# PURIFICATION AND SOME PROPERTIES OF THE QUINOLINATE SYNTHETASE SYSTEM IN *ESCHERICHIA COLI*

By

GARY RANDEL GRIFFITH

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Oklahoma State University

Stillwater, Oklahoma

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PURIFICATION AND SOME PROPERTIES OF THE

QUINOLINATE SYNTHETASE SYSTEM

IN ESCHERICHIA COLI

Thesis Approved:

Robert K. Gholson
Thesis Adviser

Elizabeth T. Gaudy

K. E. Eblen

H. Lon Lipsey

George Odell

D. P. Durham
Dean of the Graduate College

## PREFACE

The work described in this thesis involves a study of the synthesis of the vitamin niacin in the bacterium Escherichia coli. When not supplied to the bacterial cells, the vitamin and its precursors must be synthesized from small molecules in the cell. The direct precursor from these small molecules is quinolinic acid (QA). The enzymes responsible for the formation of QA in E. coli are not well characterized. The objective of this study was to purify one of the proteins that take part in QA biosynthesis and to further characterize the quinolinate synthetase system in E. coli.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ARPPR | Adenosine diphosphate ribose |
| DCC | Dicyclohexycarbodiimide |
| DEAE-Sephadex | Diethylaminoethyl-Sephadex |
| DHAP | Dihydroxyacetone-phosphate |
| $dNAD^+$ | Nicotinic acid adenine dinucleotide |
| EDAC | 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide |
| FAD | Flavin adenine dinucleotide |
| FDP | Fructose-1,6-diphosphate |
| NA | Nicotinic acid |
| $NAD^+$ | Nicotinamide adenine dinucleotide |
| $NADP^+$ | Nicotinamide adenine dinucleotide phosphate |
| NAm | Nicotinamide |
| NAMN | Nicotinic acid mononucleotide |
| NHS | N-hydroxysuccinimide |
| NMN | Nicotinamide mononucleotide |
| PRPP | 5-Phosphoribosyl-1-pyrophosphate |
| QA | Quinolinic acid |
| QRTase | Quinolinate phosphoribosyltransferase |

# CHAPTER I

## INTRODUCTION

Extensive study of the pathways of vitamin biosynthesis has been carried on since the discovery of each vitamin's nutritional significance. Often the discovery and elucidation of the biochemical function of the cofactor form of the vitamin occurred before its role in the diet was known. This was the case with niacin, and its corresponding cofactor form $NAD^+$. Research was focused upon the biosynthesis of niacin and later upon its relationship to $NAD^+$. Out of the results of such work emerged the knowledge of two general routes for niacin formation: one from tryptophan and the other from three and four-carbon molecules in the cell. Further work showed that the latter type of pathway utilizes different precursors in different organisms. It was also discovered that quinolinic acid is a common intermediate in these pathways and therefore a common precursor of $NAD^+$. Much work was also done on the many interconversions of $NAD^+$ and other nicotinyl compounds in the cell, the sum of which was found to be a cyclic mechanism operating in most organisms. The key role of $NAD^+$ in cellular metabolism in all organisms has stimulated investigation of its biosynthesis and the mechanism of its functioning as a cofactor of biological oxidation-reduction reactions.

*Escherichia coli* can synthesize $NAD^+$ by two methods: *de novo* synthesis of quinolinic acid or utilization of exogenous niacin, which

is called the "salvage pathway". This study concerns itself with the de novo synthesis of $NAD^+$, and, more specifically, with the synthesis of QA. QA synthesis occurs by the action of at least two proteins, termed "A" and "B". Genetic characterization has identified mutants which lack the active A protein, nad A mutants, and mutants which lack the active B protein, nad B mutants. An assay developed for measuring synthesis of QA in vitro has been used to show that extracts of a nad A mutant, containing the active B protein, and extracts of a nad B mutant, containing the active A protein, will synthesize QA when mixed with the necessary substrates. This system can be utilized for studying QA synthesis in E. coli.

This study was directed toward reaching a better understanding of the properties of the quinolinate synthetase system in E. coli. Primarily, this goal was approached by purifying the B protein to as great an extent as possible, in order to permit more detailed study of the intermediates, if any, the reactions, and the kinetics and mechanisms of QA biosynthesis.

# CHAPTER II

## LITERATURE REVIEW

Nicotinic acid received its name in Germany in 1867 when Huber (1) oxidized nicotine to nicotinic acid. Later he showed its structure to be pyridine-3-carboxylic acid (2). While searching for the anti beri-beri factor in 1911, Funk (3, 4) isolated nicotinic acid coincidentally from yeast and rice bran. Suzuki et al. (5) also isolated the compound from rice bran without knowing its nutritional significance.

Since the early 1700's, pellagra has been recognized as a human disease, many times occurring in epidemic proportions. Not until 1925, however, was it established that pellagra is a vitamin-deficiency disease that could be cured by yeast, which contained a "PP" or pellagra-preventative factor (6). Better results in pellagra treatment were shown by Elvehjem et al. (7) in 1937 when they isolated nicotinic acid from liver extracts and used it to cure blacktongue, the corresponding condition in dogs. Then later in 1937 and in 1938, several investigators announced conclusively that nicotinic acid would cure pellagra in humans (8, 9, 10). Furthermore, tryptophan was found to substitute for nicotinic acid in the mammalian diet (11), suggesting a precursor relationship between tryptophan and nicotinic acid. Subsequently, nicotinic acid (niacin) and nicotinamide (niacinamide) have been shown to be present in small amounts in, and essential to the function of all living systems.

Before the discovery of nicotinic acid as a vitamin, however, its biochemical function was described, beginning with the work of Harden and Young in 1904 (12). They observed that a dialyzable cofactor was required to stimulate alcohol production from glucose in yeast extracts. This cofactor ("cozymase" or "coenzyme I") was shown to contain nicotinamide by von Euler, Albus, and Schlenk (13). This conclusion was confirmed later by Warburg and Christian (14). However, Warburg and Christian (15, 16) also found another dialyzable cofactor that could not substitute for the one observed by Harden and Young. They quickly determined that this cofactor ("co-ferment" or coenzyme II") also contains nicotinamide (16).

These cofactors are now known as nicotinamide adenine dinucleotide ($NAD^+$, $DPN^+$) and nicotinamide adenine dinucleotide phosphate ($NADP^+$, $TPN^+$), respectively. Their structures were elucidated within a few years of their discovery by von Euler et al. (17, 18) and Schlenk (19, 20). Although postulated by von Euler in 1934, the hydrogen-transfer function of $NAD^+$ and $NADP^+$ was not demonstrated until the work of Warburg and his associates (14, 16, 21). Later, the reversible transfer of hydrogen was shown to take place at the 4-position of the pyridine ring of $NAD^+$ and $NADP^+$ by several investigators (22, 23, 24). The reduced forms of these cofactors were designated NADH and NADPH. The coenzyme function of $NAD^+$ and $NADP^+$ with oxidoreductase enzymes has since been observed in over 290 different biological reactions (25). The general metabolic role of $NAD^+$ is as a cofactor for energy-yielding oxidations in the cell, whereas $NADP^+$ serves as the source of biosynthetic reducing power in cellular metabolism. Figure 1 shows the structures of $NAD^+$ and related compounds.

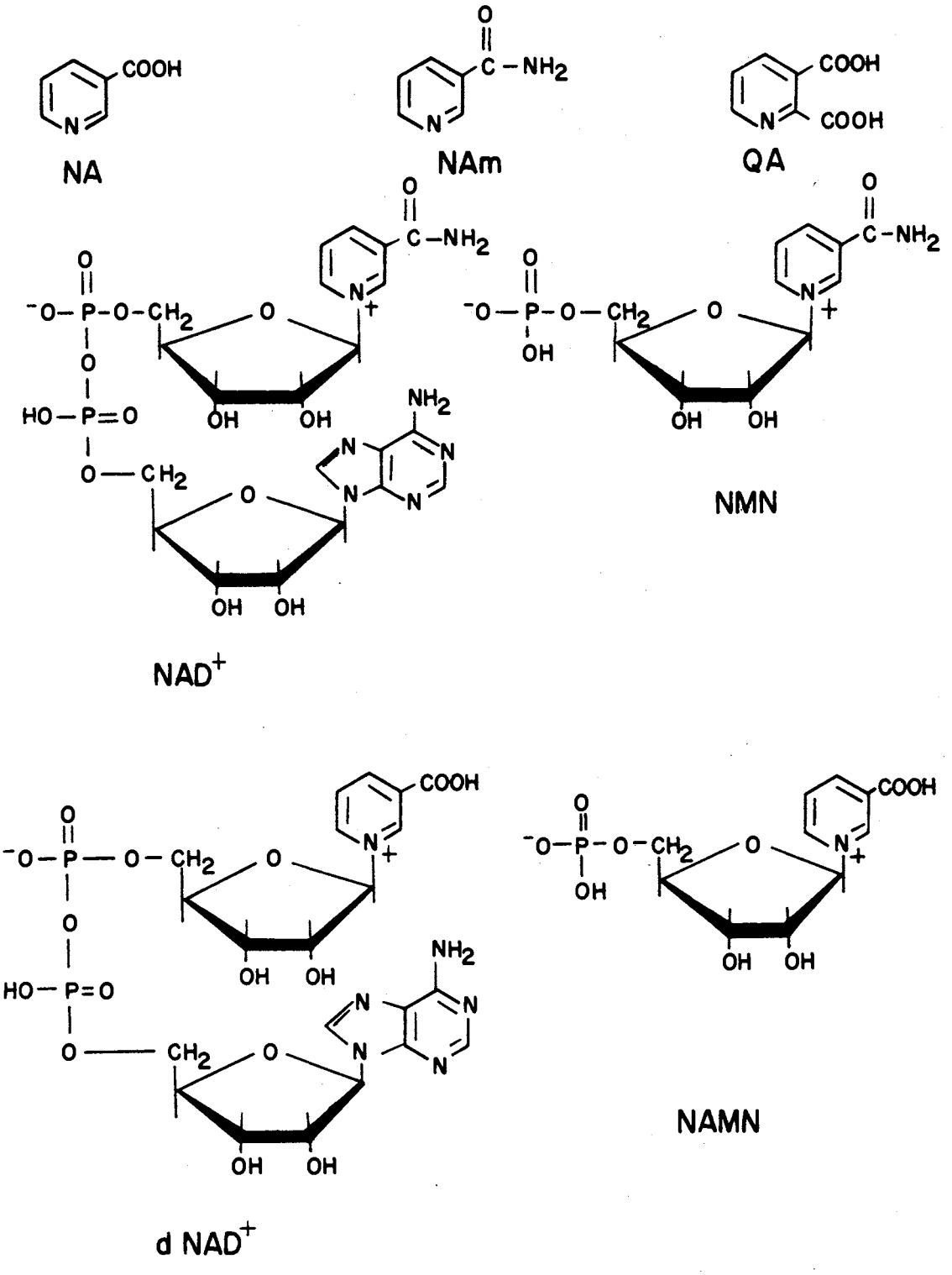



Figure 1. Structures of Nicotinic Acid, Nicotinamide, Quinolinic Acid, and Pyridine Nucleotides

## Additional Biological Roles of $NAD^+$

In addition to its major role, $NAD^+$ has been found to possess other functions in the cell. In 1967, after partial purification of a polynucleotide-joining enzyme ("DNA-joining enzyme" or DNA ligase) from Escherichia coli (E. coli) (26, 27), two groups of investigators (27, 28) promptly found $NAD^+$ to be a specifically required cofactor for the phosphodiester bond formation. In the reaction, $NAD^+$ is not reduced but is cleaved to 5'-AMP and NMN. DNA ligase has various functions in genetic recombination, repair, and DNA synthesis. The enzyme has been purified since its discovery from uninfected (29) and phage-infected (30) E. coli cells, mammalian cells (31), and higher plants (32). Only the E. coli enzyme requires $NAD^+$; others utilize ATP (29, 30).

Also, in 1964, Collier and Pappenheimer (33) reported the requirement of $NAD^+$ as an essential cofactor for the diphtheria toxin-dependent inhibition of the incorporation of amino acids into polypeptides in cell-free systems from mammalian cells. Later, Collier (34) showed the site of action of the toxin and $NAD^+$ to be the aminoacyl-transferase II (elongation factor 2) enzyme. It is now known that diphtheria toxin catalyzes the transfer of the ADP-Ribose portion of $NAD^+$ to the transferase II, thereby inactivating the transferase and releasing nicotinamide (35, 36, 37). Recently, this reversible ADPR-transferase activity of the toxin has been further characterized as to structure and action (38, 39).

The multiple functions and wide distribution of $NAD^+$, therefore, point to the key role this compound plays in the metabolism of the cell.

## De Novo Biosynthesis of $NAD^+$

Since Krehl, et al. (11) conducted nutritional studies on the tryptophan-niacin relationship in the rat, it has been found that trytophan can replace niacin as a growth requirement in many different organisms. The tryptophan-niacin relationship does not exist, however, in many species of microorganisms and several species of plants that have been tested. Thus, another pathway for niacin and therefore $NAD^+$ biosynthesis must operate in these organisms. Table I summarizes the species known to exist with or without the tryptophan pathway.

### Precursors of $NAD^+$ in E. coli

Investigation of the precursors of $NAD^+$ in E. coli began in 1960 when Ortega and Brown (60) demonstrated efficient incorporation of $^{14}C$-succinic acid and $^{14}C$-glycerol into nicotinic acid in a consistent labeling pattern. Maximum synthesis of NA in the resting cell suspension occurred when ribose and adenine were also present. The conclusion from this work was that the precursors of NA in E. coli are a 4-carbon dicarboxylic acid and glycerol or a metabolically related compound. After the demonstration that quinolinic acid (pyridine-2, 3-dicarboxylic acid) is a precursor of $NAD^+$ (61), Ogasawara et al. (62) showed incorporation of $^{14}C$-aspartic acid into carbons 2, 3, 7, and 8 of QA in cell-free extracts of E. coli. This study utilized a mutant lacking the ability to convert QA to the next intermediate in $NAD^+$ formation.

Then Chandler, et al. (63), using crude extracts of these QA-accumulating mutants (QRTase mutants), carried out incorporation of $^{14}C$-aspartic acid, $^{14}C$-glycerol, and $^{14}C$-glucose into QA. The DL-1-$^{14}C$-aspartic acid formed 7-$^{14}C$-QA, 1,3-$^{14}C$-glycerol formed 4,6-$^{14}C$-QA,

TABLE I

THE TRYPTOPHAN-NIACIN RELATIONSHIP

| Organism(s) | Reference |
|---|---|
| Tryptophan is a Precursor of Niacin | |
| Mammals and fowl | (40, 41) |
| Neurospora crassa | (42, 43) |
| Rhodotorula glutinus | (44) |
| Fusarium oxysporum | (45) |
| Claviceps purpurea | (46) |
| Saccharomyces cerevisae (aerobic) | (47) |
| Streptomyces antibioticus | (48) |
| Cyanidium caldarium | (48) |
| Karlingea rosea | (48) |
| Xanthomonas begoniae | (49) |
| Xanthomonas manihotis | (49) |
| Xanthomonas pruni | (50) |
| Tryptophan is Not a Precursor of Niacin | |
| Escherichia coli | (51) |
| Bacillus subtilus | (51) |
| Lactobacillus arabinosus | (52) |
| Leuconostoc mesenteroides | (52) |
| Streptococcus faecalis | (52) |
| Proteus vulgaris | (52) |
| Torula cremoris | (52) |
| Streptomyces venezulae | (48) |
| Anacystis nidulans | (48) |
| Chlorella pyrenoidosa | (48) |
| Saprolegnia ferax | (48) |
| Xanthomonas trifolii | (49) |
| Pseudomonas aeruginosa | (49) |
| Pseudomonas iodinium | (49) |
| Mycobacterium phlei | (49) |
| Saccharomyces cerevisae (anaerobic) | (47) |
| Mycobacterium tuberculosis | (53, 54) |
| Clostridium butylicum | (55, 56) |
| Serratia marcescens | (57) |
| Zea mays | (58) |
| Nicotiana rustica | (58) |
| Ricinus communis | (59) |

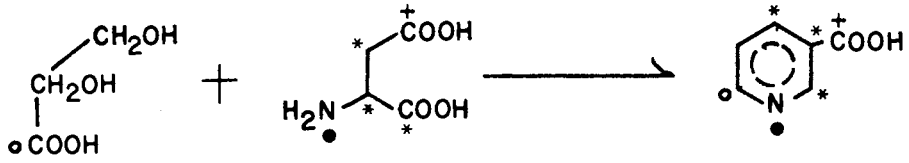
and $^{14}$C-glucose (1, 2, or 6-labeled) labeled the 4 and 6 positions most heavily, with some randomization. These results led to the suggestion that QA, as a precursor of $NAD^+$ in E. coli, is synthesized in vitro by the direct condensation of a triose-phosphate and L-aspartate. Later work (64) in the same system showed that $^{14}$C-succinate, $^{14}$C-glycerol, $^{14}$C-aspartate, $^{14}$C-fructose-1,6-diphosphate, and $^{14}$C-2,3-diphosphoglycerate are incorporated into QA, whereas $^{14}$C-glycerate, $^{14}$C-acetate, $^{14}$C-formate, $^{3}$H-glycerol, and $^{3}$H-aspartate are not. These data confirmed the role of the suggested compounds as precursors of QA. Most recently, Suzuki et al. (65) determined the triose phosphate to be dihydroxyacetone-phosphate (DHAP) and not glyceraldehyde-3-phosphate, utilizing an effective inhibitor of triose-phosphate isomerase, an enzyme present in the protein fractions.

Thus, while the precursors for de novo biosynthesis of $NAD^+$ in E. coli have been elucidated as L-aspartate and dihydroxyacetone-phosphate, the intermediates and enzymes in the pathway to QA, at least, are not yet known. Figure 2 depicts the isotope incorporation patterns known.

## Precursors of $NAD^+$ in Other Microorganisms

Two other microorganisms have been the subject of considerable investigation to determine the precursors of $NAD^+$. One of these, Mycobacterium tuberculosis (M. tuberculosis), studied because of its human pathogenicity, was shown to form niacin from aspartic acid by Mothes, et al. (53). Using DL-4-$^{14}$C-aspartic acid, they observed that the carboxyl group of nicotinic acid originates from carbon 4 of the aspartic acid. Results with unlabeled precursors (54) agreed with the

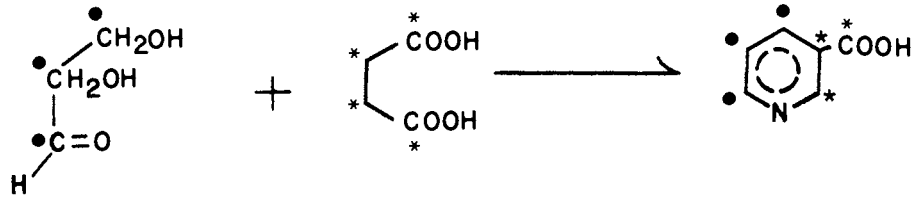
E. coli (62)



M. tuberculosis (53,66,68,69)

C. butylicum (72-74)

S. marcescens (57)



Nicotiana species (76,92)

Figure 2. Labeling Patterns from Radioactive Precursors of Nicotinic Acid or Quinolinic Acid in Bacteria and Plants

aspartate incorporation and suggested the involvement of alanine, but failed to show stimulation by glycerol or incorporation of 2-$^{14}$C-glycerol in niacin synthesis in resting cells.

Further substantiation of the role of aspartate as a precursor of NA in M. tuberculosis by Gross, et al. (66) employed DL-1,4-$^{14}$C,$^{15}$N-aspartic acid and calculation of isotope ratios. Carbons 2, 3, and 4 and the ring of nitrogen of NA are derived directly from aspartic acid. Thus, in the utilization of aspartate, carbon 1 is lost but all other carbons and the nitrogen are incorporated (67). Albertson and Moat (68) conducted experiments with 3-$^{14}$C and 4-$^{14}$C aspartic acid which were in agreement with previous results, but in contrast to one previous study (54), they demonstrated incorporation of carbon 2 of 2-$^{14}$C-glycerol into the pyridine ring. The role of glycerol (or a metabolite of glycerol) was more clearly indicated by the incorporation of 1,3-$^{14}$C-glycerol and 3-$^{14}$C-glyceric acid into carbons 4, 5, and 6 of the pyridine ring (69). Carbon 6 was exclusively derived from carbon 3 of the glyceric acid, indicating a symmetrical intermediate does not occur. In the same study, $^{14}$C-formate and $^{14}$C-acetate were shown not to be precursors of NA.

Excretion and precursor studies with isotopes in M. tuberculosis and M. bovis (the corresponding bovine pathogen) have strongly indicated that QA is an intermediate in niacin synthesis (69, 70, 71). Experiments have utilized $^{14}$C-aspartic acid, $^{14}$C-glycerol, and $^{14}$C-glyceric acid to produce $^{14}$C-QA, which was isolated and identified from media or extracts. $^{14}$C-QA was also found to be converted to $^{14}$C-NA in cell extracts.

The pathway for $NAD^+$ biosynthesis in M. tuberculosis, then, to the extent that it has been investigated, appears to have identical precursors and intermediates as the de novo system in E. coli.

Investigation has been carried out in Clostridium butylicum (C. butylicum), a strict anaerobe. Isquith and Moat (55) found both uniformly labeled aspartate and glycerol to be incorporated into nicotinic acid in crude extracts, but upon fractionation of the extract, only aspartate, acetyl CoA, and formate stimulated NA production. Incorporation of $^{14}$C-aspartate, $^{14}$C-acetate, and $^{14}$C-formate into NA was also demonstrated. Then Scott and Mattey (72) incubated resting C. butylicum cells with $^{14}$C-formate and found nearly all of the radioactivity in carbon 6 of nicotinic acid. The role of N-formyl-L-aspartate as a precursor to nicotinic acid was postulated when the compound gave enhanced rates of NA synthesis over rates for formate and L-aspartate and when carbons 2, 3, 6, and 7 of NA were labeled from $^{14}$C-N-formyl-L-aspartate (73, 74). Also in cell extracts, a specific formylation inhibitor shut off NA synthesis from formate and aspartate, but not from N-formyl-L-aspartate. The origins of carbons 4 and 5 of nicotinic acid in this organism are not known.

A different pathway, therefore, exists for $NAD^+$ biosynthesis in C. butylicum, involving N-formyl-L-aspartate and likely a 2-carbon compound. Presumably, QA is an intermediate in this process, but specific evidence is not yet available.

In Serratia marcescens, an organism more nearly like E. coli, the only work done indicates a different labeling pattern in nicotinic acid from $^{14}$C-aspartic acid. In cell suspensions, Scott and Hussey (57) observed incorporation of the aspartate carbons 3 and 4 into the

carboxyl group and ring of NA, respectively. However, carbon 2 from aspartate chiefly labeled the carboxyl group of NA, resulting in the contribution of two carbons of aspartate to one carbon of NA. It was suggested in this work that at some stage carbons 2 and 4 of aspartate become equivalent and one is lost. If this or a similar explanation is correct, nicotinic acid formation in Serratia marcescens represents a third distinct de novo pathway for $NAD^+$ biosynthesis in microorganisms without the tryptophan-niacin relationship.

The yeast Saccharomyces cerevisae (S. cerevisae) is a facultative organism and utilizes the tryptophan-nicotinic acid pathway under aerobic conditions, but not under anaerobic conditions (47). It was shown that anaerobically, the tryptophan pathway cannot operate, but that aspartic and glutamic acids are effective precursors of nicotinic acid. Aerobically, though, these dicarboxylic acids are not good precursors of NA. Quinolinic acid was also shown to be a precursor of $NAD^+$ and common to both pathways in yeast.

## Formation of Nicotinic Acid in Higher Plants

Studies on the precursors of nicotinic acid in higher plants were primarily carried out as part of the investigation of biosynthesis of the alkaloids nicotine, anabasine, and ricinine, which derive their pyridine ring from nicotinic acid. Nearly all such studies have been carried out with labeled compounds in intact plants, with subsequent isolation of labeled products. The most recent review of this work was made by Leete in 1969 (75).

Tryptophan or 3-hydroxyanthranilic acid could not serve as a precursor of the pyridine nucleus in peas, corn, or soybeans (58, 76),

nor could 7a-$^{14}$C-tryptophan be shown to form nicotine in Nicotiana tabacum or Nicotiana rustica (58, 77). Ricinine was not labeled from 7a-$^{14}$C-tryptophan in Ricinus communis (castor bean plant) (78). Therefore, work was begun to discover the precursors of NA in these species.

The incorporation of 2-$^{14}$C-acetate into nicotine and anabasine in Nicotiana species was the first demonstration of de novo biosynthesis of the pyridine ring in plants (79, 80). The use of acetate labeled in carbon 1, however, did not result in labeled alkaloids. Another study (81) demonstrated $^{14}$C incorporation into the pyridine ring of nicotine from 2-$^{14}$C-propionate, 1,3-$^{14}$C-glycerol, and 2-$^{14}$C-glycerol. Formation of the α-pyridone ring of ricinine was reported to occur from 2-$^{14}$C-acetate, 2,3-$^{14}$C-succinate, 1,3-$^{14}$C-glycerol, and 2-$^{14}$C-glycerol (78, 82, 83, 84).

When better chemical methods for carbon-by-carbon degradation of nicotinic acid and these pyridine alkaloids were developed, much more information could be obtained from labeling studies. Yang, et al. (85) and Griffith and Byerrum (86) found glycerol to be a precursor of carbons 4, 5, and 6 of the pyridine ring of nicotine. Glycerol shows similar incorporation into ricinine (59) and anabasine (87). Carbons 2 and 3 of the pyridine ring are labeled when 2,3-$^{14}$C-succinate is used in the formation of nicotine (86) and ricinine (59, 88, 89). Other dicarboxylic acids showing incorporation were 3-$^{14}$C-aspartic acid and 4-$^{14}$C-aspartic acid in ricinine (90) and 3-$^{14}$C-aspartate and 3-$^{14}$C-malate in nicotine (91).

These numerous isotopic precursor experiments indicate the source of carbons 4, 5, and 6 in higher plants is a non-symmetrical 3-carbon

compound closely related to glycerol. The remaining carbons of NA appear to be derived from a 4-carbon dicarboxylic acid that is metabolically close to succinic acid (76). The specific precursors may be aspartic acid and glyceraldehyde-3-phosphate. Fleeker and Byerrum (92) showed better incorporation of D-3-$^{14}$C-glyceraldehyde than of glycerol in Nicotiana rustica. Incorporation studies utilizing labeled aspartic acid, however, have been difficult to interpret, due to long incorporation times and rapid aspartate metabolism in the plants (76). The source of the nitrogen of the pyridine ring in Ricinus communis L. has been studied utilizing $^{15}$N-aspartate, $^{14}$C,$^{15}$N-aspartate (59, 90), and other nitrogen sources (93) with unclear results. Figure 2 indicates some incorporation patterns.

Quinolinic acid was found to be a precursor of nicotinic acid in corn (94) and castor bean plants (95). Also, QA is an intermediate in the de novo formation of nicotine in tobacco plants (85, 96) and ricinine in the castor bean plant (94).

Although much work has been done to determine the precursors of the nicotinyl portion of these pyridine alkaloids, exact pathways of biosynthesis have not been established. Confirmation of aspartic acid and glyceraldehyde-3-phosphate as the precursors awaits cell-free studies and purification of enzyme systems. Present knowledge indicates the de novo pathway of NA and thus $NAD^+$ biosynthesis in higher plants may be no different than the pathway in E. coli and M. tuberculosis.

Excluding the tryptophan-niacin pathway, then, two and perhaps three pathways for de novo $NAD^+$ biosynthesis in higher plants and microorganisms present themselves. The pathway found in E. coli may be the

one common to most plants and bacteria, but much more investigation is necessary to show this.

## Pathways of Pyridine Nucleotide Metabolism

In addition to the de novo pathway for the biosynthesis of quinolinic acid, most organisms can also utilize NA or NAm or both to synthesize $NAD^+$ (97). The pathways and interconversions involving these nicotinyl derivatives have been the subject of much study.

After identification of intermediates of these pathways using human erythrocytes, rat liver, and yeast, Preiss and Handler (98, 99) described enzyme activities that led them to propose the sequence NA → NAMN → $dNAD^+$ → $NAD^+$ for the synthesis of $NAD^+$, based upon the following reactions observed:

NA + PRPP → NAMN + PPi

NAMN + ATP → $dNAD^+$ + PPi

$dNAD^+$ + ATP + glutamine → $NAD^+$ + AMP + PPi + glutamate

These reactions were later established in other tissues (100) and their sequence became known as the Preiss-Handler pathway.

Cyclic schemes for the utilization of exogenous NA and for $NAD^+$ metabolism were suggested by Joshi and Handler (101) and Sarma, et al. (102). These were founded upon the enzyme activities then known to exist for these pathways and upon the belief that the nicotinyl portion from the de novo biosynthetic pathway entered into the cyclic reactions as nicotinic acid. However, with the discovery that the de novo pathway enters at the NAMN level through QA by the QRTase enzyme (40, 61, 94, 103), Gholson (100) summarized these interconversions as the more general pyridine nucleotide cycle existing in most organisms.

The Pyridine Nucleotide Cycle in E. coli

In E. coli, the Preiss-Handler pathway for the utilization of exogenous NA and NAm was demonstrated in 1961 when Imsande (104) reported four enzyme activities in extracts of E. coli K-12:

(1) Nicotinamidase

(2) NAMN pyrophosphorylase

(3) $dNAD^+$ pyrophosphorylase

(4) $NAD^+$ synthetase

Thus, the sequence of NAm → NA → NAMN → $dNAD^+$ → $NAD^+$ was established in E. coli. Andreoli et al. (61) detected a new enzyme activity in K-12 extracts that would convert QA to NAMN, without the involvement of NA as an intermediate. This enzyme became known as quinolinate phosphoribosyltransferase (QRTase). Nicotinic acid was, however, confirmed to be a necessary intermediate in the formation of $NAD^+$ via the Preiss-Handler pathway in studies utilizing mutants that cannot convert NAm to NA (105). Meanwhile, $NAD^+$ synthetase, which forms $NAD^+$ from $dNAD^+$, was purified from E. coli B cells and studied (106). Also, $NAD^+$ pyrophosphorylase and deamido $NAD^+$ pyrophosphorylase, which convert NMN and NAMN to $NAD^+$ and $dNAD^+$, respectively, were observed to apparently be one enzyme having both functions in E. coli B (107). The enzyme DNA ligase (27, 28) was discovered and shown to possess high activity in E. coli (108), catalyzing the hydrolysis of $NAD^+$ to NMN. $NAD^+$ is converted to $NADP^+$ by $NAD^+$ kinase (159). Table II includes these enzyme activities and their reactions.

The proposal of the pyridine nucleotide cycle in 1966 (100) furnished a frame of reference with which to evaluate these reported enzyme activities. Figure 3 outlines the cycle and its enzymes. If the cycle

TABLE II

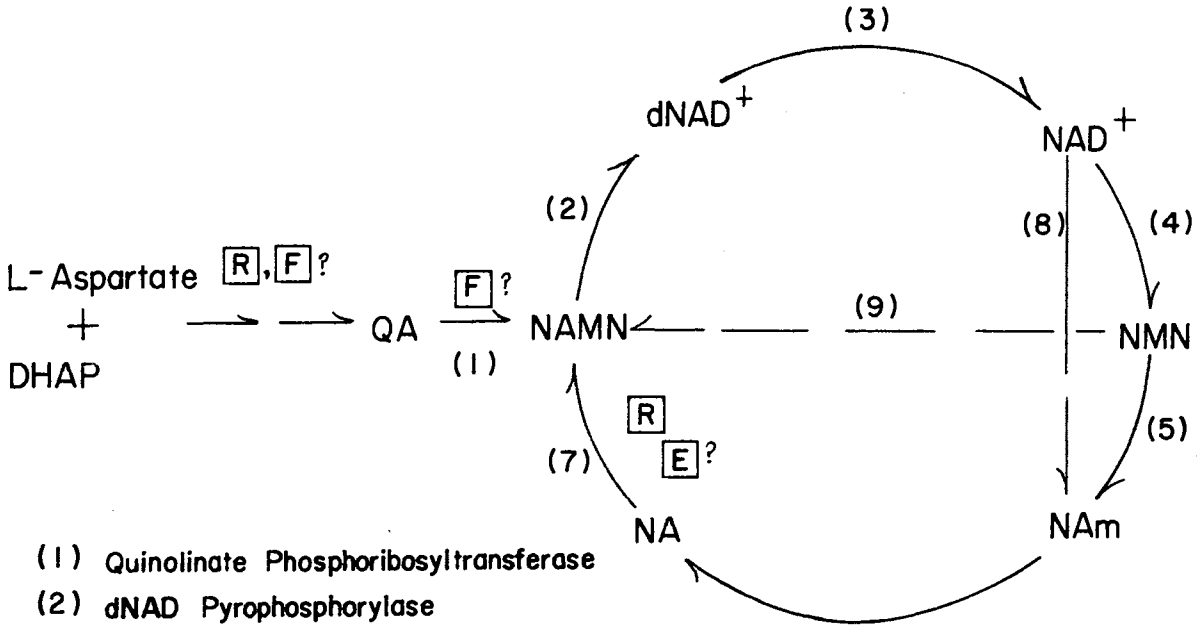
ENZYMES RELATED TO PYRIDINE NUCLEOTIDE METABOLISM IN *E. COLI*[a]

| Enzyme | Other Names | Enzyme Commission No. | *E. coli* Strain | Reaction Catalyzed | Reference |
|---|---|---|---|---|---|
| Quinolinate Phosphoribosyl-transferase | QRTase; NAMN Pyrophosphorylase (carboxylating) | E.C. 2.4.2.19 | K-12 | QA + 5-PRPP → NAMN + PPi + $CO_2$ | (61) |
| $dNAD^+$ ($NAD^+$) Pyrophosphorylase | NAMN (NMN) Adenyltransferase | E.C. 2.7.718 (E.C. 27.7.1) | K-12, B | NAMN + ATP → $dNAD^+$ + PPi (NMN + ATP → $NAD^+$ + PPi) | (104, 107) |
| NAD Synthetase | | E.C. 6.3.1.5 | K-12, B | $dNAD^+$ + ATP + $NH_3$ → $NAD^+$ + AMP + PPi | (104, 106) |
| DNA Ligase | DNA-joining enzyme; Polynucleotide Synthetase ($NAD^+$) | E.C. 6.5.1.2 | C64, B, 1100 | $NAD^+$ + (deoxyribonucleotide)$_n$ + (deoxyribonucleotide)$_m$ → NMN + AMP + (deoxyribonucleotide)$_{m+n}$ | (27, 28, 181) |
| NMN Glycohydrolase | NMNase | | K-12 | NMN + $H_2O$ → $NA_m$ + Ribose-5-P | (109) |
| Nicotinamide Deamidase | Nicotinamidase; Nicotinamide Amidohydrolase | E.C. 3.5.1.19 | K-12 | NAm + $H_2O$ → NA + $NH_3$ | (104, 168) |
| NAMN Pyrophosphorylase | NRTase; NA Phosphoribosyltransferase | E.C. 2.4.2.11 | K-12 | NAMN + PPi → NA + 5-PRPP | (104) |

TABLE II (Continued)

| Enzyme | Other Names | Enzyme Commission No. | *E. coli* Strain | Reaction Catalyzed | Reference |
|---|---|---|---|---|---|
| NMN Amidohydrolase | NMN Deamidase | | K-12 (RS 126) | $NMN + H_2O \rightarrow NAMN + NH_3$ | (115) |
| $NAD^+$ Glycohydrolase | $NAD^+$ase; $NAD^+$ Nucleosidase | E.C. 3.2.2.5 | not found | $NAD^+ + H_2O \rightarrow NA_m$ + ADP-Ribose | (109) |
| $NAD^+$ Pyrophosphatase | $NAD^+$ Phosphohydrolase | E.C. 3.6.1.22 | not reported | $NAD^+ + H_2O \rightarrow NMN + AMP$ | (110) |

[a](25)




(1) Quinolinate Phosphoribosyltransferase
(2) dNAD Pyrophosphorylase
(3) NAD Synthetase
(4) DNA Ligase
(5) NMN Glycohydrolase
(6) Nicotinamide Deamidase
(7) NAMN Pyrophosphorylase
(8) NAD Glycohydrolase
(9) NMN Amidohydrolase

Known Control Points:

R Repression
F Feedback Inhibition
E Allosteric Effector

See Table II for complete enzyme reactions.

Figure 3. The Pyridine Nucleotide Cycle in E. coli

does operate in E. coli, at least these three conditions must be met: (1) the necessary enzyme activities for each conversion must be observed, (2) production of $NAD^+$ from exogenous niacin must be found (i.e., the "salvage pathway" must operate), and (3) turnover, or cycling of the intermediates in the pathway, must be demonstrated.

Table II lists the enzymes for pyridine nucleotide interconversions that have been detected to date. All tne enzymes necessary to the cycle have been reported except one, which is $NAD^+$ glychoydrolase. This enzyme cleaves $NAD^+$ at the N-glycosyl bond to produce ARPPR and NAm and has not yet been demonstrated in E. coli in an unhibited or detectable form (109). If the cycle is to operate, then, somehow the cell must convert $NAD^+$ to NAm. $NAD^+$ could be cleaved to NMN by an enzyme such as $NAD^+$ pyrophosphatase (110), but no report of this enzyme in E. coli has been made, either. However, DNA ligase does exist in this organism (27, 28) and produces NMN from $NAD^+$. Thus, the recent report of Andreoli, et al. (109) of a membrane-localized, NMN-specific glycohydrolase presents the distinct possibility that this part of the pyridine nucleotide cycle proceeds through the steps $NAD^+ \rightarrow NMN \rightarrow NAm$, and not $NAD^+ \rightarrow NAm$ directly. If this is correct, these interconversions prescribe that a different form of the pyridine nucleotide cycle operates in E. coli compared to other microorganisms (see below).

Other evidence for the operation of the cycle consists of the demonstration that $NAD^+$ can be formed from NAm or NA, with NA as a required intermediate (100, 105), and that exogenous $NAD^+$ seems to first be cleaved and routed through the salvage pathway in order to be utilized to make cellular $NAD^+$ (111). Direct evidence for turnover was obtained

in studies in which mutants without nicotinamide deamidase (pnc A mutants) excreted NAm into the media when grown on NA (112). Thus, NA had to proceed through the cycle in order to form the excreted NAm. Also, turnover evidence has been obtained recently from in vivo studies in exponentially growing E. coli using pulse-labeling of precursors (113, 114, 115).

A new scheme for pyridine nucleotide interconversions has been proposed by Olivera, et al. (115), based upon such turnover data. In the scheme, two cycles operate, one for NAm interconversions and one for NA interconversions exclusive of NAm. $NAD^+$ is a central intermediate of both cycles. Although intermediates have not been completely demonstrated, an NMN deamidase has been found (115) that would allow NMN interconversions exclusive of NAm. Also, these cycles would involve DNA ligase and $dNAD^+$ pyrophosphorylase (see Table II) activities.

This detection of NMN deamidase in E. coli opens up the possibility of a "short cut" across the cycle by the conversion of NMN to NAMN directly, bypassing NAm and NA. Then, with the operation of DNA ligase and NMN glycohydrolase, the pattern would actually consist of a small cycle enclosed by a larger cycle containing NA and NAm. The cycle and its enzymes are depicted in Figure 3. Quinolinic acid is the precursor of NAMN and the entry point into the cycle for de novo biosynthesis. The exogenous precursors NA and NAm are incorporated into the cycle via the salvage pathway, from NAm → NA → NAMN (Figure 3).

Evidence, then, indicates that the pyridine nucleotide cycle does function in E. coli, although the exact form of the cycle needs further clarification.

## Pyridine Nucleotide Interconversions in Other Microorganisms and Plants

In other microorganisms and plants, all the enzymes have been detected for the cycle as it was originally proposed. Table III indicates the enzymes and organisms in which they have been found to date. Perhaps the most widely studied is nicotinamide deamidase, a key enzyme in the utilization of exogenous NAm and $NAD^+$, assuming the cycle is in operation. $NAD^+$ glycohydrolase appears to be widely distributed in bacteria. It is interesting to note that the $NAD^+$ glycohydrolase of Streptococcus species, in which the enzyme is excreted into the media, has recently been demonstrated to be identical with streptolysin-O, an extracellular protein with hemolytic activity produced in these bacteria (151, 152).

Also, $NAD^+$ pyrophosphatase has been found in several species and NMN amidohydrolase (NMN deamidase), newly detected in E. coli, is present in other bacteria. QRTase, included because of its role in linking the de novo pathway to the cycle, is also existent in many organisms.

It would appear, then, that most of these enzyme activities are ubiquitous in living systems, which implies the pyridine nucleotide cycle may function in all systems. Known exceptions are some Lactobacilli and Leuconostoc mesenteroides (121), which require NA or NAm for growth and seem to convert one of these to $NAD^+$ by a simple pathway.

Little work has been done on the pathways of pyridine nucleotides in plants, but quinolinic acid has been shown to be a precursor of NA and NAMN in corn and castor bean plants and a precursor of ricinine, also in the castor bean plant (94). In addition, the intermediates and

TABLE III

PRESENCE OF ENZYMES OF PYRIDINE NUCLEOTIDE METABOLISM IN OTHER MICROORGANISMS AND PLANTS

| Organism | Reference |
|---|---|
| *Quinolinate Phosphoribosyltransferase* | |
| *Mycobacterium* species | (116, 117) |
| *Pseudomonas* species | (103, 118) |
| *Clostridium butylicum* | (55) |
| *Astasia longa* | (120) |
| *Zea mays* | (94) |
| *Ricinus communis* | (94) |
| *dNAD$^+$ Pyrophosphorylase* | |
| yeast | (99) |
| *Mycobacterium tuberculosis* | (117) |
| *Astasia longa* | (120) |
| *NAD$^+$ Pyrophosphorylase* | |
| *Leuconostoc mesenteroides* | (121) |
| *Lactobacillus* species | (121) |
| *NAD$^+$ Synthetase* | |
| yeast | (99, 122) |
| *Mycobacterium tuberculosis* | (117) |
| *Astasia longa* | (120) |
| *Nicotinamide Deamidase* | |
| *Aspergillus niger* | (123) |
| *Torula cremoris* | (101) |
| *Flavobacterium peregrinum* | (124) |
| *Saccharomyoes cerevisae* | (125) |
| *Neurospora crassa* | (126, 127) |
| *Micrococcus lysodeikticus* | (128) |
| *Claviceps purpurea* | (46) |
| *Lactobacillus* species | (121, 131) |
| *Streptococcus faecalis* | (131) |
| *Mycobacterium* species | (129, 130) |
| Other bacteria | (134) |
| *NAMN Pyrophosphorylase* | |
| *Bacillus subtilis* | (135, 136) |

TABLE III (Continued)

| Organism | Reference |
|---|---|
| yeast | (137, 138) |
| *Salmonella typhimurium* | (135) |
| *Serratia marcescens* | (135) |
| *Torula cremoris* | (135) |
| *Tetrahymena pyriformis* | (135) |
| *Mycobacterium tuberculosis* | (139) |
| *Astasia longa* | (120) |
| *Leuconostoc mesenteroides* | (125, 121) |
| *Lactobaccillus plantarum* | (121) |
| *Lactobacillus casei* | (121) |
| *NMN Amidohydrolase* | |
| *Propionibacterium shermanii* | (140) |
| *Clostridium sticklandii* | (141) |
| *Azotobacter vinelandii* | (142) |
| *$NAD^+$ Glycohydrolase* | |
| *Aspergillus niger* | (123) |
| *Mycobacterium tuberculosis* | (143) |
| *Pseudomonas putida* | (144) |
| *Streptococcus species* | (145) |
| *Neurospora crassa* | (146) |
| *Mycobacterium butyricum* | (147) |
| *Bacillus subtilis* | (148) |
| *Claviceps purpurea* | (46) |
| *$NAD^+$ Pyrophosphatase* | |
| *Clostridium butylicum* | (149) |
| *Proteus vulgaris* ($NADP^+$) | (150) |
| *$dNAD^+$ Pyrophosphatase* | |
| *Clostridium butylicum* | (149) |

the operation of the pyridine nucleotide cycle have been implicated in the biosynthesis of ricinine (153, 154). Studies with barley leaves (155) and wheat leaves (156) indicate that the Preiss-Handler pathway occurs in these organisms.

If the pyridine nucleotide cycle does function in these microorganisms and plants, as the intermediates and enzyme activities indicate, it remains to be shown that the salvage pathway can operate and that turnover of $NAD^+$ occurs *in vivo*.

## Regulation of $NAD^+$ Biosynthesis in *E. Coli*

Regulation of enzyme synthesis, enzyme activities, and intracellular product levels is an important aspect of biosynthetic pathways. In general, the rate of coenzyme synthesis is about one one-thousandth of the rate of synthesis of major metabolites, such as most amino acids and nucleic acid bases. Coenzymes, however, are reused many times, thus requiring much lower cellular levels and correspondingly lower rates of synthesis. Total $NAD^+$ (oxidized and reduced) levels have been determined in *E. coli* and results range from 0.1 to 1.0 mM in the cell, depending upon nutritional and growth conditions. Consequently, control mechanisms must operate to create and maintain such levels.

One point of view that can be taken of control of $NAD^+$ biosynthesis is based upon the key roles of $NAD^+$ and $NADP^+$ in energy production and biosynthesis (157). Since such processes are so essential to the cell, careful control of $NAD^+$ synthesis and degradation would be expected so that these coenzymes are always present when needed, but not at excessively high levels.

On the other hand, regulation of coenzyme production may be considered to be loosely controlled compared to control of production of the major cell metabolites. As Tritz and Chandler (158) have pointed out, a high proportion of overproduction (and thus excretion) of $NAD^+$ precursors can occur in cultures of E. coli mutants. It would seem that the cell is indiscriminately synthesizing $NAD^+$ and wasting it, reflecting poor control of its synthesis. However, this excretion has a maximum rate, as does the excretion of major cellular metabolites, which implies an even more finely controlled system for $NAD^+$ biosynthesis, since $NAD^+$ levels are much lower than those for major metabolites.

NAMN Pyrophosphorylase

Perhaps the first enzyme of $NAD^+$ metabolism to be implicated in control was NAMN pyrophosphorylase. Imsande and Pardee (159) checked the effect of supplemental NA or $NAD^+$ in the growth medium of NA auxotrophs of E. coli upon the enzyme activities catalyzing the steps from NA to $NAD^+$. They found that above $5 \times 10^{-7}$ M supplemental NA or $NAD^+$, repression of NAMN pyrophosphorylase biosynthesis occurred. They also checked for feedback inhibition in extracts by $NAD^+$ and found none. Further work (135, 160, 161) confirmed the repression-depression regulation of NAMN pyrophosphorylase by NMN as well as NA and $NAD^+$. Compounds that fail to show feedback inhibition are NA, $NAD^+$, NAMN, and NAm at concentrations up to $1 \times 10^{-3}$ M in E. coli extracts (160). Recent in vivo studies (114) have confirmed the regulatory nature of NAMN pyrophosphorylase by showing that the rate of pyridine nucleotide biosynthesis is determined by the rate of conversion of NA to NAMN in

cultures in which the concentration of exogenous niacin is $10^{-5}$ M or greater.

Repression of NAMN pyrophosphorylase has also been demonstrated in Salmonella typhimurium (135), but not in Serratia marcescens, B. subtilis, B. megaterium, Torula cremoris, Astasia longa, or Tetrahymena pyriformis (120, 135, 160).

ATP (or another nucleoside triphosphate) is an absolute requirement for NAMN pyrophosphorylase activity in all of the above species (135, 138) except Astasia longa, in which the enzyme is ATP-independent (106). In some mammalian systems, ATP is known to act as a positive allosteric modifier of NAMN pyrophosphorylase (162). A similar role for ATP has been described for Bacillus subtilis (163, 164), thus forming a cross-linked control system for the rate of cell metabolism ($NAD^+$ levels) and the rate of energy production (ATP levels). The presence of such a control system in E. coli has been suggested (164, 165), based partially upon the ATP level of intact cells, which is apparently rate-limiting in the NAMN pyrophosphorylase reaction (166).

Nicotinamide Deamidase

Possible regulatory significance has been attributed to the first enzyme in the salvage pathway, nicotinamide deamidase. Calbreath and Joshi (167) reported feedback inhibition of the deamidase in crude extracts of several microorganisms, including E. coli. The presence of 8 mM $NAD^+$ caused up to 93% inhibition of the deamidase activity, depending upon substrate (NAm) concentration. This may or may not be actual feedback inhibition, since it is known that exogenous $NAD^+$ is utilized first by conversion to NAm in intact cells (111). The continued

operation of that enzyme system ($NAD^+ \rightarrow NAm$) in crude extracts would result in isotope dilution of the 7-$^{14}$C-NAm assay substrate used by the unlabeled NAm derived from the added $NAD^+$. More recently, McLaren, *et al.* (114) indicated that *in vivo* there is no regulation of the rate of conversion of NAm to NA (Nicotinamide deamidase activity). They observed the rapid uptake of exogenous NAm by the cells and its conversion to NA, with NA excretion occurring as long as NAm is available. Also, the study done by Pardee, *et al.* (168) showed no change in deamidase activity of cells grown on media containing NAm, NA, $NAD^+$, or $NADP^+$ at various concentrations in the $10^{-7}$ to $10^{-5}$ M range. These studies utilized an assay very similar to that of Calbreath and Joshi, but with a highly purified deamidase preparation from *E. coli* K-12, which would eliminate the potential problem of isotope dilution by production of unlabeled substrate from the exogenous compound.

Inhibition of nicotinamide deamidase by $NAD^+$ has been observed in extracts of *Clostridium acetobutylicum*, *B. stearothermophilus*, *Micrococcus lysodeikticus*, *Torula cremoris*, and *S. cerevisae* (167). In a purified preparation from *Torula cremoris* (101), $NAD^+$ and $NADP^+$ were observed to be noncompetitive feedback inhibitors of the enzyme. Independent from this inhibition, concentrations of $NAD^+$ of $10^{-4}$ M in the media of *Torula cremoris* repressed production of nicotinamide deamidase up to 50%.

## $NAD^+$ Glycohydrolase

Although not known to exist in *E. coli*, $NAD^+$ glycohydrolase has been shown to have regulatory properties in several microorganisms. Partially purified $NAD^+$ase from *M. tuberculosis* is known to possess a

heat-labile inhibitor that complexes with the enzyme to cause loss of activity (148, 169). The protein inhibitor exists in excess in crude extracts and has been purified and characterized (170). Heat-labile protein inhibitors of $NAD^+$ glycohydrolase have also been found in M. butyricum (171), Pseudomonas putida (144), and B. subtilis (148). In addition, each enzyme associated with a heat-inactivated inhibitor is itself heat-stable (144). This type of inhibitor-enzyme complex for $NAD^+$ glycohydrolase could very possibly play a role in regulating turnover and levels of $NAD^+$. Perhaps this occurs through inhibition of the formation of the enzyme-inhibitor complex by high concentrations of $NAD^+$ (144).

No basis, however, exists for this mechanism to function in E. coli. Much effort has been put into detecting $NAD^+$ glycohydrolase in an active form, but results have been negative (109). If $NAD^+$ glycohydrolase exists in E. coli, the reason for the failure in detecting it may well be a tightly bound inhibitor. Alternatively, further characterization of the recently reported NMN glycohydrolase (109) may reveal such an enzyme-inhibitor complex that may function in regulation of $NAD^+$ or NMN levels and cycle turnover.

Enzymes of the de Novo Pathway

In the de novo pathway for $NAD^+$ biosynthesis, several studies on control have been made since it was determined QA is an intermediate and that it enters the cycle at NAMN (61). Quinolinate phosphoribosyltransferase is repressed in B. subtilis and B. megaterium when these organisms are grown on media containing $10^{-5}$ M or higher NA (136, 160, 165). No repression of QRTase has been observed under these conditions in E.

coli (160, 165). Feedback inhibition could not be demonstrated in B. subtilis using NA, $NAD^+$, or $NADP^+$ (136). It has been reported in E. coli that pyridoxal-phosphate is a strong inhibitor of QRTase (158).

The enzymes for QA biosynthesis in E. coli do seem to be controlled by repression-derepression. Using mutants lacking QRTase, NA concentrations of $1 \times 10^{-5}$ M or higher in the media effectively prevented synthesis of enzymes for QA synthesis (160, 165). It is possible that NAm and $NAD^+$ at $5 \times 10^{-6}$ M can also effect this repression (64).

Inhibition of QA synthesis in extracts by addition of $NAD^+$, pyridoxal-phosphate, and pyridoxamine-phosphate was demonstrated, using concentrations of 3.33 mM in crude extracts (64). A slight inhibition was also noticed for $NAD^+$ at 0.5 mM, which is near the proposed concentration for $NAD^+$ in E. coli cells (104). In the same system, NA, NAm, and $NADP^+$ did not block QA synthesis in crude extracts. When these compounds and $NAD^+$ were added to resting cell cultures, reduction in QA synthesis was observed in the resulting crude extracts, implying the conversion of these compounds to $NAD^+$ before extracts were assayed (158).

Recent genetic work (158) suggests the existence of a gene (nad R) which may be responsible for the regulation of the two genes (nad A and nad B) coding for enzymes of quinolinic acid biosynthesis. In vitro complementation analysis has suggested this gene affects the expression of the nad A and nad B genes. Perhaps this is the mechanism by which the repression of the QA biosynthesis enzymes occurs.

Another possible control mechanism is at the cell membrane of E. coli. Olivera et al. (114) have shown that exogenous NAm is taken up rapidly into the cell and metabolized to $NAD^+$, whereas NA uptake becomes rate-limiting to $NAD^+$ synthesis at NA concentrations below $8 \times 10^{-6}$ M.

Accumulation of NA from the media has been reported (159), but recent evidence (114, 172) does not support the existence of an active transport mechanism in the membrane for niacin. It is suggested that the charged NA molecule is less permeable to the membrane than NAm.

$NAD^+$ biosynthesis in E. coli, then, appears to be regulated by known types of mechanisms, including repression-derepression, feedback inhibition, and possibly allosteric modification. The operation of these mechanisms is only partially understood and represents a complex control system that is similar but not identical to the control of $NAD^+$ biosynthesis in other microorganisms. Figure 3 indicates the known points of control of $NAD^+$ metabolism in E. coli.

## The Quinolinate Synthetase System of E. Coli

After Ogasawara, et al. (62) demonstrated incorporation of $^{14}C$-aspartate into QA in E. coli extracts, work began to further elucidate the enzymes, precursors, and intermediates of the de novo pathway. Mutants known to be blocked in the conversion of QA to NAMN were mapped by Tritz, et al. (173). These QRTase mutants (nad C gene) were very useful in studying the de novo pathway. When such mutants were grown with NA under conditions of derepression, they produced a several-fold excess of QA and excreted it into the medium (174). Further genetic characterization of the pathway using in vitro complementation established that the nad A and nad B genes code for enzymes synthesizing QA (175, 176) and that nad R is likely a regulatory gene (158).

Chandler and Gholson (177) developed an assay for isolating and measuring $^{14}C$-quinolinic acid synthesized in crude extracts of nad C

mutants and carried out numerous studies on the quinolinate synthetase system (62, 63, 64, 178). Then Suzuki, et al. (179) achieved partial purification of two soluble protein fractions, one prepared from an E. coli K-12 nad A mutant and one from a nad B mutant. Using these fractions, the triose-phosphate suggested by Chandler, et al. (63) as the 3-carbon precursor of QA was confirmed and specifically identified as dihydroxyacetone-phosphate. Furthermore, FAD was discovered as a necessary cofactor and the pH optimum for QA synthesis in vitro was shown to be 8.0 to 8.5.

A new report from Kerr and Tritz (180) shows evidence for cross-feeding of a nad A mutant by a nad B mutant grown simultaneously in liquid cultures. This indicates that the protein present in the nad B mutant extracts is the first in the sequence and produces and excretes the intermediate "prequinolinic acid". Other combinations of nad A, nad B, and nad C, however, show no cross-feeding.

On the other hand, extensive work by Chandler (178) in both in vivo and in vitro systems failed to show any evidence of an intermediate that accumulates even to the level of 3% of the QA produced. The intermediate may not be permeable to the cell membrane, or it may be enzyme-bound, or it may be unstable, or it may not exist at all. Whatever the case, much more work remains to be done before the existence or non-existence of an intermediate in QA biosynthesis is completely understood.

The protein fraction from the nad B mutant, the A protein, is heat-stable and was purified about 40-fold. The protein from the nad A mutant, the B protein, was purified about 15-fold. Molecular weights and other properties of the two proteins are different and both are

required for the synthesis of $^{14}$C-QA from $^{14}$C-aspartate (179). Figure 4 depicts the QA synthetase system as it is now known.

For any further work on the intermediate, kinetics, regulation, or enzyme mechanisms of the de novo biosynthesis of QA, a highly or completely purified preparation of at least one of the proteins is required. Therefore, this work began at this point, with the objective of purifying the B protein produced by the nad A mutant PA-2-18 and of further characterizing the quinolinate synthetase system using more highly purified enzyme preparations from E. coli K-12.

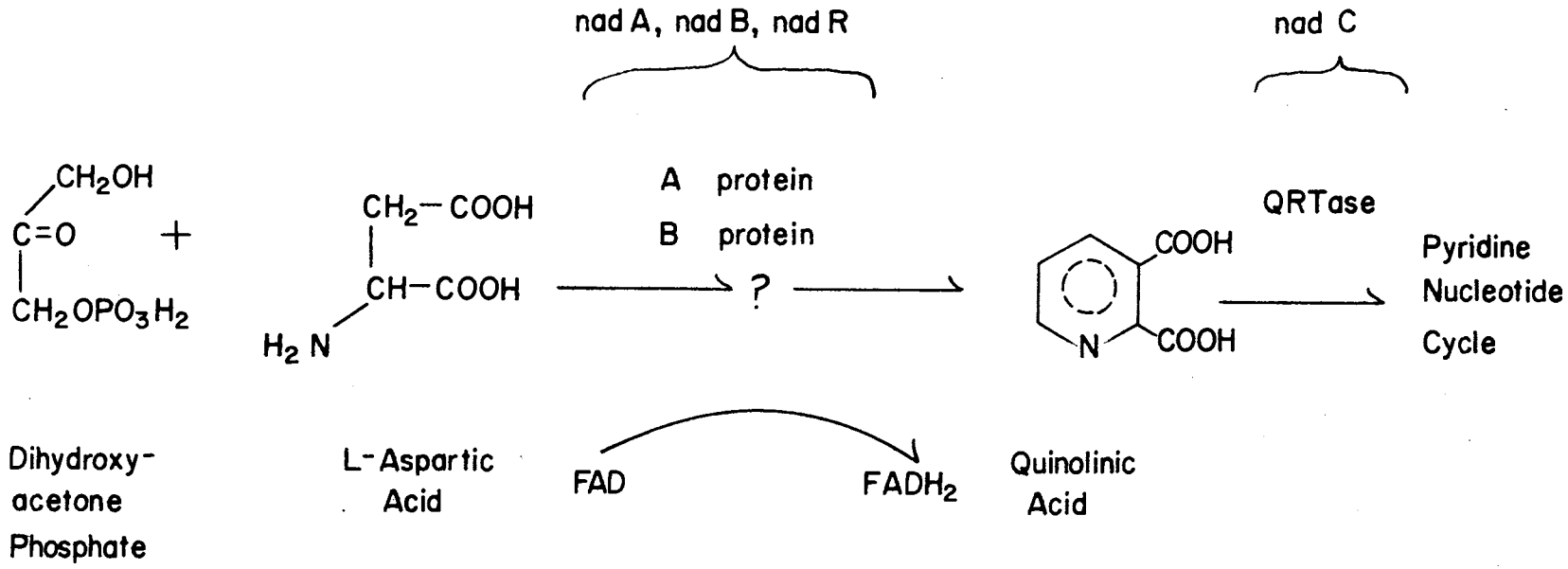



Figure 4. The Quinolinate Synthetase System in E. coli

# CHAPTER III

# METHODS AND MATERIALS

## Cultures

Mutant strains of E. coli K-12 requiring niacin and other growth factors were routinely marker checked to insure appropriate strains (see Table IV) were being used. Stock cultures were maintained in nutrient agar stab cultures and new nutrient agar plate cultures used for inoculation were prepared monthly. Genetic characteristics of mutants used in these studies are listed in Table IV (108). Cells were grown in the minimal medium of Yates and Pardee (182). Additions to the medium were $5 \times 10^{-7}$ M NA, $2 \times 10^{-6}$ M thiamine, and $1 \times 10^{-3}$ M concentrations of the appropriate amino acids for each strain. All cultures were grown at 37°C with vigorous shaking or aeration for 12 to 14 hours. When only crude extracts were checked for enzyme activities, 100-ml cultures were grown using a 5-ml inoculating culture. These were harvested by centrifugation at 8000 x g for 15 minutes and yielded five to seven grams wet weight of cells for each 100-ml culture.

For enzyme purification, 10-liter cultures were grown using a New Brunswick Fermentor and successive inoculation cultures of 5 and 100 ml. Cells were harvested with a Sharples centrifuge. The wet cell paste was washed by resuspension in 0.9% NaCl (4 ml per gram wet cells) and centrifuged at 10,000 x g for 15 minutes. After weighing, cells were stored at -15°C. PA-2-18 and SB-16 10-liter cultures yielded 50 to 60

TABLE IV

GENETIC CHARACTERISTICS OF MUTANTS OF *E. COLI* K-12[a]

| Strain | Sex | λ Phage Sensitivity | Mutations Present | Stock No. (Univ. Texas Houston) |
|---|---|---|---|---|
| PA-2-18 | $F^-$ | - | nad $A_{19}$ arg A, thi, str A, xyl, mtl, mal, lac, gal | UTH 4460 |
| AT-11-23 | $F^-$ | + | nad $A_{28}$, thr, leu | UTH 4455 |
| SB-16 | Hfr | - | nad $B_{15}$, met | UTH 4674 |
| W-3899 | $F^-$ | - | nad $B_{30}$ | UTH 4464 |
| E-126 | $F^+$ | - | nad $C_{13}$ | UTH 4673 |
| SB-99 | Hfr | - | nad $C_{27}$, met | UTH 4143 |
| W-4516 | $F^-$ | - | nad $C_{32}$ | UTH 4466 |
| AT-13-3 | $F^+$ | - | nad $C_{22}$, thr, leu | UTH 4678 |

[a](108)

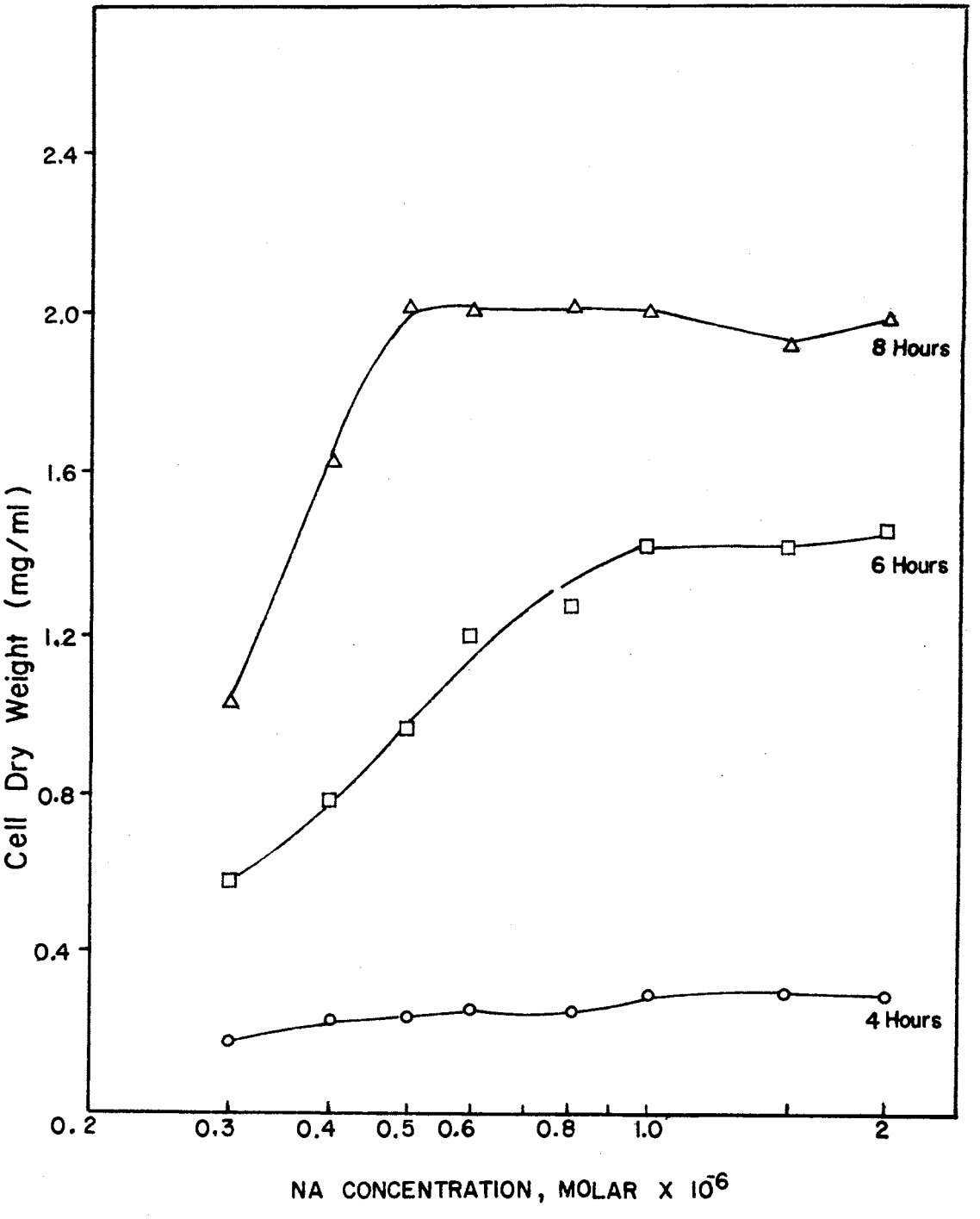
grams and 30 to 40 grams of wet cells, respectively. Activity of QA-synthesizing enzymes in frozen cells remained stable up to four months or more after harvesting.

## Nicotinic Acid Concentration

Since all strains used require nicotinic acid, the optimum NA concentration for maximum cell growth was determined. Twenty-five-ml cultures of PA-2-18 were grown at 37°C in sidearm flasks with NA concentrations from $3 \times 10^{-7}$ M to $2 \times 10^{-6}$ M in the media. Cell density was checked at four hours, six hours, and eight hours using absorbance at 420 nm, as measured by a Coleman Jr. spectrophotometer. A standard curve of $A_{420}$ versus cell dry weight in mg per ml was prepared. Then the cell dry weight was plotted against the logarithm of the NA concentration used, as shown in Figure 5. Such experiments indicated that the lowest NA concentration that allows maximum cell growth is $5 \times 10^{-7}$ M for 25-ml cultures of the PA-2-18 strain. This concentration of NA was used in the 10-liter cultures of PA-2-18 and SB-16 strains.

## Preparation of Crude Extracts

The crude extract of *E. coli* K-12 PA-2-18 for enzyme purification was prepared by suspension of 60 grams of frozen cells in five volumes per gram of 0.05 M Bicine buffer, pH 8.0. When a homogenous suspension was obtained, a volume of 350 ml was sonicated for 18 minutes in a large rosette cell with a Branson sonifier set at 10 D.C. amperes power. The rosette cell was set in an ice bath to maintain the temperature of the solution at 0 to 4°C during sonication. The sonicate was centrifuged at 40,000 x g for 45 minutes to remove cell debris, yielding about 325 ml




Twenty-five-ml cultures of PA-2-18 were grown at 37°C at NA concentrations from 3 x $10^{-7}$M to 2 x $10^{-6}$M. Cell density was determined by absorbance at 420 nm at 4, 6, and 8 hours and converted to cell dry weight.

Figure 5. Dependence of the Growth of PA-2-18 Cultures on the Concentration of Nicotinic Acid in the Media

of crude extract (supernatant). When prepared for purification, crude extracts of other strains were prepared in an identical manner, except that SB-16 cells were suspended in 0.05 M $KPO_4$ buffer, pH 8.0, instead of the bicine buffer.

Routinely, crude extracts of the SB-16 strain were used in smaller volumes only as a source of A protein for QA synthesis. Cells were suspended in 0.05 M $KPO_4$, pH 8.0 (5 volumes per gram), and sonicated 15 minutes at 0 to 4°C using a Raytheon sonic oscillator unit. The sonicate was centrifuged for 15 minutes at 20,000 x g. Ten grams of cells yielded about 52 ml of crude extract.

Experiments were conducted with PA-2-18 and E-126 strains to determine the optimum length of time and the optimum power (D.C. amperes) and 10 to 18 minutes sonication time, depending upon the volume sonicated would insure maximum release of active protein with very little denaturation. These parameters were employed in all subsequent preparations of crude extracts.

## Preparation of the A Protein for Assay

The source of A protein for the QA synthesis assay was the SB-16 strain. It was partially purified by heat treatment and salt fractionation before use in the assay. The crude extract, prepared as described above, was mixed for five minutes in a 55° water bath and subsequently cooled 10 minutes in a water-ice bath. After centrifugation for 15 minutes at 20,000 x g, about 50 ml of heat-treated supernatant were obtained. Solid ammonium sulfate was added gradually over 15 minutes to this supernatant and stirred at 0 to 4°C to reach 40% saturation of ammonium sulfate (148). After mixing for 30 minutes, the solution was

centrifuged at 20,000 x g for 15 minutes. The supernatant was treated with ammonium sulfate in the same manner to bring it to 60% saturation. After 30 minutes mixing and 15 minutes centrifugation at 20,000 x g, the supernatant was discarded and the pellet was redissolved in 0.005 M $KPO_4$ buffer, pH 7.5, with 0.05 M KCl (0.2 ml per mg pellet). The solubilized pellet containing the A protein was placed in dialysis tubing (boiled three times in 1 mM EDTA) and dialyzed 16 hours at 4°C against 1 liter of 0.005 M $KPO_4$, pH 7.5, with 0.05 M KCl. Thus, the SB-16 40 to 60% ammonium sulfate fraction prepared one day can be ready for the QA assay the next day. Heat treatment and subsequent ammonium sulfate fractionation results in a seven-fold final increase in specific activity over the crude extract.

## Enzyme Assay for Quinolinic Acid Synthesis

### Assay Procedure

Measurements of the enzymatic activities of the A and B proteins were made using the procedures described by Suzuki, et al. (65) and Chandler and Gholson (177), with some modifications.

Reaction mixtures were prepared as follows: each assay sample contained 0.25 µmole of L-aspartate, 1 µmole of FDP or DHAP, 0.01 µmole FAD, and 30 µmoles of bicine buffer, pH 8.0 (adjusted with KOH) in a volume of 0.1 ml. Also, 0.1 ml of a 5 µCi-per-ml U-$^{14}$C-aspartic acid solution (specific activity 160 mCi/mMole was added to each tube. Enzyme preparations totaling 0.3 ml were added, making final volume in the assay tubes of 0.5 ml, unless reagent or inhibitor effects were studied, in which case final volumes were 0.6 ml.

Assay of the B protein was carried out using an excess of the A protein, such that the amount of B protein activity present limited the amount of QA synthesized. Routinely, 0.2 ml of the SB-16 40 to 60% ammonium sulfate fraction and 0.1 ml of diluted enzyme fraction from PA-2-18 were added to each assay tube. These volumes represent 0.3 to 0.5 mg and 0.01 to 3 mg protein, respectively. When activity of the A protein was measured, B protein was added in excess as 0.1 ml of the 0 to 50% sodium citrate (see Results Chapter) fraction and the A protein was added as a limiting amount in a final volume of 0.2 ml. In this case, these volumes represent 1 to 2 mg and 0.02 to 4 mg protein, respectively. The use of FDP as a substrate necessitates, of course, the presence of FDP-aldolase in the assay. This enzyme was present in the 40 to 60% ammonium sulfate fraction used in the B protein assay (192).

The assay reaction was begun by adding the protein fraction which was to be in excess to the other assay components in order to make a complete mixture for <u>in vitro</u> QA synthesis. After gentle mixing, tubes were incubated at room temperature (23°C) without shaking. After 20 minutes, 0.5 ml of 15% perchloric acid was added to stop the reaction. Then 0.5 ml of 2 mM unlabeled QA was added, and the tubes were mixed and centrifuged to compact the precipitated protein. After decantation, the supernatants were neutralized by addition of 0.5 ml of 2.5 N KOH, with subsequent adjustment to pH 7.0 using indicator paper. Tubes were again centrifuged to compact the precipitated $KClO_4$.

These deproteinized and neutralized reaction mixtures were applied to 0.7 x 12-cm Dowex 1-X8 $Cl^-$ columns. Tubes were rinsed with 2 ml of deionized water, which was also applied to the columns. After a wash of

15 ml of 0.2 M LiCl, a 6-ml fraction of 0.6 M LiCl was collected from each column. These fractions contained the QA and were applied to 0.9 x 18-cm Dowex 50-X8 $H^+$ columns. After washing with 20 ml of 0.01 N HCl, a 10-ml fraction of 0.01 N HCl was collected from each column. These fractions contained the synthesized $^{14}$C-QA and the unlabeled carrier QA, and QA recovery was monitored by checking absorbance at 268 nmeters. Collecting one 10-ml fraction allowed greater recovery of the total $^{14}$C-QA synthesized than did the previous method of collecting four or five 2-ml fractions and pooling two of them after checking absorbance. Dowex 1 and Dowex 50 columns were regenerated by washing with ten and five column volumes of 3 N HCl, respectively, and then with equal volumes of deionized water. Figure 6 summarizes the assay procedure.

Quantification of the Assay

Determination of $^{14}$C-QA synthesized was carried out by counting two ml of the 10-ml fraction from Dowex 50 with 10 ml of Bray's scintillation cocktail using a Liquid Scintillation Counter. The samples were counted for 10 minutes each and the counts per 10 minutes values were converted to net disintegrations per minute by channels ratio quench correction and background subtraction. Then the net dpm values for each 10-ml Dowex 50 fraction were calculated and converted to the number of nanomoles of QA synthesized that they represent. The overall specific activity of the U-$^{14}$C-L-aspartic acid used for incorporation, including the unlabeled aspartate in the assay, was 2 µCi per µmole. Thus, one µmole of L-aspartic acid incorporated into one µmole of quinolinic acid is represented by 2 µCi of $^{14}$C-QA, which is $4.4 \times 10^6$

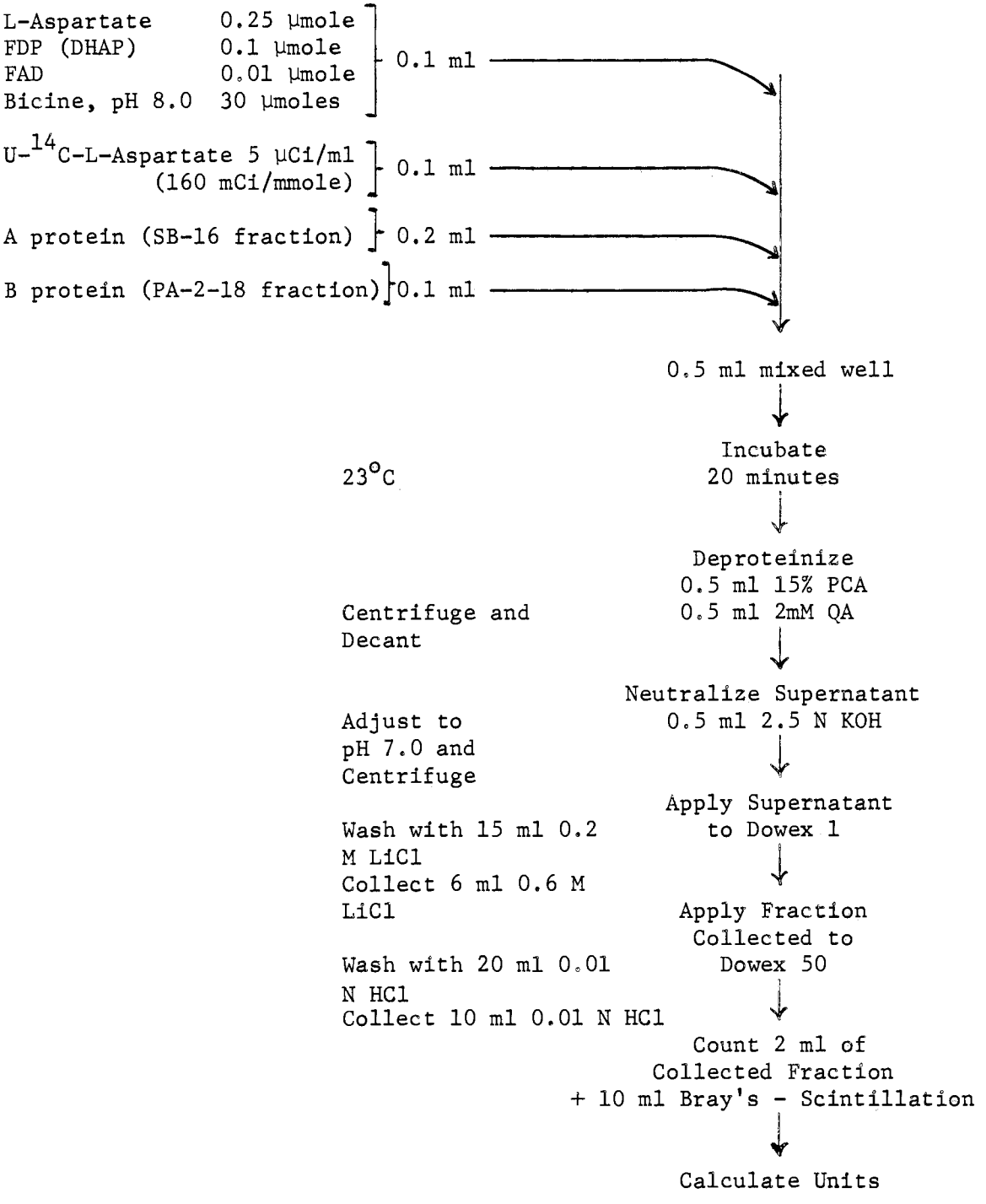



Figure 6. Assay Procedure for Quinolinic Acid Biosynthesis

dpm. Thus,

$$\text{nmoles QA synthesized (per assay tube)} = \frac{\text{total net dpm (per 10-ml fraction)}}{\text{4400 dpm/nmole QA}}$$

A unit of enzyme activity, then, is defined as the amount of enzyme (either the A or B protein) that can form one nmole of quinolinic acid from aspartic acid and DHAP at 23°C in 20 minutes, in the assay system used. Time-course studies (Suzuki, unpublished results) showed that the rate of QA synthesis remains constant during this period. Activity in assays is usually expressed as units per ml of undiluted enzyme fraction, thereby including the dilution factor required to make the protein assayed (usually B) rate-limiting in the synthesis of QA. Specific activity is expressed as units per mg of protein. Protein was determined spectrophotometrically by 280/260 nmeter absorbance ratios and by the method of Lowry, et al. (179).

The A protein is unstable and decreases to 50% or less of its original activity in one week when stored as the 40 to 60% ammonium sulfate fraction at -15°C (Suzuki, unpublished observations; see Results Chapter). Therefore, the 40 to 60% ammonium sulfate fraction was prepared the day before each time the B protein was assayed. Stabilization of the A protein at -15°C with 33% glycerol was achieved, thus allowing B protein assays to be carried out with the same 40 to 60% ammonium sulfate preparation over about one week. Care had to be taken, however, to insure A protein activity was in excess, since assay samples could not be quantitatively compared if total units depended upon the amount of A protein present and not the amount of B protein present. Hence, fresh preparations (one to four days) of the A protein were routinely used. Linear dependence of total QA synthesis on the amount of B protein was observed, as discussed in Chapter IV. The B protein

activity presented little problem concerning stability when stored at high protein concentrations (i.e., 10 to 20 mg per ml) at -15°C. Under such conditions, the 0 to 50% sodium citrate fraction retained from 50 to 75% of its original activity after six months. Thus, assay samples for A protein activity could be compared quantitatively.

## Preparation of Affinity Columns

The technique of affinity chromatography was examined for its value in purification of the proteins of the quinolinate synthetase system. Specifically selected ligands were covalently bound to agarose, a polygalactan polymer, through coupling reactions with sidearms attached to the polymer. These coupling reactions involve formation of a peptide bond with the elimination of water, using a carbodiimide coupling agent. The two coupling agents used in these preparations were 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) and dicyclohexyl-carbodiimide (DCC). Agarose derivatives with various sidearm functional groups are available commercially from Bio-Rad Laboratories and are termed Affinose gels. Affinose 102 and 202, containing a terminal amino group and a terminal carboxyl group, respectively, in the sidearms, were utilized for coupling reactions. Sidearm structures are shown in Figures 7 and 8.

### Preparation of Carboxyl-Linked Aspartate Affinity Gel

Since L-aspartate is a known substrate for QA synthesis, it was checked for its ability to bind the B protein to an affinity gel. The general technique employed was patterned after specific procedures

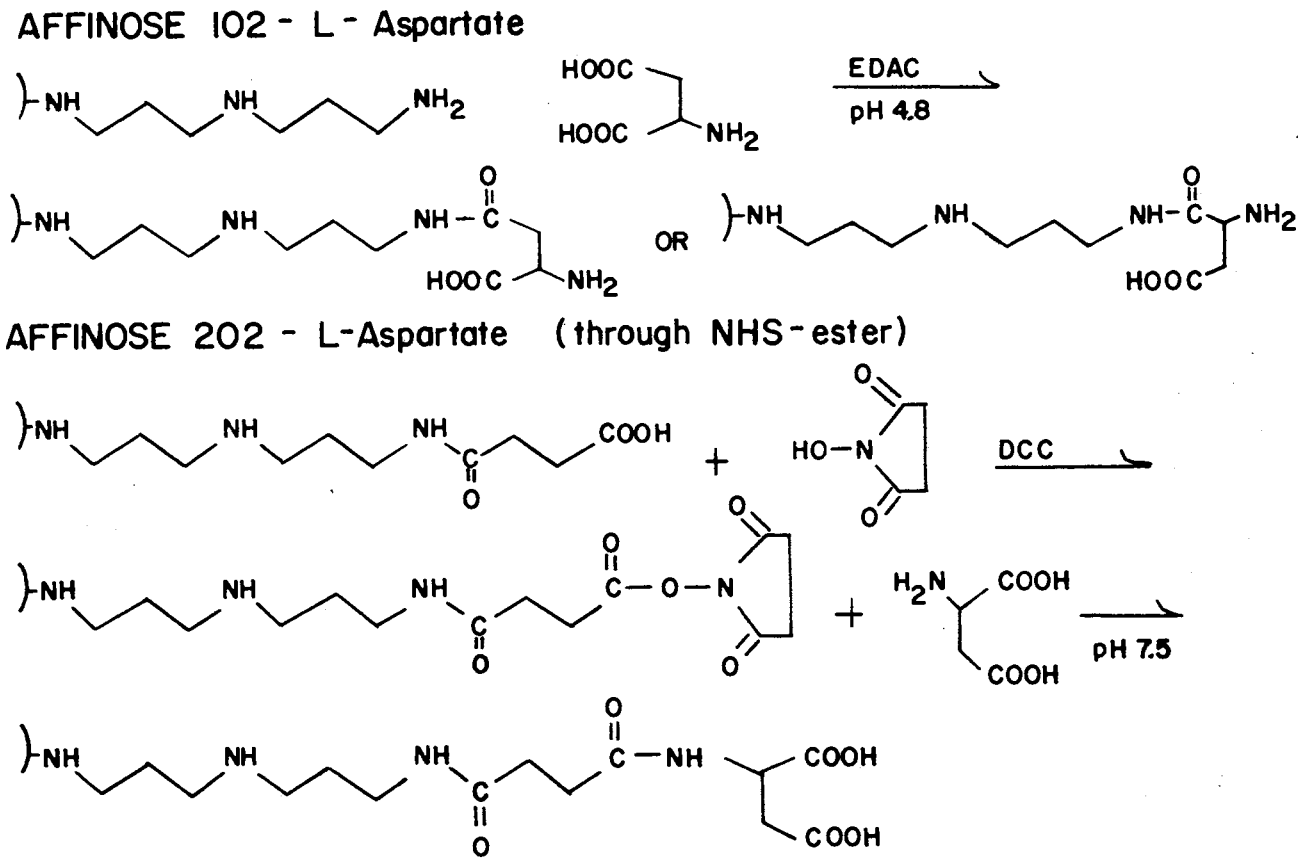



Figure 7. Coupling Reactions for the Preparation of Aspartate-Linked Affinity Gels

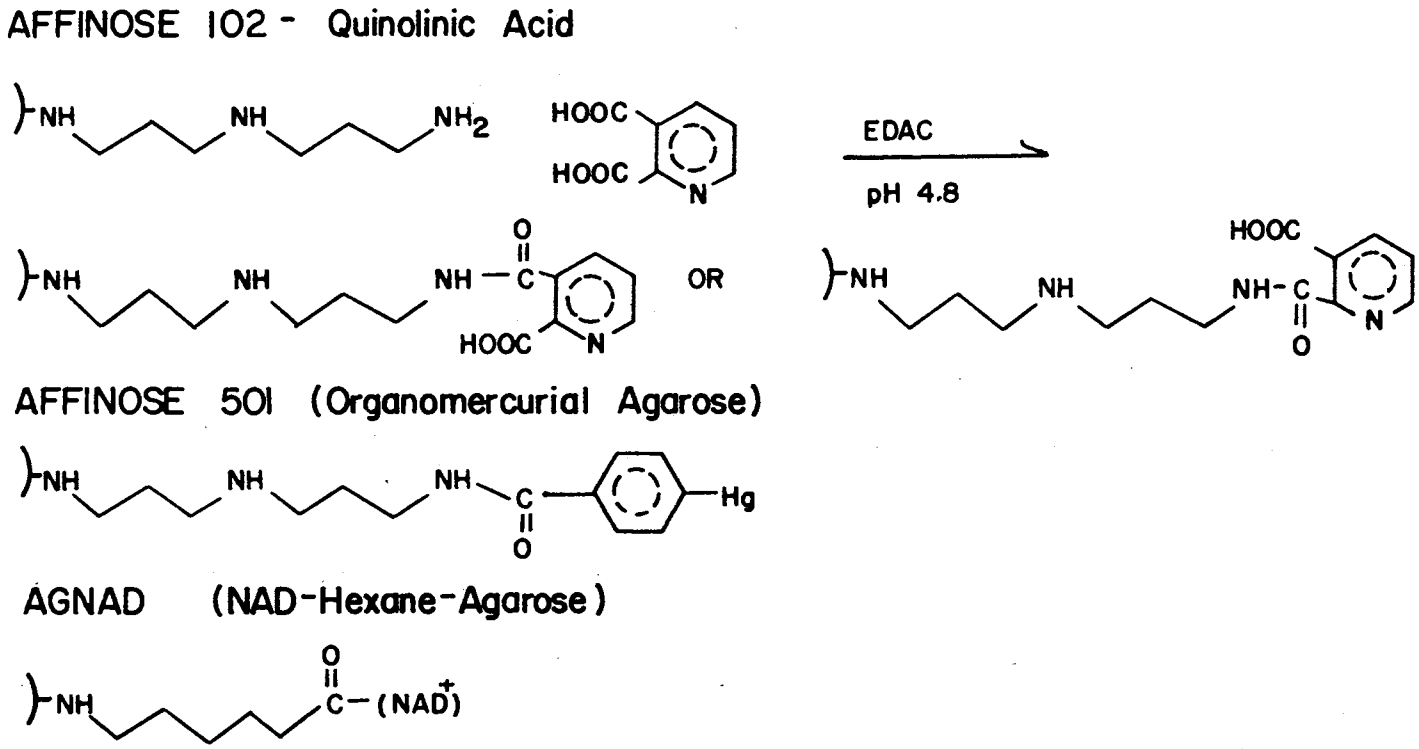



Figure 8. Coupling Reaction for the Preparation of QA-Linked Affinity Gel and Structures of pCMB-Agarose and NAD-Agarose

described by Cuatrecasas and Anfinsen (183). Affinose 102, an ω-aminoalkyl agarose, was washed with 20 volumes of 0.1 M NaCl and then deionized water to remove sodium azide (a bacterial growth inhibitor) from the gel. To 10 ml (packed volume) of the gel, 0.5 mmoles of unlabeled L-aspartate and 0.44 μCi of U-$^{14}$C-L-aspartate were added. The labeled aspartate served to monitor the extent of coupling obtained by counting the radioactivity of the gel. The resulting 20-ml solution was adjusted to pH 4.8 with 1 N HCl. Magnetic stirring, which can fracture the gel beads, was avoided. Over a period of five minutes, 1.3 mmoles (a 2.6-fold excess) of EDAC, a water-soluble carbodiimide, in a volume of 1.5 ml, were added with stirring. The pH was adjusted to 4.8 and checked periodically to maintain that value. The coupling reaction thus begun was allowed to proceed 20 hours at room temperature (23°C) with no stirring. Subsequently, the gel was packed in an appropriate column and washed thoroughly with more than 20 column volumes of the buffer to be used in subsequent experiments. This wash was monitored using a scintillation counter and when radioactivity had dropped to background levels, all of the excess, i.e., unreacted aspartate was considered to have been removed.

A 10 to 20-μl sample of the gel was taken and counted in Bray's solution with a scintillation counter. Based upon the gel capacity for coupling of 8 to 10 μmoles of amino groups per ml of packed gel and upon the known amount of $^{14}$C-aspartate added to the gel, the extent of the reaction was calculated. Data indicated that at least 2% of the total aspartate available was coupled to the gel. This represents ten μmoles of L-aspartate bound, which meant that 10 to 12% of the available amino

groups in 10 ml of the Affinose gel were covalently bound to a carboxyl group of L-aspartate.

As to which carboxyl group is linked, no absolute conclusion can be made. Both groups may be bound in some proportion. At pH 4.5, the Zwitterion of the amino group and the α-carboxyl does exist, since the α-carboxyl would not be protonated and the amino group would be protonated. This internal salt may sufficiently involve the α-carboxyl such that it is unavailable and thus most of the coupling reaction takes place at the β-carboxyl. Since it is not known which protein, A or B, actually binds aspartate or the functional groups involved in that binding, the significance for the success of this affinity column of which carboxyl is coupled to the gel cannot be evaluated. Figure 7 depicts the coupling reaction carried out.

Preparation of Amino-Linked Aspartate Affinity Gel

It was also decided to prepare an affinity column with L-aspartate as the ligand in which both carboxyl groups were free and the peptide bond was made through the amino group. For this purpose, Affinose 202, a succinylaminoalkyl agarose, which has a carboxyl terminal group, was employed. To insure coupling with the amino group, the procedures outlined by Cuatrecasas and Parikh (184) were followed. This method involves the initial formation of the N-hydroxysuccinimide (NHS) ester of the terminal carboxyl group, which in turn is quite reactive and specific for the amino group of amino acids.

Affinose 202 was washed as before (as with the amino-terminal agarose) and then washed extensively (300 ml) with p-dioxane, since the

NHS-ester formation must be carried out in a non-aqueous medium. The 10-ml packed volume of gel was added to 20 ml dioxane and 3 mmoles of N-hydroxysuccinimide were stirred in as a solid. Again, magnetic stirring was avoided. Then 3 mmoles of dicyclohexylcarbodiimide were stirred in as the coupling agent. The mixture was stirred gently for 70 minutes at room temperature (23°C) using an Omni-mixer with a micro attachment, set at low rpm. The agarose was afterwards washed over a Buchner funnel with six to eight reaction mixture volumes of dioxane (6-8 x 30 ml) for 10 minutes, three to four volumes of methanol over five minutes while stirring the gel, and three more volumes of dioxane. The activated gel was air-dried for one to two minutes with suction.

This NHS-ester of the carboxyl-terminal agarose was then immediately used in the coupling reaction with L-aspartate. No carbodiimide coupling agent was required, since the NHS-ester is highly reactive toward amino groups. Using the ratio of 5 ml of solution per gram (ml) of gel, the 10-ml dried gel cake was mixed into 50 ml of 0.05 M $KPO_4$, pH 7.5, containing 5 mmoles of L-aspartate. The solution was adjusted to pH 7.5 after addition of the aspartate and before addition of the gel. Also, 0.5 μCi of U-$^{14}$C-L-aspartate (160 mCi/mmole) was added to monitor the degree of coupling. This mixture was kept at 0 to 4°C before gel addition. Then, for five hours, with stirring as described above, the coupling reaction was allowed to proceed at 0 to 4°C. Reaction termination and complete reaction of all ester groups was carried out by addition of 50 mmoles of glycine and continued stirring at room temperature for three hours. The gel was washed extensively with 0.05 M $KPO_4$, pH 7.5, to remove excess reactants and reaction by-products.

It was not realized at the time of the addition of the U-$^{14}$C-L-aspartate that much more labeled aspartate would be needed for monitoring the reaction than that which was added. This is because of the much larger amount of unlabeled aspartate used in this reaction in comparison to the Affinose 102 coupling reaction. Thus, the $^{14}$C-aspartate was diluted such that, theoretically, if the gel were completely coupled with aspartate at all available sites, only two to three times background counts would be observed in counting 20 µl of the gel. Actual counts of this volume of gel after reaction were no higher than background levels. No validation, then, of the existence of amino-coupled L-aspartate in the gel or the extent of coupling was made, since this method of monitoring the reaction failed and other methods were not attempted. The high reactivity of the NHS-ester and the demonstrated reliability of the coupling method (184) suggest that at least partial linking of the carboxyl groups of the gel did occur. Figure 7 shows the ester formation and coupling reactions.

Preparation of Carboxyl-Linked QA Affinity Gel

The product of the enzymatic action of the A and B proteins, quinolinic acid, was found to inhibit the reaction when added in excess. Thus, carboxyl-linked QA affinity gel was prepared from QA and Affinose 102. Five ml (packed volume) of the ω-aminoalkyl agarose was washed as before and added to 0.5 mmoles (a 10-fold excess) of quinolinic acid and 3.5 ml of deionized water. The pH of the mixture was adjusted to 4.8 with KOH with hand mixing. Then 1.3 mmoles of EDAC in 1.5 ml water was mixed in over a period of six minutes and the pH was brought back to 4.8. The final reaction volume of 10.3 ml was allowed to stand 20 hours

at 23°C with no mixing. Afterwards, the gel was washed extensively with the equilibrating buffer to be used in the experiments.

The spectrophotometric method of Failla and Santi (185) was employed to ascertain the extent of binding of QA to the gel that was achieved. Samples of unreacted and reacted gel were prepared as 4% suspensions (0.2 ml packed gel for 5 ml) in 50% acetic acid. Two suspensions of uncoupled gel and one of coupled gel were heated at 80°C in a water bath for four hours. The resulting clear solutions of solubilized agarose were cooled and their pH was adjusted to neutrality without visible precipitation. Then 0.2 μmoles of QA (as a 2 mM solution) was added to one of the samples with uncoupled gel. Spectra of each solution were made by scanning the ultraviolet region from 320 to 200 nmeters using a Varian Model 635 spectrophotometer. A standard 40 μM QA solution showed the known absorbance maximum at 268 nm for QA. Comparing scans of the coupled gel, uncoupled gel, and uncoupled gel containing 40 μM QA, it was calculated that the difference of 0.15 absorbance units at 268 nm in the coupled and uncoupled gel without QA was representative of a QA concentration of approximately 40 μM QA in the coupled gel. The spectrum of the uncoupled gel with added QA was very similar to that of the coupled gel, both in overall shape and in amplitude at 268 nm. Thus, if 5 ml of a solution containing 0.2 ml of gel had 40 μM or 0.04 μmoles per ml QA, then 0.2 ml of coupled gel contained 0.2 μmoles of QA. The entire 5 ml of gel would contain 5 μmoles of bound QA, then, which represents 10 to 12% coupling, based upon the 10 to 12 μmoles-per-ml capacity of the agarose gel. This amount of coupling is quite reasonable and the method seems to be very sensitive and relatively fast. It was fortuitous that the amount of added QA in the uncoupled

gel turned out to be very near the amount of QA actually bound to the gel. Figure 8 shows the coupling reaction.

## Other Methods

Scintillation counting was done by adding 2 ml of the 10-ml Dowex 50 fraction (0.01 NHCl) from assays to 10 ml of Bray's solution in glass scintillation vials. Bray's solution contained 100 ml of methanol, 20 ml of ethylene glycol, 60 grams of naphthalene, 4 grams of 2,5-diphenyloxazole (PPO), 2 grams of 1,4-bis[2-(5-phenyloxazolyl)]-benzene (POPOP), and p-dioxane to make one liter. Counting was done on a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3320. Quench correction by channels ratio and determination of counting efficiency were carried out using Nuclear Chicago quenched standards, a prepared quenched standard set with Bray's solution, and known methods for calculations (186). Counting efficiencies normally were 71 to 75%.

A Beckman DB Spectrophotomer was used to monitor protein from column effluents at 280 nm and to check QA recovery from Dowex columns at 268 nm. A Varian Techtron Model 635 Spectrophotometer and an A-25 recorder were employed for making scans in the UV region.

Protein determinations were made with the Biuret, Lowry, or 280/260 ratio methods (179) depending upon the type of experiment conducted. Routine purification utilized the 280/260 ratio method and the Biuret and Lowry methods were used in sonication and crude extract assay experiments. In each case, standard curves were prepared from 10 mg-per-ml solutions of bovine serum albumin. Absorbance was checked with the Beckman Spectrophotometer in all methods. Determination of cell density

in growing cultures was made at 420 nm with a Coleman Jr. Spectrophotometer, Model 6D.

Concentration methods utilized a Bio-Rad Beaker Ultrafilter b-Hfu-1 hollow fiber device and Amicon Ultrafiltration cells Models 50 and 202. The latter type of equipment was used primarily and both UM-10 and UM-20E membranes were useful.

Thin layer chromatography for checking the radiochemical purity of the U-$^{14}$-C-L-aspartate was accomplished with Eastman #6064 Cellulose 160-micron chromatogram sheets. After spotting 0.002, 0.02, and 0.2 μCi of the $^{14}$C-aspartate, sheets were developed six hours in n-butanol-acetic acid-water, 25:4:10, and dried. Sheets were then placed under GAF X-ray film for 12 hours and purity was checked after development of the film.

## Chemicals

The U-$^{14}$C-L-aspartic acid (160 mCi/mmole) utilized in these studies was obtained from New England Nuclear and ICN Isotope and Nuclear Division. Quinolinic acid, FAD, DHAP, iodoacetamide, aldolase, protamine sulfate, pyridoxamine-5'-phosphate·HCl, pyridoxal-5'-phosphate, the pyridine nucleotides, and L-aspartate and its derivatives were all purchased from the Sigma Chemical Company. The Calbiochem Company was the source of Bicine, FDP, pCMB, and Aquacide. PPO and POPOP were obtained from the Packard Instrument Company, Inc. Polyethylene glycol was bought from Matheson, Coleman, and Bell and N-ethyl-maleimide was obtained from Eastman Organic Chemicals. DCC was purchased from K and K Laboratories, Inc., and EDAC from the Ott Chemical Company. All other chemicals were obtained from major supply houses.

## Chromatography Materials

Dowex 1-X8 chloride form, 200-400 mesh, and Dowex AG 50-X8 hydrogen form, 200-400 mesh, were purchased from Bio-Rad Laboratories. Also, Bio-Rad was the source of Bio-Gel P-150 and P-300, Agarose 5m and 15m, DEAE-cellulose, and Affinose 102, 202, and 501. Sephadex G-100 and G-200, DEAE-Sephadex A-50, carboxymethyl-cellulose, carboxymethyl-Sephadex, and chromatographic columns were purchased from Pharmacia Fine Chemicals. Hydroxyapatite was bought from the Clarkson Chemical Company and Celite 545 was bought from Fisher Scientific Company. NAD-Agarose, or AGNAD, was obtained from P-L Biochemicals, Inc.

# CHAPTER IV

# RESULTS AND DISCUSSION

## Purification of the B Protein

Many techniques and methods of protein fractionation were utilized in this study to effect purification of the B protein from the PA-2-18 strain. Methods which resulted in the highest recovery of total activity with a corresponding decrease in total protein (i.e., removal of unwanted protein) were incorporated into the purification scheme. Simple trial and error and modification of techniques resulted in the final purification scheme that is shown in Table V.

### Protamine Sulfate Fractionation

Removal of nucleic acids was accomplished by addition of protamine sulfate to the crude extract. A 2% (20 mg/ml) suspension was prepared by adding 550 mg protamine sulfate to 27.4 ml 0.05 M $KPO_4$, pH 7.5, using the ratio of 2 mg protamine sulfate to every 10 mg crude extract protein (187). The suspension was added dropwise with mixing to 415 ml crude extract kept at 0 to 4°C. After the addition was complete and the mixture had been stirred another 30 minutes, it was centrifuged 15 minutes at 8,000 x g. The pellet was discarded and 443 ml of supernatant were ready for further purification. In all purification steps, 0.5 or 1-ml samples were removed and stored at -15°C for assay and protein determination.

TABLE V

PURIFICATION OF THE B PROTEIN OF THE QUINOLINATE SYNTHETASE SYSTEM IN *E. COLI*

| Step | Total Volume (ml) | Total Protein (mg) | Total Activity (units) | Specific Activity (units/mg)$\times 10^{-3}$ | Enrichment (-fold) | Recovery (%) |
|---|---|---|---|---|---|---|
| Crude Extract | 416 | 111659 | 12242 | 1.05 | 1 | 100% |
| Protamine Sulfate Supernatant | 443 | 5039 | 15514 | 3.08 | 2.9 | 127 |
| 0 to 50% Sodium Citrate Pellet | 23 | 837.2 | 10803 | 12.9 | 12.3 | 88 |
| Sephadex G-200 (Pooled Fractions) | 650 | 335.0 | 15470 | 47.6 | 45.3 | 126 |
| DEAE-Sephadex (Pooled Fractions) | 502 | 55.2 | 10542 | 191 | 182 | 86 |
| Hydroxyapatite (Pooled Fractions) | 156 | 8.3 | 2900 | 361 | 344 | 24 |

Frequently, the total activity recovered in the protamine sulfate supernatant was greater than the assayed activity in the crude extract. This enhancement may be due to the removal of inhibiting substances by precipitation with the protamine sulfate or it may occur as a result of the action of proteinases in the crude extract that destroy B protein activity during storage at -15°C before assay.

Routinely, this step resulted in recovery of total activity of over 90% and a one to three-fold increase in specific activity over that of the crude extract. Its use, therefore, was quite satisfactory as a purification step.

Sodium Citrate Fractionation

Fractionation based upon solubility was achieved with sodium citrate addition to the protamine sulfate supernatant. A saturated solution of sodium citrate was prepared at room temperature (23°C) and adjusted to pH 7.0 with saturated citric acid. Then an equal volume (443 ml) of this solution was added dropwise to the 443 ml of protamine sulfate supernatant which was stirring at 0 to 4°C. After completion of this addition and further mixing for 20 minutes, the suspension was centrifuged 15 minutes at 8,000 x g. The supernatant was discarded and the protein pellet was redissolved in 11 to 12 ml 0.005 M $KPO_4$, pH 7.5, with a final volume of 23 ml. Twenty-two ml of this fraction were utilized for further purification and 0.5 ml was frozen for assay.

Dialysis was usually necessary for the redissolved pellet in order to remove sodium citrate, but in this case the gel filtration used in the subsequent step effectively separated the protein from the salt.

When dialysis was done, the redissolved pellet was sealed in 25 mm dialysis tubing (boiled three times in 1 mM EDTA) and stirred overnight against 3 liters of 0.005 M $KPO_4$, pH 7.5.

Recovery from sodium citrate fractionation was regularly above 50% and protein was reduced to one-sixth of the original amount. Assay of the supernatant after dialysis showed less than 5% of the total activity from the supernatant was present in that fraction. Sodium citrate was found to inhibit the QA synthesis assay and reduce the total amount of activity in preparations. Dialysis, however, may contribute significantly to this loss of activity, since the B protein is somewhat unstable at 4°C. Length of time of exposure of the protein to sodium citrate was kept at a minimum in any case.

Gel Filtration

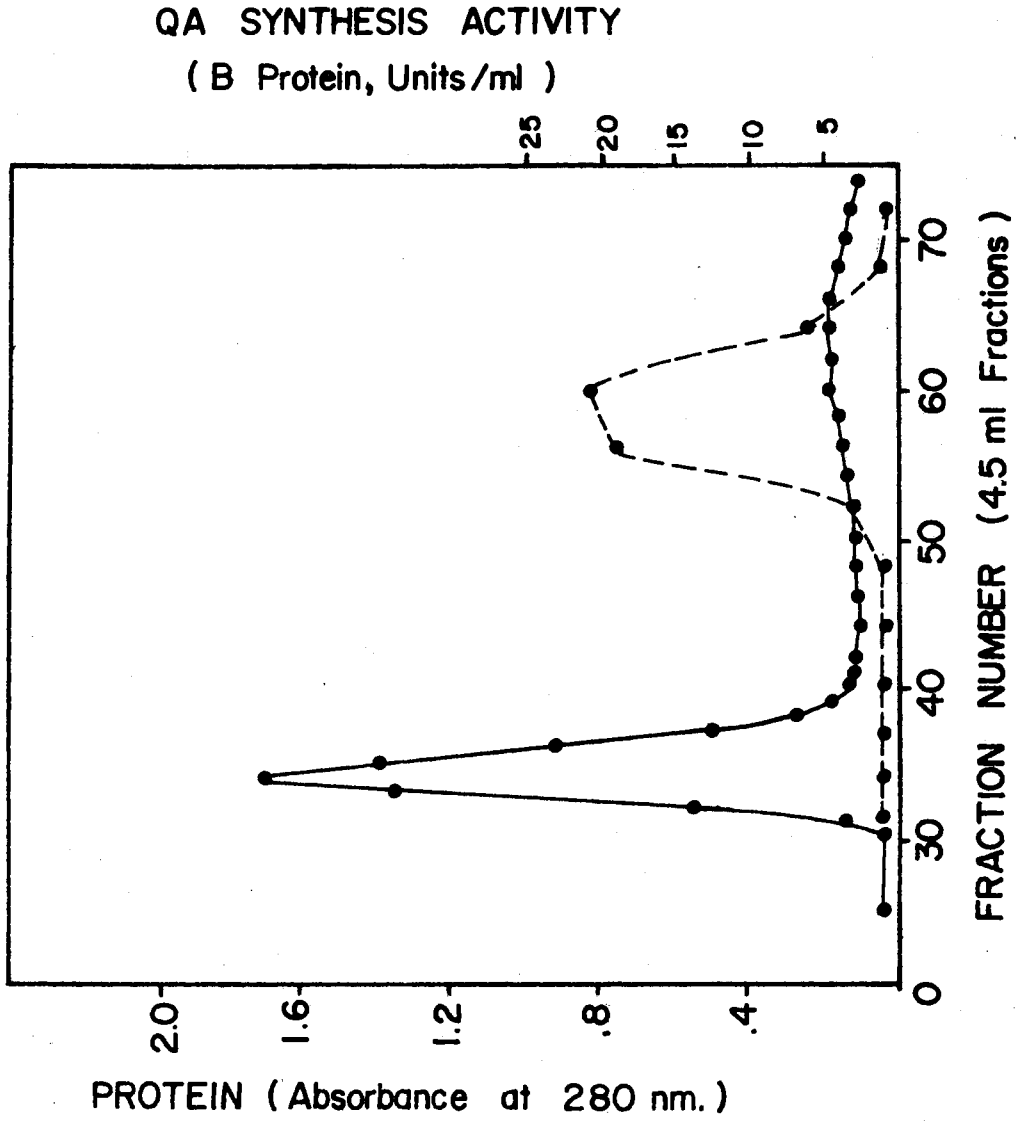
A rough separation according to molecular weight and geometry was carried out with Sephadex G-200. A 4.5 x 88-cm column which was packed at 4°C with 1400 ml of swelled, degassed G-200 was equilibrated with 2 liters of 0.005 M $KPO_4$, pH 7.5. Then 22 ml of the 0 to 50% saturation sodium citrate fraction was mixed with 1 ml of saturated sucrose solution and layered under the buffer on top of the gel bed. Elution was effected by washing the column with 0.005 M $KPO_4$ buffer, pH 7.5, using a 25-cm head and collecting 9-ml fractions at 4°C. Flow rate of the column was about 37 ml per hour and the column was eluted 40 hours. Previous determinations of the behavior of the B protein on G-200 allowed pooling of fractions containing the B protein without first assaying the effluent. Fractions 68 to 140 were thus pooled, making the pooled G-200 eluate 650 ml in volume. Void volume of the column was

approximately 410 ml. An elution profile using a smaller column is shown in Figure 9.

Use of gel filtration as a later purification step involving much less protein could have been done with much better fractionation of proteins. Also, a smaller column size such as 3 x 64 cm gives better resolution. The difficulty of using a smaller column with less protein is the tendency of the B protein to lose activity in dilute solution. Thus, more protein was required for the applied sample which necessitated a large column. As the purification table indicates, a three- to four-fold enrichment was obtained with good recovery.

Anion Exchange Chromatography

Efficient fractionation of protein was obtained using DEAE-Sephadex. A 4 x 13-cm column of DEAE-Sephadex was packed at 4°C and equilibrated with 700 ml of 0.005 M $KPO_4$, pH 7.5. Then 647 ml of the pooled G-200 effluent was passed through the column and the absorbance of the effluent was monitored at 280 nm. Monitoring indicated all of the protein was bound to the charged groups of the DEAE-Sephadex. The column was washed at 4°C with 0.20 M KCl in 0.005 M $KPO_4$, pH 7.5. After 600 ml, protein had dropped to below 0.05 absorbance units at 280 nm in the effluent. This wash was discarded and the column was eluted with 375 ml of 0.25 M KCl, 0.005 M $KPO_4$ buffer, pH 7.5, after which the protein absorbance was again reduced to 0.05 absorbance units. This effluent was a fairly dilute protein solution, so 25 and 50-ml portions of glycerol, a stabilizing agent, were added as the 375 ml effluent was collected. The solution was continuously stirred at 4°C during collection and the final volume was 504 ml, containing 25% glycerol.




A 3-ml sample containing 45 mg of protein the 0 to 50% saturation sodium citrate fraction was applied to the 3 x 64-cm column and eluted at 4°C with 0.005 M $KPO_4$, pH 7.5, collecting 4.5-ml fractions, using a 22-cm head.

Protein absorbance at 280 nm

B protein activity, units per ml

Figure 9. Elution Profile of the B Protein on Sephadex G-200

Anion exchange gave a clean separation of activity, with almost no activity being found in the 0.20 M KCl wash. Utilizing the short, wide column, fast stepwise elution can be carried out in 8 to 10 hours. Again, a three to four-fold enrichment with good recovery is obtained.

Hydroxyapatite Chromatography

Further purification beyond DEAE-Sephadex involved very low protein levels as compared to initial steps. Thus, 25% glycerol was used to stabilize the B protein by adding it to all buffers. At 4°C, however, flow rates of 25% glycerol solutions are quite low, so adsorption chromatography using hydroxyapatite was done at room temperature. A 1.9 x 30-cm column was packed with hydroxyapatite and equilibrated with 500 ml 0.005 M $KPO_4$, pH 7.5, and then 200 ml 0.01 M $KPO_4$, pH 7.5. Both equilibrating solutions contained 25% glycerol. Then 500 ml of the 0.25 M KCl DEAE-Sephadex effluent were passed through the column, and all the protein was bound to the hydroxyapatite. The B protein activity was subsequently eluted with 0.01 M $KPO_4$, pH 7.5, in 25% glycerol by collecting 9.8-ml fractions. The protein peak as monitored by absorbance at 280 nm was pooled and resulted in 78 ml of protein solution.

Although about two-thirds of the total activity of the previous fraction from DEAE-Sephadex was lost, sufficient protein was removed so that nearly a two-fold increase in specific activity resulted. The 0.25 M KCl in the protein solution added to the column might have influenced protein binding to the hydroxyapatite. Also, glycerol could have had an effect on adsorption, but under the conditions used, no adverse effects upon adsorption occurred. Sequential use of DEAE-Sephadex and

hydroxyapatite steps was convenient, as it eliminated the need for an intermediate concentration step.

## Evaluation of Purification in the QA Synthetase System

One aspect of purification of the proteins of the QA synthetase system is the low level of protein at which the activities exist. Since little QA is needed, enzymes are either not synthesized at high levels or they are strongly inhibited. Whatever the case, very small amounts of protein are available for such purifications. This often necessitates enrichment values of over 1000-fold before a pure protein can be obtained.

Another difficulty is the apparent instability of the protein. Many purification steps beyond the DEAE-Sephadex step failed because of denaturation. Glycerol was finally found to reduce the rate of this denaturation (as discussed later). Hydroxyapatite columns with this stabilizing agent were found to work well in spite of their operation at room temperature and dilution of protein solutions below 1 mg/ml. Addition of glycerol to buffers for running gel filtration columns does not allow good resolution or flow rate, but a system in which glycerol would be mixed with the effluent of the gel column as it is being eluted might be worked out.

Finally, the characteristics of the assay system for quinolinic acid biosynthesis made immediate evaluation of purification steps impossible. Minimum time required to complete an assay was 24 hours. If a column needed to be monitored for activity elution or a decision as to procedure needed to be made as an experiment was conducted, only

previous information about the behavior of the protein could be used. Thus, the overall procedure of selection of purification steps was greatly slowed as compared to systems in which a direct spectrophotometric assay such as NADH formation can be used. The necessity of the presence of two proteins in the assay complicated the evaluation of the effect of various reagents employed in purification procedures upon the B protein. For example, sodium citrate and potassium chloride are known to reduce QA synthesis. However, it is not known upon which protein, A or B, or both, that this effect occurs. It may be that use of these reagents involves a very satisfactory purification step, but appears to give low recovery according to the QA assay.

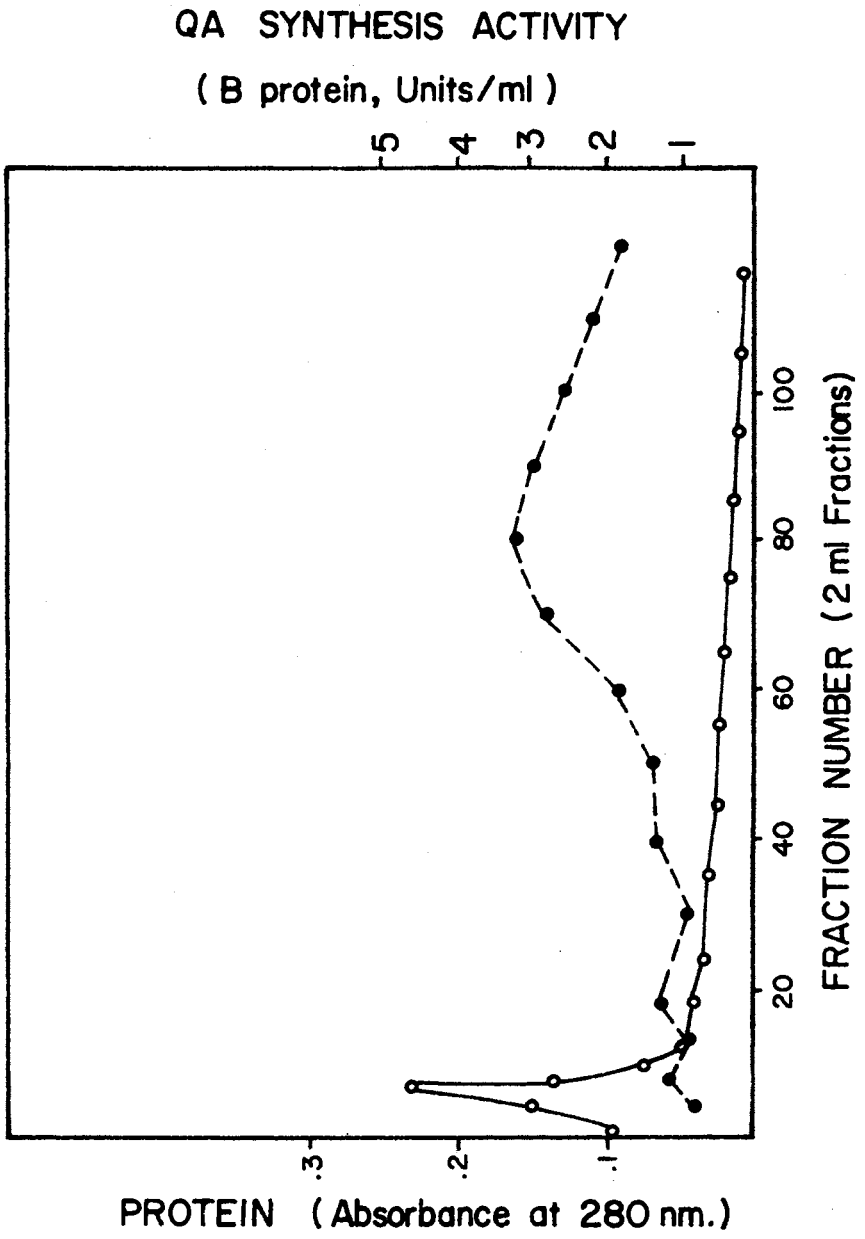
Progress was made in purification to the point of over 340-fold enrichment of specific activity with approximately one-fourth of the enzyme activity being recovered from the crude extract, as shown in Table V. With modification of present techniques and introduction of new ones, it is felt that further purification can be obtained. No estimate was made of how pure the B protein is at the 340-fold stage.

Affinity Chromatography with NAD-Agarose

Agarose-hexane-NAD (AGNAD) was available commercially from P-L Biochemicals, Inc., and was ready to use after washing with deionized water. The structure of AGNAD is shown in Figure 8. Ten ml of the washed gel was packed in a 0.7 x 32-cm column that had been coated with dichlorodimethylsilane (2%) to reduce wall effects. Equilibration was carried out at 23°C by washing the gel with 100 ml of 0.05 M $KPO_4$ in 25% glycerol.

Thirty-eight ml of a pooled hydroxyapatite 0.01 M $KPO_4$, pH 7.5, effluent was concentrated to 6.5 ml with an Amicon 50-ml Ultrafiltration cell. Concentration was done at 4°C with a UM-20E membrane with 18 pounds of nitrogen for four hours. Then 1.5 ml of this concentrated hydroxyapatite fraction was applied to the prepared AGNAD column. Elution was carried out with the equilibrating buffer at 23°C for about 24 hours. Two-ml fractions were collected and stored at -15°C as soon as possible to insure retention of as much B protein activity as possible. When 120 fractions, or 22 to 24 column volumes had been collected, tubes were thawed, monitored for protein, and assayed with a fresh 40 to 60% saturation ammonium sulfate A protein preparation. Figure 10 shows the protein and activity profiles of the effluent.

As shown, the AGNAD does sufficiently retard the movement of the B protein through the column such that it elutes much later than the bulk of the protein applied to the column. The protein levels in the area of the activity peak appear to be quite low, also. Fractions 70 to 100, at the top of the activity peak, were pooled and then concentrated from 60 ml to 5.8 ml by ultrafiltration. Protein determination and assay of these preparations and those of previous purification steps indicated only a 65-fold enrichment in the specific activity of the concentrated AGNAD fraction over that of the crude extract. Total protein, however, was about 0.5 mg in the concentrated fraction from the affinity gel, which was only 0.008% of the original protein of the crude extract. Thus, very large decreases in the total activity of the B protein present occurred, down to 0.5% of the original activity in the crude extract.




A 1.5-ml volume of the B protein from a pooled, concentrated hydroxyapatite column effluent. The column was eluted with 0.05 M $KPO_4$, pH 7.5, in 25% glycerol, collecting 2-ml fractions.

———— Protein, absorbance at 280 nm

— — — B protein activity (units/ml)

Figure 10. Elution of the B Protein from NAD-Agarose

Several possible reasons exist for the low specific activity of the AGNAD fraction. Most likely, denaturation of the B protein during elution of the AGNAD column occurred both from temperature and high dilution effects. It is possible that glycerol could not compensate for these conditions. A contributing factor may be inaccurate protein determination. The method of Lowry, et al. (179) was used and should be able to distinguish protein concentrations as low as 50 µg per ml. Protein determinations of the AGNAD fractions containing the activity were not consistent and showed similar results for the pooled and concentrated (10-fold) fractions. The glycerol or the phosphate may have interfered with the protein determination. If the protein concentration is actually 0.1 mg per ml, as found for the pooled AGNAD fraction, then much of the B protein present is in denatured form.

The significance of this type of separation is, of course, the possibility of purification of the B protein to homogenicity. This type of elution from AGNAD was repeated many times on one-ml gel columns to achieve better separation and recovery. No set of conditions was found, however, better than the one discussed. The length of the ten-ml column allowed much better resolution and separation of protein and activity peaks than that for the one-ml columns. If denaturation problems can be overcome, it is felt that the desired purification can be realized.

The binding of the B protein to AGNAD suggests that $NAD^+$ is an effector of some type of the B protein activity. Unless the binding is in the adenine portion of $NAD^+$ (which is the portion to which the Agarose sidearm is thought to be bound) as an analog of FAD, the affinity of the B protein for $NAD^+$ seems only to be explainable by feedback

inhibition. Inhibition studies discussed below appear to support this hypothesis.

## Other Purification Methods

### Nucleic Acid Removal

Three reagents were tested for their efficiency of nucleic acid removal with corresponding retention of B protein activity. Streptomycin sulfate (10%), protamine sulfate (2% suspension), and $MnCl_2$ (1 M) were used according to known methods (27, 187, 188). Each addition was made in a dropwise manner with stirring to 10-ml volumes of crude extract (16 mg protein per ml) at $4^{o}C$. After 30 minutes mixing, the suspensions were centrifuged and the supernatants were assayed. Streptomycin sulfate and $MnCl_2$ did not cleanly fractionate the activity or give good recovery.

Protamine sulfate, however, appeared to precipitate much protein and nucleic acids without destroying activity or removing the B protein from solution. Experiments were conducted to determine the optimum amount of protamine sulfate to add as the 2% suspension. To separate 20-ml volumes of crude extract, increasing volumes of the protamine sulfate suspension were added to obtain concentrations of 2, 3, 4, and 5 mg of protamine sulfate per 10 mg of protein. Assay of the resulting supernatants indicated that 2 mg protamine sulfate per 10 mg protein was the best concentration for maximum recovery, which was often above 90%.

### Fractionation by Solubility

Initially, Suzuki, et al. (65) found that the B protein precipitated in the 0 to 40% saturation fraction using ammonium sulfate. Such

fractionation usually resulted in a five to seven-fold increase in specific activity and 25 to 50% recovery, based on the crude extract. Then sodium citrate was tested as a substitute for ammonium sulfate and found to give better fractionation of the protamine sulfate supernatant when added to 50% saturation. Routinely, the 0 to 50% saturation sodium citrate fraction yielded 10 to 15-fold enrichment and 50 to 75% recovery.

Reverse gradient solubilization (189) was attempted to perhaps further fractionate the 0 to 50% sodium citrate pellet. The pellet was suspended in 50% saturated sodium citrate and mixed with the top layers of a Celite 545 column equilibrated with 50% saturated sodium citrate, pH 7.0. The Celite acted as a solid support upon which continual solubilization and precipitation of protein could occur as the percent of sodium citrate in the eluant decreased. A reverse gradient such as 50% to 20% was applied, separating the 0 to 50% sodium citrate pellet into two large protein peaks. Activity appeared to be located in one of the peaks, but difficulty was encountered when it was discovered that high sodium citrate completely inhibited the QA synthesis assay. Dialysis was not a solution, since dilution of protein caused large amounts of denaturation. Also, attempts to scale up this type of chromatography were not successful in duplicating the separation of peaks obtained with smaller Celite columns. If modified properly, this method might work, but it appeared that the greatest retention of the B protein activity accompanied the shortest exposure time to high salt concentrations.

Acetone fractionation was tested by adding acetone dropwise at -15$^{o}$C to the PS supernatant. Successive additions resulting in 0 to

33%, 33 to 50%, and 50 to 75% acetone were made. Each solution was mixed well and centrifuged and the redissolved pellets were assayed. Although some activity was recovered in the 50 to 75% fraction, no increase in specific activity was observed.

Another technique used for precipitation of proteins from solution is polyethylene glycol fractionation (190). Polyethylene glycol with a molecular weight range from 6000 to 7500 daltons was added as a 50% solution to separate volumes of protamine sulfate supernatant to yield 5, 10, 15, 20 and 25% solutions of polyethylene glycol. After mixing and centrifugation, supernatants were assayed for B protein activity. No clear fractionation was obtained and the protein seemed to distribute itself between supernatants and pellets, with gradual precipitation up to 25% polyethylene glycol. Also, it was observed that polyethylene glycol inhibited QA synthesis and interfered with protein determinations at 280 and 260 nm.

Heat Treatment

The B protein was found to be heat-labile (Suzuki, unpublished data), so heat treatment was carried out in the presence of possible stabilizing agents. Crude extracts were treated for 5, 10, and 15 minutes at 55°C in the presence of 5 mM L-aspartate or 1 mM riboflavin. Treated mixtures were cooled, centrifuged, and put on dialysis as resuspended pellets and supernatants to remove the aspartate and riboflavin. Assays revealed that no increases in specific activity had occurred and that recovery was well below 30% for most of the fractions.

In addition, heat treatment with agents known to stabilize B activity (see below) was done. Diluted 0 to 50% sodium citrate fraction

solutions that were 50% glycerol or 50% ethylene glycol were heated at five minutes at 50°C. A solution with 2 mM mercaptoethanol was also heated. Ethylene glycol and mercaptoethanol solutions lost almost all their activity. Very little protein was precipitated in the 50% glycerol solution and most of the activity was retained. No feasible purification step was suggested by these experiments.

Acid Treatment

The B protein was known to be stable at neutral pH, so attempts were made to incorporate acid treatment as a purification method. Aliquots of the protamine sulfate supernatant fraction were dialyzed 16 to 18 hours at 4°C against potassium phosphate, sodium citrate, or sodium acetate buffers at pH 4.5, 5.0, 5.5, and 6.0. Pellets showed a three to seven-fold increase in specific activity but many would not redissolve in buffer even when neutralized to pH 7.0. Recovery of activity was below 25% for the pellets, also. Neutralized supernatants retained more than 50% of the total activity in the starting fraction, but showed little or no increase in specific activity. Direct addition of 7% acetic acid resulted in even poorer retention of activity. A gradual denaturation of the B protein seemed to be occurring as the pH was lowered.

A combination of sodium citrate and acid fractionation was accomplished by addition of 1 M citric acid to the protamine sulfate supernatant until pH 6.0 was reached. Then the solution was brought to 40% saturation by the addition of solid sodium citrate and mixed well. No improvement in purification was observed. Direct addition of 0.1 N

HCl to the 0 to 50% sodium citrate fraction until the pH of 6.0 was reached yielded similar results.

Thus, an apparently irreversible denaturation of the B protein occurs at pH of 6.0 and below. This gradual loss of activity with decreasing pH makes such treatment impractical as a purification step.

Gel Adsorption

Calcium triphosphate and Alumina Cγ gels were checked for their ability to adsorb the B protein selectively or not adsorb it at all. Either case would provide a useful tool for the removal of unwanted protein. The protamine sulfate supernatant fraction was treated without dilution and with 1:10 dilution at gel concentrations from 1 to 25 mg dry gel per mg protein. After mixing the protein and the gel and allowing adsorption to occur, the supernatants from centrifugation were assayed. Adsorption of activity to both types of gel paralleled the adsorption of protein, such that specific activity was not increased. Most protein adsorption occurred in the 1 to 5 mg dry gel to mg protein range. Elution of gels with 0.20 M KCl released no activity from the gel.

Ion Exchange and Adsorption Chromatography

Anion exchange chromatography proved to be quite useful in purification of the B protein. DEAE-cellulose columns routinely equilibrated with 0.005 M $KPO_4$, pH 7.5, were eluted stepwise with phosphate buffer or KCl solutions after application of the dialyzed 0 to 50% sodium citrate fraction. After washing with 0.10 M $KPO_4$, pH 7.5, or 0.15 M KCl, activity was eluted with 0.20 M $KPO_4$ or 0.20 M KCl,

respectively. These procedures resulted in a two to three-fold increase in specific activity and a 10 to 15% recovery.

DEAE-Sephadex A-50, which has a higher capacity for protein than DEAE-cellulose, was found to give 3 to 10-fold enrichment in specific activity over the sodium citrate fraction applied with an accompanying overall recovery of 20 to 30%. Therefore, it was used in place of DEAE-cellulose.

Linear gradient elutions on DEAE-Sephadex columns were attempted, but high dilution and low flow rates resulted in high activity losses. Stabilizing agents were employed, but often the activity levels were too low to detect. The faster stepwise elution required 8 to 10 hours and was preferred.

No binding of the protein on the cation exchange media carboxy-methyl-cellulose or carboxymethyl-Sephadex was observed. These were tested at pH 6.5 and 7.5, respectively, and might have bound the protein at lower pH, but denaturation would have occurred at those pH values.

Hydroxyapatite was first employed using a stepwise elution at 4°C after application of a dialyzed 0 to 40% saturation salt fractionation preparation. Activity was eluted with 0.05 M $KPO_4$, pH 7.5. When glycerol was found to stabilize the B protein, hydroxyapatite columns were run at room temperature with 25% glycerol in the buffer. The B protein then eluted in 0.01 M $KPO_4$, pH 7.5, with an overall recovery of up to 25% and with a two to three-fold increase in specific activity over the previous purification step.

Gel Filtration

Three major protein peaks were observed on an Agarose 5 m

(exclusion limit - 5,000,000 daltons) column, 2.6 x 35 cm. The middle peak, with intermediate molecular weights, consistently contained the B protein. Agarose 15 m (exclusion limit - 15,000,000 daltons) showed a similar elution pattern. High dilution caused overall recovery for such steps to drop to less than 5%. The use of 25% glycerol in the eluting buffer of the Agarose 5 m column apparently decreased resolution of the gel, although recovery was increased to 25 to 30%. Broader peaks with more protein resulted in only a doubling of specific activity.

Other gels used were P-60, P-150, P-300, and G-100. Of these, only P-300 gave good separation of the activity from the bulk of the protein in the effluent. However, P-300 flow rates were extremely slow. G-200 gave the best elution results, as previously discussed and as shown in Figure 9.

Concentration

In order to employ gel filtration steps after such steps as DEAE-Sephadex, concentration of pooled fractions 10 to 100-fold became essential. A hollow fiber device was employed but bubbling and frothing and other denaturation caused a 97% loss in activity. Sodium citrate precipitation with 60% saturation yielded over 50% of the activity of the dilute solution. However, dialysis was required with this method, requiring time and reducing activity. Lyophilization of pooled fractions required 16 to 20 hours for 10-fold concentration with 35 to 45% recovery. Aquacide III, a flake polyethylene glycol (molecular weight ~ 20,000), removed up to 20 to 30 ml of water per hour from dilute solutions in dialysis tubing. It was discovered, however, that the

polyethylene glycol passed through the dialysis membrane and caused high "protein" levels in the solution concentrated, as well as inhibiting the assay for activity.

Ultrafiltration was the most successful method of concentration utilized. The UM-20E (exclusion limit - 15-25,000 daltons) membrane was used with 18 to 20 pounds nitrogen, with usually over 50% recovery. The important advantage of this technique is that it can be used at 4°C or 23°C, with or without 25% glycerol. At 4°C without glycerol, 380 ml can be concentrated to 5.5 ml in 11 hours. Slower rates are observed with 25% glycerol.

## Affinity Chromatography

The binding of a specific protein by a specific ligand attached to a solid support has great potential for the purification of proteins. Considering the low levels of proteins responsible for QA synthesis and their ease of denaturation, the technique is especially attractive for use in isolating the proteins of the QA synthetase system. Thus, several types of affinity columns were prepared (Chapter III) or purchased and employed in attempts to purify the B and A proteins.

### Carboxyl-Linked Aspartate Affinity Chromatography

The first Affinose 102-Aspartate columns used had 10 ml of gel and were run at 4°C with 0.005 M $KPO_4$, pH 7.5. Application of the B protein preparations resulted in complete loss of activity and binding of all protein applied, some of which was eluted by 0.20 M and 0.40 M KCl concentrations. Elution of the B protein with 0.05 M and 0.10 M L-aspartate

also failed to recover any activity from the column. Identical results were obtained with KCl elution of the A protein.

Then a one-ml column was packed and equilibrated with 0.05 M $KPO_4$, pH 7.5, to reduce the apparent non-specific binding which was occurring. The buffer also had 25% glycerol to stabilize the protein. Experiments conducted with this column indicated that the B protein did not bind specifically or non-specifically to the aspartate column in 0.05 M $KPO_4$, pH 7.5, since the column wash immediately showed a protein peak and an activity peak. Failure of the B protein to bind the carboxyl-linked affinity gel suggested that one or both of the carboxyl groups are required for aspartate to bind the B protein, or that the B protein does not bind aspartate even as a substrate.

Amino-Linked Aspartate Affinity Chromatography

The Affinose 202-aspartate column was also initially used in a 10-ml volume at 4°C with 0.005 M $KPO_4$, pH 7.5. Non-specific binding was shown to occur by the addition of bovine serum albumin (BSA) to a column of uncoupled Affinose 202. The BSA was bound to the column and subsequently was eluted with KCl. Since many free carboxyl groups exist in the gel after the coupling reaction (from aspartate, glycine, and the sidearm), ion exchange may be occurring with these groups.

Therefore, a one-ml column of the gel was packed and equilibrated with 0.05 M $KPO_4$, pH 7.5, to eliminate non-specific binding. It was assumed that failure to observe elution of the B protein was caused by very tight binding of the protein to the gel. Elution was attempted with 0.05 M L-aspartate and 0.05 M N-acetyl-L-aspartate and 0.5 M KCl. The KCl fraction contained small but definite activity.

The next experiment was carried out at 23°C with 25% glycerol, using a stepwise elution of KCl with increments of 0.20 M KCl from 0 to 0.80 M. Activity was found in the 0.20 M KCl fraction. Further experiments with the gel at room temperature failed to show any binding at all of the B protein to the gel. Finally, an experiment in which 0.02 mM FAD was present in the eluting buffer as a possible aid to binding also failed to show any attachment of the B protein to the gel. No binding was observed when the A protein was applied to the column at 23°C with 0.05 M $KPO_4$, pH 7.5 and 25% glycerol.

Therefore, what at first appeared to be specific binding at 4°C was demonstrated to be non-specific binding at room temperature. Eventually, perhaps with saturation of some kind of the charged groups on the gel, the binding ceased completely. Glycerol had been established, at least, as a stabilizer that would enable recovery of some activity to monitor the affinity gel. It seems probable that at least one of the proteins, A or B, must bind aspartate. All possibilities for that binding, however, were not explored, and aspartate binding to A or B may be only a matter of ionic strength, steric orientation, sidearm length, or other variables. Also, it is possible that both carboxyl groups and the amino group are required for binding of aspartate to one of the proteins, in which case an affinity column prepared from unmodified aspartate cannot be successful. Inhibition of QA synthesis by N-acetyl-L-aspartate suggests that a substrate can bind with only the two carboxyl groups, with the amino group blocked to cause inhibition. This must be substantiated by much more detailed study, however.

Carboxyl-Linked QA Affinity Chromatography

The Affinose 102-QA was equilibrated with 0.05 M $KPO_4$, pH 7.5, with 25% glycerol. Both A and B proteins were applied to the column in separate experiments and elution was carried out with KCl and QA. Neither protein was bound to the column, but passed through with the protein peak.

This result is not surprising, since QA is probably bound to the proteins that synthesize it through one or both carboxyl groups. Even if only one carboxyl group is required for binding, it is likely that steric hindrance by the sidearm and the gel itself would block the protein from access to that side of the QA molecule.

Based on these ideas, it was thought that if 6-aminoquinolinic acid (6-AQA) could be linked to Affinose 102, the sidearm and gel attachment to the other side of the molecule than the carboxyl groups would leave the carboxyl groups free to bind the protein or proteins. However, 6-AQA was not available commercially, so preparation of the compound was attempted, based on similar preparations of 2, 3-pyrazinedicarboxylic acid (191). The permanganate oxidation of 2-aminoquinoline was done by the addition of 6.6 mmoles of $KMnO_4$ in 75 ml of water to 1.12 mmoles of 2-aminoquinoline in 25 ml of water. The addition was made over a period of 3.5 hours and the mixture was kept at 90°C with mechanical stirring and refluxing. Subsequent isolation and analysis of the reaction product(s) gave unclear information about the identity of the product. Various methods, including thin layer chromatography, could not conclusively identify the product as 6-AQA, so the preparation was not continued. Therefore, this avenue of affinity chromatography remains open if 6-AQA synthesis can be carried out successfully.

Organomercurial Agarose Affinity Chromatography

Affinose 501 (pCMB-Agarose) was purchased commercially and was used based on the observation that $10^{-5}$ M pCMB inhibited QA synthesis (Suzuki, unpublished observations). This suggests that free sulfhydryl groups are present somewhere in the protein and are somehow necessary for activity. A one-ml column was packed and washed with 0.05 M $KPO_4$, pH 7.5 at 4°C. After application of the B protein preparation, elution with buffer, mercaptoethanol, and KCl was carried out. Fractionation of proteins was achieved, but no activity was detected in any effluents. The column was run again at room temperature in buffer with 25% glycerol, using cysteine as the eluting ligand. Mercaptoethanol may inhibit QA synthesis, whereas cysteine does not at the concentrations used. Again, however, no activity was detected in the effluent fractions. It appears that denaturation was occurring when the B protein was binding to the pCMB-Agarose. If the protein did not bind, it would have passed through the column and retained some activity, at least, when assayed, since 25% glycerol was used. The presence of a sulfhydryl group might explain the binding that seems to occur on the pCMB-Agarose as well as the effect of various sulfydryl reagents and possibly the instability of the B protein in dilute solutions.

## Linearity of Assay and Protein Stabilization

Dependence of QA Synthesis on the Amount of B Protein

Since almost all assays were conducted in the presence of excess A protein for quantitatively determining B protein activity, it was

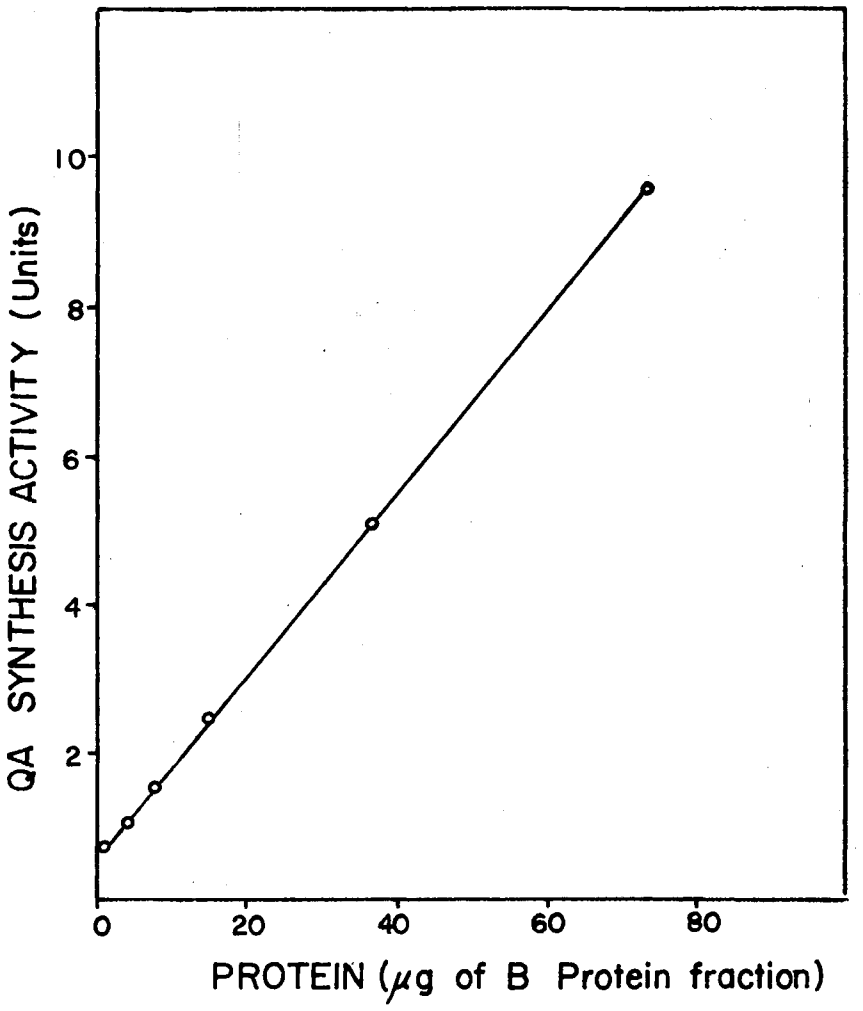
important to insure that the levels of the B protein were actually rate-limiting to QA synthesis. A 0 to 50% sodium citrate fraction from PA-2-18 was assayed in dilutions from 1:10 to 1:200 using a fresh 40 to 60% ammonium sulfate preparation of SB-16. The protein concentration of the 0 to 50% sodium citrate fraction was determined to be 7.35 mg per ml by 280 and 260 nm ratios. The units of QA synthesis activity were calculated for each assay tube and averaged for duplicate samples. Units were plotted against μg of B protein in the assay tubes.

As shown in Figure 11, linear dependence of QA synthesis on the amount of B protein was achieved. Presumably, then, by conducting assays with B protein amounts from 1 to 10 units per assay tube and with an excess of A protein activity, accurate quantitative evaluation of B protein activity may be made.

Glycerol Stabilization of the B Protein

The problem of denaturation of the B protein in dilute solutions and at room temperature made the use of a stabilizing agent necessary for further purification steps and for running columns at 23°C. Glycerol was found to do this job when added to the buffer solutions. Experiments were conducted to determine the optimum percent glycerol necessary to stabilize the B protein activity at -15°, 4°, and 23°C.

Solutions of 0 to 50% sodium citrate fraction from PA-2-18 were prepared with glycerol and without glycerol to a final dilution of 1:20. Glycerol-containing solutions were 0, 10, 15, 20, and 25% glycerol. Corresponding solutions without glycerol substituted 0.05 M $KPO_4$ buffer, pH 7.5. Small volumes of each type of solution were stored in tubes ready to assay at each temperature. After 24 hours, tubes were assayed




Dilutions from 1:10 to 1:200 of a 0 to 50% saturation sodium citrate fraction (7.35 mg per ml) were assayed in duplicate units per assay tube and plotted against μg protein.

_____ Units for each assay

Figure 11. Linear Dependence of the Rate of QA Synthesis on the Concentration of B Protein

with excess A protein. Remaining activity was calculated as a percentage of the 0 to 50% sodium citrate fraction diluted just before assay.

Table VI shows the effect of temperature and the presence of glycerol on remaining enzyme activity. Glycerol stabilized and even enhanced B protein activity when assayed after storage at -15°C and 4°C. At room temperature, however, 20 or 25% glycerol was required before complete retention of activity was achieved. Activity remaining in samples with buffer varied considerably, but the effect of room temperature was again quite pronounced. Such information was quite useful in choosing temperatures and glycerol concentrations for particular columns. More dilution than used here, of course, would have much greater effect on the stability of the B protein, and thus increasing the amounts of glycerol that would have to be used.

Glycerol Stabilization of the A Protein

A greater problem in loss of activity was found in the A protein fractions for both storage of the 40 to 60% ammonium sulfate for assay and for purification of the A protein itself. Most immediate was the need to maintain high activity in fractions stored for use in the B protein assay. Thus, experiments were conducted over two weeks in order to allow the decay of A activity with and without 33% glycerol. The buffer and glycerol-containing solutions were assayed on days 1, 2, 3, 6, 8, 10, and 13, using an excess of B protein. Remaining activity was expressed as a percent of the initial units existing on day one in the 33% glycerol fraction.

Table VII shows that glycerol again enhanced QA synthesis and protected the A activity for nearly two weeks. Rapid decay of activity

TABLE VI

STABILIZATION OF THE B PROTEIN WITH GLYCEROL[a]

| | Percent of Activity Remaining (% of Units of Undiluted B Protein) | | |
|---|---|---|---|
| | Temperature | | |
| | 23°C | 4°C | -15°C |
| No Dilution (0 to 50% Sodium Citrate Fraction at -15% diluted just before assay) | 27.9 | 92.2 | 100[b] |
| With Glycerol | | | |
| 10% | 76.5 | 110 | 104 |
| 15% | 80.4 | 111 | 103 |
| 20% | 115 | 116 | 105 |
| 25% | 110 | 119 | 104 |
| With Buffer | | | |
| 10% | 23.6 | 91.9 | 98.6 |
| 15% | 11.8 | 61.0 | 102 |
| 20% | 22.1 | 78.8 | 84.6 |
| 25% | 22.0 | 88.9 | 89.0 |

[a]See text for experimental details.

[b]Actual activity of preparation used as 100% activity remaining was 905 units/ml B fraction.

was noted after three days in the fraction with buffer. If this fraction is not used within three days after preparation, then glycerol should be included with the solution when it is frozen. The convenience of high activity A protein is thus afforded and assays could be carried out for one week with the same preparation.

TABLE VII

STABILIZATION OF THE A PROTEIN WITH GLYCEROL[a]

| Time of Storage (Days) | Percent of Activity Remaining (% of Units of 33% Glycerol on Day 1) Addition to A Protein | |
|---|---|---|
| | Buffer, 33% | Glycerol, 33% |
| 1 | 99.6 | 100[b] |
| 2 | 81.3 | 96.6 |
| 3 | 83.8 | 107 |
| 6 | 52.2 | 97.8 |
| 8 | 54.6 | 96.1 |
| 10 | 53.2 | 106 |
| 13 | 18.4 | 87.3 |

[a]See text for experimental details.

[b]Actual activity of preparation used as 100% activity remaining was 8.5 units/ml A fraction.

## The Effect of Cofactors and Inhibitors on QA Synthesis

### Pyridine Nucleotides

The first enzyme in a biosynthetic pathway is often subject to control mechanisms such as feedback inhibition. The results of Chandler and Gholson (64) suggested the possibility of feedback inhibition of the QA synthetase system in *E. coli* by $NAD^+$. Experiments, therefore, were conducted to determine the effect of pyridine nucleotides upon the QA synthetase system using a more highly purified preparation of B protein.

Assays were conducted using a fresh 40 to 60% ammonium sulfate preparation from SB-16 and a concentrated hydroxyapatite effluent from PA-2-18. The B protein preparation had a specific activity of 167 units per mg and was purified 100-fold from the crude extract. Assays were done as usual with the exception of the addition of 0.1 ml of the reagent tested, making final assay volumes 0.6 ml. Pyridine nucleotide stock solutions at concentrations of 20 mM were prepared in deionized water and adjusted to neutral pH. Stock solutions were diluted appropriately for obtaining the desired concentrations in the assay tubes.

Table VIII shows the QA synthesis and inhibition data. Marked inhibition of QA synthetase activity occurred with the addition of $NAD^+$ or NADH. NADH showed slightly less inhibition than $NAD^+$ at all concentrations used. Much less inhibition by $NADP^+$ and NADPH occurred and the action appeared to be much less dependent on concentration of these compounds. NMN was unique in its enhancement of QA synthesis by as much as 5%.

TABLE VIII

THE EFFECT OF PYRIDINE NUCLEOTIDES ON QA SYNTHESIS[a]

| Addition to Assay | QA Synthesis Activity (Units/ml B Protein Fraction) | | | Percent Inhibition (of Control) | | |
|---|---|---|---|---|---|---|
| | Final Concentration in Assay | | | | | |
| | 3.33 mM | 1.0 mM | .33 mM | 3.33 mM | 1.0 mM | .33 mM |
| $H_2O$ | 513 | 513 | 513 | 0 | 0 | 0 |
| $NAD^+$ | 22.6 | 237 | 437 | 95.6 | 53.9 | 10.9 |
| NADH | 41.3 | 217 | 414 | 92.0 | 57.8 | 19.2 |
| $NADP^+$ | 368 | 366 | 436 | 28.3 | 28.6 | 15.0 |
| NADPH | 393 | 408 | 487 | 23.5 | 20.5 | 5.0 |

[a]See text for experimental details.

It appears that $NAD^+$ is an effective inhibitor of QA synthesis, as previously reported in a crude system (64). These experiments showed 100% inhibition of QA synthesis with 3.33 mM $NAD^+$ (final concentration), which is very similar to present results. It is difficult to say if $NAD^+$ is an inhibitor of QA synthesis in vivo, at cellular $NAD^+$ levels, but the 50% inhibition by 1 mM $NAD^+$ suggests the possibility, since cellular levels are in the 0.1 to 1.0 mM range (104).

In an attempt to localize the site of $NAD^+$ inhibition, the reactions for NAD inhibition were repeated, using limiting amounts of the A protein. In this experiment, $NAD^+$ was added to make the final concentration 1 mM. Excess B protein was added and 2, 10, and 40-fold reductions in the A protein achieved rate-limiting conditions for the A protein. Table IX shows that less inhibition by $NAD^+$ occurred under these conditions. A possible interpretation of results is that $NAD^+$ acts on the B protein and not the A protein.

The existence of a complex between A and B that is responsible for catalysis is a possibility that cannot be disregarded. The failure of the demonstration of a free intermediate may be evidence for this. Inhibition effects observed would then be results of inhibiting the complex and not individual proteins, thus invalidating attempts to determine which protein binds $NAD^+$ by excess and rate-limiting levels of one protein.

Pyridoxal-Phosphate and Pyridoxamine-Phosphate

Assays were conducted to check the effect of these coenzymes at concentrations 3.33, .33, and .033 mM. Stock solutions of 20 mM were

prepared in the same way as those for the pyridine nucleotides. Assays were also the same.

TABLE IX

$NAD^+$ INHIBITION IN CONDITIONS OF EXCESS B PROTEIN

| Addition to Assay | Total QA Synthesis (Units per Assay) | | Percent Inhibition (of Control) | |
|---|---|---|---|---|
| | $H_2O$ | $NAD^+$ | $H_2O$ | $NAD^+$ |
| Excess B Protein<br>A Protein Added (mg) | | | | |
| .1 | 1.32 | 1.02 | 0 | 22.7 |
| .4 | 5.08 | 4.64 | 0 | 8.7 |
| 2.0 | 27.6 | 21.3 | 0 | 22.8 |
| Excess A Protein<br>B Protein Added (mg) | | | | |
| .017 | 24.2 | 12.4 | 0 | 51.2 |

Table X indicates a greater inhibition by pyridoxal-phosphate as compared to pyridoxamine-phosphate. A previous report (64) indicated equal inhibition by these, but one is only 10% of the other in these results. It is quite possible that a non-specific chemical reaction of the aldehyde group of pyridoxal-phosphate is occurring that results in a decrease in QA synthesis. This would explain the difference observed with pyridoxamine-phosphate, which has no aldehyde group. Multivalent

TABLE X

THE EFFECT OF NMN, PYRIDOXAL-PHOSPHATE, AND PYRIDOXAMINE-PHOSPHATE ON QA SYNTHESIS[a]

| Addition to Assay | QA Synthesis Activity (Units/ml B Fraction) | | | Percent Inhibition (of Control) | | |
|---|---|---|---|---|---|---|
| | Final Concentration in Assay | | | | | |
| | 3.33 mM | .33 mM | .033 mM | 3.33 mM | .33 mM | .033 mM |
| $H_2O$ | 513 | 513 | 513 | 0 | 0 | 0 |
| NMN | 533.5 | 537.4 | 526.3 | -4.0 | -4.8 | -2.6 |
| Pyridoxal-5'-P | 163.5 | 383.9 | 422.3 | 68.1 | 25.2 | 17.7 |
| Pyridoxamine-5'-P .HCl | 476.3 | 385.1 | 445.7 | 7.2 | 24.9 | 13.1 |

[a]See text for experimental details.

inhibition of QA synthesis by pyridine-containing cofactors might also be considered as a possible mechanism.

Aspartic Acid and Related Compounds

In the same assay system, the effect of several derivatives of L-aspartate was tested. The apparent inhibition by 5 mM L-aspartate is due to isotope dilution in the assay, since the addition of 2.5 μmoles of unlabeled L-aspartate represented a 10-fold increase in the aspartate levels in the assay. Thus, an inhibition of 90% would be expected for QA synthesis if isotope dilution was occurring, and 82.7% was observed, as shown in Table XI.

TABLE XI

THE EFFECT OF L-ASPARTATE AND RELATED COMPOUNDS ON QA SYNTHESIS[a]

| | QA Synthesis Activity (Units/ml B Fraction) | | Percent Inhibition (of Control) | |
|---|---|---|---|---|
| | Final Concentration in Assay | | | |
| Addition to Assay | 5 mM | .5 mM | 5 mM | .5 mM |
| $H_2O$ | 461.9 | 461.9 | 0 | 0 |
| L-Aspartate | 79.8 | 313.0 | 82.7 | 32.2 |
| D-Aspartate | 415.4 | 433.9 | 10.1 | 6.1 |
| N-Acetyl-L-Aspartate | 404.9 | 449.1 | 12.3 | 2.8 |
| DL-Aspartate Dimethyl Ester | 348.9 | 385.9 | 24.5 | 16.5 |

[a]See text for experimental details.

Of the others shown in Table XI, the dimethyl ester showed the greatest affect on QA synthesis. It is possible that these inhibitory effects by the aspartate analogs are the result of their conversion to L-aspartate and subsequent isotope dilution in the assay. However, all the enzymes for such conversions are likely not present in the protein preparations used. At 5 mM, the inhibition by the dimethyl ester may indicate the relative importance of the carboxyl groups in the binding involved in QA biosynthesis. Previous experiments resulted in over 50% inhibition of the assay by 20 mM N-acetyl-L-aspartate, and these results are consistent with that amount of inhibition. Again, interpretation of the action of particular inhibitors on the significance of particular groups of the substrate in binding is difficult in this system.

### Sulfhydryl Reagents

Three types of sulfhydryl reagents were tested in this assay system, as shown in Table XII. These preliminary results suggest that $10^{-4}$ or $10^{-5}$ M concentrations of N-ethylmaleimide and pCMB would also give significant inhibition, since 10 mM and 1 mM are both highly inhibitory to QA synthesis. The iodoacetamide was pre-incubated about five minutes at $23^{\circ}C$ with the B protein, resulting in a large amount of inhibition. These results, the behavior on pCMB-Agarose, and the lability of the B protein in dilute solutions all suggest the involvement of sulfhydryl groups in the synthesis of quinolinic acid.

## Other Sources of Enzyme Activity

### E. coli K-12 Strains

Other mutants of E. coli K-12 were checked for A and B protein

activity. Strains PA-2-18 (nad A), AT-11-23 (nad A), SB-16 (nad B), W-3899 (nad B), E-126 (nad C), SB-99 (nad C), W-4516 (nad C), and AT-13-3 (nad C) were grown in 100-ml cultures at 37°C with the appropriate growth factors (see Table IV). After 12 hours, cultures were harvested by centrifugation at 8000 x g for 15 minutes. Crude extracts were prepared as described previously and assayed for the A protein, the B protein, and then both activities, using PA and SB extracts. Protein was determined by absorbance at 280 and 260 nm. Table XIII shows the comparison of the specific activities of the mutants.

TABLE XII

THE EFFECT OF SULFHYDRYL REAGENTS ON QA SYNTHESIS[a]

| | QA Synthesis Acitivity (Units/ml B Fraction) | | Percent Inhibition (of Control) | |
|---|---|---|---|---|
| | Final Concentration in Assay | | | |
| Addition to Assay | 10 mM | 1 mM | 10 mM | 1 mM |
| $H_2O$ | 461.9 | 461.9 | 0 | 0 |
| Iodoacetamide | 37.2 | 200 | 92.0 | 56.7 |
| p-Chloromercuri-benzoate | 3.5 | 35.9 | 99.2 | 92.2 |
| N-Ethylmaleimide | 5.8 | 75.0 | 98.7 | 83.8 |

[a]See text for experimental details.

Qualitatively, these results would be expected from genetic knowledge of these strains. The differences in the amounts of these

TABLE XIII

A AND B PROTEIN ACTIVITIES IN *NAD A*, *NAD B*, AND *NAD C*, STRAINS OF *E. COLI*[a]

| Mutant | Specific Activity (Units/mg) x $10^{-3}$ | | | Percent of Activity (Based upon PA-2-18 and SB-16) | |
|---|---|---|---|---|---|
| | Enzyme Activity | | | | |
| | A Protein | B Protein | A and B Protein | A Protein | B Protein |
| *nad A* | | | | | |
| PA-2-18 | 48.2 | 964 | 44.9 | 5.0 | 100 |
| AT-11-23 | 29.6 | 952 | 22.7 | 2.3 | 73.3 |
| *nad B* | | | | | |
| SB-16 | 1200 | 31.7 | 16.7 | 100 | 2.7 |
| W-3899 | 718 | 31.8 | 23.8 | 53.4 | 2.4 |
| *nad C* | | | | | |
| E-126 | 1150 | 566 | 664 | 101.5 | 50.3 |
| SB-99 | 600 | 382 | 404 | 51.9 | 33.2 |
| W-4516 | 920 | 672 | 748 | 67.6 | 49.7 |
| AT-13-3 | 79.7 | 532 | 19.0 | 5.8 | 39.1 |

[a]See text for experimental details.

activities may or may not have significance. It is interesting to note that all the activities for the A proteins in nad B mutants, for example, are well within an order of magnitude of each other. This is true for activities present in the nad A and nad C mutants as well. The strain AT-13-3 is genetically classed as a nad C mutant, but has very low A protein activity, similar to nad A mutant, as observed in these studies.

E-126 Strain

E. coli E-126 was used as a source for the partial purification of the A and B proteins. Cultures were grown and harvested in an identical manner as the PA-2-18 strain, except for growth supplements. Sonication and crude extract preparation was also done as before. Simultaneous purification of both activities from the strain was not possible, since the purification steps for one protein would inactivate or exclude the other protein. Table XIV indicates the B protein purification from E-126. No significant differences appear to exist in the behavior of this activity. The A protein purification, also shown in Table XIV, appears to be quite similar to other A preparations. Both E-126 preparations had somewhat lower activities than those observed for PA-2-18 and SB-16, respectively.

Mung Bean Plant

Crude extracts of the mung bean plant Phaseolus vulgaris were checked for their ability to synthesize QA. Plants were grown from seed for eight days in a controlled humidity growth chamber, after which the

TABLE XIV

PURIFICATION OF THE A AND B PROTEINS FROM *E. COLI* STRAIN E-126

| Step | Total Volume (ml) | Total Protein (mg) | Total Activity (units) | Specific Activity (units/mg)$^{x10^{-3}}$ | Enrichment (-fold) | Recovery (%) |
|---|---|---|---|---|---|---|
| B Protein | | | | | | |
| Crude Extract | 194 | 4656 | 16199 | 3.5 | 1 | 100 |
| Protamine Sulfate Supernatant | 212 | 3286 | 14522 | 4.4 | 1.3 | 89.6 |
| 0 to 50% Sodium Citrate Pellet | 7.1 | 234 | 5495 | 23.5 | 6.7 | 33.9 |
| DEAE-Cellulose (Pooled Fractions) | 173 | 52 | 3689 | 71.0 | 20.4 | 22.8 |
| A Protein | | | | | | |
| Crude Extract | 95 | 2260 | 1000 | 0.44 | 1 | 100 |
| Heat Treatment Supernatant | 93 | 2840 | 804 | 0.28 | 0.6 | 80.4 |
| 40 to 60% Ammonium Sulfate Pellet | 18.4 | 258 | 405 | 1.57 | 3.6 | 40.5 |

first set of leaves besides the cotyledons had formed. Entire seedlings were washed, dried, cut into large pieces, and frozen in a large mortar with liquid nitrogen. The pieces were ground to a fine powder with continued addition of liquid nitrogen and then to a pulpy slurry by addition of 50 ml of 0.05 M $KPO_4$, pH 7.5. The well-mixed slurry was sonicated as described previously for small volumes of bacterial crude extracts and centrifuged 15 minutes at 20,000 x g. The decanted supernatant was assayed for both A and B protein activities according to methods described previously.

The assays were conducted with FDP or DHAP. Aldolase was added to the assay tubes when FDP was used as substrate in the event the native enzyme was not active or present in the extracts. Results were negative for all substrates and all assays. Thus, it appears that in this assay system no QA is synthesized. Both A and B protein activities appear to be lacking. No known reports on $NAD^+$ or NA biosynthesis in this organism have been published, but it is assumed that de novo synthesis of QA occurs as in other higher plants, from a three-carbon unit and a four-carbon dicarboxylic acid (75). Many possible explanations could be given for the failure to demonstrate $^{14}C$-aspartate incorporation into QA. The substrates may be different or the proteins themselves sufficiently different in structure or action to prevent the substitution of a bacterial enzyme for the plant enzyme. Or, if the enzymes are present in these extracts, it may be inhibited by another protein or effector or completing enzymes rapidly utilize the $^{14}C$-aspartate present. The enzymes may have been denatured by freezing, sonication, or even assay incubation; whatever the case, much more work will be needed to clarify QA synthesis in this organism.

# CHAPTER V

## CONCLUSIONS

The elucidation of the enzymatic activities and of the intermediates of the pathways of biosynthesis of vitamins appears to present a special problem in that only very low levels of the vitamins are required. Particularly, in $NAD^+$ biosynthesis, the enzymes for biosynthesis comprise only a small fraction of the total protein in the E. coli cell. Much greater purification from crude extracts is necessary, therefore, to obtain a homogenous protein in this pathway.

The purification data presented for the B protein in this study represent the purest preparation of an enzyme activity for QA synthesis that has been reported to date. Rearrangement and modification of these purification techniques should improve the purification from 340-fold to perhaps 500-fold. Many different methods for purification were attempted and most of these were eliminated as unsatisfactory techniques. Perhaps purification of the B protein from other strains of E. coli or even other microorganisms may be carried out with the methods that were utilized in this study. This would greatly speed elucidation of the QA synthesis system in these organisms.

The most promising result for purification, however, is the behavior of the B protein on the $NAD^+$ affinity column. Proper modification of this type of elution seems to provide a method of obtaining a highly purified B protein. A large volume of PA-2-18 crude extract

(1 to 2 liters) is required for such a purification. Since the $NAD^+$-Agarose is less specific for the B protein than an aspartate affinity column, for example, the most purified protein preparation from other methods should be applied to the column to reduce to a minimum the number of proteins that bind $NAD^+$ (dehydrogenases). Perhaps a modification of the temperature (to $4^oC$) or the glycerol concentration is necessary.

The aspartate and QA affinity columns that failed to bind the B protein might do so under different conditions, but this does not seem likely. The functional groups of L-aspartate and QA that were used to couple these ligands to the Affinose gels are probably necessary for their binding to the protein. Alternatively, *in vivo*, these molecules may not bind at all to the B protein. If the process of QA synthesis is a two-step reaction, then aspartate should bind as a substrate to one protein and QA should bind as a product to the other. The existence of a complex between the A and B proteins would further complicate the possibilities, since both proteins would then need to be applied to the affinity column to observe binding of the ligand. If the A protein is to be isolated by affinity chromatography, some of these possibilities will need to be investigated in order to prepare the proper affinity column to obtain binding.

All evidence indicates that $NAD^+$ binds the B protein and effects its activity for *in vitro* synthesis of QA. This is demonstrated by the behavior of the B protein on $NAD^+$-Agarose as well as inhibitor studies with pyridine nucleotides. This binding is apparently specific for $NAD^+$ or NADH, and suggests a regulatory function for $NAD^+$ in the quinolinate synthetase system. Obviously, since $NAD^+$ is the end product of the

pathway in a functional sense, at least, this suggests classical feedback inhibition is occurring. Alternatively, $NAD^+$ may function as an FAD analog, suggesting FAD binds the B protein. Either possibility may be true at this point and only further inhibition studies with highly purified enzyme preparations will distinguish between the two.

Inhibition by aspartate derivatives suggests an apparent specificity for L-aspartate at levels below 0.5 mM, at least. Partial inhibition by both N-acetyl-L-aspartate and the dimethyl ester of aspartate implies that both carboxyl groups and the amino group of L-aspartate are involved in binding to the protein involved. This appears to agree with incorporation data for labeled L-aspartate, in which all carbon atoms and the nitrogen atom form part of QA. Stability characteristics and sulfhydryl reagent effects indicate that some type of sulfhydryl group is important in the A or B protein activities.

It must be kept in mind in all these studies, however, that one is looking not just at the B protein as far as synthesis of QA is concerned. This holds true especially for the inhibitor studies, since both proteins (and many others) are present in the assay with the cofactor or inhibitor. Effects observed on QA synthesis may be a result of action upon the A or the B protein or both, or neither protein, in which case side reactions involving other enzymes are occurring. The effects are on the overall system, i.e., on the assay, and not on the specific proteins. In this sense, the assay for QA is a limiting factor for characterizing the system further.

The A and B proteins of the system appear to be identical in other strains of *E. coli*. Likely, they are present in all microorganisms that

utilize the aspartate-DHAP pathway to make QA. No activity of this kind could be demonstrated in the mung bean plant, however.

These studies and their results, then, have answered a few questions, but, typical of most research, they have generated many more questions and possibilities to explore. A step has been made, though, that eventually will enable study of the B protein of the quinolinate synthetase system in a pure form and perhaps may culminate in the final elucidation of this pathway in E. coli.

A SELECTED BIBLIOGRAPHY


(1) Huber, C. (1867) "Vorlaufige Notiz Uber Einige Derivate Des Nicotins." Justus Liebigs Ann. Chem., 141, 271.

(2) Huber, C. (1870) "Vorlaufige Mittheilung." Ber. Deut. Chem. Ges., 3, 849.

(3) Funk, C. (1911) "On the Chemical Nature of the Substance Which Cures Polyneuritis in Birds Induced by a Diet of Polished Rice." J. Physiol., (London) 43, 395.

(4) Funk, C. (1913) "Studies on Beri-Beri. VII. Chemistry of the Vitamine-Fraction From Yeast and Rice-Polishing." J. Physiol., (London) 46, 173.

(5) Suzuki, U., Shimamura, T., and Odake, S. (1912) "Uber Oryzamin, Ein Bestandteil Der Reiskleie und seine Physiologische Bedeutung." Biochem. Z., 43, 89.

(6) Goldberger, J., and Tanner, W. F. (1925) "A Study of the Pellagra-Preventive Action of Dried Beans, Casein, Dried Milk, and Brewers' Yeast, With a Consideration of the Essential Preventive Factors Involved." U. S. Public Health Report, 40, 54.

(7) Elvehjem, C. A., Madden, R. J., Strong, F. M., and Woolley, D. W. (1937) "Relation of Nicotinic Acid and Nicotinic Acid Amide to Canine Black Tongue." J. Am. Chem. Soc., 59, 1967.

(8) Spies, T. D., Cooper, C., and Blackenhorn, M. A. (1938) "The Use of Nicotinic Acid in the Treatment of Pellagra." J. Am. Med. Assoc., 110, 622.

(9) Fouts, P. J., Helmer, O. M., Lepkovsky, S., and Jukes, T. H. (1937) "Treatment of Human Pellagra With Nicotinic Acid." Proc. Soc. Exptl. Biol. Med., 37, 405.

(10) Smith, D. T., Ruffin, J. M., and Smith, S. G. (1937) "Pellagra Successfully Treated With Nicotinic Acid: A Case Report." J. Am. Med. Assoc., 109, 2054.

(11) Krehl, W. A., Sarma, P. S., Teply, L. J., and Elvehjem, C. A. (1946) "Factors Affecting the Dietary Niacin and Tryptophane Requirement of the Growing Rat." J. Nutr., 31, 85.

(12) Harden, A., and Young, W. (1909) "The Hexosephosphate Formed by Yeast Juice From Hexose and Phosphate." Proc. Roy. Soc., (London) Ser. B 81, 528.

(13) Albus, H., Schlenk, F., and Von Euler, H. (1935) "Uber Die Co-Zymase." Z. Physiol. Chem., 237, I.

(14) Warburg, O., and Christian, W. (1936) "Pyridin, Der Wasserstoffubertragende Bestandteil Von Garungsfermenten (Pyridin-Nucleotide)." Biochem. Z., 287, 291.

(15) Warburg, O., and Christian W. (1931) "Uber Aktivierung Der Robisonschen Hexose-Mono-Phosphorsaure in Roten Blutzellen und die Gewinnung Aktivierender Fermentlosungen." Biochem. Z., 242, 206.

(16) Warburg, O., and Christian, W. (1934) "Co-Fermentproblem." Biochem. Z., 274, 112.

(17) Von Euler, H., Schlenk, F., and Vestrin, R. (1937) "Adenosin-Diphosphorsaure Aus Co-Zymase." Naturwissenschaften, 25, 318.

(18) Von Euler, H., Karrer, P., and Bocker, B. (1936) "Charakterisierung von Zuckerphosphorsauren und Konstitution der Pentose-Phosphorsaure Aus Cozymase." Helv. Chim. Acta., 19, 1060.

(19) Schlenk, F. (1937) "Die Einwirkung von Phosphoroxychlorid auf Cozymase." Naturwissenschaften, 25, 668.

(20) Schlenk, F. (1943) "Nicotinamide Riboside." Arch. Biochem., 3, 93.

(21) Warburg, O., and Christian, W. (1935) "Co-Fermentproblem." Biochem. Z., 275, 112 and 446.

(22) Pullman, M. E., San Pietro, A., and Colowick, S. P. (1954) "On the Structure of Reduced Diphosphopyridine Nucleotide." J. Biol. Chem., 206, 129.

(23) Loewus, F. A., Vennesland, B., and Harris, D. L. (1955) "The Site of Enzymatic Hydrogen Transfer in Diphosphopyridine Nucleotide." J. Am. Chem. Soc., 77, 3391.

(24) Rafter, G. W., and Colowick, S. P. (1954) "On the Structure of Reduced N-Methylnicotinamide." J. Biol. Chem., 209, 773.

(25) Florikin, M., and Stotz, E., Ed. (1973) Comprehensive Biochemistry, Vol. 13, 3rd Ed., Enzyme Nomenclature Recommendations - 1972, Elsevier Publishing Co., Inc., New York.

(26) Olivera, B. M., and Lehman, I. R. (1967) "Linkage of Polynucleotides Through Phosphodiester Bonds by an Enzyme From Escherichia coli." Proc. Natl. Acad. Sci. U. S., 57, 1426.

(27) Zimmerman, S. B., Little, J. W., Oshinsky, C. K., and Gellert, M. (1967) "Enzymatic Joining of DNA Strands: A Novel Reaction of Diphosphopyridine Nucleotide." Proc. Natl. Acad. Sci. U. S., 57, 1841.

(28) Olivera, B. M., and Lehman, I. R. (1967) "Diphosphopyridine Nucleotide: A Cofactor for the Polynucleotide-Joining Enzyme From Escherichia coli." Proc. Natl. Acad. Sci. U. S., 57, 1700.

(29) Gellert, M. (1967) "Formation of Covalent Circles of Lambda DNA by E. coli Extracts." Proc. Natl. Acad. Sci. U. S., 57, 148.

(30) Weiss, B., and Richardson, C. C. (1967) "Enzymatic Breakage and Joining of Deoxyribonucleic Acid. I. Repair of Single-Strand Breaks in DNA by an Enzyme System From Escherichia Coli Infected with T4 Bacteriophage." Proc. Natl. Acad. Sci. U. S., 57, 1021.

(31) Spadari, S., Ciarrocchi, G., and Falaschi, A. (1971) "Purification and Properties of a Polynucleotide Ligase From Human Cell Cultures." Eur. J. Biochem., 22, 75.

(32) Kessler, B. (1971) "Isolation, Characterization and Distribution of a DNA Ligase From Higher Plants." Biochim. Biophys. Acta. 240, 496.

(33) Collier, R. J., and Pappenheimer, A. M., Jr. (1964) "Studies on the Mode of Action of Diptheria Toxin. II. Effect of Toxin on Amino Acid Incorporation in Cell-Free Systems." J. Exptl. Med., 120, 1019.

(34) Collier, R. J. (1967) "Effect of Diptheria Toxin on Protein Synthesis: Inactivation of One of the Transfer Factors." J. Mol. Biol., 25, 83.

(35) Honjo, T., Nishizuka, Y., Hayaishi, O., and Kato, I. (1968) "Diptheria Toxin-Dependent Adenosine Diphosphate Ribosylation of Aminoacyl Transferase II and Inhibition of Protein Synthesis." J. Biol. Chem., 243, 3553.

(36) Gill, D. M., Pappenheimer, A. M., Jr., Brown, R., and Kurnick, J. T. (1969) "Studies on the Mode of Action of Diphtheria Toxin. VII. Toxin-Stimulated Hydrolysis of Nicotinamide Adenine Dinucleotide in Mammalian Cell Extracts." J. Exptl. Med., 129, 1.

(37) Goor, R. S., and Maxwell, E. S. (1970) "The Diphtheria Toxin-Dependent Adenosine Diphosphate Ribosylation of Rat Liver Aminoacyl Transferase II: General Characteristics and Mechanism of the Reaction." J. Biol. Chem., 245, 616.

(38) Honjo, T., Nishizuka, Y., Kato, I., and Hayaishi, O. (1971) "Adenosine Diphosphate Ribosylation of Aminoacyl Transferase II and Inhibition of Protein Synthesis by Diphtheria Toxin." J. Biol. Chem., 246, 4251.

(39) Gill, D. M., and Pappenheimer, A. M., Jr. (1971) "Structure-Activity Relationships in Diphtheria Toxin." J. Biol. Chem., 246, 1492.

(40) Collier, R. J., and Kandel, J. (1971) "Structure and Activity of Diphtheria Toxin. I. Thiol-Dependent Dissociation of a Fraction of Toxin into Enzymically Active and Inactive Fragments." J. Biol. Chem., 246, 1496.

(41) Nishizuka, Y., and Hayaishi, O. (1963) "Studies on the Biosynthesis of Nicotinamide Adenine Dinucleotide. I. Enzymic Synthesis of Niacin Ribonucleotides From 3-Hydroxyanthranilic Acid in Mammalian Tissues." J. Biol. Chem., 238, 3369.

(42) Hundley, J. M. (1954) "XIII. Requirements and Factors Influencing Animals and Man" in "Niacin" in The Vitamins, Vol. II, W. H. Sebrell, Jr. and R. S. Harris, Ed., Academic Press, Inc., N. Y., p. 578.

(43) Beadle, G. W., Mitchell, H. K., and Nyc, J. F. (1947) "Kynurenine as an Intermediate in the Formation of Nicotinic Acid From Tryptophane by Neurospora." Proc. Natl. Acad. Sci. U. S., 33, 155.

(44) Birnbaum, D., Boettcher, F., and Samsonova, I. A. (1972) "Nicotinic Acid Biosynthesis in Rhodotorula glutinus." Z. Allg. Mikrobiol., 12, 365.

(45) Desaty, D., and Vining, L. C. (1967) "Biosynthesis of Nicotinic Acid by Fusarium oxysporum Schlecht." Can. J. Biochem., 45, 1953.

(46) Audettte, R. C. S., Purvis, J. L., Worthen, L. R., and Youngken, H. W., Jr. (1973) "Pyridine Nucleotide Biosynthesis in Claviceps." Arch. Biochem. Biophys., 154, 173.

(47) Ahmad, F., and Moat, A. G. (1966) "Nicotinic Acid Biosynthesis in Prototrophs and Tryptophan Auxotrophs of Saccharomyces cerevisiae." J. Biol. Chem., 241, 775.

(48) Lingens, F., and Vollprecht, P. (1964) "Zur Biosynthese Der Nicotinsaure in Streptomyceten, Algen, Phycomyceten and Hefe." Z. Physiol. Chemie., 339, 64.

(49) Lingens, F., Vollprecht, P., and Gildemeister, V. (1966) "Zur Biosynthese Der Nicotinsaure in Xanthomonas- und Pseudomonas-Arten, Mycobacterium phlei und Rotalgen." Biochem. Z., 344, 462.

(50) Davis, C., Henderson, L. M., and Powell, D. (1951) "The Niacin-Tryptophan Relationship in the Metabolism of Xanthomonas Pruni." J. Biol. Chem., 189, 543.

(51) Yanofsky, C. (1954) "The Absence of a Tryptophan-Niacin Relationship in Escherichia coli and Bacillus subtilis." J. Bacteriol., 68, 577.

(52) Volcani, B. E., and Snell, E. E. (1948) "Specificity of the Response of Various Assay Organisms to Nicotinic Acid." Proc. Soc. Exptl. Biol. Med., 67, 511.

(53) Mothes, E., Gross, D., Schutte, H. R., and Mothes, K. (1961) "Zur Biosynthese der Nicotinsaure bei Mycobacterium tuberculosis (Stamm BCG)." Naturwissenschaften, 48, 623.

(54) del Rio-Estrada, C., and Patiño, H. (1962) "Nicotinic Acid Biosynthesis by Mycobacterium tuberculosis." J. Bacteriol., 84, 871.

(55) Isquith, A. J., and Moat, A. G. (1966) "Biosynthesis of NAD and Nicotinic Acid by Clostridium butylicum." Biochem. Biophys. Res. Commun., 22, 565.

(56) Isquith, A. J., and Moat, A. G. (1965) Bacteriol. Proc., 1965, 74.

(57) Scott, T. A., and Hussey, H. (1965) "The Biosynthesis of Nicotinic Acid in Serratia marcescens." Biochem. J., 96, 9c.

(58) Henderson, L. M., Someroski, J. F., Rao, D. R., Wu, P. H. L., Griffith, T., and Byerrum, R. U. (1959) "Lack of a Tryptophan-Niacin Relationship in Corn and Tobacco." J. Biol. Chem., 324, 93.

(59) Yang, K. S., and Waller, G. R. (1965) "Biosynthesis of the Pyridine Ring of Ricinine from Quinolinic Acid Glycerol and Aspartic Acid." Phytochem., 4, 881.

(60) Ortega, M. V., and Brown, G. M. (1960) "Precursors of Nicotinic Acid in Escherichia coli." J. Biol. Chem., 235, 2939.

(61) Andreoli, A. J., Ikeda, M., Nishizuka, Y., and Hayaishi, O. (1963) "Quinolinic Acid: A Precursor to Nicotinamide Adenine Dinucleotide in Escherichia coli." Biochem. Biophys. Res. Commun., 12, 92.

(62) Ogasawara, N., Chandler, J. L. R., Gholson, R. K., Rosser, R. J., and Andreoli, A. J. (1967) "Biosynthesis of Quinolinic Acid in a Cell-Free System." Biochim. Biophys. Acta, 141, 199.

(63) Chandler, J. L. R., Gholson, R. K., and Scott, T. A. (1970) "Studies on the de novo Biosynthesis of NAD in Escherichia coli. I. Labelling Patterns from Precursors." Biochim. Biophys. Acta, 222, 523.

(64) Chandler, J. L. R., and Gholson, R. K. (1972) "Studies on the Biosynthesis of NAD in Escherichia coli. III. Precursors of Quinolinic Acid in Vitro." Biochim. Biophys. Acta, 264, 311.

(65) Suzuki, N., Carlson, J. P., Griffith, G. R., and Gholson, R. K. (1973) "Studies on the de novo Biosynthesis of NAD in Escherichia coli. V. Properties of the Quinolinic Acid Synthetase System." Biochim. Biophys. Acta, 304, 309.

(66) Gross, D., Schutte, H. R., Hubner, G., and Mothes, K. (1963) "The Biosynthesis of Nicotinic Acid by Mycobacterium tuberculosis." Tetrahedron Letters, No. 9, 541.

(67) Leete, E. (1965) "Biosynthesis of Alkaloids." Science, 147, 1000.

(68) Albertson, J. N., Jr., and Moat, A. G. (1965) "Biosynthesis of Nicotinic Acid by Mycobacterium tuberculosis." J. Bacteriol., 89, 540.

(69) Feige, A., Gross, D., Zureck, A., and Schutte, H. R. (1970) "Beitrage zur Biosynthese von Nicotinsaure und Chinolinsaure bei Mycobacterium bovis Stamm BCG." Arch. Mikrobiol., 75, 80.

(70) Gross, D., Banditt, P., Zureck, A., and Schutte, H. R. (1968) "Chinolinsaure als Zwischenstufe in der Nicotinsaure-Biosynthese bei Mycobacterium bovis Stamm BCG." Z. Naturforsch, 23, 390.

(71) Konno, K., Oizumi, K., Shimizu, Y., Tamagawa, S., and Oka, S. (1966) "Niacin Metabolism in Mycobacteria: Mechanism of Excess Niacin Production by Human Tubercle Bacilli." Am. Rev. Resp. Dis., 93, 41.

(72) Scott, T. A., and Mattey, M. (1968) "The Incorporation of Formate into Nicotinic Acid by Clostridium butylicum." Biochem. J., 107, 606.

(73) Scott, T. A., Bellion, E., and Mattey, M. (1968) "The Synthesis of Nicotinic Acid from N-Formyl-L-Aspartate by Extracts of Clostridium butylicum." Biochem. J., 107, 23P.

(74) Scott, T. A., Bellion, E., and Mattey, M. (1969) "The Conversion of N-Formyl-L-Aspartate into Nicotinic Acid by Extracts of Clostridium butylicum." Eur. J. Biochem., 10, 318.

(75) Leete, E. (1969) "Alkaloid Biosynthesis," in Advances in Enzymology, Vol. 32, F. F. Nord, Ed., Interscience, N. Y., p. 373.

(76) Aronoff, S. (1956) "Experiments on the Biogenesis of the Pyridine Ring in Higher Plants." Plant Physiol., 31, 355.

(77) Leete, E. (1957) "The Biogenesis of the Pyridine Ring of Nicotine." Chem. Ind. (London), 1957, No. 38, 1270.

(78) Waller, G. R., and Henderson, L. M. (1961) "Biosynthesis of the Pyridine Ring of Ricinine." J. Biol. Chem., 236, 1186.

(79) Griffith, T., and Byerrum, R. U. (1959) "Biogenesis of Nicotine." Science, 129, 1485.

(80) Leete, E. (1958) "Biogenesis of Anabasine: Preferential Incorporation of Acetate into the Pyridine Ring." Chem. Ind. (London), 1958, No. 45, 1477.

(81) Griffith, T., Hellman, K. P., and Byerrum, R. U. (1960) "Studies on the Biogenesis of the Ring Systems of Nicotine." J. Biol. Chem., 235, 800.

(82) Waller, G. R., and Henderson, L. M. (1961) "Biosynthesis of the Pyridine Ring of Ricinine from Succinate and Other Labeled Compounds." Biochem. Biophys. Res. Commun., 5, 5.

(83) Juby, P. F., and Marion, L. (1961) "The Biogenesis of Ricinine." Biochem. Biophys. Res. Commun., 5, 461.

(84) Anwar, R. A., Griffith, T., and Byerrum, R. U. (1961) "Biosynthesis of Ricinine in Ricinus communis L." Federation Proc., 20, 374.

(85) Yang, K. S., Gholson, R. K., and Waller, G. R. (1965) "Studies on Nicotine Biosynthesis." J. Am. Chem. Soc., 87, 4184.

(86) Griffith, T., and Byerrum, R. U. (1963) "Biosynthesis of the Pyridine Ring of Nicotine from Succinate and Acetate." Biochem. Biophys. Res. Commun., 10, 293.

(87) Leete, E., and Friedman, A. R. (1964) "Biosynthesis of the Nicotiana Alkaloids. X. The Incorporation of Glycerol-2-$^{14}$C into the Pyridine Ring of Anabasine." J. Am. Chem. Soc., 86, 1224.

(88) Juby, P. F., and Marion, L. (1963) "The Origin of Carbon Atoms 2, 3, and 7 of Ricinine." Can. J. Chem., 41, 117.

(89) Essery, J. M., Juby, P. F., Marion, L., and Trumbull, J. (1963) "The Origin of Carbon Atoms 4, 5, and 6 of Ricinine." Can. J. Chem., 41, 1142.

(90) Johns, S. R., and Marion, L. (1966) "The Role of Aspartic Acid in the Biosynthesis of Ricinine." Can. J. Chem., 44, 23.

(91) Jackanicz, T. M., and Byerrum, R. U. (1966) "Incorporation of Aspartate and Malate into the Pyridine Ring of Nicotine." J. Biol. Chem., 241, 1296.

(92) Fleeker, J., and Byerrum, R. U. (1967) "Incorporation of Glyceraldehyde into the Pyridine Ring of Nicotine." J. Biol. Chem., 242, 3042.

(93) Waller, G. R., and Yang, K. S. (1967) "The Origin of the Nitrogen Atoms of Ricinine Produced by Ricinus communis L." Phytochem., 6, 1637.

(94) Hadwiger, L. A., Badiei, S. E., Waller, G. R., and Gholson, R. K. (1963) "Quinolinic Acid as a Precursor of Nicotinic Acid and its Derivatives in Plants." Biochem. Biophys. Res. Commun., 13, 466.

(95) Gross, D., Banditt, P., Kurbatow, J. W., and Schutte, H. R. (1968) "Zur Biosynthese des Ricinins." Z. Pflanzenphysiol., 58, 410.

(96) Frost, G. M., Yang, K. S., and Waller, G. R. (1967) "Nicotinamide Adenine Dinucleotide as a Precursor of Nicotine in Nicotiana rustica L." J. Biol. Chem., 242, 887.

(97) Kaplan, N. O. (1961) "Metabolic Pathways Involving Niacin and its Derivatives," in Metabolic Pathways, Vol. II, D. M. Greenberg, Ed., Academic Press, Inc., N. Y., Ch. 22.

(98) Preiss, J., and Handler, P. (1958) "Biosynthesis of Diphosphopyridine Nucleotide. I. Identification of Intermediates." J. Biol. Chem., 233, 488.

(99) Preiss, J., and Handler, P. (1958) "Biosynthesis of Diphosphopyridine Nucleotide. II. Enzymatic Aspects." J. Biol. Chem., 233, 493.

(100) Gholson, R. K. (1966) "The Pyridine Nucleotide Cycle." Nature, 212, 933.

(101) Joshi, J. G., and Handler, P. (1962) "Purification and Properties of Nicotinamidase from Torula cremoris." J. Biol. Chem., 237, 929.

(102) Sarma, D. S. R., Rajalakshmi, S., and Sarma, P. S. (1961) "Deamidation of Nicotinamide and NMN." Biochem. Biophys. Res. Commun., 6, 389.

(103) Packman, P. M., and Jakoby, W. B. (1965) "Quinolinate Phosphoribosyltransferase." Biochem. Biophys. Res. Commun., 18, 710.

(104) Imsande, J. (1961) "Pathway of Diphosphopyridine Nucleotide Biosynthesis in Escherichia coli." J. Biol. Chem., 236, 1494.

(105) Sundaram, T. K. (1967) "Biosynthesis of Nicotinamide-Adenine Dinucleotide in Escherichia coli." Biochim. Biophys. Acta, 136, 586.

(106) Spencer, R. L., and Preiss, J. (1967) "Biosynthesis of Diphosphopyridine Nucleotide: The Purification and the Properties of Diphosphopyridine Nucleotide Synthetase from Escherichia coli B." J. Biol. Chem., 242, 385.

(107) Dahmen, W., Webb, B., and Preiss, J. (1967) "The Deamido-Diphosphopyridine Nucleotide and Diphosphopyridine Nucleotide Pyrophosphorylases of Escherichia coli and Yeast." Arch. Biochem. Biophys., 120, 440.

(108) Tritz, G. J. (1970) Genetic Analysis of Mutants of E. coli Defective in the Biosynthesis of Nicotinamide Adenine Dinucleotide. Ph.D. Dissertation, the University of Texas at Houston.

(109) Andreoli, A. J., Okita, T. W., Bloom, R., and Grover, T. A. (1972) "The Pyridine Nucleotide Cycle: Presence of a Nicotinamide Mononucleotide-specific Glycohydrolase in Escherichia coli." Biochem. Biophys. Res. Commun., 49, 264.

(110) Schwartz, N. M., Kaplan, N. O., and Frech, M. E. (1956) "Significance of 'Heat-Activated' Enzymes." Science, 123, 50.

(111) Gholson, R. K., Tritz, G. J., Matney, T. S., and Andreoli, A. J. (1969) "Mode of Nicotinamide Adenine Dinucleotide Utilization by Escherichia coli." J. Bacteriol., 99, 895.

(112) Andreoli, A. J., Grover, T., Gholson, R. K., and Matney, T. S. (1969) "Evidence for a Functional Pyridine Nucleotide Cycle in Escherichia coli." Biochim. Biophys. Acta, 192, 539.

(113) Lundquist, R., and Olivera, B. M. (1973) "Pyridine Nucleotide Metabolism in Escherichia coli. II. Niacin Starvation." J. Biol. Chem., 248, 5137.

(114) McLaren, J., Ngo, D. T. C., and Olivera, B. M. (1973) "Pyridine Nucleotide Metabolism in Escherichia coli. III. Biosynthesis from Alternative Precursors in Vivo." J. Biol. Chem., 248, 5144.

(115) Manlapaz-Fernandez, P., and Olivera, B. M. (1973) "Pyridine Nucleotide Metabolism in Escherichia coli. IV. Turnover." J. Biol. Chem., 248, 5150.

(116) Konno, K., Oizumi, J., and Oka, S. (1965) "Biosynthesis of Niacin Ribonucleotide from Quinolinic Acid by Mycobacteria." Nature, 205, 874.

(117) Kasarov, L. B., and Moat, A. G. (1972) "Metabolism of Nicotinamide Adenine Dinucleotide in Human and Bovine Strains of Mycobacterium tuberculosis." J. Bacteriol., 110, 600.

(118) Packman, P. M., and Jakoby, W. B. (1965) "Crystalline Quinolinate Phosphoribosyltransferase." J. Biol. Chem., 240, PC4107.

(119) Ahmad, F., and Moat, A. G. (1965) "Nicotinic Acid Biosynthesis in Prototrophs and Certain Yeast Auxotrophs." Federation Proc., 24, 480.

(120) Kahn, V., and Blum, J. J. (1968) "Studies of Nicotinic Acid Metabolism in Astasia longa. II. Pathway and Regulation of Nicotinamide Adenine Dinucleotide Biosynthesis in Cell-free Preparations." J. Biol. Chem., 243, 1448.

(121) Ohtsu, E., Ichiyama, A., Nishizuka, Y., and Hayaishi, O. (1967) "Pathways of Nicotinamide Adenine Dinucleotide Biosynthesis in Nicotinic Acid or Nicotinamide Requiring Microorganisms." Biochem. Biophys. Res. Commun., 29, 635.

(122) Yu, C. K., and Dietrich, L. S. (1972) "Purification and Properties of Yeast Nicotinamide Adenine Dinucleotide Synthetase." J. Biol. Chem., 247, 4794.

(123) Sarma, D. S. R., Rajalakshmi, S., and Sarma, P. S. (1964) "Studies on the Enzymes Involved in Nicotinamide Adenine Dinucleotide Metabolism in Aspergillus niger." Biochim. Biophys. Acta, 81, 311.

(124) Tanigawa, Y., Shimoyama, M., Dohi, K., and Ueda, I. (1972) "Purification and Properties of Nicotinamide Deamidase from Flavobacterium peregrinum." J. Biol. Chem., 247, 8036.

(125) Sundaram, T. K., Rajagopalan, K. V., Pichappa, C. V., and Sarma, P. S. (1960) "Studies on Pyridine Nucleotide Biosynthesis." Biochem. J., 77, 145.

(126) Rajagopalan, K. V., Sundaram, T. K., and Sarma, P. S. (1960) "Metabolism of Nicotinamide and Nicotinic Acid in Corcyra cephalonica St." Biochem. J., 74, 355.

(127) Sundaram, T. K. Rajagopalan, K. V., and Sarma, P. S. (1958) "Metabolism of Nicotinamide and Nicotinic Acid in Neurospora crassa." Biochem. J., 70, 196.

(128) Luchka, E., and Johnson, W. J. (1965) "Inhibition of Nicotinamide Deamidase Activity in Bacteria and Pigeon Liver." Federation Proc., 24, 480.

(129) Grossowicz, N., and Halpern, Y. S. (1956) "Inhibition of Nicotinamidase Activity in Cell-free Extracts of Mycobacterium phlei by 3-Acetylpyridine." Biochim. Biophys. Acta, 20, 576.

(130) Halpern, Y. S., and Grossowicz, N. (1957) "Hydrolysis of Amides by Extracts from Mycobacteria." Biochem. J., 65, 716.

(131) Shimoyama, M., Tanigawa, Y., Ito, T., Murashima, R., Ueda, I., and Tomoda, T. (1971) "Nicotinamide Deamidation of Microorganisms in Rat Stomach." J. Bacteriol., 108, 191.

(132) Hughes, D. E., and Williamson, D. H. (1952) "The Synthesis of Cozymase from Nicotinic Acid and its Derivatives by Lactobacillus arabinosus 17-5." Biochem. J., 51, 330.

(133) Joshi, J. G., and Handler, P. (1960) "Biosynthesis of Trigonelline." J. Biol. Chem., 235, 2981.

(134) Hughes, D. E., and Williamson, D. H. (1953) "The Deamidation of Nicotinamide by Bacteria." Biochem. J., 55, 851.

(135) Imsande, J. (1964) "A Comparative Study of the Regulation of Pyridine Nucleotide Formation." Biochim. Biophys. Acta, 82, 445.

(136) Gholson, R. K., and Kori, J. (1964) "Enzyme Repression in the Regulation of Nicotinamide Adenine Dinucleotide Biosynthesis in Bacillus subtilis." J. Biol. Chem., 239, PC2399.

(137) Ogasawara, N., and Gholson, R. K. (1966) "The Non-identity of Yeast Nicotinic Acid Mononucleotide Pyrophosphorylase and Nicotinic Acid Mononucleotidase." Biochim. Biophys. Acta, 118, 422.

(138) Kosaka, A., Spivey, H. O., and Gholson, R. K. (1971) "Nicotinate Phosphoribosyltransferase of Yeast." J. Biol. Chem., 246, 3277.

(139) Dudley, M. A., and Willett, H. P. (1965) "Nicotinamide Adenine Dinucleotide Synthesis by Mycobacterium tuberculosis var. bovis." Proc. Soc. Exptl. Biol. Med., 119, 807.

(140) Friedmann, H. C., and Garstki, C. (1973) "The Pyridine Nucleotide Cycle: Presence of a Nicotinamide Mononucleotide-specific Amidohydrolase in Propionibacterium shermanii." Biochem. Biophys. Res. Commun., 50, 54.

(141) Fyfe, J. A., and Friedmann, H. C. (1969) "Vitamin $B_{12}$ Biosynthesis: Enzyme Studies on the Formation of the α-Glycosidic Nucleotide Precursor." J. Biol. Chem., 244, 1659.

(142) Imai, T. (1971) "Studies on Pyridine Nucleotides. I. Nicotinamide Mononucleotide Amidohydrolase (NMN Deamidase) and a New Pathway for 'Salvage Synthesis' of Nicotinamide-adenine Dinucleotide." Seikagaku, 43, 336; Chem. Abstr., 75, 105066c.

(143) Gopinathan, K. P., Sirsi, M., and Ramakrishnan, T. (1963) "Nicotinamide-adenine Nucleotides of Mycobacterium tuberculosis $H_{37}$ Rv." Biochem. J., 87, 444.

(144) Mather, I. H., and Knight, M. (1972) "A Heat-Stable Nicotinamide-Adenine Dinucleotide Glycohydrolase from Pseudomonas putida KB1: Partial Purification and Some Properties of the Enzyme and an Inhibitory Protein." Biochem. J., 129, 141.

(145) Fehrenbach, F. J. (1969) "Gel-Filtration Behaviour and Molecular Weight of NAD-Glycohydrolase (EC 3.2.2.5) from Streptococci in Column Chromatography on Sephadex Gels." J. Chromatog., 41, 43.

(146) Kaplan, N. O., Colowick, S. P., and Nason, A. (1951) "Neurospora Diphosphopyridine Nucleotidase." J. Biol. Chem., 191, 473.

(147) Kern, M., and Natale, R. (1958) "A Diphosphopyridine Nucleotidase and its Protein Inhibitor from Mycobacterium butyricum." J. Biol. Chem., 231, 41.

(148) Green, A. A., and Hughes, W. L. (1955) "Protein Fractionation on the Basis of Solubility in Aqueous Solutions of Salts and Organic Solvents," in Methods in Enzymology, Vol. I, S. P. Colowick and N. O. Kaplan, Ed., Academic Press, N. Y., p. 67.

(149) Kasarov, L. B., and Moat, A. G. (1973) "Metabolism of the Pyridine Nucleotides Involved in Nicotinamide Adenine Dinucleotide Biosynthesis by Clostridium butylicum." J. Bacteriol., 115, 35.

(150) Nakajima, Y., Fukunaga, N., Sasaki, S., and Usami, S. (1973) "Purification and Properties of NADP Pyrophosphatase from Proteus vulgaris." Biochim. Biophys. Acta, 293, 242.

(151) Fehrenbach, F. J. (1971) "Identity of Streptolysin-O and NAD-Glycohydrolase (EC 3.2.2.5)." Z. Naturforsch, 26, 1336.

(152) Fehrenbach, F. J. (1971) "Reinigung und Kristallisation der NAD-Glykohydrolase aus C-Streptokokken." Eur. J. Biochem., 18, 94.

(153) Waller, G. R., Yang, K. S., Gholson, R. K., and Hadwiger, L. A. (1966) "The Pyridine Nucleotide Cycle and Its Role in the Biosynthesis of Ricinne by Ricinus communis L." J. Biol. Chem., 241, 4411.

(154) Hiles, R. A., and Byerrum, R. U. (1969) "The Role of the Pyridine Nucleotide Cycle in the Biosynthesis of Ricinine." Phytochem., 8, 1927.

(155) Ryrie, I. J., and Scott, K. J. (1969) "Nicotinate, Quinolinate and Nicotinamide as Precursors in the Biosynthesis of Nicotinamide-Adenine Dinucleotide in Barley." Biochem. J., 115, 679.

(156) Godavari, H. R., and Waygood, E. R. (1970) "Nicotinamide Adenine Dinucleotide Metabolism in Plants. I. Intermediates of Biosynthesis in Wheat Leaves and the Effect of Benzimidazole." Can. J. Bot., 48, 2267.

(157) Lundquist, R., and Olivera, B. M. (1971) "Pyridine Nucleotide Metabolism in Escherichia coli. I. Exponential Growth." J. Biol. Chem., 246, 1107.

(158) Tritz, G. J., and Chandler, J. L. R. (1973) "Recognition of a Gene Involved in the Regulation of Nicotinamide Adenine Dinucleotide Biosynthesis." J. Bacteriol., 114, 128.

(159) Imsande, J., and Pardee, A. B. (1962) "Regulation of Pyridine Nucleotide Biosynthesis in Escherichia coli." J. Biol. Chem., 237, 1305.

(160) Saxton, R. E., Rocha, V., Rosser, R. J., Andreoli, A. J., Shimoyama, M., Kosaka, A., Chandler, J. L. R., and Gholson, R. K. (1968) "A Comparative Study of the Regulation of Nicotinamide-Adenine Dinucleotide Biosynthesis." Biochim. Biophys. Acta, 156, 77.

(161) Witholt, B. (1972) "Method for Isolating Mutants Overproducing Nicotinamide Adenine Dinucleotide and Its Precursors." J. Bacteriol., 109, 350.

(162) Smith, L. D., and Gholson, R. K. (1969) "Allosteric Properties of Bovine Liver Nicotinate Phosphoribosyltransferase." J. Biol. Chem., 244, 68.

(163) Imsande, J. (1964) "A Cross-Linked Control System. I. Properties of a Triphosphate-dependent Nicotinic Acid Mononucleotide Pyrophosphorylase from Bacillus subtilis." Biochim. Biophys. Acta, 85, 255.

(164) Imsande, J., and Prestidge, L. S. (1964) A Cross-Linked Control System. II. Control of Pyridine Nucleotide Formation in Vivo." Biochim. Biophys. Acta, 85, 265.

(165) Gholson, R. K. (1968) "Regulation of NAD Biosynthesis via the Pyridine Nucleotide Cycle." J. Vitamin, 14, 114.

(166) Imsande, J. (1961) "Pathway of Diphosphopyridine Nucleotide Biosynthesis in Escherichia coli." J. Biol. Chem., 236, 1494.

(167) Calbreath, D. F., and Joshi, J. G. (1971) "Inhibition of Nicotinamidase by Nicotinamide Adenine Dinucleotide." J. Biol. Chem., 246, 4334.

(168) Pardee, A. B., Benz, E. J., Jr., St. Peter, D. A., Krieger, J. N., Meuth, M., and Trieshmann, H. W., Jr. (1971) "Hyperproduction and Purification of Nicotinamide Deamidase, A Microconstitutive Enzyme of Escherichia coli." J. Biol. Chem., 246, 6792.

(169) Gopinathan, K. P., Sirsi, M., and Vaidyanathan, C. S. (1964) "Nicotinamide-Adenine Dinucleotide Glycohydrolase of Mycobacterium tuberculosis $H_{37}Rv$." Biochem. J., 41, 277.

(170) Gopinathan, K. P., Ramakrishnan, T., and Vaidyanathan, C. S. (1966) "Purification and Properties of an Inhibitor for Nicotinamide-Adenine Dinucleotide from Mycobacterium tuberculosis $H_{37}Rv$." Arch. Biochem. Biophys., 113, 376.

(171) Suzuki, N. (1968) "Partial Purification and Properties of Nicotinamide-Adenine Dinucleotide Nucleosidase and Interaction Between Enzyme and Its Protein Inhibitor." J. Vitamin, 14, 121.

(172) Olivera, B. M., and Lundquist, R. (1971) "DNA Synthesis in Escherichia coli in the Presence of Cyanide." J. Mol. Biol., 57, 263.

(173) Tritz, G. J., Matney, T. S., Chandler, J. L. R., and Gholson, R. K. (1970) "Chromosomal Location of the C Gene Involved in the Biosynthesis of Nicotinamide Adenine Dinucleotide in Escherichia coli K-12." J. Bacteriol., 104, 45.

(174) Chandler, J. L. R., and Gholson, R. K. (1972) "De Novo Biosynthesis of Nicotinamide Adenine Dinucleotide in Escherichia coli: Excretion of Quinolinic Acid by Mutants Lacking Quinolinate Phosphoribosyl Transferase." J. Bacteriol., 111, 98.

(175) Tritz, G. J., Matney, t. S., and Gholson, R. K. (1970) "Mapping of the nad B Locus Adjacent to a Previously Undescribed Purine Locus in Escherichia coli K-12." J. Bacteriol., 102, 377.

(176) Taylor, A. L. (1970) "Current Linkage Map of Escherichia coli." Bacteriol. Rev., 34, 155.

(177) Chandler, J. L. R., and Gholson, R. K. (1972) "Studies on the de novo Biosynthesis of NAD in Escherichia coli. II. Quantitative Method for Isolating Quinolinic Acid from Biological Materials." Anal. Biochem., 48, 529.

(178) Chandler, J. L. R. (1969) Quinolinic Acid Biosynthesis in Escherichia coli, Ph.D. Thesis, Oklahoma State University, Department of Biochemistry, Stillwater, Oklahoma.

(179) Layne, E. (1957) "Spectrophotometric and Turbidimetric Methods for Measuring Proteins," in Methods in Enzymology, Vol. III, S. P. Colowick and N. O. Kaplan, Ed., Academic Press, Inc., N. Y., p. 447.

(180) Kerr, T. J., and Tritz, G. J. (1973) "Cross-Feeding of Escherichia coli Mutants Defective in the Biosynthesis of Nicotinamide Adenine Dinucleotide." J. Bacteriol., 115, 982.

(181) Feldberg, R. S., Young, H., Morrice, L. A. F., and Keir, H. M. (1972) "Some Observations on the Cofactor Requirement for Partially Purified DNA Ligase from Escherichia coli." FEBS Letters, 27, 345.

(182) Yates, R. A., and Pardee, A. B. (1956) "Pyrimidine Biosynthesis in Escherichia coli." J. Biol. Chem., 221, 743.

(183) Cuatrecasas, P., and Anfinsen, C. B. (1971) "Affinity Chromatography," in Methods in Enzymology, Vol. XXII, W. B. Jacoby, S. P. Colowick and N. O. Kaplan, Ed., Academic Press, Inc., N. Y., p. 345.

(184) Cuatrecasas, P., and Parikh, I. (1972) "Adsorbents for Affinity Chromatography: Use of N-Hydroxysuccinimide Esters of Agarose." Biochemistry, 11, 2291.

(185) Failla, D., and Santi, D. V. (1973) "A Simple Method for Quantitating Ligands Covalently Bound to Agarose Beads." Anal. Biochem., 52, 363.

(186) Wang, C. H., and Willis, D. L. (1965) "Measurement of Radioactivity by the Liquid (Internal-Sample) Scintillation Method," in Radiotracer Methodology in Biological Science, Prentice-Hall, Inc., Englewood Cliffs, N. J., p. 104.

(187) Weitzman, P. D. J. (1969) "Citrate Synthase from Escherichia coli," in Methods in Enzymology, Vol. XIII, J. M. Lowenstein Ed., Academic Press, Inc., N. Y., p. 22.

(188) Davis, B. D., Gilvarg, C., and Mitsuhashi, S. (1955) "Enzymes of Aromatic Biosynthesis," in Methods in Enzymology, Vol. II, S. P. Colowick and N. O. Kaplan, Ed., Academic Press, Inc., N. Y., p. 300.

(189) King, T. P. (1972) "Separation of Proteins by Ammonium Sulfate Gradient Solubilization." Biochemistry, 11, 367.

(190) Polson, A., Potgieter, G. M., Largier, J. F., Mears, G. E. F., and Joubert, F. J. (1964) "The Fractionation of Protein Mixtures by Linear Polymers of High Molecular Weight." Biochim. Biophys. Acta, 82, 463.

(191) Jones, R. G., and McLaughlin, K. C. (1963) "2, 3-Pyrazinedicarboxylic Acid," in Org. Syn. Collective, Vol. IV, N. Rabjohn, Ed., p. 824.

(192) Rutter, W. J., Hunsley, J. R., Groves, W. E., Calder, J., Rajkumar, T. V., and Woodfin, B. M. (1966) "Fructose Diphosphate Aldolase," in Methods in Enzymology, Vol. IX, W. A. Wood, Ed., Academic Press, N. Y., p. 479.

VITA

Gary Randel Griffith

Candidate for the Degree of

Doctor of Philosophy

Thesis: PURIFICATION AND SOME PROPERTIES OF THE QUINOLINATE SYNTHETASE SYSTEM IN ESCHERICHIA COLI

Major Field: Biochemistry

Biographical:

Personal Data: Born in Oklahoma City, Oklahoma, December 1, 1946, the son of Mr. and Mrs. H. R. Griffith.

Education: Graduated from Guthrie High School in May, 1964; received Bachelor of Science degree in Biochemistry from Oklahoma State University in 1969; completed requirements for the Doctor of Philosophy degree at Oklahoma State University in December, 1973.

Professional Experience: Laboratory technician, Department of Biochemistry, Oklahoma State University, September, 1967 to May, 1969; Graduate Research Assistant, Department of Biochemistry, Oklahoma State University, June, 1969 to December, 1973.

Professional and Honorary Societies: American Scientific Affiliation, Phi Lambda Upsilon.