QUINOLINIC ACID BIOSYNTHESIS

IN *ESCHERICHIA COLI*

By

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CHAPTER I

INTRODUCTION

In 1867, Huber (1) oxidized nicotine with chromic acid and obtained a white, crystalline solid, m.p. 233°, which he called nicotinic acid. He later demonstrated the structure of nicotinic acid to be pyridine-3-carboxylic acid (2). In 1911, Funk (3) isolated nicotinamide from rice bran. It is now known that nicotinic acid and its derivatives are ubiquitously distributed in biological material.

The first evidence for the biological function of nicotinic acid was obtained by Harden and Young (4) in 1906, who found that the dialysate (cozymase) obtained from boiled yeast extract stimulated fermenting yeast juice (zymase) to produce more alcohol. The lengthy efforts to identify "cozymase" and the closely related compound, "coferment", culminated with the identification of "cozymase" as nicotinamide adenine dinucleotide (NAD)* and "coferment" as nicotinamide adenine dinucleotide phosphate (NADP) (5,6,7). Concomitant with the

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*All abbreviations used are those specified in the Journal of Biological Chemistry. For those not appearing in this journal, the following are used: NA, nicotinic acid; NAm, nicotinamide; NAMN, nicotinic acid mononucleotide; des-NAD, desamido NAD; ARPPR, adenosine diphosphate ribose; PRPP, 5-phosphoribosyl-1-pyrophosphate; QA, quinolinic acid; DEAE-cellulose, diethylaminoethylcellulose; QRTase, quinolinate phosphoribosyl transferase (decarboxylating); NRTase, nicotinic acid mononucleotide pyrophosphorylase.

isolation and identification of the pyridine nucleotides was the observation that the nicotinamide moiety was involved in hydrogen transfer reactions. Loewus et al. (8) demonstrated that hydrogen was reversibly transferred to and from the 4-position of the pyridine ring. By 1962, over 120 different biochemical reactions had been identified in which NAD or NADP functioned as a coenzyme in oxidation-reduction reactions (9).

In 1937, Elvehjem et al. (10) reported that NA would cure black tongue, a dietary disease of dogs. It was subsequently demonstrated that the four "D's" of the human nutritional disease of pellagra; dermatitis, diarrhea, delirium, and death; could be cured by the addition of NA to the diet (11). Further nutritional studies by Krehl et al. (12) led to the conclusion that tryptophan could substitute for niacin in the diet of mammals. Furthermore, the injection of tryptophan-7a-^{14}C resulted in the production of nicotinic acid-^{14}C, demostrating that tryptophan is a precursor of niacin. The enzymes which catalyze the conversion of tryptophan to NAD and hence to NA have been purified and studied in vitro (13).

Other Functions of NAD

In addition to the reaction of NAD in oxidation-reduction processes, three new reactions have recently been discovered. Mandel et al. (14) discovered that eukaryotic cells contain a nuclear enzyme which polymerizes the ARPPR moiety of NAD with release of nicotinamide. The units of the ARPPR polymer are covalently linked by a glycosidic bond between the terminal ribose and the hydroxyl group at the two

position of the ribose attached to the adenine (15). The polymer is attached to histones *in vivo* (16). The enzyme requires the presence of DNA for activity. The function of this polymer is not known. Koch *et al.* (17) have recently shown that the polymerase is probably identical to the previously reported nuclear NAD nucleosidase.

In bacteria, the enzyme DNA ligase, which covalently links short, hydrogen-bonded DNA strands, utilizes NAD as a cofactor (18, 19). NAD is stoichiometrically cleaved to NMN and AMP during the course of the reaction. It apparently functions in a similar manner *in vivo* since a temperature-sensitive mutant of bacterial phage T4 accumulates short pieces of DNA rather than a complete genome after infection (20).

The mechanism of action of diphtheria toxin in inhibiting protein synthesis has recently been explained on the basis of the transfer of the ARPPR moiety from NAD to amino-acyl transferase II thus inactivating the enzyme and stopping protein synthesis (21).

The discovery of these three diverse reactions of NAD further emphasizes the unique and complex role of NAD in cellular metabolism.

Precursors of NAD *in vivo*

Although tryptophan is an effective precursor of NAD in mammals, fowl, *Neurospora crassa*, *Fusarium oxysporum*, and *Xanthomonas pruni* (22, 23), no evidence for the tryptophan pathway has been found in *Escherichia coli*, *Bacillus subtilis* (24), *Mycobacterium tuberculosis* (25), *Clostridium butylicum* (26) or higher plants (27); this indicates that these organisms synthesize NAD by a pathway different from that used in animals. The first evidence for the nature of the *de novo*

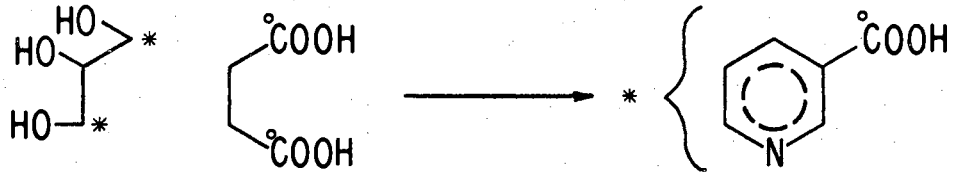
pathway for the synthesis of NA in several bacteria and plants, which will be referred to as the aspartate pathway, was obtained by Ortega and Brown (28). The presence of alanine, aspartate, glutamate, pyruvate and oxalacetate in the growth medium of E. coli stimulates the production of niacin. In resting cells, synthesis of niacin is obtained when both a 3-carbon compound (glycerol or a closely related metabolite) and an acid from the tricarboxylic acid cycle are present. Adenine, ribose and pyruvate stimulate the production of niacin, while glucose inhibits the synthesis. The carbon chains of both ^{14}C-labeled glycerol and ^{14}C-labeled succinate are efficiently incorporated into newly synthesized niacin. Nicotinic acid produced from succinate-1, 4-^{14}C contains ^{14}C in the carboxyl group, while nicotinic acid produced from succinate-2,3-^{14}C or glycerol-1(3)-^{14}C is labeled mainly in the pyridine ring with only relatively small amounts of radioactivity in the carboxyl group. (See Figure 1). Neither radioactive pyruvate nor radioactive propionate is incorporated into the vitamin.

In Germany, Mothes, Gross and co-workers have conducted extensive studies on the precursors of nicotinic acid in M. tuberculosis. This bacterium excretes large quantities of NA (up to 60 μg/ml) and 3-(hydroxymethyl)pyridine into the culture medium (29, 30). They observed that aspartic acid, glutamic acid, asparagine or glycine can serve as good nitrogen sources for nicotinic acid (31). When DL-aspartate-4-^{14}C was incubated with M. tuberculosis cells, the isotope was found in the carboxyl group of nicotinic acid (32). Experiments with aspartic acid-1,4-^{14}C,^{15}N indicate that the C-1 carboxyl group of aspartic acid is lost and the remainder of the molecule is incor-

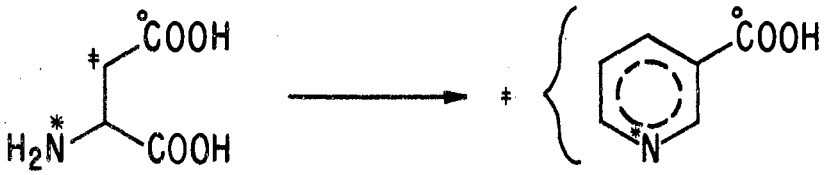
Figure 1

The Incorporation of Radioactive Precursors into Nicotinic Acid in Bacteria and Plants

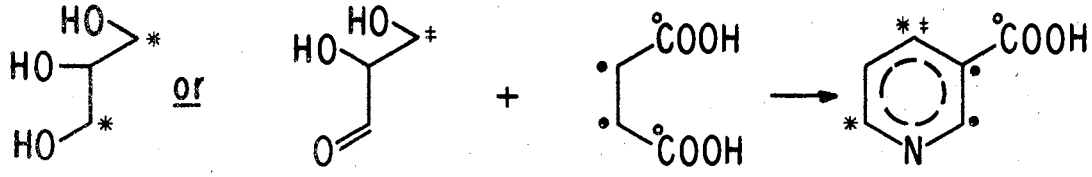
PRECURSOR'S OF NICOTINIC ACID IN BACTERIA AND PLANTS



E. coli (Ortega and Brown, 1960)

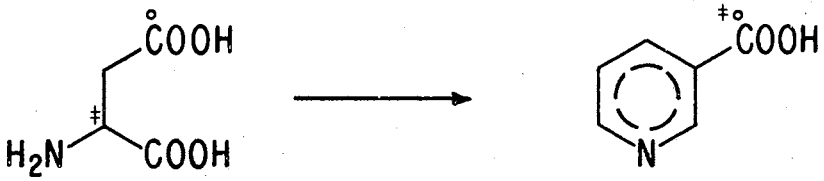


M. tuberculosis (Mothes, et al., 1964, Moat and Albertson, 1964)



Nicotiana species (Yang, et al., 1965, Flecker and Byerrum, 1967)

C. butylicum (Scott, et al., 1968)



S. marcescens (Scott and Hussy, 1965)

porated into NA (33). Moat and Albertson also studied the precursors of nicotinic acid in M. tuberculosis (34). Aspartate-3-^{14}C labeled the pyridine ring while aspartate-4-^{14}C labeled the carboxyl group. Glutamate-1-^{14}C was not incorporated; however, glutamate-5-^{14}C labeled the carboxyl group of NA.

The biosynthesis of pyridine compounds in higher plants has been studied extensively by determining labeling patterns which characterize the incorporation of various isotopically labeled precursors into the pyridine alkaloids nicotine, anabasine, and ricinine. Review articles by Ramstad and Agurell (35) and by Leets (29) summarize recent work.

Nicotinic acid is a precursor of the pyridine alkaloids nicotine, anabasine, and ricinine without randomization of the carbon atoms (36, 37, 38). Since the aspartate pathway appears to be the same in plants as in E. coli and M. tuberculosis, isotopic labeling studies on the pathway of synthesis of these compounds appear to be pertinent. Yang et al. (39) and Griffith and Byerrum (40) found that glycerol is a precursor of positions 4, 5 and 6 of the pyridine ring of nicotine. Glycerol-1(3)-^{14}C labeled the 4- and 6-positions while glycerol-2-^{14}C labeled the 5-position of the pyridine ring. Similar labeling patterns from glycerol-^{14}C have been obtained with ricinine and anabasine (41, 42). In 1967, Flecker and Byerrum (43) observed that D-glyceraldehyde-3-^{14}C was incorporated into the 4-position of the pyridine ring of nicotine. Griffith and Byerrum (40) demonstrated the incorporation of succinate-2,3-^{14}C into carbons 2 and 3 of the pyridine ring. The observed labeling patterns are shown in Figure 1.

Quinolinic acid is a better precursor of either nicotine (39) or ricinine (44) than either aspartate or glycerol, indicating that it is an intermediate on the de novo biosynthetic pathway of these alkaloids. Waller et al. (45) made the interesting observation that NA, NAm, QA, NAMN and NAD are incorporated into ricinine with similar efficiencies.

In summary, these in vivo labeling studies with E. coli, M. tuberculosis, and higher plants are all consistent with the direct incorporation of asymmetrical 3- and 4-carbon units into the family of pyridine ring compounds related to nicotinic acid. Since both intact organism and enzymatic studies conclusively demonstrate that QA is converted into NAMN and NAD, it follows that the 3- and 4-carbon moieties are converted into QA, which then enters the pyridine nucleotide cycle (vide infra) at the NAMN level.

In addition to the tryptophan and aspartate pathways, available evidence indicates the presence of a third and perhaps a fourth pathway for niacin biosynthesis. Isquith and Moat (46) have reported the synthesis of NAD and NA in cell-free extracts of C. butylicum. Fractionation of the crude extract yielded an enzyme preparation which incorporated aspartate, acetate and formate into NA. Glycerol was not incorporated into pyridine ring compounds in this preparation. Scott et al. (47) have found that N-formyl-L-aspartate-5-^{14}C is converted into nicotinic acid-6-^{14}C by extracts of this organism.

After incubating Serratia marcescens cells with aspartate labeled in the 2-, 3- or 4-positions, Scott and Hussey (48) isolated the nicotinic acid and degraded it to pyridine and CO_2. The CO_2 released from NA derived from both aspartate-2-^{14}C and aspartate-4-^{14}C contained greater than 80% of the total radioactivity found in the NA, while the

^{14}C from aspartate-3-^{14}C was retained in the pyridine moiety of NA, indicating aspartate must pass through a symmetrical 3-carbon precursor before conversion to niacin. The observed labeling patterns are shown in Figure 1. The role, if any, of QA in the synthesis of niacin in C. butylicum and S. marcescens remains to be demonstrated.

The yeast Saccharomyces cerevisiae appears to be unique in that this organism is capable of utilizing the tryptophan pathway under aerobic conditions and the aspartate pathway under anaerobic conditions (49).

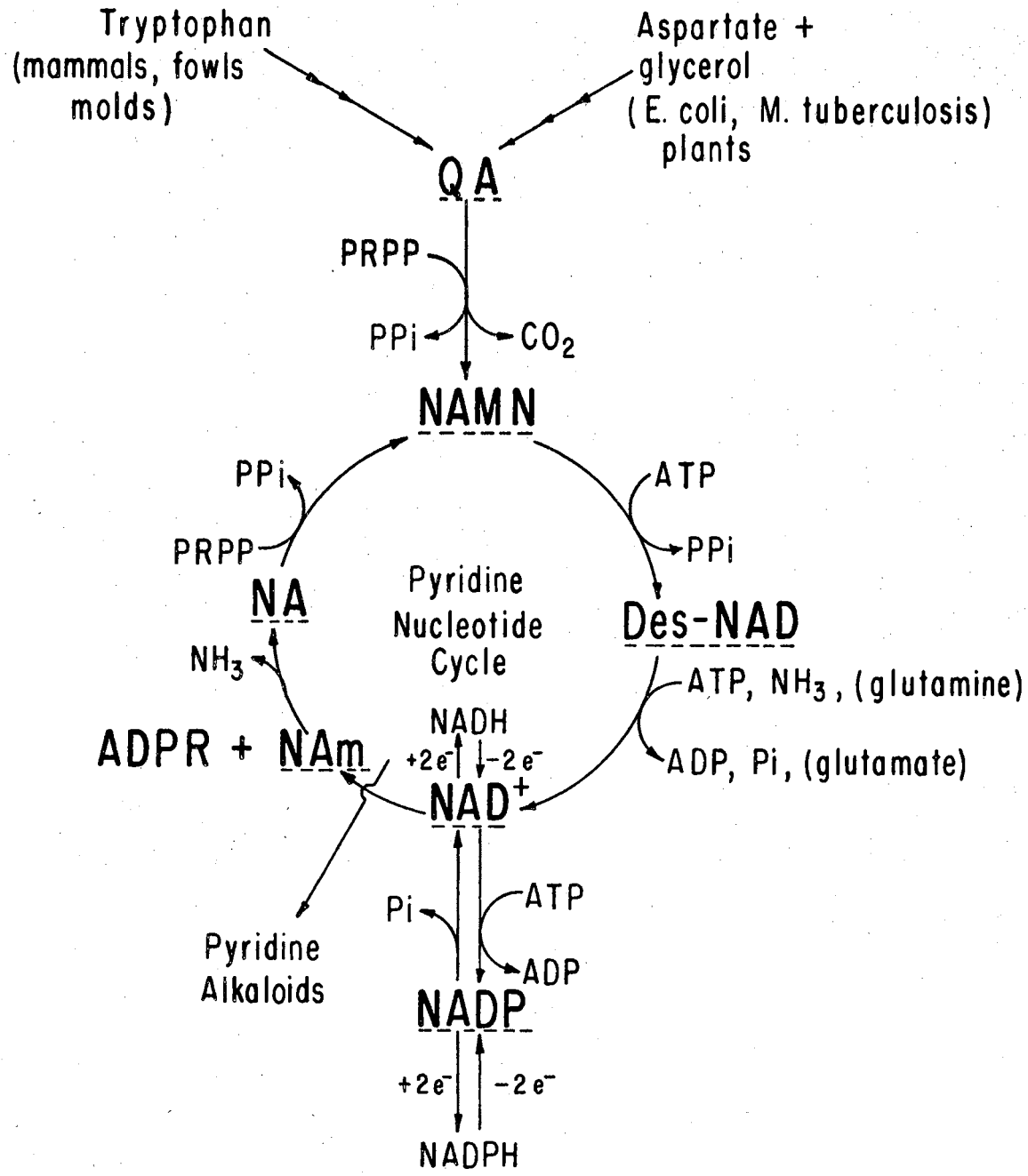
Pathways of Pyridine Ring Metabolism in E. coli and Other Microorganisms

Cells of E. coli appear to metabolize external sources of niacin by reactions common to most organisms. Nicotinamide is hydrolyzed to nicotinic acid by the enzyme nicotinamide deamidase (Figure 2). Nicotinamide deamidase activity has been observed in crude extracts of E. coli (50), yeast (51), castor beans (52), Aspergillus niger (53), Astasia longa (54), Lactobacillus plantarum (55), N. crassa (56) and several other organisms (57). Sundaram (58), starting with an E. coli niacin auxotroph, isolated a mutant unable to utilize nicotinamide. Extracts from this mutant did not deamidate nicotinamide, indicating the obligatory role of NA in NAD formation from NAm. While most bacteria can utilize either nicotinamide or nicotinic acid, Lactobacillus fructosis is an exception. It absolutely requires nicotinamide for growth, and extracts lack the nicotinamide deamidase but contain nicotinamide mononucleotide pyrophosphorylase and thus synthesize NAD at the amide level (55).

Figure 2

The Metabolism of Pyridine Ring Compounds and the Pyridine Nucleotide Cycle

PYRIDINE RING METABOLISM



Nicotinic acid mononucleotide pyrophosphorylase (NRTase) catalyzes the condensation of nicotinic acid with 5-phosphoribosyl-1-pyrophosphate (PRPP) to form nicotinic acid mononucleotide (NAMN) (59). NRTase activity has been observed in crude extracts from E. coli (50), A. longa (54), Lactobacillus mesenteroides, L. plantarum (55), B. subtilis, Salmonella typhimurium, S. marcescens, Torula cremonis, Tetrahymena pyriformis (60), and several other organisms. This enzyme appears to be subject to regulation and will be discussed later.

In the absence of external sources of niacin, NAMN is formed from QA and PRPP by the action of quinolinate phosphoribosyl transferase (decarboxylating) (QRTase) (57).

NAMN reacts with adenosine triphosphate (ATP) in the presence of nicotinate phosphoribosyl transferase to form desamidonicotinamide adenine dinucleotide (desNAD). DesNAD, in the presence of ammonia or glutamine, ATP, and NAD synthetase, is amidated to form NAD (59). Nicotinate phosphoribosyl transferase and NAD synthetase have been observed in most organisms containing NRTase activity (57). NAD and NADP are interconvertible in the cell by the action of NAD kinase and NADP phosphatase (57). The synthesis of NAD from nicotinic acid was first observed by Preiss and Handler (59) in erythrocytes and consequently is usually called the Preiss-Handler pathway. These reactions are shown in Figure 2.

Imsande and Pardee (61) reported that NAD-(nicotinamide-7-^{14}C) was relatively stable metabolically, i.e., its rate of degradation was only 2-3% per hour. Similar experiments by Iizuka et al (62) also indicated very little turnover in E. coli. However, the recent

discovery and purification of DNA ligase in E. coli casts these results in doubt. The intracellular concentration of DNA ligase is extremely high, approximately 3000 molecules per cell (63), indicating that it is probably vital to cellular functions. Since NAD pyrophosphorylase would convert NMN into NAD without the intermediate formation of free nicotinamide, it is quite conceivable that NAD is rapidly cleaved to AMP and NMN and resynthesized. This form of NAD turnover would not be detected by Imsande and Pardee's experiments.

NAD glycohydrolase, which is present in several bacterial species (64), catalyzes the breakdown of NAD to ARPPR and nicotinamide. This reaction completes the pyridine nucleotide cycle in E. coli (57). The existence of enzymes with the capacity to catalyze a cyclic process within the cell strongly suggests that the cycle is functioning, although definitive experimental evidence for its operation in E. coli has not been obtained.

Control Mechanisms Influencing Pyridine Ring Metabolism in E. coli

From a chemical viewpoint, one of the most fascinating aspects of living organisms is the delicate integration of reaction rates of a vast and yet undetermined number of diverse reactions necessary for growth and reproduction. During the past decade, extensive investigations have revealed that the phenomena of induction, repression, and end product inhibition are ubiquitously distributed in biological systems. Various models have been proposed to explain these phenomena at both the macromolecular and molecular levels (65, 66, 67). Most of these studies have been concerned with the mechanisms which

control the synthesis and utilization of major cellular metabolites (precursors of proteins, nucleic acids, cell wall, etc.) (68, 69). E. coli growing on a defined glucose-salts medium with a generation time of 60 minutes synthesizes amino acids and nucleic acids at a rate of approximately 5 μmoles/g (dry weight)/100 sec which is approximately equal to 7,500 molecules per cell per second (70). In general, coenzymes, unlike the major cellular metabolites, are reutilized time and again during the normal course of cellular metabolism and hence the rate of synthesis for a typical coenzyme is about 1/1000 as fast as that of other metabolites (72). The synthesis of NAD at a rate of about 85 molecules per cell per second would be sufficient to maintain an in vivo concentration of about 5×10^{-4} M, which approximates the normal cellular concentration in E. coli growing on an inorganic salts-glucose medium (61, 71).

This slow rate of synthesis of coenzyme has led other authors to consider what mechanisms are used to prevent a coenzyme from being synthesized as fast as an amino acid or nucleic acid base. Wilson and Pardee (72) suggested four plausible models for explaining the low rate of coenzyme synthesis:

1) The number of enzyme molecules is very low because of severe repression by the coenzyme.
2) The number of enzyme molecules is very low as a result of a constitutively low rate of synthesis.
3) The catalytic rate of the enzymes is very slow as a result of feedback inhibition by the coenzyme.
4) The catalytic activity of the enzymes is inherently low.

These four models assume that under normal growth conditions the rate of coenzyme synthesis is independent of other cellular processes.

Imsande and Pardee (61) examined the possible control mechanisms of the nicotinic acid pathway of NAD synthesis in E. coli. Nicotinic acid mononucleotide pyrophosphorylase was the only enzyme of those examined which was repressed when the organism was grown in the presence of 10^{-4} M NA or NAD. No repression of NRTase was observed in S. marcescens, B. subtilis, T. cremonis or T. pyriformis (60). NAD did not inhibit the enzyme, which suggests this pathway is not controlled by feedback inhibition.

NRTase isolated from different sources exhibits diverse responses to ATP. NRTase from yeast and E. coli requires ATP for activity (59, 61); however, the same enzyme from A. longa does not (54). Beef liver NRTase shows an intermediate response to ATP. It is not required for activity; however, in the presence of ATP, the K_m is reduced and catalytic rate is considerably increased (73). The activities of other enzymes were sufficient to make the catalytic rate of NRTase the rate limiting step of NAD biosynthesis (58, 59, 60).

On the de novo pathway, QRTase is not repressed in E. coli when the cells are grown in the presence of high concentrations (10^{-4} M) of NA or NAD (74). This implies that the de novo pathway is controlled in reactions preceding QRTase and presumably at the first step of the reaction sequence leading to QA.

Another mechanism which could contribute to control the level of NAD in the cell is through degradation of NAD by NAD glycohydrolase or a pyrophosphatase. In M. tuberculosis, an active pyrophosphatase

which cleaves NAD to AMP and NMN is present when the organism is grown in yeast extract-supplemented medium. However, when the organism is grown on a minimal-salts medium, the pyrophosphatase is totally inhibited by a second protein; the inhibitor can be inactivated by a brief heat treatment (75). M. butylicum and B. subtilis contain a heat-activated NAD glycohydrolase. The role, if present, of these two enzymes is NAD metabolism in E. coli remains to be determined.

Still another possible control site of the Preiss-Handler pathway is at the cellular membrane. It is known that E. coli can concentrate NA from the medium but the process has not been examined in detail (61). Niacin auxotrophs can utilize NAD, NADP, and nicotinamide ribotide as well as NA and NAm, but it is not known whether the ribotides are hydrolyzed before entering the cell. It seems probable that the ribotides can be transported across the bacterial membrane in view of the fact that Hemophilus influenzae can only utilize niacin vitamers containing the preformed pyridine-ribotide bond (64).

Quinolinic Acid as an Intermediate in NAD Biosynthesis

Quinolinic acid was first isolated from mammalian sources after feeding overloading doses of tryptophan by Henderson in 1949 (76). In 1951, Preist et al. (77) reported a soluble enzyme system from liver which catalyzed the conversion of 3-hydroxyanthranilic acid to QA. However, the primary product of the oxidation is thought to be 2-amino-3-acryloyl-fumaric acid, which cyclizes, apparently spontaneously, to QA. The discovery of quinolinate phosphoribosyl transfer-

rase (decarboxylating) (QRTase) in 1963 culminated the 17-year search for the complete sequence for the conversion of tryptophan to niacin in mammals and certain other species (78, 79).

Since dicarboxylic acids had been found to be precursors of NA derivatives in bacteria and plants (35, 42), the discovery of QRTase suggested that QA may also serve as a precursor of NAD (or its derivatives) in other organisms. This was rapidly established by Gholson et al. (80) with corn seedlings and by Andreoli et al. (81) with E. coli. Thus it appears that pathways of synthesis of NAD from tryptophan and from aspartate converge at QA as shown in Figure 2.

QRTase has been purified from liver (78, 79), from a soil pseudomonad (82) and from E. coli (83). Available evidence indicates a concerted reaction mechanism, i.e., the loss of the alpha-carboxyl group and condensation with PRPP appear to occur simultaneously, since evidence for exchange reactions could not be obtained (84).

The presence of QRTase in E. coli strongly suggests that the product of the aspartate-glycerol pathway is QA, which then enters the pyridine nucleotide cycle at the NAMN level. To test this hypothesis, Ogasawara et al. (85) incubated L-aspartate-^{14}C with an extract from an E. coli mutant lacking QRTase. QA-^{14}C was isolated by ion-exchange and paper chromatography. With the demonstration of QA synthesis in vitro, the molecular basis for the de novo NAD synthesis in E. coli could now be examined at the enzymatic level.

More specifically, studies could be directed toward the identification of the chemical compounds which are precursors of QA and the biological control mechanisms, if any, which control the rate of QA and NAD synthesis in E. coli.

A large number of biochemical pathways in microorganisms have been elucidated by studying auxotrophic organisms which require a specific nutrient for growth. In general, such mutants usually lack one enzyme in the series of enzymes catalyzing the reactions leading to the synthesis of the nutrient required for growth. Hence, *E. coli* mutants which will grow in the presence of a minimal salts medium supplemented with NA were used for this exploratory study on the biochemical synthesis of quinolinic acid.

CHAPTER II

METHODS AND MATERIALS

Cultures

Niacin-requiring strains of E. coli were obtained from Drs. John Imsande, E. A. Adelberg, R. Lavalle, A. L. Taylor, T. K. Sundarum and the American Type Culture Collection. A mutant of E. coli resistant to 6-aminonicotinamide was donated by Dr. A. J. Andreoli. All cultures were derived from the K-12 strain of E. coli except strain 9726b from the American Type Culture Collection. The properties of these strains are listed in Table I. The abbreviations used follow the recommendations of Demerec, Taylor and Adelberg (86). Stock cultures were maintained at 4° on potato extract agar slants.

Growth Conditions

The minimal salts medium formula of Yates and Pardee (87) supplemented with 2×10^{-6} M thiamine was used as basic medium. In Medium "N" 0.5% glycerol was used as the carbon source and nicotinic acid was added at either 6×10^{-7} or 10^{-6} M. In Medium "D", the minimal salts were supplemented with 1.0% glucose and 1.0% vitamin-free Casamino Acids with or without 10^{-6} M niacin. The bacterial subcultures, axenically grown overnight in minimal medium supplemented with 0.05%

TABLE I

GENETIC CHARACTERISTICS OF NIC$^-$ MUTANTS OF *E. coli*

Strain	Sex	Phage	Genetic Markers
E-2	Hfr		nic 2, met
E-3	Hfr		nic 3, met, (?)
E-13	F$^-$	λ^-	nic A1, pro A2, his 4, arg E3, thi 1, lac Y1 gal K2, xyl 1, mtl 1, tsx 1
E-18	F$^-$	λ^+	nic 18, thi 1, mtl 1, xyl 5, ara 14, gal 2, lac Y1, str 20(r)
E-25			nic 25
E-30			nic B2, pur F66, arg H1, thi 1, str A1
E-36			nic 36
E-51	F$^-$	λ^+	nic 51, lac 1, gal 2, ara 14, xyl 5, mtl 1, thi 1, +Ar 5, +6r 57, str 20(r)
E-52	F$^-$	λ^+	nic 52, lac 1, gal 2, ara 14, xyl 5, mtl 1, thi 1, +Ar 5, +6r 57, str 20(r)
E-53	F$^-$	λ^+	nic 53, lac 1, gal 2, ara 14, xyl 5, mtl 1, thi 1, +Ar 5, +6r 57, str 20(r)
E-55	F$^-$	λ^+	nic 55, lac 1, gal 2, ara 14, xyl 5, mtl 1, thi 1, +Ar 5, +6r 57, str 20(r)
E-56	F$^-$	λ^+	nic 56, lac 1, gal 2, ara 14, xyl 5, mtl 1, thi 1, +Ar 5, +6r 57, str 20(r)
E-57	F$^-$	λ^+	nic 57, lac 1, gal 2, ara 14, xyl 5, mtl 1, thi 1, +Ar 5, +6r 57, str 20(r)
E-97			nic 97, ATCC 9723b
E-126	Hfr		nic 126
P4 X SB-16	Hfr		nic 16, met
P4 X SB-41	Hfr		nic 41, met
P4 X SB-84	Hfr		nic 84, met
P4 X SB-99	Hfr		nic 99, met
PA-217	F$^-$		nic 17, arg 1, thi 1, xyl, mal, mtl, lac, gal, strR
PA-218	F$^-$		nic 21, arg 1, thi 1, xyl, mal, mtl, lac, gal, strR
PA-2355	F$^-$		nic 35, try, arg 1, thi 1, xyl, mal, mtl, lac, gal, strR
AT-1123	Hfr		nic 23, thr, leu, thi 1, strR
AT-1124	Hfr		nic 24, thr, leu, thi 1, strR
AT-133	Hfr		nic 33, thr, leu, thi 1, strR
AT-1323	Hfr		nic 32, thr, leu, thi 1, strR
W-3899	F$^-$	λ^+	nic 38
W-3899N	F$^-$	λ^+	nic 38, nad A1
W-4516	F$^-$	λ^+	nic 45, gal
3440	F$^-$		nic 34, met

vitamin-free Casamino Acids, were used to inoculate 10 liters of media in carboys. The cultures were vigorously aerated with compressed air and maintained at 37° in a water bath. After the cultures had reached late log phase, the bacteria were harvested with a refrigerated Sharples Continuous-flow Centrifuge. This procedure usually yielded 25 to 50 g of wet cell paste per 10-liter carboy, depending on the strain employed. The cell paste was washed with 0.9% NaCl solution and stored at -20° until used. Enzymatic activity slowly decreased during storage under these conditions; after eight months approximately 65% of the original activity remained.

To test the effect of different medium supplementations on QA synthesis *in vitro*, one-liter cultures were axenically grown in 2-liter Fernbach flasks and aerated on a New Brunswick Rotary Shaker at 37°. The cells were harvested in the late log phase and stored as described above.

The effect of various compounds on the growth of niacin-requiring strains was determined in 5-ml cultures in 16 x 150 mm test tubes, aerated in a slanted position on a reciprocal shaker. Growth was measured by determining the optical density at 660 mμ on a Coleman Jr. Spectrophotometer. Subcultures for inoculum were grown overnight in Medium "D" containing 10^{-6} M NA. The cultures were washed with 0.9% NaCl solution and resuspended in the same solution. One drop was used to inoculate the test media.

Crude extracts of the cell pastes were obtained by ultrasonic disruption. The frozen cells, about 4 g, were suspended in 20 ml of either 0.10 M potassium phosphate, pH 8.0 or 0.10 M bicine, pH 8.0,

buffer in a rosette cell and sonicated for 5 min, in 30-sec bursts, at full power on a Branson SS-2 Sonifier. By submerging the rosette cell in a salt-ice water mixture, the temperature was maintained below 10°. Unbroken cells and cell debris were removed from the extract by centrifugation for 15 min at 10,000 x g. The composition of the reaction solutions is given in the text.

Assay for Quinolinic Acid

No routine assay for determining either ^{14}C-labeled or unlabeled QA from bacterial sources has previously been reported. Gholson, et al. (79) and Ijichi et al. (88) have reported the isolation of ^{14}C-labeled QA from mammalian sources by ion-exchange chromatography on Dowex-1-formate columns. However, when the reported procedures for the purification of QA were applied to bacterial extracts, several other compounds were found in the fractions containing QA. Numerous alterations of the elution conditions on Dowex-1-formate did not yield a homogeneous sample. Therefore, I explored other methods for purifying QA. The retention of pyridinedicarboxylic acids on cationic exchange resins has not previously been reported. It was hypothesized that QA might be retained at low pH if a significant amount of pyridinium ion existed at moderately low concentrations of acid since it is known that pyridine compounds are easily eluted with M HCL (89). To test this hypothesis, a series of Dowex-50-H^+ columns were eluted with different concentrations of HCl. QA was retained on Dowex-50-H^+ columns in the range of pH 0.5 to 4.0. Maximum retention on these columns occurred in the range of pH 1.7 to 2.5.

Hence, the assays for QA which I developed are based on chromatography on both anionic and cationic exchange chromatography. In the early large-scale experiments, 100-ml columns of Dowex-1-formate and 14-ml columns of Dowex-50-H^+ were utilized. Since the large volumes of solvent required to elute QA from the columns necessitated the use of a fraction collector and required lengthy periods for elution, this assay, which will be referred to as Assay I, could not be used for multiple samples. The complete procedure required about four days. When, during the course of these studies, it became apparent that an extensive characterization of the enzymatic system for QA synthesis would be necessary, a second, simpler, faster assay was developed.

To avoid the use of large volumes of solvents, the column sizes were reduced to 5- and 13-ml anion and cation exchange columns, used because a) QA was less strongly retained on the chloride form of the resin and thus could be eluted with lower concentrations of the counter ion and b) it allowed the use of lithium chloride as the eluting agent. It was especially useful to use a lithium salt since all other metal ions form stable salts with sulfonic acid residues of the cation exchange resin while lithium ions do not. Hence, this allowed the partially purified QA fractions from the anion exchange columns to be chromatographed directly on the cation exchange resin without removing the solvent or concentrating the sample. The columns were eluted manually. With Assay II, as described below, up to 24 samples could be assayed for QA within 24 hours. Less than 10^{-12} moles of QA-^{14}C or 10^{-7} moles of unlabeled QA could be quantitatively determined with Assay II.

Assay I

At the end of the incubation 10 μmoles of carrier quinolinate were added to the reaction solution. The protein was removed by treatment with 3 M perchloric acid and centrifugation at 1,000 x g for 10 min. Then the supernatant was neutralized with M KOH and precipitated $KClO_4$ was removed by centrifugation. The precipitates were washed with 1/10 volume of H_2O and combined with the original supernatants.

The sample was then applied to a 100-ml (2.2 x 30 cm) Dowex-1-formate column. The column was eluted with formic acid as shown in Figure 3. After determining the radioactivity and absorbancy at 260 mμ, the fractions containing authentic quinolinic acid were pooled and reduced to dryness on a rotary evaporator. The residue was dissolved in 5 ml of H_2O, adjusted to pH 2 with HCl, applied to a Dowex-50-H^+ column (100 ml, 2.2 x 30 cm) and eluted with 0.01 M HCl. The fractions were analyzed for 260 mμ-absorbing material and radioactivity. A typical chromatogram is shown in Figure 4.

Assay II

A deproteinized sample containing 2 μmoles of authentic QA was applied to a Dowex-1-Cl^- column (0.7 x 12 cm, 4.5 ml) and washed with 3 ml of H_2O. The column was washed with three 7-ml aliquots of 0.20 M LiCl, which removed approximately 90% of the radioactivity produced in *E. coli* extracts from aspartate-^{14}C. The QA was then removed from the column in a single fraction with 5 ml of 0.60 M LiCl. This fraction was applied directly to a 13-ml (0.9 x 18 cm) Dowex-50-H^+ column and eluted with 0.01 M HCl. The first 15-ml fraction was dis-

Figure 3

Preliminary Separation of QA-^{14}C from Other Aspartate Metabolites by Ion-Exchange Chromatography on a Dowex-1-Formate Column

The preparation of the extract, the enzymatic synthesis of QA-^{14}C and the preparation of the sample for chromatography are described in "Methods" and Table XV. After applying the sample to the Dowex-1-formate column (2.2 x 33 cm, 100 ml), the column was washed with 100 ml of H_2O, then eluted stepwise with increasing concentrations of formate ion. The flow rate 1.2 ml/min and the fraction volume was 10 ml. Each fraction was monitored for ultraviolet absorbing material at 260 mμ (-∘-∘) and for radioactivity (-∘-∘) as described in methods.

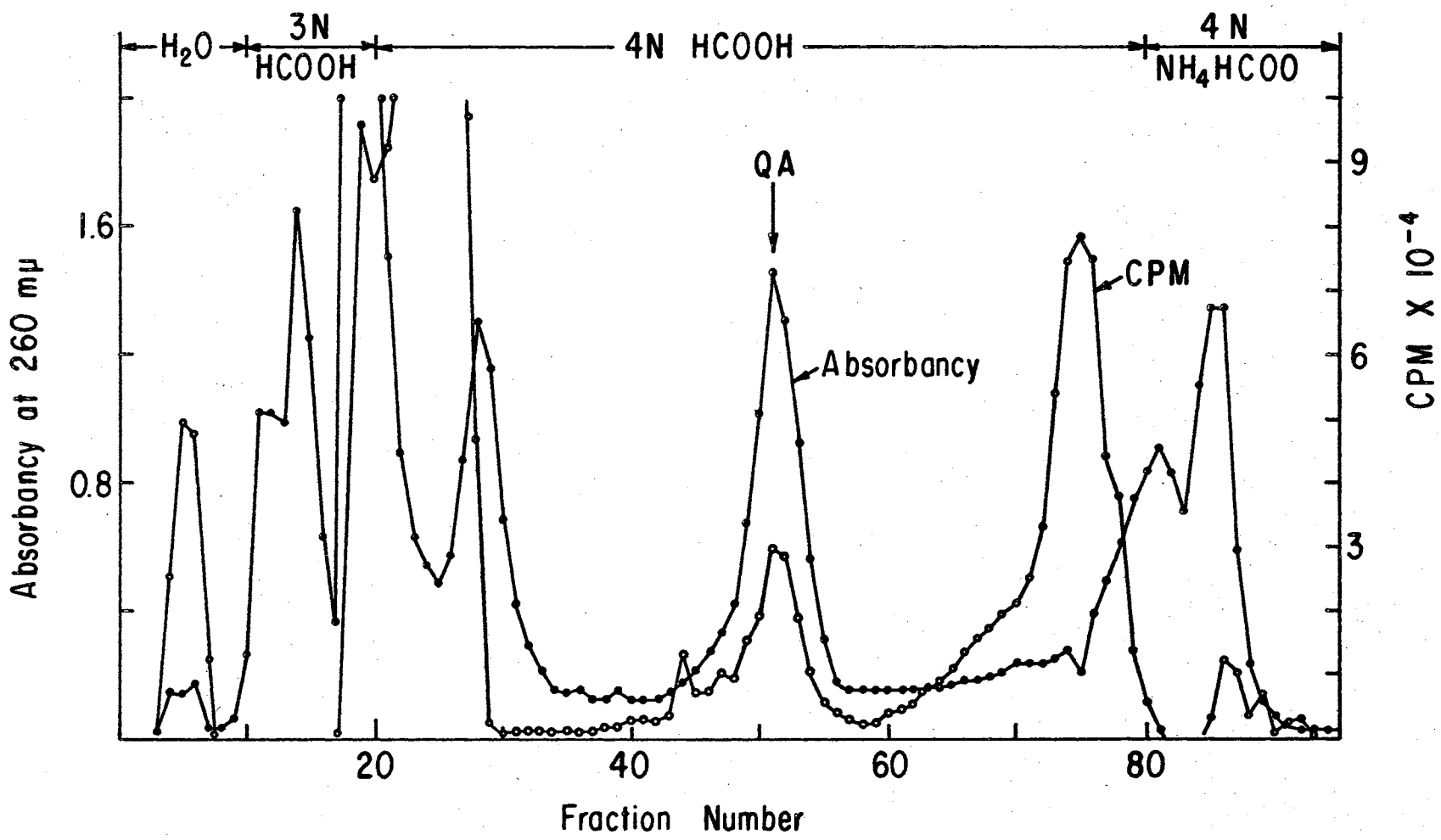
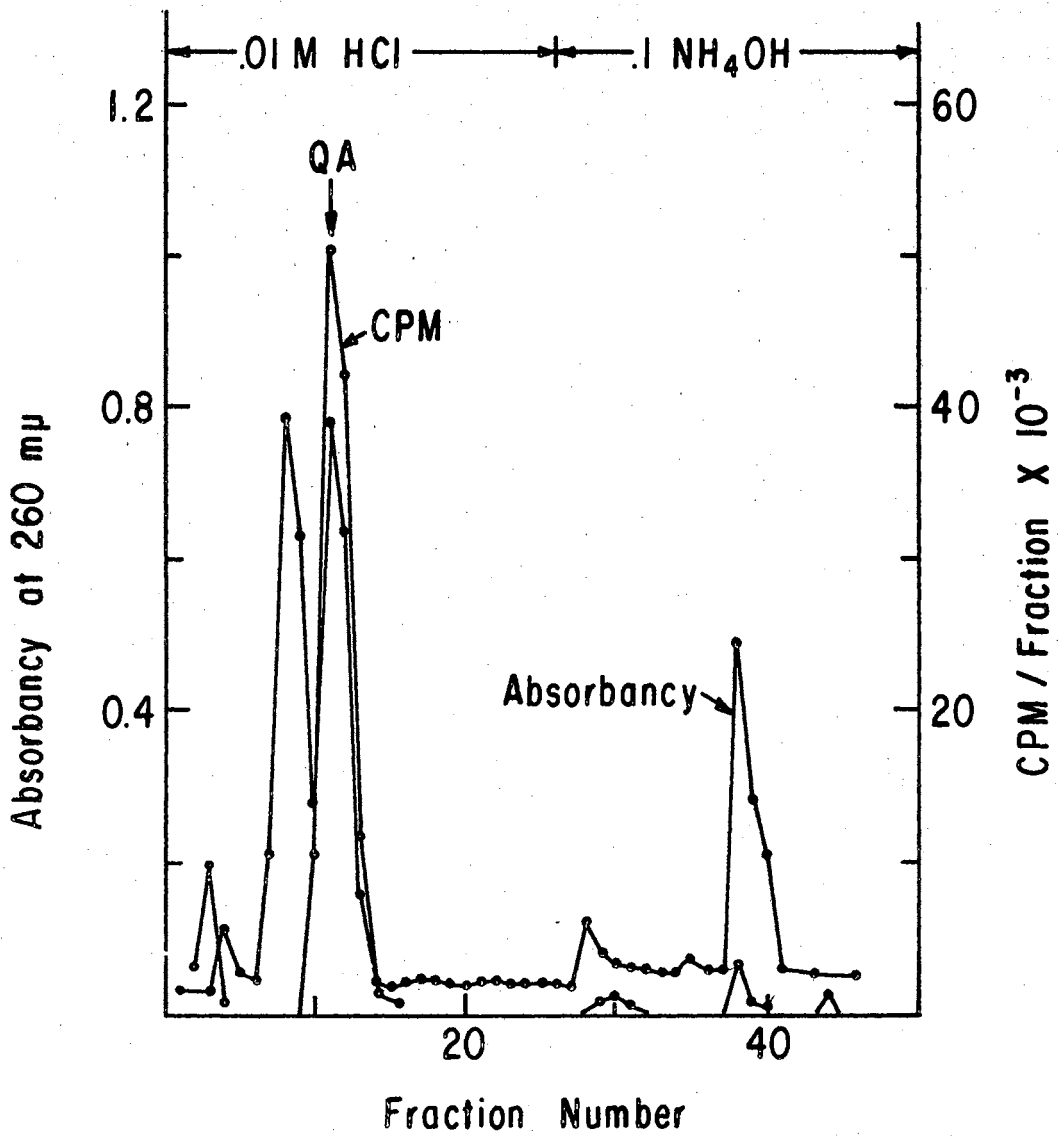

H_2O
3N HCOOH
4N HCOOH
4 N NH_4HCOO
QA
CPM
Absorbancy
Absorbancy at 260 mμ
1.6
0.8
CPM X 10^{-4}
9
6
3
20
40
60
80
Fraction Number

Figure 4

Chromatography of QA on Dowex-50-H^+ Column

The QA-containing fractions obtained from Dowex-1-formate chromatography were reduced to dryness on a rotary evaporator and dissolved in 4 ml of 0.01 M HCl. The sample was applied to a 13 ml (0.9 x 18 cm) Dowex-50-H^+ column. The sample was eluted stepwise with 100 ml of 0.01 M HCl and 75 ml of 0.10 M NH_4OH. Each 4 ml fraction was monitored for 260 mμ absorbancy (-∘-∘) and radioactivity (-∘-∘).


.01 M HCl
.1 NH4OH
1.2
0.8
0.4
60
40
20
QA
CPM
Absorbancy
20
40
Fraction Number
Absorbancy at 260 mμ
CPM / Fraction X 10-3

carded, and nine 4-ml fractions were then manually collected. The absorbancy at 268 mμ and ^{14}C-content were determined for each fraction. The recovery of authentic QA, which was calculated from its extinction coefficient (88), was usually greater than 95%. Since Dowex-50 slowly bleeds small amounts of UV-absorbing material, it was necessary to carry out the manual elution as rapidly as possible.

When ^{14}C-labeled carbohydrates were used to study the source of the 3-carbon moiety of QA, an acid-labile, ^{14}C-labeled compound (possibly a sugar nucleotide) was eluted slightly ahead of the QA peak from the Dowex-50 column. This compound was destroyed by adjusting the LiCl fraction from the Dowex-1-Cl^- columns to pH 1 with HCl and heating the solution for one hour in a boiling water bath. After neutralization with M LiOH, the samples were applied to the Dowex-50 columns and eluted as previously described.

Dowex-1-formate columns were regenerated after use by washing with 5 column volumes of 3 M HCl and then with 4 N ammonium formate until the effluent was free of chloride ion as detected with acidic silver nitrate. Before the sample was applied, the column was thoroughly flushed with deionized water.

Dowex-1-chloride and Dowex-50-hydrogen were regenerated by washing with 5 column volumes of 3 M HCl and then with deionized water until the effluent was neutral. Periodically, the columns were emptied and the resins recycled with HCl and NaOH as recommended by the manufacturer (90).

Validity of Assay for QA

Since the precursors of quinolinic acid are metabolized to numerous other compounds in cell-free extracts, it was necessary to rigorously prove that QA purified by ion-exchange chromatography was chemically and radiochemically pure. The following evidence was obtained for the purity of either ^{14}C-labeled or unlabeled QA.

The isolated material migrated with authentic QA on Dowex-50-H^+ and Dowex-1-Cl^- columns. The constant specific activity of the isolated material across the QA peak eluted from a Dowex-50 column is shown in Figure 5. A comparison of the paper chromatographic R_f's of isolated QA-^{14}C and authentic QA is shown in Table II. The isolated material migrated as a single spot in the five different solvent systems. Decarboxylation of the isolated product by refluxing in glacial acetic acid for 30 minutes produced nicotinic acid which migrated with authentic NA during either paper chromatography in three different solvent systems or ion-exchange chromatography on Dowex-1-formate or DEAE cellulose.

After I discovered that resting cell cultures of certain niacin mutants would excrete micromolar quantities of quinolinic acid into the medium (vide infra), the biological activity of the isolated product was determined. QA isolated from the culture medium was decarboxylated and the product was tested for the support of two non-dichotomistic niacin mutants. Equivalent growth was obtained from the isolated material and authentic NA. As shown in Figure 6, QA isolated from the medium and authentic material had identical ultraviolet absorption spectra. Pyridine-3,4-dicarboxylic acid and

Figure 5

Evidence for the Radiochemical Purity of

QA-^{14}C Purified by Assay I

A sample of enzymatically synthesized QA-^{14}C which had been purified by Assay I, was dissolved in 2 ml of 0.01 M HCL and rechromatographed on a 14 ml column of Dowex-50-H^+ resin. The column was eluted with 0.01 M HCl at a flow rate of 0.4 ml/min. The fraction volume was 2.0 ml. The dotted bars represent the absorbancy at 260 mμ and the cross-hatched bars the radioactivity. The specific activity of the QA-^{14}C was essentially constant in all the fractions.

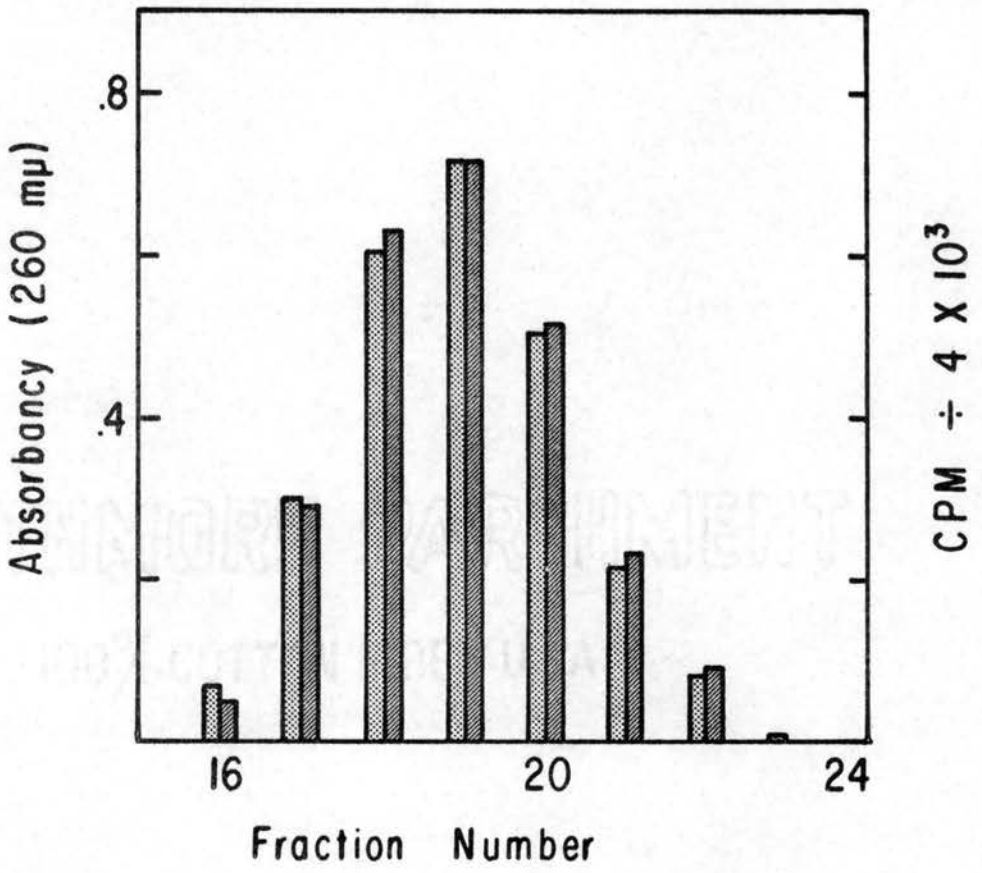

.8
.4
Absorbancy (260 mμ)
16
20
24
Fraction Number
CPM ÷ 4 X 10³

TABLE II

DESCENDING PAPER CHROMATOGRAPHY OF AUTHENTIC QUINOLINIC ACID-2,3,7,8-^{14}C AND THE ENZYMATIC PRODUCT OF MUTANT E-126

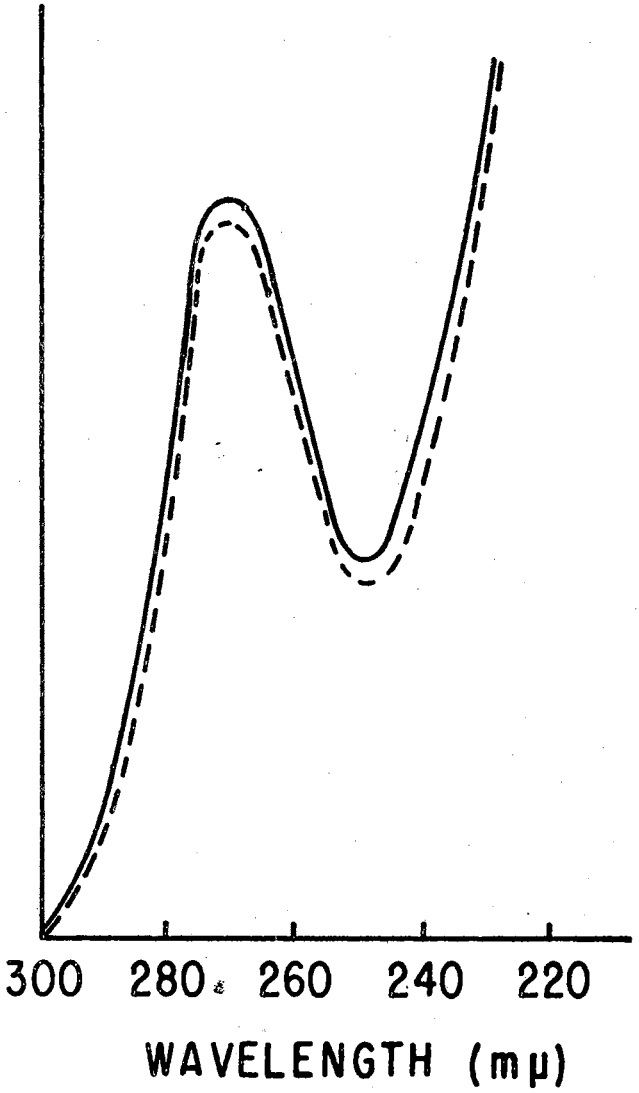
	Solvent System	R_f of QA-^{14}C	
		Authentic	Enzymatic
I	t-Butanol-Formic Acid-H_2O (75:15:10)	.49	.51
II	n-Propanol-H_2O (60:40)	.67	.69
III	n-Butanol-Acetic Acid-H_2O (4:1:2)	.43	.42
IV	Isobutyric Acid-NH_4OH-H_2O (66:1:33)	.36	.37
V	Isopropanol-H_2O (80:20)	.79	.81

Figure 6

The Ultraviolet Spectra of Authentic and Isolated QA

QA was isolated from the growth medium of mutant E-126 by the ion-exchange chromatography procedure of Assay II. The spectrum of the QA isolated from the medium (solid line) was determined on the peak fraction obtained from the ion-exchange column. Authentic quinolinic acid (1×10^{-4} M) (dashed line) was dissolved in the same solvent (0.01 M HCl). The ultraviolet spectra of the isolated material and authentic QA was also identical at pH 7.5 and pH 12.

UV SPECTRA OF AUTHENTIC AND ISOLATED QA



pyridine-2,5-dicarboxylic acid, isomers of QA, also are easily decarboxylated to form NA. However, it was found that these two isomers are readily separated from QA by chromatography on Dowex-1-chloride columns. Thus physiochemical and biological evidence was obtained for the purity of the isolated product.

Paper Chromatography

Descending paper chromatography following the procedures recommended by Block, Durrum and Zweig (91) was routinely employed for separation of compounds from the culture fluid and extracts and to check the purity of various compounds. The solvent systems employed and the R_f values obtained are given in Table II. The location of various compounds was detected either by chemical methods, using the reagents recommended by Stahl (92), or by UV fluorescence or quenching. The location of ^{14}C-containing compounds was detected on a Nuclear Chicago 4π strip counter.

The migration of quinolinic acid varied with the amount of QA applied to the paper and the pH of the QA solution. Hence, to obtain reproducible results, 1 μmole of quinolinic acid and 10 μmoles of HCl were spotted directly over the sample of QA-^{14}C.

Other Methods

UV spectra were determined on a Cary-14 automatic recording spectrophotometer using quartz cells with a 1-cm light path. Column chromatography fractions were routinely monitored at 260 or 268 mμ with a Beckman DU Spectrophotometer.

The radioactivity of liquid solutions was determined by pipetting 0.2-ml aliquots into 10-ml of scintillation fluid and counting with a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3950. The efficiency of counting was periodically checked by adding an internal standard and ranged from 66.1 to 67.3%. The scintillation fluid was composed of 600 ml toluene, 400 ml ethanol, 4.0 g of 2,5-diphenyl-oxazole and 0.2 g of 1,4-bis[2-(5-phenyloxazolyl)]-benzene.

Chemicals

Aspartic acid-U-^{14}C, acetic acid-2-^{14}C, and glycerol-1-^{14}C were obtained from Nuclear Chicago; fructose-1,6-diphosphate-U-^{14}C from Schwartz Biochemicals; and glycerol-1(3)-^{3}H, glycerol-2-^{3}H, glucose-6-^{14}C, glucose-1-^{14}C, glucose-2-^{14}C, and 2,3-diphosphoglycerate-U-^{14}C from Calbiochem Laboratories. The L-amino acids were obtained from Sigma Chemical Co. Microgranular DEAE-cellulose was purchased from the Reave-Angel Corporation. Dowex-1-chloride and Dowex-50-sodium were purchased from Bio-Rad Laboratories. NAD was very generously donated by Dr. D. Brusca of the Enzymedic Laboratories, Seattle, Washington. 5-Fluoronicotinic acid and 5-fluoronicotinamide were gifts from Dr. P. McGuire of Eli Lilly Company, Indianapolis, Indiana. All other chemicals were of reagent grade and were obtained from local supply houses.

CHAPTER III

RESULTS

Growth Experiments

During the course of this study I discovered that certain E. coli mutants previously characterized as niacin auxotrophs would grow in the total absence of niacin. This dichotomistic character was discovered while searching for the biosynthetic relationship between niacin and other growth factors. When the salts-glycerol medium was supplemented with either NA or vitamin-free casein hydrolysate (Casamino Acids) the medium supported the growth of mutant E-18, as shown in Table III. The amount of growth obtained monotonically increased with the amount of Casamino Acids added. In order to determine whether the character of strain E-18 was a unique mutation or if it was a commonly occurring genetic event, all available niacin mutants were inoculated into media supplemented with 1% Casamino Acids in the presence and absence of NA. The results of this experiment are shown in Table IV. After incubating 25 hours at 37°, eight mutants had obtained essentially full growth in the Casamino Acid medium, eight mutants gave an intermediate response, and nine others appeared to be classical niacin auxotrophs and did not grow. All cultures grew in the presence of Casamino Acids supplemented with niacin. In minimal salts-glycerol media supplemented with the specific growth requirements

TABLE III

GROWTH OF DICHOTOMISTIC MUTANT E-18 IN THE PRESENCE OF EITHER NICOTINIC ACID OR VITAMIN-FREE CASAMINO ACIDS

Additions	Absorbancy (660 mμ)
None	.02
Nicotinic Acid (5 μM)	1.12
Casamino Acids (0.05%)	.20
Casamino Acids (0.10%)	.38
Casamino Acids (0.25%)	.66
Casamino Acids (0.50%)	.85
Casamino Acids (1.0%)	1.18

The experimental conditions are defined in the text. Growth was determined after incubating for 22 hours.

TABLE IV

GROWTH RESPONSE OF NIACIN AUXOTROPHS TO 1% VITAMIN-FREE CASAMINO ACIDS

Mutant	Absorbancy (660 mμ) Additions to Medium D	
	None	10^{-6} M NA
E-2	.90	1.00
E-18	1.08	1.08
E-25	.85	1.05
E-36	.38	1.01
E-126	.09	1.13
SB-16	.28	1.10
SB-41	.16	1.00
SB-84	.55	1.10
SB-99	.02	.91
W-4516	.04	1.02
W-3899	.40	1.05
AT-13-3	.20	1.03
AT-13-23	.05	.90
AT-11-23	.05	1.05
AT-11-24	.73	1.01
PA-2-18	.05	1.05
PA-2-35-5	.54	1.15
3440	1.10	1.10
E-51	1.05	1.35
E-52	1.10	1.20
E-53	1.25	1.08
E-56	.35	1.20
E-57	.29	1.30

The experimental conditions are defined in the text. Growth was determined after incubating for 25 hours.

for each strain, the NA concentration required for optimum growth was similar for both the dichotomistic and non-dichotomistic niacin mutants. This suggests that the growth observed in the presence of Casamino Acids was due to factors other than trace amounts of niacin which might be present in the protein hydrolysate.

To ascertain the active principle contained in Casamino Acids and to obtain definitive proof that the apparent niacin auxotrophs were indeed dichotomistic mutants, a systematic study of the effects of amino acids on the growth of these mutants was undertaken. The amino acids were divided into families based on their biosynthetic relationships as determined by the classic studies of Roberts et al. (70). The composition of these families are as follows: aspartate family - methionine, isoleucine, threonine, lysine and aspartate; glutamate family - arginine, proline and glutamate; aromatic family - phenylalanine, tyrosine and tryptophan; pyruvate family - alanine, valine and leucine; serine family - glycine, cysteine and serine. Initial experiments indicated that the aspartate and pyruvate families would support the growth of mutant E-18, and the glutamate family stimulated growth in the presence of the aspartate and pyruvate families; the aromatic and serine families had little effect on the growth.

Next, the growth-stimulating effects of the individual amino acids of these families were tested. Typical data are shown in Table V. The well known sparing effects of biosynthetically related amino acids make these data difficult to interpret. However, glutamate consistently stimulated growth more than earlier proline or arginine; thus it appears to be the growth-stimulating amino acid of its family.

TABLE V

GROWTH OF DICHOTOMISTIC MUTANT E-18 ON DEFINED L-AMINO ACID MEDIA

Additions	Absorbancy (660 mμ)
Experiment I	
None	---
Aspartate family	.07
Aspartate family + pyruvate family	.52
Aspartate family + leucine	.22
Aspartate family + valine	.24
Aspartate family + alanine	.22
Aspartate family + histidine	.16
Aspartate family + glutamate family	.32
Aspartate family + proline	.17
Aspartate family + arginine	.24
Aspartate family + glutamate	.22
Experiment II	
None	.01
NA (5 μM)	1.25
Aspartate family + pyruvate family	1.30
Aspartate family (10 mM) + pyruvate family (10 mM)	1.35
Aspartate + methionine + alanine (10 mM)	.91
Aspartate + methionine + valine (10 mM)	1.10
Aspartate + methionine + leucine (10 mM)	.90
Aspartate + methionine + glutamate (10 mM)	.95
Aspartate + methionine + arginine (10 mM)	.85
Aspartate + methionine + proline (10 mM)	.85

The experimental conditions are defined in the text. Unless otherwise indicated, the substrate concentration was 1 mM in Experiment I and 5 mM in Experiment II. The turbidity was determined after about 24 hours of growth.

The growth-promoting amino acid(s) of the pyruvate family remains obscure. L-Alanine, L-leucine and L-valine were about equally effective. Isoleucine, which reverses the growth inhibition due to valine in E. coli K-12, was added to the media in this experiment (93). In several experiments L-alanine and sodium pyruvate were equally effective when added to the medium containing aspartate and methionine. Among members of the aspartate family, aspartate and methionine were both required for the growth of dichotomistic mutant E-18, although threonine, an intermediate in the pathway of methionine synthesis, would partially replace methionine. Intermediate compounds between threonine and methionine were not tested.

Mutant E-18 grew at normal rates when only niacin was added to the salts-glycerol medium; therefore it is quite apparent that the organism is capable of synthesizing the amino acids which allow growth in the absence of niacin. Hence, the growth-promoting effect of these amino acids depends on having excess quantities of amino acids present. The data in Table VI show the effects of different concentrations of amino acids on E-18 growth. The final cell concentration of the added amino acids was 5 or 10 millimolar, which is about 10 times the amount of a typical amino acid that the organism would incorporate into its protein under normal growth conditions (70). Table VI also demonstrates the relatively specific requirement for methionine and aspartate. Other dicarboxylic acids are relatively ineffective in promoting the growth of E-18 in the absence of aspartate.

When other dichotomistic mutants were tested for growth in the presence of various combinations of amino acids, the response was

TABLE VI

EFFECT OF ORGANIC ACID CONCENTRATIONS ON THE GROWTH OF DICHOTOMISTIC MUTANT E-18

Concentration (mM)						
Aspartate Family	Pyruvate Family	Aspartate	Methionine	Alanine	Other Additions	Absorbancy (660 mμ)
					None	.02
					NA (5 μM)	1.15
1	1					.57
5	1					1.04
10	1					1.10
1	5					.61
1	10					.76
	1	1	1			.35
	1	5	1			.87
	1	10	1			1.03
	1		10			.02
			10	1	Glutamate Family (10 mM)	.10
			10	10	Glutamate (10 mM)	.05
			10		Succinate (10 mM)	.07
			10		Succinate + Glutamate (10 mM each)	.13
			10		Glutamate + Acetate (10 mM each)	.06

The experimental conditions were the same as those used in Table IV.

generally less pronounced than with E-18 except with mutant E-52. This apparent niacin auxotroph grew in the presence of either aspartate and methionine or aspartate and guanine, as shown in Table VII.

While these growth experiments clearly indicate the pronounced effect of different media supplements on the growth of niacin auxotrophs, they do not define but only hint at the possibility that amino acids influence the synthesis of QA and NAD in the cell. The effects of amino acids on QA biosynthesis *in vitro* are discussed later.

Accumulation of Quinolinic Acid in the Culture Medium

It was discovered that both growing and resting cells of mutant E-126, which lacks QRTase (85), would excrete QA into the culture fluid. The factors which effect the excretion of QA by this organism were studied in considerable detail for two reasons. First, the culture conditions which result in the maximum production of QA should be directly applicable to the production of unknown precursors of QA in niacin auxotrophs lacking enzymes catalyzing earlier steps in the pathway. This should greatly facilitate the isolation and identification of these intermediates since resting cell cultures excrete micromolar quantities of QA while *in vitro* synthesis results in only nanomolar quantities of QA. Secondly, QA excretion was studied to gain insight into the control mechanisms which govern its biosynthesis and to compare the *in vivo* results with *in vitro* experiments.

The yield of QA obtained from growing cells of mutant E-126 is shown in Table VIII. Since a 5-ml culture of a niacin mutant requires only 5 nanomoles of NA for growth (50), it is apparent that this

TABLE VII

GROWTH OF DICHOTOMISTIC MUTANT E-52 IN SUPPLEMENTED MEDIA

Additions	Absorbancy (660 mμ)
None	.05
Aspartate	.11
Aspartate + methionine	1.02
Aspartate + methionine + alanine	1.15
Aspartate + alanine	.09
Aspartate + adenine	.05
Aspartate + cytosine	.08
Aspartate + uracil	.08
Aspartate + guanine	.64

The concentration of the substrates was 5 m<u>M</u>. Growth was determined after incubating for 24 hours.

TABLE VIII

EXCRETION OF QA BY GROWING CELLS
OF MUTANT E-126

Additions	Absorbancy (660 mμ)	QA Yield (μM)
Experiment I		
None	.96	<.10
Aspartate	.87	.51
Malate	.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
Experiment II		
NA (1 μM)	.85	.69
NA (2 μM)	.72	.29
NA (10 μM)	.70	<.05

Growth Media:

Experiment I - Medium N + 1 μM + 5 mM substrates. The incubation time was 30 hours.

Experiment II - Medium N with 0.5% glucose as the carbon source, supplemented with 0.1% Casamino Acids and 5 mM aspartate and NA as given.

mutant excretes a hundredfold excess of QA into the medium. The highest yield of QA obtained from growing cultures was from media supplemented with 0.5% Casamino Acids and aspartate. Experiment II in Table VIII indicates that the pathway for QA synthesis is controlled. When excess NA was present in the medium, no QA was detected in the culture medium. The interrelationships between growth rate, the final concentration of cells, and medium supplements complicate the interpretation of these data; therefore, further experiments were conducted with heavy cell suspensions. Although a nitrogen source was present, the initial density of the suspension would allow little growth even if the cells were not completely devoid of niacin.

A preliminary experiment which demonstrates the accumulation of QA in the medium of these cell suspensions of mutant E-126 is shown in Table IX. After incubating 29.5 mg (dry wt) of cells for twenty hours in minimal medium supplemented with 0.5% glycerol and 0.02 M aspartate, 1.07 μmoles of QA were present in the culture. Control experiments indicated that less than 10% of the QA was retained within the cells. In this long term experiment, citrate, Casamino Acids and glucose enhanced QA production. The QA obtained from this experiment was characterized by its biological activity after conversion to nicotinic acid, by paper chromatography, and by its UV spectrum as described in Methods.

The optimum pH for accumulation of QA in cell suspensions occurred between pH 7.8 and 8.2 (Table X). All further experiments were conducted at pH 8.0.

TABLE IX

THE SYNTHESIS OF QA BY CELL SUSPENSIONS OF A NIC⁻ MUTANT LACKING QRTase

Additions to the Medium			QA Yield
Glycerol (%)	Aspartate (mM)	Other	(μmoles)
0.5	10		.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)	.97

A 500-ml culture of mutant E-126 was grown on minimal medium, supplemented with 10^{-6} M NA. After the bacteria were harvested by centrifugation at 4°, the cells were washed once with 0.9% NaCl and resuspended in 10 ml of H_2O. Aliquots of the cell suspension (0.5 ml, 18.5 mg dry weight) were added to 16 x 150 mm test tubes containing 5 ml of minimal medium and the additions given in the table. The suspensions were aerated on a reciprocal shaker for 26 hours. The cells were removed by centrifugation and washed once with H_2O. The QA content of the combined supernatants was determined by Assay II.

TABLE X

EFFECT OF pH ON QA SYNTHESIS BY CELL SUSPENSIONS OF MUTANT E-126

pH	QA Yield (μmoles)
6.0	.42
6.5	.61
7.0	.74
7.5	.87
7.8	1.01
8.0	1.08
8.2	1.18
8.5	.93

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

The effect of various organic bases on QA synthesis is shown in Table XI. NA and NAm were effective inhibitors of QA synthesis. The potent bacterial growth inhibitors 5-fluoronicotinic acid, 5-fluoronicotinamide, 6-aminonicotinic acid, and 6-aminonicotinamide were also found to inhibit QA synthesis, while 6-chloronicotinic acid, which does not inhibit bacterial growth, does not inhibit synthesis of QA (64). These data suggest that the enzymes catalyzing QA synthesis are subject to feedback inhibition in vivo.

During the course of cross-feeding experiments which will be discussed later, it was observed that nucleic acid bases stimulated the growth of some niacin auxotrophs on solid medium. However, adenine, guanine, cytosine, and uracil only very slightly stimulated the production of QA under these experimental conditions. Similar effects of these purines and pyrimidines were observed when added at 5×10^{-3} M, except that adenine was slightly inhibitory at this level.

While it has been observed that glycerol and D-glyceraldehyde are effective precursors of carbons 4, 5, and 6 of the pyridine ring and that pyruvate and propionate are not incorporated into these positions, the incorporation of other precursors has not been reported (28, 43). Thus it was of considerable interest when it was observed that higher yields of QA were obtained in the presence of ribose rather than glycerol. When parallel cell suspensions were incubated in the presence of glycerol and ribose, it was found that the rate of QA synthesis was slightly faster from the latter, as shown in Table XII. At this time, the enzymatic basis of this phenomenon remains obscure.

TABLE XI

EFFECT OF ORGANIC BASES ON QA SYNTHESIS BY CELL SUSPENSIONS OF MUTANT E-126

Additions	QA Yield (μmoles)
None	.93
5-Fluoronicotinic Acid	.13
5-Fluoronicotinamide	.14
6-Aminonicotinic Acid	.16
6-Aminonicotinamide	.19
6-Chloronicotinic Acid	1.06
Nicotinic Acid	.18
Nicotinamide	.19
Guanine	1.03
Adenine	1.03
Cytosine	1.05
Uracil	1.04

The experimental conditions are described in Table IX. 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 μmoles of the pyridine derivatives or 2.5 μmoles of the pyrimidine or purine derivatives.

TABLE XII

TIME COURSE OF QA SYNTHESIS WITH CELL SUSPENSIONS OF MUTANT E-126 IN THE PRESENCE OF GLYCEROL OR RIBOSE

Incubation Time (minutes)	QA Yield (μmoles)	
	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
360	----	1.18
480	1.37	1.38

The experimental conditions are described in Table IX. Mutant E-126 cells (24.5 mg dry weight) were incubated in minimal medium supplemented with 0.02 M L-aspartate and either 0.02 M glycerol or 0.02 M ribose on a reciprocal shaker at 37°. After the reaction had proceeded for the desired time, the cells were removed by centrifugation and the QA in the medium was determined by Assay II.

While studying the effects of adenine and ribose on QA accumulation which were reported to stimulate NA synthesis (28), it was observed that intermediate levels of ribose strongly inhibited QA synthesis. The effect of glycerol and ribose concentrations on QA accumulation is shown in Table XIII. Surprisingly, higher yields of QA were obtained when the carbohydrate concentration in the medium was either 0.5 mM or less or 5 mM or greater. When the carbohydrate concentration was about 1 mM, the QA production was severely inhibited. The enzymatic nature of this inhibition also remains obscure.

After discovering the dichotomistic character of certain niacin mutants, a study of the effect of growth conditions on QA synthesis in cell suspensions was undertaken. Parallel cultures were grown in minimal medium with 0.5% glycerol as the carbon source (Medium N) and complex medium containing 1% Casamino Acids and 1% glucose (Medium D). The effect of carbohydrate source and different potential feedback inhibitors on QA accumulation from these cells is shown in Table XIV. The capacity of the cells from different growth conditions to produce QA and their response to inhibitors was identical within the limits of experimental error. Preliminary experiments conducted with regard to the effect of amino acids on QA synthesis *in vivo* indicated that some amino acids stimulated vitamer synthesis. However, these experiments were not pursued in detail since it seemed more profitable to examine this phenomenon *in vitro*.

In addition to mutant E-126, mutants W-4516, AT-133 and SB-99 excreted QA into the medium. The quantity of QA produced by mutants W-4516 and AT-133 was similar to that produced by E-126, while QA production by strain SB-99 was sharply reduced.

TABLE XIII

THE EFFECT OF GLYCEROL AND RIBOSE CONCENTRATIONS ON QA SYNTHESIS IN CELL SUSPENSIONS OF MUTANT E-126

Additions	Concentration (mM)	QA Yield (μmoles)
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

The experimental conditions are described in Table IX. 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 hours.

TABLE XIV

COMPARISON OF GROWTH MEDIA ON THE SYNTHESIS OF QA BY CELL SUSPENSIONS OF MUTANT E-126

Additions	QA Yield (μmoles) Media N	Media D
Glycerol	.58	.52
Glucose	.62	.68
Ribose	.69	.62
Glycerol + NAD	.14	.12
Glycerol + NA	.12	.11
Glycerol + NAm	.12	.12
Glucose + NAD	.21	.18
Glucose + NA	.17	.13

The experimental conditions are described in Table IX except parallel cultures were grown on medium N and Medium D. Aliquots of cell suspensions (13.1 and 14.2 mg dry weight, respectively) were added to pH 8.0 minimal medium supplemented with 0.02 M L-aspartate, 0.02 M carbohydrate and, where indicated, .001 M pyridine derivatives and incubated for 4 hours.

The Enzymatic Synthesis of Quinolinic Acid in Cell-Free Extracts

The preliminary observations of Ogasawara et al. (85) which demonstrated that cell-free extracts of mutant E-126 would incorporate aspartate-U-^{14}C into QA served as the starting point in these studies. Unless otherwise indicated, extracts of mutant E-126 were used in these experiments. The initial experiments were designed to find the pH optimum of quinolinate-synthesizing enzymes and to find which compounds were the most effective precursors of QA in vitro. In these experiments, QA was determined by the procedure of Assay I. The effect of pH on the incorporation of uniformly labeled L-aspartate-^{14}C is shown in Table XV. The highest yield of QA-^{14}C was obtained at pH 8.0. Unless otherwise stated, all further incubations were conducted at pH 8.0.

It should be emphasized that this is the pH optimum for the complete enzyme system for QA synthesis in freshly prepared crude extracts and may not reflect the optimum pH for any specific enzyme. Also, since aspartate and glycerol are rapidly metabolized by these crude extracts, the observed pH optimum may only reflect a balance between rates of the utilization of the precursors for QA synthesis and degradation of the precursors by competing pathways. However, a similar pH optimum was observed for accumulation of QA in cell suspensions.

The incorporation of L-aspartate-U-^{14}C, DL-aspartate-1-^{14}C, L-aspartate-4-^{14}C, DL-aspartate-^{3}H and succinate-U-^{14}C into QA is shown in Table XVI. The experiments were conducted with individually pre-

TABLE XV

EFFECT OF pH ON ENZYMATIC SYNTHESIS OF QA

pH	QA-^{14}C mμC	% Yield
7.2	2.4	0.048
7.6	18.1	0.362
8.0	66.7	1.330
8.4	1.2	0.024

In a volume of 12 ml, 1 μmole L-aspartate-U-^{14}C (specific activity = 5 μC/μmole), 20 μmoles of ATP, 2 μmoles of $MgCl_2$, 2 μmoles of glycerol, 10 ml of crude extract in 0.10 M K_2HPO_4 buffer were incubated for 8 hours at 24°. QA-^{14}C was determined by Assay I.

TABLE XVI

INCORPORATION OF DL-ASPARTATE-1-^{14}C, L-ASPARTATE-4-^{14}C, DL-ASPARTATE-G-^{3}H AND SUCCINATE-U-^{14}C INTO QA

Precursor	Specific Activity (μC/μmole)	μC	QA-^{14}C % Radiochemical Yield
L-Aspartate-U-^{14}C	5.00	5.0	1.28
DL-Aspartate-1-^{14}C	2.03	15.0	0.34
L-Aspartate-4-^{14}C	19.50	10.0	0.88
DL-Aspartate-G-^{3}H	287.50	50.0	----
Succinate-U-^{14}C	4.00	8.0	0.15

Each incubation contained 20 μmoles of ATP, 20 μmoles of glycerol, 2 μmoles of $MgCl_2$ and 10 ml of crude extract in 0.10 M K_2HPO_4 buffer, pH 8.0. NH_4Cl (10 μmoles) was added to the succinate-U-^{14}C incubation. The extracts were incubated for 10 hours at room temperature. Separate extracts were prepared for each precursor. QA-^{14}C was determined by Assay I.

pared extracts from different batches of cells. Also, the specific activity and the concentrations of the precursors varied; hence the results are not strictly comparable. However, in several experiments, the radiochemical yield of QA-^{14}C was always considerably higher from L-aspartate-U-^{14}C than from succinate-U-^{14}C.

After the reaction conditions were improved, a sample of QA-^{14}C was synthesized *in vitro* from DL-aspartate-1-^{14}C. This QA-^{14}C (15.1 mμC) was converted to NA by refluxing in glacial acetic acid as described in the Methods section. Only 2.5% (.32 mμC) of the radioactivity was retained in NA and 97.5% (14.8 mμC) was lost as CO_2. Therefore, DL-aspartate-1-^{14}C is converted into QA-7-^{14}C without randomization.

The incorporation of potential 3-carbon precursors into QA is shown in Table XVII. The highest radiochemical yield was obtained from FDP-^{14}C and 2,3-diphosphoglycerate-^{14}C. However, since the specific activity of the precursors was different, the data are not conclusive. Acetate-2-^{14}C and formate-^{14}C were not incorporated into QA. Thus, the pathway for QA synthesis in *E. coli* appears to be different from the pathway in *C. butylicum* (47). It is quite significant that glycerol-2-^{3}H and glycerol-1(3)-^{3}H are not incorporated into QA. This suggests either that glycerol is converted into D-glyceraldehyde-3-phosphate (or dihydroxyacetone phosphate) before incorporation into QA or that all of the hydrogen atoms become exchangeable during the course of QA synthesis.

TABLE XVII

INCORPORATION OF THREE-CARBON PRECURSORS
INTO QUINOLINATE IN VITRO

Compound	Specific Activity (μC/μmole)	μC	mμC	QA-^{14}C % Radiochemical Yield
Glycerol-1-^{14}C	5.2	5.0	5.3	0.106
Fructose-1,6-diphosphate-^{14}C	2.4	5.0	7.1	0.140
2,3-Diphosphoglycerate-^{14}C	6.0	10.0	14.4	0.144
Glycerate-^{14}C	15.0	10.0	(?)	-----
Glycerol-1(3)-^{3}H	244.0	50.0	0	-----
Glycerol-2-^{3}H	86.5	50.0	0	-----
Acetate-2-^{14}C	8.6	10.0	0	-----
Formate-^{14}C	22.0	10.0	0	-----

Each incubation contained 0.1 mM L-aspartate, 20 μmoles of ATP, 2 μmoles of $MgCl_2$, 11 to 16 ml of crude extract and the radioactive precursor. After incubation for about 8 hours, the reaction was terminated and QA-^{14}C was determined by Assay I. Separate, freshly-prepared extracts were used for each experiment.

Properties of the QA-Synthesizing Enzyme System

After the development of Assay II which greatly reduced the time required to assay for QA-^{14}C, it became feasible to study the enzymatic characteristics of the QA synthesis in greater detail The time course of QA synthesis in crude extracts is shown in Table XVIII. The reaction was about 90% complete after incubating for two hours and this time period was used for all further experiments where QA synthesis was determined by Assay II.

To be assured of operating at saturating substrate levels, it was necessary to determine the optimum concentration of aspartic acid for the synthesis of QA. These data are shown in Table XIX. The data demonstrate that the quantity of QA synthesized increased until the aspartate concentration was 0.67 mM. Higher concentrations of aspartate did not further increase the yield of QA and apparently diluted the radioactive aspartate, reducing the incorporation of ^{14}C into QA.

Before initiating fractionation experiments, it was desirable to examine the stability of enzymatic activity in crude extracts. The decay of enzymatic activity during storage at 4° is shown in Table XX. The enzyme system is very labile; 59% of the activity was lost in nine hours. The presence of dithiothreitol, a sulfhydryl-protecting agent, did not preserve enzymatic activity. The enzyme system was also readily denatured by a very mild heat treatment. After heating 1 ml of extract in a test tube in a 50° water bath for 4 minutes, no QA-synthesizing activity was observed in the resulting solution.

TABLE XVIII

TIME COURSE OF QUINOLINIC ACID SYNTHESIS IN VITRO

Incubation Time (minutes)	Radiochemical Yield (%)
0	0.00
30	1.47
60	1.89
90	2.02
120	2.45
180	2.68
240	2.75

In a volume of 1.5 ml, 0.2 μmoles of L-aspartate-U-^{14}C (2.5 μC/μM), 1 μmole of ATP, 1 μmole of $MgCl_2$, 1 μmole of glycerol and 1 ml of crude extract were incubated at 4° for 2 hours. QA-^{14}C synthesis was determined by Assay II.

TABLE XIX

EFFECT OF L-ASPARTATE CONCENTRATION ON THE ENZYMATIC SYNTHESIS OF QUINOLINIC ACID

Aspartate Concentration (mM)	QA-^{14}C cpm x 10^{-3}	QA-^{14}C % Yield	QA-^{14}C nmoles
0.133	16.35	1.47	2.95
0.330	18.15	1.63	8.15
0.670	20.40	1.84	18.40
1.330	10.10	0.91	17.90
8.330	5.10	0.46	18.3

In a total volume of 1.5 ml, 1 ml of crude extract, 1.0 μmole of ATP, 1.0 μmole of $MgCl_2$, 1.0 μmole of glycerol and 0.5 μC of L-aspartate-U-^{14}C were incubated for 2 hours. The specific activity of L-aspartate was adjusted by the addition of unlabeled L-aspartate. QA-^{14}C was determined by Assay II.

TABLE XX

DECAY OF QUINOLINATE SYNTHETASE ACTIVITY AT 4°
IN EXTRACTS OF MUTANT E-126

Storage Time (hours)	QA-^{14}C +DTT dpm x 10^{-3}	% Control	QA-^{14}C -DTT cpm x 10^{-3}	% Control
"0" Time Control	21.0	100	23.4	100
4	14.1	67	17.1	69
6	12.7	60	13.5	59
9	8.1	41	7.0	30
24	2.3	11	3.2	12

Parallel crude extracts were prepared in 100 m$\underline{M}$ K_2HPO_4 buffer, pH 8.0, in the presence and absence of 0.5 m$\underline{M}$ dithiothreitol (DTT) and stored at 4° before incubating with substrates for 2 hours at 24°. Each reaction mixture contained 0.5 μmoles L-aspartate-U-^{14}C (specific activity = 1 μC/μmole), 1 μmole ATP, 1 μmole $MgCl_2$, 0.1 μmole fructose-1,6-diphosphate and 1 ml of crude extract in a total volume of 1.5 ml. QA-^{14}C was determined by Assay II.

As stated earlier, in vivo labeling studies concerning the nature of the 3-carbon precursor of niacin are limited to the determination of the relative efficiency of incorporation of various precursors and to the determination of which carbon atoms are labeled. These studies have indicated that glycerol and D-glyceraldehyde-3-^{14}C are incorporated into the pyridine ring and that the carbon atoms of D-glyceraldehyde are not appreciably randomized (43). Pyruvate was not incorporated into pyridine compounds. Hence, the data from in vivo experiments are consistent with the incorporation of any of the glycolytic compounds from glucose-6-phosphate to phosphoenolpyruvate.

When aspartate-U-^{14}C was incubated with various glycolytic intermediates without adding any ATP, the highest yield of QA-^{14}C was obtained from fructose-1,6-diphosphate and 3-phosphoglyceraldehyde (Table XXI, Experiment I). When the ratio of ATP to 3-carbon precursors was one to one, similar results were obtained with considerably higher yields of QA. When the ATP to 3-carbon precursor was increased to three to one, the yield of QA-^{14}C was further increased and the various 3-carbon precursors were nearly equally effective in promoting QA-^{14}C synthesis at both low and high ATP levels. Since triose phosphate isomerase and aldolase are constitutive enzymes known to be very active in crude extracts, one would expect an equilibrium to be rapidly established between D-glyceraldehyde phosphate and fructose-1,6-diphosphate. Therefore, a priori, one would anticipate these two compounds to be about equally effective precursors of any cellular metabolite. In other experiments, higher concentrations of either 3-carbon precursors or ATP did not further increase the yield of QA-^{14}C.

TABLE XXI

EFFECT OF VARIOUS THREE-CARBON PRECURSORS AND ATP CONCENTRATION ON THE INCORPORATION OF L-ASPARTATE-U-^{14}C INTO QUINOLINATE

Additions	ATP (μmoles)	QA-^{14}C (cpm x 10^{-3})
Experiment I		
None	---	2.2
Glucose-6-phosphate	---	2.5
Fructose-1,6-diphosphate	---	15.8
3-Phosphoglyceraldehyde	---	13.1
3-Phosphoglycerate	---	5.2
Phosphoenolpyruvate	---	2.8
Ribose-5-phosphate	---	0.5
Glycerol	---	5.2
Glycerol	1	11.9
Experiment II		
None	---	6.9
Glycerol	1	15.7
Glycerol	2	21.3
Glucose-6-phosphate	---	4.8
Glucose-6-phosphate	1	24.8
Fructose-1,6-diphosphate	1	26.8
3-Phosphoglycerate	1	14.0
Glycerol + NaF	1	23.7
Experiment III		
Glycerol	3	34.2
Glycerol	4	29.6
Glucose-6-phosphate	3	29.7
Fructose-1,6-diphosphate	3	32.4
3-Phosphoglycerate	3	23.4
Glycerol + NaF	3	48.9
Fructose-1,6-diphosphate + NaF	3	46.3
3-Phosphoglycerate + NaF	3	45.7

In a volume of 1.5 ml, 1 ml of crude extract, 1 μmole of L-aspartate, (specific activity = 0.5 μC/μmole), 1 μmole of $MgCl_2$, 1 μmole of the three-carbon precursor and the indicated amount of ATP were incubated for 2 hours at room temperature. QA-^{14}C was determined by Assay II.

It should be noted that the highest yields of QA-^{14}C obtained during the course of these studies are shown in the last three lines of Experiment III. The calculated radiochemical yield was about 6%, which is equivalent to the net synthesis of 60 nanomoles per milliliter of extract in two hours.

In view of the above results, it was decided to reexamine the synthesis of QA-^{14}C from radioactive 3-carbon precursors with specifically labeled glucose. As shown in Table XXII, glucose-1-^{14}C, glucose-2-^{14}C and glucose-6-^{14}C are incorporated into QA with similar radiochemical yields. The experiment was run in triplicate and an average of the three values is reported. The incorporation of ribose-1-^{14}C and ribose-U-^{14}C into QA under similar conditions was so low that it was barely detectable. This indicated that ribose is an inefficient precursor of QA *in vitro*.

The Control of QA Synthesis in *E. coli*

To test for the possible repression of QA synthesis activity, parallel cultures of mutant E-126 were grown on minimal media supplemented with 10^{-4} M and 10^{-6} M NA. No QA synthesis was observed in the crude extract prepared from the cells grown in the presence of excess NA, as shown in Table XXIII. This strongly suggests the pathway is subject to repression.

To test for possible feedback inhibition of QA synthesis, the effect of NA, NAm, NAD and NADP on the incorporation of aspartate-U-^{14}C into QA was examined. Of the vitamers tested, only NAD inhibited QA synthesis (Table XIV, Experiment I). The data in Experiment II of

TABLE XXII

INCORPORATION OF GLUCOSE-1-^{14}C, GLUCOSE-2-^{14}C, GLUCOSE-6-^{14}C AND GLYCEROL-1(3)-^{14}C INTO QUINOLINATE

Precursor	QA-^{14}C (% Yield)
Glucose-1-^{14}C	1.52
Glucose-2-^{14}C	1.51
Glucose-6-^{14}C	1.45
Glycerol-1(3)-^{14}C	1.32

In a volume of 1.5 ml, 1.0 μmoles of L-aspartate, 2 μmoles of ATP, 2 μmoles of $MgCl_2$, 1.0 ml of crude extract and 0.50 μmole of glucose-^{14}C or glycerol-^{14}C were incubated for 2 hours at room temperature. The specific activity of the radioactive precursors was adjusted to 2 c/mole by addition of the appropriate ^{12}C compound. The results represent the average of three separate experiments. QA-^{14}C was determined by Assay II.

TABLE XXIII

REPRESSION OF QUINOLINATE SYNTHETASE ACTIVITY
IN MUTANT E-126

Growth Medium	QA-^{14}C (mμC)
NA Concentration	
10^{-6} M	41.2
10^{-4} M	< 0.2

Crude extracts were prepared from mutant E-126 cells grown on medium N supplemented with either 10^{-6} M or 10^{-4} M nicotinic acid. The extract (10 ml) was incubated with 1 μmole L-aspartate-U-^{14}C (5 μC/μmole), 20 μmoles ATP, 2 μmoles $MgCl_2$ and 10 μmoles glycerol for 8 hours at room temperature. After termination of the reaction, QA-^{14}C was determined by Assay I.

Table XXIV indicate that the active forms of vitamine B_6, pyridoxal phosphate and pyridoxamine phosphate, also inhibit QA synthesis.

The growth experiments with the dichotomistic niacin mutants suggested that amino acids may influence QA synthesis. However, when the effect of the amino acid families and the growth promoting amino acids on QA synthesis in extracts of mutant E-126 was examined, very little effect was found, as shown in Table XXV. Neither the amino acid families nor the individual amino acids significantly stimulated or inhibited the synthesis of QA in this experiment. It should be emphasized that only a few of the many possible combinations of amino acids were tested and that the experiment was carried out with crude extracts. Further experimentation, preferably with purified enzymes, will be required to clarify the role of amino acids as growth factors for dichotomistic niacin mutants.

Attempted Cross-Feeding Experiments

The well known technique of cross-feeding of auxotrophic mutants with the same nutritional requirement has frequently been used to establish the synthetic sequence of reactions leading to production of a metabolite (94). The results of attempted cross-feeding experiments with niacin auxotrophs, under a wide variety of experimental conditions, were confusing and inconclusive. In general, the amount of cross-feeding observed was barely significant. Furthermore, the observed direction of cross-feeding was inconsistent within an experiment and between experiments. Therefore, these experiments did not establish either the number or the sequence of the biochemical events

TABLE XXIV

EFFECT OF POTENTIAL FEEDBACK INHIBITORS ON QUINOLINATE SYNTHETASE ACTIVITY

Additions	$QA\text{-}^{14}C$ cpm x 10^{-3}	% Yield
Experiment I		
None	24.6	3.31
Nicotinic Acid	21.0	2.92
Nicotinamide	25.1	3.38
NADP	24.2	3.27
NAD	0.6	0.08
Experiment II		
None	26.4	3.53
NAD	0.5	0.07
Pyridoxalphosphate	0.7	0.08
Pyridoxaminephosphate	0.6	0.08

In a volume of 1.5 ml, 1 µmole of L-aspartate-U-^{14}C, (specific activity = 0.5 µC/µmole), 1 µmole of glycerol, 2 µmoles of ATP, 2 µmoles of $MgCl_2$ and 1 ml of crude extract in 0.10 M Bicine buffer were incubated with 5 µmoles of the pyridine derivatives for 2 hours at room temperature.

TABLE XXV

EFFECT OF L-AMINO ACIDS ON QUINOLINATE SYNTHETASE ACTIVITY IN CRUDE EXTRACTS OF MUTANT E-126

Additions	QA-^{14}C (cpm x 10^{-3})
None	13.0
Aspartate family	11.9
Pyruvate family	13.2
Aromatic family	13.9
Serine family	11.4
Methionine	12.2
Isoleucine	12.2
Lysine	12.0
Threonine	12.0
Alanine	12.1
Valine	10.5
Leucine	10.7

In a volume of 1.5 ml, 1 μmole of L-aspartate-U-^{14}C (specific activity = 0.5 μC/μmole), 1 μmole of fructose-1,6-diphosphate, 2 μmoles of ATP, 2 μmoles of $MgCl_2$, 10 μmoles of L-amino acid(s), and 1 ml of crude extract in 0.10 M Bicine buffer, pH 8.0, were incubated for 2 hours at room temperature. QA-^{14}C was determined by Assay II.

leading to QA formation in *E. coli*. At this time, no satisfactory explanation is available for these results.

Attempted Detection of the Immediate Precursors of QA

From the results presented in this thesis and *in vivo* labeling studies, it is apparent that the anabolism of QA is initiated by the formation of an unknown compound containing the carbon atoms of aspartate and D-glyceraldehyde phosphate (or closely related compounds). Presumably, this unknown compound is then converted to QA by a series of unknown enzymatic reactions. Before the postulated unknown compounds can be isolated and identified, a qualitative assay must be developed for determining the existence of these compounds. To this end, experiments which attempted to detect the immediate precursors of QA were performed with niacin auxotrophs. Two basic assay systems were employed in attempting to demonstrate the existence of the unknown compounds - biological activity as a niacin replacing factor for other *E. coli* auxotrophs *in vivo* or enzymatic conversion to QA *in vitro*.

It was hoped that niacin auxotrophs, which contained QRTase activity and therefore were blocked earlier in the pathway, would excrete the precursors of QA into the medium. These precursors, if present and if the cell were permeable to them, should then serve as niacin replacing-factors for the growth of other niacin auxotrophs which were blocked still earlier in the pathway. The attempted accumulation of intermediates was carried out with about twenty different niacin auxotrophs under the experimental conditions used for optimum synthesis

of QA with resting cells of mutant E-126. The medium, after sterilization by either filtration or autoclaving, was then tested for niacin-replacing activity in growth experiments with several other niacin mutants. In general, very little, if any, niacin-replacing activity was observed. When weakly positive results were obtained attempts to fractionate the medium by gel filtration, ion exchange chromatography, or paper chromatography resulted in a total loss of activity.

Numerous experiments attempting to enzymatically synthesize the unknownintermediates from isotopically labeled aspartate and glycerol were also conducted. Crude extracts of mutants E-36, E-25, E-2, E-4, E-97, SB-16 and SB-41 were prepared and incubated with either aspartate-U-^{14}C or aspartate-G-^{3}H and glycerol-^{14}C under the experimental conditions employed for optimal QA synthesis with mutant E-126. The radioactive reaction products obtained from these incubations were fractionated by ion exchange chromatography on either Dowex-1-formate or DEAE-cellulose columns. After removal of the solvent from the radioactive fractions by lyophilization, the reaction products were incubated with extracts of mutant E-126. If the unknown intermediates were synthesized under these conditions, then the E-126 extract should have converted the intermediates to QA-^{14}C. Very little, if any, QA-^{14}C was obtained from these fractions.

In summary, these *in vivo* and *in vitro* experiments did not provide evidence for the existence of the immediate precursors of QA in *E. coli*. The enzymatic basis of these puzzling results remains obscure.

CHAPTER IV

DISCUSSION

Very little was known about the de novo biosynthesis of NAD in Escherichia coli when this study was initiated. Indeed, only three reports suggesting the nature of this pathway had appeared. Ortega and Brown (28) observed the incorporation of succinate-^{14}C and glycerol-^{14}C into niacin with resting cells of this organism. Andreoli et al. (81) detected QRTase activity in extracts of E. coli, which suggested that QA was an intermediate in the de novo pathway of NAD synthesis. And finally, Ogasawara et al. (85) observed the incorporation of aspartate-U-^{14}C into quinolinate in cell-free extracts prepared from an E. coli mutant lacking QRTase. In addition to these reports, evidence from other organisms which appear to contain the same pathway seemed pertinent to this study. In brief, these studies provided convincing evidence that asymmetrical 3- and 4-carbon units derived from D-glyceraldehyde and L-aspartate (or closely related metabolites) are converted into nicotinic acid and its derivatives (43, 95).

Before biosynthetic studies could be initiated, it was necessary to develop a convenient method for obtaining radiochemically pure QA-^{14}C from bacterial extracts. (Since only nanogram quantities of QA were synthesized in crude extracts, classical colorimetric assays,

which usually require microgram quantities of material, could not be used.)

Aspartate is a precursor of numerous major cellular metabolites. It is a precursor of the amino acids lysine, diaminopimelate, isoleucine, threonine, β-alanine and methionine; the pyrimidines uracil and cytosine, and their derivatives; and fumarate and other Krebs cycle compounds (70). In addition, aspartate derivatives are intermediates in the arginine and purine biosynthetic pathways (96). Thus, after incubating E. coli extracts with radioactive aspartate, a very large number of radioactive compounds can be detected, of which QA is only a minor component. This is also true for glycerol or glycolytic compounds. Thus, even though the assay described in Methods is both tedious and laborious, it usually gives reliable, reproducible results for the isolation of trace quantities of QA from a very heterogeneous mixture. One inherent limitation of using ion-exchange chromatography is the relatively lengthy time required to obtain the assay results. This delay, combined with the extreme lability of the enzyme system, seriously hampered attempts to fractionate the crude extract.

The data presented in this thesis suggest that L-aspartate and D-glyceraldehyde phosphate or dihydroxyacetone phosphate are the direct precursors of QA. This hypothesis is based on the following observations: The highest yields of QA were obtained, both in vitro and in vivo, when L-aspartate was the substrate. Other dicarboxylic acids were less efficient precursors of QA in parallel experiments. L-Aspartate-1-^{14}C was converted into QA-7-^{14}C. If one of the Krebs cycle dicarboxylic acids was the immediate precursor of QA, one would

expect to obtain randomization of the carbon atoms of L-aspartate-1-^{14}C to form succinate-1,4-^{14}C and hence QA-7,8-^{14}C. It should be noted that L-asparagine, L-aspartyl-γ-semialdehyde, and L-homoserine would give the same labeling pattern observed in these experiments. However, one would expect the incorporation of the latter two compounds to be influenced by the presence of reducing agents and this was not observed. While L-asparagine can not be ruled out by this reasoning, it seems more likely that the organism would initiate the synthesis of a dicarboxylic acid, QA, from the dicarboxylic acid, L-aspartate, rather than its half amide, L-asparagine. The lack of incorporation of tritium from DL-aspartate-U-^{3}H into QA was not surprising in view of the fact that C-2 and C-3 of aspartate were precursors of the carboxylate-substituted C-2 and C-3 positions of the pyridine ring of QA, which do not contain hydrogen atoms.

3-Phosphoglyceraldehyde or dihydroxyacetone phosphate is proposed as the 3-carbon precursor of QA from the incorporation of ^{14}C-labeled precursors and from the effect of glycolytic intermediates on the incorporation of L-aspartate-U-^{14}C into QA. No radioactivity could be detected in QA after incubating crude extracts with acetate-2-^{14}C or formate-^{14}C. Therefore, the E. coli pathway for QA synthesis appears to differ from the pathway in C. butylicum (46). Of the available ^{14}C-labeled 3-carbon precursors, fructose-1,6-diphosphate gave the highest radiochemical yield of QA-^{14}C. Considerably lower radiochemical yields were obtained with 2,3-diphosphoglycerate-U-^{14}C and glucose-U-^{14}C. Since other ^{14}C-labeled glycolytic compounds were not available for testing, the effect of unlabeled glycolytic intermediates

on the incorporation of L-aspartate-U-^{14}C into QA was studied in some detail. Since this type of experiment does not directly measure the precursor-product relationship from the 3-carbon precursor, the data can only be considered as indirect evidence.

When no ATP was added to the extracts the highest yields of QA-^{14}C were obtained in the presence of fructose-1,6-diphosphate and 3-phosphoglyceraldehyde. When excess amounts of ATP were present in the reaction mixture, i.e., under conditions which should allow rapid interconversion of glycolytic compounds, glycerol, fructose-1,6-diphosphate, and glucose-6-phosphate were about equally effective. Sodium fluoride, which specifically inhibits the conversion of 2-phosphoglyceric acid to phosphoenol pyruvate, stimulated QA synthesis in the extracts. Presumably, the presence of sodium fluoride should increase the concentrations of the glycolytic compounds in the extracts, although other indirect mechanisms of stimulation can not be ruled out.

As previously mentioned, fructose-1,6-diphosphate, D-glyceraldehyde-3-phosphate and dihydroxyacetone phosphate are readily interconverted in extracts by aldolase and triose phosphate isomerase. If fructose-1,6-diphosphate were the immediate precursor of QA, then one would expect that glucose-6-^{14}C and glucose-1-^{14}C which would be converted to fructose-1,6-diphosphate-6-^{14}C and fructose-1,6-diphosphate-1-^{14}C, respectively, would give different radiochemical yields of QA-^{14}C. Such differences were not observed. Presumably, therefore, the intermediate 3-carbon precursor of QA is one of the triose phosphates.

No evidence was obtained, either for or against, the participation of a nucleotide diphosphate-carbohydrate as a "carrier molecule".

Physical Properties of the Enzymatic System

In general, the conversion of a compound into a distantly related compound in cell-free extracts implies the existence of a series of enzymes catalyzing the stepwise conversion of the precursor to the product. Thus, one would predict the existence of several enzymes in E. coli extracts which catalyze the conversion of L-aspartate and triose phosphate (or closely related compounds) to QA. Observations made concerning the nature of the enzymes of a multiple step synthesis in crude extracts are of limited value because of the existence of competing enzyme systems which may affect the levels of substrates, promote the formation of inhibitors or activators, or consume cofactors. This is especially true for QA synthesis since QA is a minor cellular metabolite and both precursors are central cellular metabolites which are rapidly metabolized in cell-free extracts.

Nevertheless, it is highly desirable to partially characterize the enzyme system in crude extracts to serve as a guide for later fractionation and purification studies and to determine the cofactor requirements.

To this end, several experiments were carried out with extracts of mutant E-126. Unfortunately, the lability of the enzyme system greatly hampered these studies. The enzymatic activity was rapidly destroyed by a mild heat treatment and was slowly lost on standing at 4°. Dialysis of the extract rapidly decreased the incorporation of aspartate into QA and the activity could not be restored by addition of the common cofactors. The presence of aspartate or other potential substrates in the dialysate did not appreciably preserve the enzymatic

activity. These results, although discouraging, should not be considered conclusive since further studies may well indicate that the enzymes can be stabilized in the appropriate environment.

The Control Mechanism Influencing QA Synthesis in E. coli

It has been suggested that the rate of coenzyme synthesis, which is only about one-thousandth the rate of which the average amino acid or nucleic acid base is made, is relatively poorly adjusted by comparison with the tightly controlled synthesis of amino acids and nucleic acid bases (72). This concept was based on several criteria: overproduction (excretion), uncoupling of growth and coenzyme synthesis, and disadvantages of precise adjustments. The overproduction and excretion of the end product of a biosynthetic pathway indicates that the control mechanisms in the pathway are too weak to prevent the compound from being overproduced (97, 98). The ratio of end product excreted to end product retained has been used as a measure of the weakness of these control mechanisms (72). By this criterion, large-scale biosynthetic pathways are strongly controlled; their end products are not found in easily detectable amounts outside of the cells of bacteria growing exponentially in minimal medium. For example, the extent of overproduction for many of the amino acids synthesized by E. coli is about 0.01 or less, for the porphyrins synthesized by the photosynthetic bacterium Rhodopseudomonas spheroides it is less than 0.01 and for the nucleic-acid bases synthesized by E. coli it is about 0.05 or less (99, 72). By comparison, the flavins are greatly overproduced by E. coli; the ratio of excreted to restrained flavin ranged between 0.8

and 8 during exponential growth, depending on the growth conditions. For each of the pyridine nucleotides, the ratio was about 0.4 (50). Biotin, pantothenic acid and vitamin B_6 are also considered to be overproduced by this criterion (72).

Further evidence for the imprecise adjustment of coenzyme synthesis to physiological needs is postulated from experiments which showed that growth and coenzyme synthesis are not tightly coupled to each other. Frequently, there is residual growth in the absence of a required vitamin, and furthermore with some vitamins, synthesis continues after growth has been interrupted. Wilson and Pardee (72) found the rate of flavin synthesis is constant throughout exponential growth and beyond, up to the onset of maximum stationary phase. Moreover, McIlwain (100) found that resting cells of E. coli would synthesize pantothenic acid at about the same rate as exponentially growing bacteria when suspended in fresh medium lacking the nitrogen source. Vitamin B_6 and niacin are also excreted by exponentially growing E. coli cultures (101, 28). Wilson and Pardee (72) concluded that bacteria would probably not benefit from stricter controls of coenzyme synthesis since the quantity of metabolic energy and materials entailed in the overproduction of coenzymes is relatively small.

However, it should be emphasized that the absolute rate of excretion of coenzymes is very similar to the absolute rate of excretion of an average amino acid or nucleic acid base (72). Therefore, from this viewpoint, the control mechanism for the synthesis of coenzymes are as strict as those of quantitatively more important pathways. Furthermore, for the organism to prevent this low absolute rate of

excretion, the control mechanisms would have to be even stricter than those employed for major metabolites.

On the other hand, it is now slowly becoming apparent that the complexity of control mechanisms governing the production of a metabolite reflect the complexity of the metabolic role of that metabolite. For example, consider the synthesis of PRPP which supplies the 5-phosphoribosyl moiety for the five nucleoside triphosphates, NAD and NADP, and which is an intermediate in the de novo pathway of histidine and tryptophan synthesis. PRPP synthetase activity from E. coli has been found to be feedback regulated by ATP, CTP, UTP, GTP, NAD, tryptophan and histidine (102).

A very elaborate mechanism has been discovered in E. coli for controlling the rate of synthesis of glutamine which serves as a nitrogen donor for the synthesis of glucosamine, purines, CTP, histidine, para-aminobenzoic acid and carbamyl phosphate (103). In this case, the kinetic properties of glutamine synthetase are dependent on the growth conditions employed. The enzyme is subject to feedback inhibition by eight metabolites when the organism is grown on a glycerol-glutamate medium, while no such inhibition is observed when the organism is grown on a glucose-ammonium chloride medium. The kinetic differences have been ascribed to the presence of twelve bound AMP residues on the inhibitable form of the enzyme.

In light of the above phenomena, we can examine the control of NAD synthesis in E. coli. When grown on minimal medium, niacin is excreted into the culture fluid, the excretion ratio varying between 0.15 and 0.45 (28). In the presence of excess nicotinic acid, cultures

of E. coli excrete 30 to 50% of their NAD and NADP into the medium (62). The level of intracellular NAD is increased four fold after growth in the presence of a twenty-fold excess of NA (71). In contrast to the results obtained with flavin and pantothenate synthesis, resting-cell cultures of E. coli do not continue to synthesize niacin in minimal medium (28). As previously mentioned, the addition of QA precursors to resting cells of prototrophic E. coli resulted in a three fold increase in niacin.

The influence of growth stage and generation time on niacin synthesis has not been reported.

In summary, these in vivo experiments demonstrate that the enzymatic capacity of E. coli for the synthesis of NAD and NADP is more than sufficient to meet the physiological needs of the organism, and both the internal and external concentrations can be increased three to four fold when the precursors are present in excess. The control mechanisms are not exceedingly strict; nevertheless, in terms of absolute amounts, the overproduction is small.

As previously observed, only scanty data were available concerning the control mechanism for NAD biosynthesis in E. coli. Imsande and Pardee (62) had observed the repression of NRTase on the Preiss-Handler pathway and Saxton et al. (74) observed the lack of repression of QRTase on the de novo pathway.

From the data presented in this thesis, it is apparent that several control mechanisms govern the de novo synthesis of QA and hence NAD in E. coli. First, the pathway for QA synthesis appears to be repressible. When the organism was grown in the presence of excess

nicotinic acid, no QA synthesis was observed either *in vivo* or *in vitro*. It is not possible to obtain definitive proof for the absence of the QA synthesizing enzymes in crude extracts: For example, the formation of a non-dialysable inhibitor when excess nicotinic acid is present would give similar results. Further experiments on this aspect of the control mechanisms await purification of the enzymes. Regardless of the actual mechanism this apparent repression of the pathway may be considered to be a coarse adjustment mechanism.

Secondly, the pathway appears to be subject to end product inhibition. In resting-cell cultures, the accumulation of QA was stopped by the addition of NA, NAm, NAD and NADP. Furthermore, the nicotinic acid antagonists 5-fluoronicotinic acid, 5-fluoronicotinamide, 6-aminonicotinic acid and 6-aminonicotinamide also inhibited QA excretion. In crude extracts, however, the only niacin vitamer which inhibited QA synthesis was NAD. This suggests that the other niacin vitamers and analogs were converted to NAD or NAD analogs which then effected the inhibition.

The NAD concentration required to totally inhibit QA synthesis *in vitro*, 5 mM, was approximately ten fold higher than the normal *in vivo* concentration of NAD, 0.5 mM.

The phenomena of repression and feedback inhibition have been extensively studied in major metabolic pathways in bacteria, and it appears *E. coli* uses the same or similar mechanisms for controlling the rate of synthesis of NAD even though the absolute rate of synthesis is, comparatively, extremely slow.

In addition, preliminary evidence was obtained for two further control mechanisms. Pyridoxal phosphate and pyridoxamine phosphate, the coenzyme forms of vitamin B_6, also effectively inhibit QA synthesis. In conjunction with the observation of Andreoli (83) that pyridoxal phosphate is a potent inhibitor of QRTase, this strongly suggests a biosynthetic relationship between coenzymes containing the pyridine ring. Since aspartate does not serve as a precursor of pyridoxine and the biosynthetic pathway for pyridoxine is unknown, the nature of the biosynthetic relationship remains obscure (104). Several plausible mechanisms could be advanced for the relationship, such as one involving an enzyme common to both pathways or reciprocal control mechanisms. Further investigation of this aspect of pyridine ring synthesis may lead to a very interesting and novel control relationship among the B vitamins.

The fact that amino acids will support the growth of dichotomistic niacin mutants suggests that an amino acid may exert an influence on NAD synthesis. The physiochemical mechanism and the overall role of the influence of amino acids on NAD synthesis remain to be demonstrated. However, several mechanisms for the influence of amino acids on the growth of nic$^-$ mutants can be postulated. For example, the presence of excess amino acids may lead to the production of an enzyme with altered kinetic or regulatory properties.

The nic 18 mutation maps adjacent to the well known threonine-leucine loci (105), separate from the nic loci reported by Taylor (106), Lavelle (107) and Matney (108). It is possible that this mutation occurs in a gene which regulates NAD synthesis. However, since

other nic loci are not clustered in E. coli and occur at at least four different chromosomal loci (108) it is not possible to eliminate a structural role for the nic 18 gene. The nature of mutation nic 52 remains equally obscure. This problem will be clarified when the structural gene products have been identified.

One of the most puzzling and frustrating aspects of this research has been the apparent lack of accumulation of intermediate compounds which proceed QA either in vivo or in vitro as determined by biological activity or by enzymatic conversion to QA-^{14}C, respectively. The sensitivity of the experiments attempting to detect intermediates was such that if the yield of intermediates was 3% or less of the yield of QA produced under identical conditions with mutant E-126, the compound(s) should have been detected. As previously mentioned, the accumulation of intermediates in auxotrophic mutants has been widely used in the study of microbial pathways, including the vitamins biotin and thiamin (109, 110). A priori, there is no apparent reason why intermediates should not accumulate, unless one postulates an exceptional case such as the occurrence of enzyme-bound forms or exceedingly labile compounds. The negative results of the in vivo experiments could be attributed to cellular impermeability to the vitamers; however, this seems unlikely in view of the similar results obtained from in vitro experiments.

In addition to the four suggested mechanisms for controlling coenzyme synthesis (72), this work suggests that two other mechanisms may influence coenzyme synthesis--stimulation or inhibition by other cellular metabolites or reciprocal control systems among the coenzymes.

While this research has posed several formidable questions concerning the control of NAD biosynthesis, the pathway of QA synthesis in *E. coli* remains a mystery.

SUMMARY

The biosynthesis of quinolinic acid (QA) in E. coli has been studied both in vivo and in vitro. The assay used for measuring QA synthesis was based on both cation- and anion-exchange chromatography. Crude extracts from a nic⁻ mutant of E. coli which lacks quinolinate phosphoribosyl transferase activity incorporated both ^{14}C-labeled 4-carbon dicarboxylic acids and glycerol or closely related compounds into QA. L-Aspartate-1-^{14}C was incorporated directly into QA-7-^{14}C without randomization. Tritium from glycerol-1(3)-^{3}H, glycerol-2-^{3}H or DL-aspartate-G-^{3}H was not incorporated into quinolinate. Optimum synthesis of QA in these extracts occurred in the presence of L-aspartate, D-glyceraldehyde-3-phosphate (or fructose-1,6-diphosphate), ATP and $MgCl_2$. Quinolinate synthesis was stimulated by NaF and was not inhibited by iodoacetate.

Heavy cell suspensions of this mutant also excreted excessive quantities of quinolinate into the medium when the cells were incubated with aspartate and a carbohydrate. The optimum pH for enzymatic synthesis of QA, either in vivo or in vitro, was about 8.0.

Despite numerous attempts, no definite evidence was obtained for the postulated unknown compounds in this pathway.

The pathway for QA synthesis appears to be controlled by several mechanisms. Evidence was obtained from in vivo and in vitro experi-

ments for both the repression and feedback inhibition of QA synthesis. The biologically active forms of vitamin B_6, pyridoxalphosphate and pyridoxaminephosphate inhibited QA synthesis in crude extracts.

It was discovered that several E. coli mutants which had been previously characterized as niacin auxotrophs would grow in the absence of niacin when the minimal medium was supplemented with certain L-amino-acids. The nature of these unusual mutations and their relationship(s) to QA biosynthesis or the regulation of QA biosynthesis remains to be determined.

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