the last non-nucleotidic compound on the de novo pathway of NAD biosynthesis in aerobic microorganisms and plants (4), have now been established. This work suggests that similar experiments may be useful to produce postulated earlier intermediates in the pathway by utilizing *nadA* and *nadB* mutants.

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