STUDIES ON THE BIOSYNTHESIS OF SOME PYRIDINE CARBOXYLIC ACIDS

By

Sue Ellen Badiei

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Oklahoma State University

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STUDIES ON THE BIOSYNTHESIS OF SOME PYRIDINE CARBOXYLIC ACIDS

Thesis Approved:

Robert K. Gholson
Thesis Adviser

Kurt E. Ekner

D. D. Durham
Dean of the Graduate College

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CHAPTER I

INTRODUCTION

Since the discovery in 1945 that tryptophan can replace the niacin required in the diet of the rat (63), there has been an accumulation of considerable information on the detailed pathways leading to nicotinic acid, quinolinic acid, and picolinic acid in mammals, higher plants, and bacteria.

The major metabolic pathway for the formation of nicotinic acid in mammals, Neurospora, and perhaps the chicken has been found to be from tryptophan via 3-hydroxyanthranilic acid and quinolinic acid, and is referred to as the "quinolinate pathway." Under certain conditions, decarboxylation of the primary oxidation product of 3-hydroxyanthranilic acid occurs giving rise to picolinic acid.

"Most available evidence supports the view that in higher plants and most bacteria, nicotinic acid is formed by the condensation of glycerol (or a closely related compound) and a four carbon dicarboxylic acid" (104). Moreover, there is evidence supporting the view that quinolinic acid is an intermediate in this pathway (104). One of the objectives of the present study is to determine whether or not quinolinic acid acts as a precursor of nicotinic acid in corn.

There is little information at this time on the synthesis of pyridine carboxylic acids in lower animals. However, studies on the metabolism of N-methylpicolinic acid, which has been found in large quantities

in marine invertebrates, may lead to further information. In view of this and the fact that tryptophan is converted to picolinic acid in mammals, the other objective of the present study is to determine whether or not tryptophan or N-methylpyridine is a precursor of N-methylpicolinic acid (homarine) in the lobster.

CHAPTER II

LITERATURE REVIEW

A. Quinolinate Pathway for Niacin Biosynthesis

In 1945, Krehl et al. (63) found that tryptophan could replace the nicotinic acid required in the diet of the rat; in 1946, Rosen et al. (89) reported a significant drop in nicotinic acid excretion by rats which were fed a tryptophan-deficient diet; and in 1949, Heidelberger et al. (45) demonstrated by isotopic experiments that the carbon-3 of the indole ring of tryptophan becomes the carboxyl-carbon of nicotinic acid. Hence, it was established with certainty that tryptophan is converted to nicotinic acid in the rat. However, nicotinic acid does not appear to be a direct product of tryptophan catabolism since evidence presented by Nishizuka and Hayaishi (82) excludes free niacin as an intermediate in the conversion of tryptophan to nicotinamide adenine dinucleotide (NAD). Nicotinic acid is converted to NAD by another pathway discovered by Preiss and Handler (87). The enzyme NADase (40) cleaves NAD releasing nicatinamide which is in turn deaminated to nicotinic acid (86) accounting for the formation of radioactive nicotinic acid obtained in experiments utilizing administration of radioactive tryptophan and its metabolites.

In mammals, the fungus Neurospora, and perhaps Xanthomonas pruni and the chicken, the eventual formation of nicotinic acid occurs through the following known intermediates: N-formylkynurenine, kynurenine,

3-hydroxy-kynurenine, 3-hydroxyanthranilic acid, 2-amino-3-acroleylfumaric acid (Compound I), quinolinic acid, nicotinic acid mononucleotide, deamidonicotinamide adenine dinucleotide, nicotinamide adenine dinucleotide, and nicotinamide.

1. Formation of Kynurenine

The first reactions are the conversion of L-tryptophan to formylkynurenine and formylkynurenine to kynurenine. Beadle et al. (12) reported in 1947 the utilization of kynurenine for growth by mutants of Neurospora crassa; and in 1949, Heidelberger et al. (45) isolated ^{14}C-labeled kynurenine from the urine of rats which had been administered tryptophan-3-^{14}C by stomach tube. The next year, Knox and Mehler (59) showed that kynurenine will substitute for nicotinic acid in rat growth and demonstrated the formation of kynurenine from tryptophan in liver homogenates under aerobic conditions. They described the initial oxidation of tryptophan to formylkynurenine to be catalyzed by a "physiological coupled oxidation system consisting of a tryptophan peroxidase and an oxidase which forms peroxide" (59) and named the enzyme tryptophan peroxidase-oxidase.

In 1962, Greengard and Feigelson (32) purified this enzyme system 1000-fold, obtaining the apoenzyme tryptophan pyrrolase and its cofactor hematin. Mehler and Knox (74) identified and partially purified formylase which catalyzes the hydrolysis of formylkynurenine to kynurenine and formate. They found formylkynurenine to accumulate in the absence of formylase during the initial oxidation of tryptophan by the liver enzyme.

2. Formation of 3-Hydroxykynurenine

In 1955, Henderson et al. (51) administered hydroxykynurenine to

rats and noted an enhanced excretion of quinolinic acid and nicotinic acid. They also showed that hydroxykynurenine will substitute for niacin in the diet of the rat (52).

In 1956, deCastro, Price and Brown (24) demonstrated *in vitro* in liver mitochondria of cats the hydroxylation of L-kynurenine to form 3-hydroxy-kynurenine by a NADPH dependent oxygen requiring hydroxylase. The enzyme was partially purified in 1957 (90).

3. Formation of 3-Hydroxyanthranilic Acid

The next known intermediate in the pathway from tryptophan to nicotinic acid is 3-hydroxyanthranilic acid. The kynureninase which catalyzes the removal of the side chain from 3-hydroxykynurenine to yield 3-hydroxyanthranilic acid was studied in 1941 by Kotake and Nakayama (61). They reported a pyridoxal phosphate requirement for this enzyme. In 1952 and 1953, studies *in vitro* showed that the side chain of 3-hydroxykynurenine is removed as alanine (44, 76). Furthermore, Gholson *et al.* (30) showed in 1959 by isotopic studies that the side chain is removed as alanine or its metabolic equivalent by administering DL-tryptophan-α-^{14}C to living rats and determining the labeling pattern of some nonessential amino acids which they isolated.

In 1948, Alberto *et al.* (8) administered 3-hydroxyanthranilic acid to niacin deficient rats and observed an increase in the excretion of pyridine carboxylic acids and their metabolites. Mitchell *et al.* (78) observed that rats can utilize 3-hydroxyanthranilic acid as a substitute for their niacin requirements. It was also established that 3-hydroxyanthranilic acid is an intermediate in the tryptophan to niacin pathway in *Neurospora* (14, 43, 77, 107). Davis *et al.* (22) observed that 3-hydroxyanthranilic acid is capable of replacing nicotinic acid for

growth in Xanthomonas pruni.

Early isotopic evidence for the intermediate role played by 3-hydroxyanthranilic acid was obtained in 1954 by Hankes and Urivetsky (42) who injected a rat which had been on an 18% casein diet for several months with ^{14}C-carboxyl-labeled 3-hydroxyanthranilic acid. They isolated ^{14}C-labeled N-methylnicotinamide from the urine.

4. Formation of Compound I and Quinolinic Acid

In 1950, Bokman and Schweigert (13) reported that the immediate oxidation product of 3-hydroxyanthranilic acid is a very unstable aliphatic compound detectable by its high absorption at 360 mμ. Suhadolnik et al. (94) observed that the liver extracts of 10 mammalian species oxidized ^{14}C-carboxyl labeled 3-hydroxyanthranilic acid to this oxidation product--Compound I. Wiss and Weber (102) prepared derivatives of Compound I and observed their infrared spectra. Their results indicated the structure of Compound I to be 1-amino-4-formyl-1,3-butadiene-1,2-dicarboxylic acid. The absorption spectrum, the oxidation state, and the major product of Compound I also support this structure (50).

In 1946, Singal et al. (92) found a compound in the urine of rats after tryptophan administration which yielded nicotinic acid upon heating with acid. Henderson and co-workers (48, 49) reported in 1949 that this substance was probably quinolinic acid. They demonstrated a marked increase of quinolinic acid in rat urine following tryptophan feeding. Henderson (46) further reported that high levels of quinolinic acid can relieve nicotinic acid deficiency in rats and that quinolinic acid is rather poorly utilized by some Neurospora mutants as a source of nicotinic acid and accumulates in the medium of other Neurospora mutants.

He discussed the possibility that quinolinic acid may be an intermediate in the tryptophan to niacin pathway.

Furthermore, Davis et al. (22) found that quinolinic acid replaces nicotinic acid for growth of Xanthomonus pruni. On the other hand, Krehl et al. (62) and Bonner and Yanofsky (15) proposed that quinolinic acid is not an intermediate in tryptophan metabolism due to the inefficiency of exogenous quinolinic acid in replacing nicotinic acid.

In 1957, Hankes and Segel (41) injected rats with quinolinic acid labeled with tritium in the 4, 5, and 6 positions and isolated N-methylnicotinamide-^{3}H from the urine; and in 1960, Wilson and Henderson (101) injected quinolinic acid-^{3}H into developing chick embryos and isolated nicotinic acid-^{3}H. These experiments established with certainty that quinolinic acid is a precursor of nicotinic acid in the rat and the chicken.

It was reported in 1954 by Long et al. (72) that Compound I is converted almost quantitatively and spontaneously into quinolinic acid. The enzyme involved in this conversion was first purified by Stevens and Henderson (93) in 1949 from ox liver; and in 1962, Vescia and dePrisco (96) succeeded in purifying it 3500-fold. It is a "phenyolytic oxygenase," i.e., it catalyzes the oxidation of 3-hydroxyanthranilic acid with the uptake of one mole of oxygen per mole of substrate, and has a ferrous iron requirement (50). 3-Hydroxyanthranilic oxidase appears to be present in most vertebrates but not in the invertebrates studied (64, 65, 94).

The conversion of Compound I to quinolinic acid might proceed via "Schiff's base formation from the cis-form of the aldehyde amine" (50). There is no report of an enzyme which catalyzes the isomerization at

the double bond which is essential to bring the amino and carboxyl groups close together (50).

Mehler (73) was the first to report the formation of picolinic acid from Compound I catalyzed by an enzyme concentrated from beef liver extracts. He stated that Compound I can go in two directions--spontaneously to quinolinic acid and enzymatically to picolinic acid. In 1964, Lan (64) obtained an 83-fold purification of this picolinic carboxylase from beef liver.

Isotopic evidence for these two reactions was obtained in 1957 by Suhadolnik et al. (94) and Mehler and May (75). They reported that the decarboxylase which competes with the non-enzymatic reaction leading to quinolinic acid catalyzed the loss of $^{14}CO_2$ from Compound I which in turn had been formed from 3-hydroxyanthranilic acid-carboxyl-^{14}C. The final product was non-radioactive picolinic acid. The same series of reactions yielded ^{14}C-labeled quinolinic acid.

Further evidence was presented by Henderson (47) who administered tritium-labeled 3-hydroxyanthranilic acid to rats and cats and recovered most of the isotope in quinolinic acid in the rat and in picolinic acid in the cat.

Lan and Gholson (65) reported a relatively low 3-hydroxyanthranilic acid oxidase picolinic carboxylase ratio in cat liver and proposed that this may be the basis for the absence of the tryptophan-niacin relationship in cats. It was demonstrated earlier (23) that the cat possesses all the enzymes required to convert tryptophan to 3-hydroxyanthranilic acid, but tryptophan cannot replace the niacin required in the diet of the cat. Hayaishi and co-workers (31) discovered the metabolic pathway in cat liver which appears responsible for the observed oxidation of

Compound I to glutarate and CO_2. Lan and Gholson (65) proposed that too much Compound I is diverted to the glutarate pathway by picolinic carboxylase to allow NAD formation in the cat (29, 82, 83).

Gholson et al. (31) reported that the major portion of 3-hydroxyanthranilic acid is not metabolized via picolinic acid, nicotinic acid, nor quinolinic acid, rather via picolinic carboxylase to glutarate and carbon dioxide. On the other hand, Dalgliesch (18) assumed in 1955 that the major route of tryptophan metabolism was via kynurenine and 3-hydroxyanthranilic acid to nicotinic acid.

5. Formation of Nicotinamide Ademine Dinucleotide.

In 1963, Nishizuka and Hayaishi (82, 83) reported that niacin ribonucleotide is formed from 3-hydroxyanthranilic acid in the presence of 5-phosphoribosyl-1-pyrophosphate (PRPP) by a soluble enzyme system from rat liver. They found that Compound I and quinolinic acid are intermediates in this conversion, while deamido NAD is produced from the niacin ribonucleotide in the presence of adenosine triphosphate (ATP) by a pyrophosphorylase. NAD is then formed from the amidation of deamido NAD by NAD synthetase in the presence of glutamine and ATP. Gholson et al. (29) purified more than 1000-fold the enzyme responsible for the conversion of quinolinic acid in the presence of PRPP to niacin ribonucleotide. The K_m for quinolinic acid was found to be 6×10^{-5}M and for PRPP to be 5×10^{-5}M.

Nishizuka and Hayaishi (82) found no evidence that free niacin is an intermediate in the formation of NAD from 3-hydroxyanthranilic acid. However, in 1957, Preiss and Handler (87) demonstrated the biosynthesis of NAD from nicotinic acid in the presence of PRPP; and Imsande and Handler (56) isolated an enzyme from beef liver which catalyzes the

conversion of nicotinic acid to nicotinic acid monomucleotide (NaMN) in the presence of PRPP. The name proposed for this enzyme is nicotinic acid mononucleotide pyrophosphorylase.

B. Alternate Pathways for Niacin Biosynthesis

Many attempts have been made to show a positive tryptophan-nicotinic acid relationship in plants (10, 11, 37, 80, 81, 91, 95).

In 1949, Gustafson (37) supplied intact cabbage, broccoli, and tomato leaves with tryptophan through the petioles and reported an increase in nicotinic acid with increase in trytophan concentration, concluding that tryptophan is a precursor of niacin.

The same year, Nason (80) reported a tryptophan-niacin relationship in corn. He observed a significant increase in nicotinic acid synthesis in corn embryos upon addition of L-tryptophan to the nutrient solution. Banerjee and Banerjee (11) reported in 1950 that DL-tryptophan enhanced the niacin content of germinating Phaseolus mungo, and in another study on corn the same year, Nason (81) reported that "this strengthened the similarity of the biosynthesis mechanism (of nicotinic acid in corn) to that of mammals and Neurospora."

However, studies by Dawson and co-workers (19, 20) with carboxyl-^{14}C-labeled nicotinic acid and ring-^{14}C-labeled tryptophan failed to show either nicotinic acid or tryptophan to be precursors of nicotine in tobacco. Also, Leete et al. (68) reported that tryptophan-3-^{14}C did not give rise to radioactive trigonelline when fed to pea seedlings.

Moreover, observations have been made which preclude a tryptophan-nicotinic acid relationship in bacteria (97, 105). As early as 1948, Volcani and Snell (97) reported that nicotinic acid does not arise from tryptophan in the five different bacteria species with which they

experimented. In 1954, Yanofsky (105) found that several known intermediates in the tryptophan to niacin pathway were incapable of supporting growth of eight niacin auxotrophs of Bacillus subtilus and three niacin auxotrophs of Escherichia coli. He also observed that tryptophan is not a precursor of nicotinic acid in E. coli and B. subtilus by isotopic studies with tryptophan labeled uniformly in the indole ring.

Then in 1956, Dawson et al. (21) reported that the pyridine ring of nicotinic acid is utilized in the formation of the pyridine ring of nicotine. By supplying ring-labeled nicotinic-^{3}H acid and nicotinic acid containing ^{14}C in both ring and carboxyl positions to sterile cultures of excised roots of tobacco, they found considerable amounts of radioactivity in the nicotine produced.

The next year Leete (66) administered DL-tryptophan-7a-^{14}C to tobacco growing in an inorganic nutrient solution. The nicotine was isolated from the plants and was found completely inactive. This led to the conclusion that tryptophan is not a precursor of nicotinic acid in tobacco. Positive evidence for this conclusion was presented in 1959 by Henderson et al. (53). Their addition of tryptophan-7a-^{14}C and tritium labeled 3-hydroxyanthranilic acid to corn seedlings through the germinating medium resulted in no radioactive niacin.

In 1957, Leete and Leitz (67) found that nicotinic acid is also a precursor of the alkaloid ricinine (N'-methyl-3-cyano-4-methoxy-2-pyridone) in castor plants.

Once it had been established that the tryptophan-nicotinic acid relationship does not exist in some plants and bacteria, numerous experiments were carried out to determine the precursors of nicotinic acid. Among the first was the study of Ortega and Brown (85) in 1959 in which

they incubated resting cells of E. coli in reaction mixtures which included succinic acid-2,3-^{14}C, glycerol-1,3-^{14}C, and pyruvic acid-2-^{14}C. Succinate and glycerol were both incorporated into nicotinic acid, but the incorporation of pyruvate was negligible. Fumarate, oxalacetate, or malate could substitute for succinate, and glyceric acid or dihydroxyacetone could substitute for glycerol. The next year they demonstrated that in E. coli glycerol and succinic acid are precursors of the pyridine ring of nicotinic acid and that tryptophan is not a precursor of nicotinic acid in this organism (86).

Moreover, in 1960 and 1962, Griffith et al. (33, 34) fed acetate-1-^{14}C, acetate-2-^{14}C, propionate-1-^{14}C, propionate-3-^{14}C, glycerol-1,3-^{14}C, glycerol-2-^{14}C, and aspartic acid-3-^{14}C to Nicotiana rustica and found that radioactive nicotinic acid resulted from each, while the pyridine ring was labeled only by propionate-2-^{14}C and glycerol-1,3-^{14}C. Propionate-3-^{14}C was not utilized at all, which indicates that propionate must be converted to acetate first. Glycerol produced radioactive nicotinic acid with the greatest specific activity.

Waller and Henderson (98, 99) further demonstrated that nicotinic acid is an efficient precursor of the pyridine ring of ricinine in castor plant seedlings by isotopic experiments and that propionate-^{14}C is incorporated into the pyridine ring of ricinine. Moreover, it was found (26, 100) that succinic acid and glycerol were also precursors of the ring carbons of ricinine in Ricinus communis.

Gross et al. (36) showed that in Mycobacterium tuberculosis aspartic acid inclusive of its nitrogen is directly incorporated in the nicotinic acid molecule as atoms 2, 3, and 4 and the other three atoms of the pyridine ring come via glycerol or glyceraldehyde.

Hence, recent evidence indicates that C_3-compounds such as glycerol and C_4-compounds such as aspartic acid or succinic acid are precursors in the biosynthesis of the pyridine ring in some higher plants and bacteria.

In 1963, Yang (103) noted that the incorporation of succinate-^{14}C and glycerol-^{14}C suggest that C_-^3 unit and C_-^4 unit dicarboxylic acids might form quinolinic acid which in turn may be decarboxylated to form nicotinic acid and hence be incorporated into ricinine. Andreoli (9) demonstrated the same year with isotopic studies the formation of nicotinic acid mononucleotide from quinolinic acid by a PRPP dependent reaction in E. coli. However, he observed that nicotinic acid is not an intermediate in this conversion. The conversion of nicotinic acid to nicotinic acid mononucleotide in E. coli was earlier shown by Imsande and Handler (57) to be a separate PRPP-ATP dependent reaction.

Injection of quinolinic acid-2,3,7,8-^{14}C into castor plants and isolation of nicotinic acid and ricinine showed that quinolinic acid is incorporated into ricinine without going through free nicotinic acid (39). Incubation of a castor cotyledon extract with quinolinic acid-2,3,7,8-^{14}C and PRPP and/or ATP showed that PRPP is required for decarboxylation of quinolinic acid while ATP inhibits it. With an incubation period of only one hour, the only major radioactive compound formed was nicotinic acid mononucleotide which led to the conclusion that the other products formed, which included nicotinic acid, arise from further reactions of nicotinic acid mononucleotide (39).

Yang et al. (104) injected equimolar amounts of quinolinic acid-2,3,7,8-^{14}C and nicotinic acid-2,3,7-^{14}C into Ricinus communis seedlings and isolated the ricinine 96 hours later. Less than 3 percent of the

nicotinic acid was incorporated while 10 percent of the labeled quinolinic acid was incorporated. Moreover, simultaneous injection of quinolinic acid ^{14}C and a 10-fold excess of nonradioactive nicotinic acid did not depress the incorporation of radioactivity into ricinine. They concluded that quinolinic acid is probably not converted to pyridine alkaloids via nicotinic acid.

Evidence that quinolinic acid is not a direct precursor of nicotinic acid in bacteria was reported by Moat and Albertson (79) who observed with labeling studies that aspartic acid appeared to be an effective precursor of nicotinic acid in Mycobacterium tuberculosis, but they reported that quinolinic acid was not decarboxylated by this organism.

From the majority of evidence it appears that nicotinic acid mononucleotide, which is a precursor of NAD and pyridine alkaloids in some bacteria and higher plants, is formed from C_3- and C_4-compounds like glycerol and succinate or aspartate via quinolinic acid.

C. Biosynthesis of Pyridine Carboxylic Acids in Lower Animals

The pathways for the synthesis of these pyridine carboxylic acids are as yet unknown in lower animals. However, a derivative of picolinic acid has been found in large quantities in marine invertebrates, and studies on this compound may eventually lead to information on these pathways in lower animals. This derivative, homarine or N-methylpicolinic acid, was discovered in 1933 by Hoppe-Seyler (54) who extracted it from the lobster Homarus vulgaris and the sea urchin Arbacia pustolosa.

In 1953, Kalckar et al. (58) isolated homarine from the foot muscle of Busycon canaliculatum. They reported a maximum absorbance of 272 mμ for this compound which was not affected between pH 0 and pH 14. It was also unaffected by treatment with sodium nitrite and by heating to about

200°C in concentrated hydrochloric acid. This compound was absorbed on the cation exchange resin Dowex 50-H^+ and eluted with 2 N hydrochloric acid, but it was not retained on the anion exchange resin Dowex-2-acetate even at pH 12.

In 1955, Koechlin (60) reported to have found homarine in squid. The compound he isolated had an absorbance maximum at 274 mμ, was precipitated with heavy metal ions, and its absorption was not affected by pH. It was also retained by strong cation exchangers but not strong anion exchangers.

From 1953 to 1961, Ackermann and co-workers (1-7, 70, 71) identified homarine and often trigonelline in the tissues of the sea anemone Arca sulcala, the sea snail Patella sp., the marine snail Arenicola marina, the crab Crangon vulgaris, the king crab Limulus polyphemus, the leather coral Alcyonium digitatum, the sponge Calyx nicacensis, and the fungus Polyporus sulphureus. Moreover, Deffner and Hafter (25) reported that the dialyzable portion of the axoplasm of the squids Loligo pealie and Dosidicus gigas contained homarine in weight percent 2.93 and 3.41 respectively.

Since homarine is found in high concentrations in nerve tissue and has characteristics that suggest possible roles in nerve function, Gasteiger et al. (27) made an extensive investigation of homarine distribution in tissues and phyla and made perfusion studies with homarine of the lobster heart. They concluded that it is unlikely that homarine is involved in nerve function. However, since homarine is found in marine invertebrates but has not been found in fresh water invertebrates, Gasteiger et al. (27) did postulate that homarine may serve as an osmoregulator because of its ability to diffuse from the cells inspite of

its form as a quaternary base. They suggested that homarine was lost from the metabolic pathway with the evolution of fresh water species resulting in a desirably lessening in cellular osmotic pressure.

The unusually large quantities of homarine in marine invertebrates and the unusually high rate of decarboxylation of homarine compared to similar compounds lead Haake and Mantecon (38) to hypothesize that the physiological role of homarine is to act as a sink to store CO_2 during periods of metabolic activity and that it is a product of the carboxylation of the N-methyl pyridinium ion.

However, the function as well as the pathway of biosynthesis of homarine are as yet unknown.

The specific objectives of the studies presented here are:

1. To determine whether or not quinolinic acid acts as a precursor of nicotinic acid in corn seedlings.

2. To determine whether or not tryptophan acts as a precursor of homarine in the lobster Homarus americanus and to determine whether or not homarine results as a radioactive metabolite of N-methylpyridine-^{14}C.

CHAPTER III

EXPERIMENTAL PROCEDURE

A. Quinolinic Acid-Nicotinic Acid Relationship in Corn

1. Materials

Franklin Yellow Dent corn seeds were obtained from Dr. J. S. Brooks of the Department of Agronomy at Oklahoma State University. The quinolinic acid-2,3,7,8-^{14}C was prepared by Gholson, Ueda, Ogaswara, and Henderson in 1963 in the Department of Biochemistry at Oklahoma State University.

2. Experimental Methods

a. Germination of Corn Seeds

Franklin Yellow Dent corn seeds were germinated under sterile conditions similar to those reported by Henderson et al. (52) in 1959. A disk of #5 Whatman filter paper, 11 cm in diameter, was placed with 15 ml distilled water in each of 9 Erlenmeyer flasks; the flasks were sterilized for one hour under 15 lbs./sq. in. pressure at about 120°C.

The seeds were washed about five times with distilled water to remove the fungicide. The seeds were soaked for three minutes in a 50% ethanol solution containing 0.2 percent $HgCl_2$ and rinsed four or five times with cool sterile distilled water under an ultraviolet-light hood. The seeds were then soaked for 24 hours in a minimum of sterile distilled water under the ultraviolet-light hood.

While under the sterile hood, fifteen seeds were transferred to each of the nine sterile flasks with flamed tongs. The flasks were then placed in a dark cupboard for germination at about 25°C.

b. Addition of Labeled Compound

After five days, five of the flasks with very little or no microbial growth evident were selected. To each of four flasks, 2.5 μc of quinolinic acid-2,3,7,8-^{14}C were added with sterile pipettes under the ultraviolet light hood. To the fifth flask, approximately 2 μc of the same solution was added. The seedlings were then grown in the dark for four more days.

c. Termination of Germination

After a total of nine days of germination and growth, one of the five flasks which had been treated with the labeled compound was discarded because of excessive bacterial growth. The embryo, shoot, and roots of each seedling in the remaining four flasks were removed from the endosperms, washed with a minimum of distilled water, and lyophilized overnight. Table I indicates the number of seedling used.

TABLE I

CORN SEEDLINGS UTILIZED FOR EXTRACTION

Flask Number	Number Germinated	Number Lyophilized	
1	9	6	small amount of bacteria in flask
2	11	9	small amount of bacteria in flask
3	10	9	small amount of bacteria in flask
4	12	11	flask with 4 ml labeled compound

The unabsorbed ^{14}C-labeled quinolinic acid solution in each flask was pooled with the water used to rinse the seedlings for each respective flask. From each, a 0.2 ml aliquot was counted on the Tri-Carb Liquid Scintillation Counter, and the results are presented in Table II.

TABLE II

RADIOACTIVITY UNABSORBED BY CORN SEEDLINGS

Flask Number	Total Volume (ml)	Total Counts/Min
1	25	304,000
2	28	436,000
3	23	240,000
4	35	120,000

d. Alcohol Extraction of 9-Day Corn Seedlings

The 20 dry seedlings from flasks 2 and 4 were pooled. Then the corn was ground in a mortar to a fine powder after addition of a few milliliters of liquid nitrogen. This powder was extracted twice for 15 minutes with boiling 95 percent ethanol. The combined supernatant liquids were then evaporated to dryness and the residue was dissolved in 50 ml water. This solution was filtered and adjusted to a pH of about 7.5 after the addition of 2 mg each of trigonelline, quinolinic acid, and nicotinamide adenine dinucleotide as carriers.

e. Elution of Extract

The solution was put on a Dowex 1-8X (100-200 mesh, 3.2 cm^2 x 27 cm) column which was in the formate form. The column was eluted stepwise with the following reagents, and 10 ml fractions were collected.

75 ml H_2O

120 ml 0.05 N formic acid

130 ml 0.10 N formic acid

100 ml 0.25 N formic acid

180 ml 1.00 N formic acid

180 ml 2.00 N formic acid

200 ml 4.00 N formic acid

250 ml 4.00 N ammonium formate

Figure 1 shows a plot of radioactivity and absorbance versus faction number.

f. Determination of Peak 1

The fractions of peak 1 were pooled and evaporated to dryness on a rotary evaporator. The residue was dissolved in a minimum volume of absolute ethanol, and about 1 ml of absolute ethanol saturated with picric acid was added to the solution. Yellow crystals weighing 30.1 ml dry weight were formed. The supernatant liquid was condensed and more picric acid solution was added yielding a 24.3 mg dry weight crystal crop. The two crops were pooled and combined with 115.6 mg of nonradioactive trigonelline picrate. The mixture was then recrystallized yielding 122.4 mg of trigonelline picrate. A melting point determination was made with the Bach apparatus. The trigonelline picrate was recrystallized five times from absolute ethanol to constant specific activity.

g. Determination of Peaks 2, 3, and 4

The fractions of peaks 2, 3, and 4 were pooled separately and lyophylized to dryness, and the residues were dissolved in minimum amounts of absolute ethanol. Peak 4 contained considerable ammonium formate which was separated from the sample by lyophilizing the peak fractions

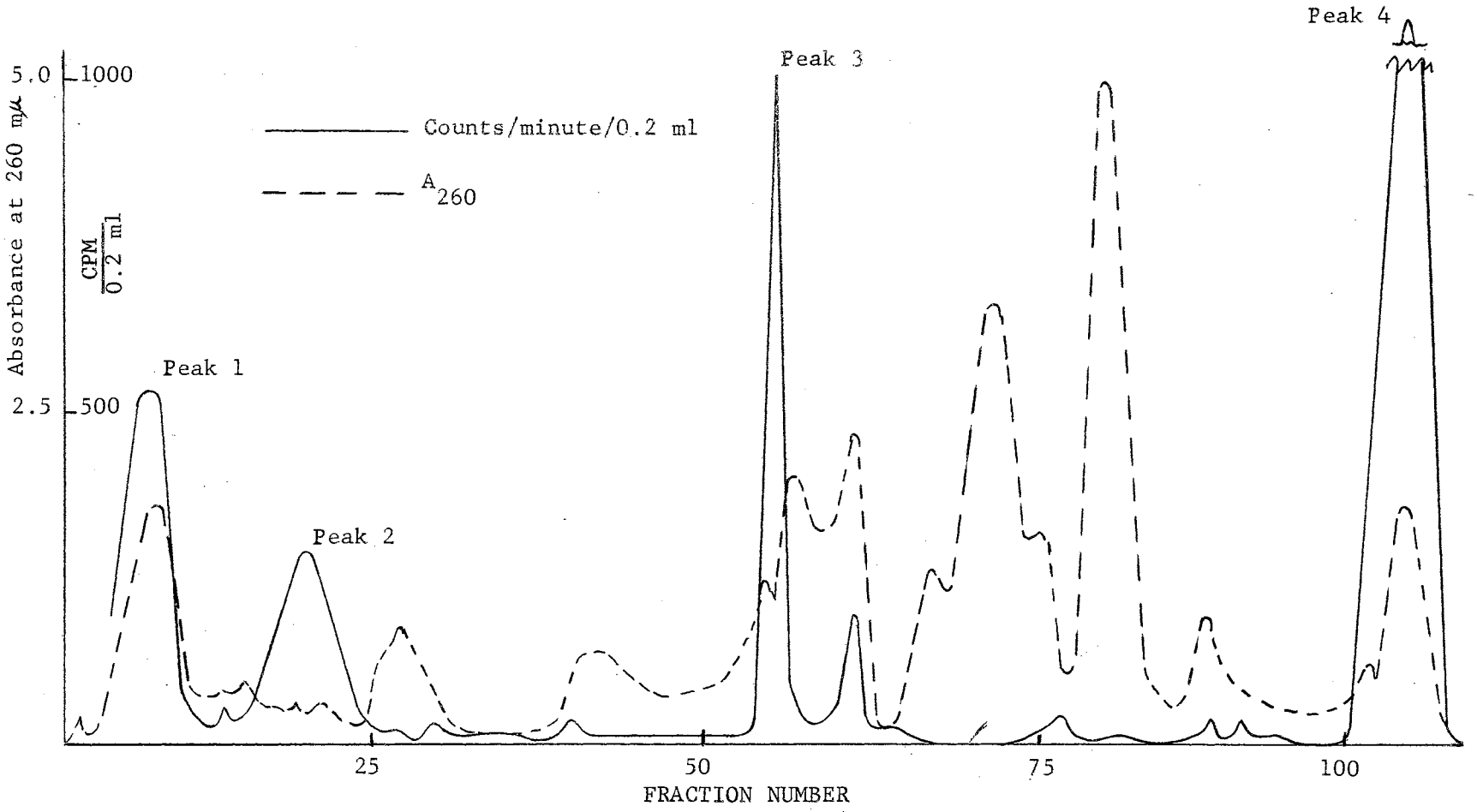

Figure 1. Alcohol Extraction of Quinolinic Acid-2,3,7,8-^{14}C Fed Corn Seedlings

to dryness and extracting the residue with absolute ethanol.

About 100λ of each sample plus 0.625 mg of standard quinolinic acid, nicotinic acid, and diphosphopyridine nucleotide were spotted on Whatman No. 1 paper and developed by descending chromatography in five separate solvent systems as shown in Table III.

TABLE III

ALCOHOL EXTRACTION OF 20 QUINOLINIC ACID-2,3,7,8-^{14}C FED CORN SEEDLINGS

CHROMATOGRAPHY OF PEAKS 2, 3, AND 4

Solvent System	R_f Value Std. QA	Std. NA	Std. DPN	Peaks 2	3	4
Butanol saturated with 15% NH_4OH	.00	.30	.00	.75	.30 .00*	.00
Top layer of water: n-butanol:acetic acid 25:25:6	.30	.76	.72	.37 .52*	.79	.09 .53*
95% ethanol:1 M ammonium acetate adjusted to pH 5 with HCl, 7:3	.47	.68	.18	.70	.69	.55
85% isopropanol stored in glass	.13	.53	.00	.76 .88*	.56 .05*	.14
60% propanol	.70	.78	--	.88	.86	.85

*The smaller of 2 peaks.

h. Water Extraction of 15 Additional Seedlings

A total of 15 seedlings from flasks 1 and 3 were ground to a powder in liquid nitrogen. The powder was stirred in about 150 ml cold water and filtered. Thirty μmoles each of diphosphopyridine nucleotide, quinolinic acid, and trigonelline were added as carriers to the supernatant liquid. The solution was then put on another Dowex 1-X8 column

and eluted as before. The radioactivity and absorbance of each fraction were measured and plotted versus fraction number as shown in Figure 2.

i. Determination of Peak 1

Fractions 4-30 were pooled, and 20 mg non-radioactive trigonelline were added as a carrier. The solution was put on a Dowex 50-X8 column in the hydrogen form. The pH of the solution was not adjusted, and the following reagents were used to elute the column, with 10 ml fractions being collected.

20 ml water

100 ml 0.1 N HCl

100 ml 0.5 N HCl

100 ml 1.0 N HCl

400 ml 2.0 N HCl

200 ml 3.0 N HCl

The fractions were then measured for activity on a Tri-Carb Liquid Scintillation Spectrometer, and the radioactive fractions were pooled and evaporated to a small volume. The solution was identified as trigonelline by the formation of fine needle and plate precipitates upon addition of 0.5 ml sample to 0.2 ml $AuCl_3$ in one percent HCl and to 0.2 ml KI_3 in 3N H_2SO_4 (16, 17), respectively.

j. Determination of Peaks 2, 3, 4, and 5

The fractions of peaks 2, 3, and 4 were lyophilized, and the residues were dissolved in minimum amounts of distilled water.

Fractions included in peak 5 were pooled, lyophilized to about 50 ml, and put on a Dowex 50 column which was in the hydrogen form. The column was washed with several hundred milliliters of distilled water. The counts per minute in each fraction were measured as described above.

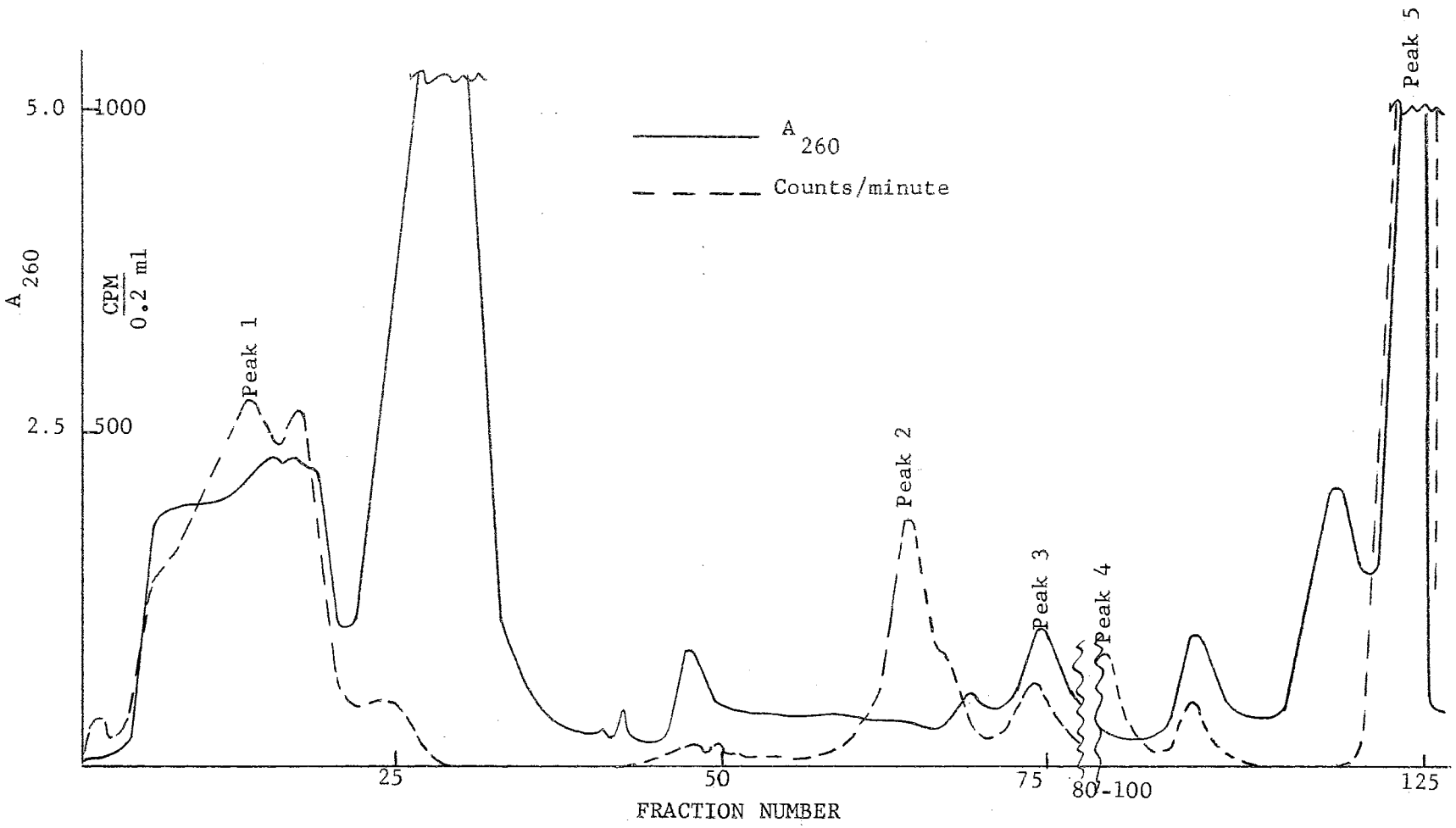

Figure 2. Water Extraction of Quinolinic Acid-2,3,7,8-^{14}C Fed Corn Seedlings

After condensing the radioactive fractions collected from the Dowex 50 column, the residues of peaks 2, 3, 4, and 5 were chromatographed as before with the solvent systems shown in Table IV.

TABLE IV

WATER EXTRACTION OF 15 QUINOLINIC ACID-2,3,7,8-^{14}C FED CORN SEEDLINGS

CHROMATOGRAPHY OF PEAKS 2, 3, 4, AND 5

Solvent System	R_f Value				Peaks			
	Std. QA	Std. NA	Std. DPN	Std. N_aMN*	2	3	4	5
Butanol saturated with 15% NH_4OH		.32	.36	.00	.30	.30	.00	
Top layer of water: n-butanol:acetic acid 25:25:6	.71	.73	.03	.15	.75	.70	.25	.68
95% ethanol:1 M ammonium acetate adjusted to pH 5 with HCl, 7:3		.73	.25	.40	.73	.67	.59	
85% isopropanol stored in glass		.47	.87	.39	.50	.04	.23	
60% propanol	.52	.63	.72	.70	.81	.80	.83	.47

*Nicotinic acid mononucleotide

B. Some Studies on the Metabolism of Homarine in the Lobster

1. Materials

Live lobsters were obtained from the Mid-Central Fish Company in Oklahoma City, Oklahoma. The standard homarine was purchased from K & K Laboratories in Plainview, New York. The DL-tryptophan-benzene-U-^{14}C was obtained from Nuclear Chicago. Homarine-^{14}C and N-methyl-pyridine-^{14}C were synthesized by Bih-Jing Jeng for this study in this laboratory.

The synthesis of homarine-CH_3-^{14}C·HCl consisted of heating alpha picolinic acid with $CH \cdot I$-^{14}C at 70-80°C for 18 hours, and the synthesis of N-methylpyridine-CH_3-^{14}C consisted of heating pyridine with $CH_3 \cdot I$-^{14}C under the same conditions. The products were recrystallized from methanol and ether.

2. Experimental Methods

The lobsters were kept alive in the cold room at 4°C in artificial sea water prepared as reported by Prudden (88). It consisted of the following:

357 gm NaCl

86.4 gm $MgSO_4 \cdot 7H_2O$

70.4 gm $MgCl_2 \cdot 6H_2O$

17.2 gm $CaCl_2 \cdot 2H_2O$

8.1 gm KCl

These specified amounts were dissolved in 19.4 liters of tap water which had previously been bubbled with air approximately twelve hours to remove the chlorine.

a. Detection of Homarine

The detection of homarine was based on the comparison of the behavior of standard and extracted homarine in column and paper chromatography.

A modified form of the ion exchange method used by Gasteiger and his associates (27) for the purification of lobster homarine extracts was used. A 100 mg sample of standard homarine in 5 ml of 0.2N HCl was put on a 47 cm column (2.6 cm in diameter) of Dowex 50W-X8 resin which had been recycled with 1N NaOH and 2N HCl to the H^+ form. The column was eluted with 300 ml 0.2N HCl, 300 ml 1N HCl, 450 ml 2N HCl, and 500 ml

3N HCl. Ten milliliter fractions were collected, and the ultraviolet absorbance at 272 mμ of each fraction was read on a Beckman DU Photospectrometer as shown in Figure 3. The fractions in the peak having maximum absorbance were pooled and evaporated to dryness in vacuo at about 50°C with the Flash Evaporator.

The residue was dissolved in a minimum of hot methanol, and the crystals obtained were redissolved in 0.2N HCl and chromatographed beside standard homarine dissolved in 0.2N HCl using 95 percent ethanol:NH_4OH (95:5, v/v) as the solvent system.

Lobster chela and tail muscle tissue was homogenized in two volumes of distilled water at top speed in a Waring Blender and then made to 5 percent perchloric acid with 70-72 percent perchloric acid. This mixture was then centrifuged at 16,000 x g for 15 minutes after which the supernatant solution was decanted and neutralized with 20% KOH and centrifuged to remove the $KClO_4$. The supernatant solution was lyophilized to dryness and dissolved in 0.2N HCl, the insoluble portion being removed by centrifugation.

The solution was put on the Dowex 50W-X8 column and eluted with 250 ml 0.2N HCl, 250 ml 1N HCl, 280 ml 2N HCl, and 500 ml 3.5N HCl as shown in Figure 4. The fractions corresponding to the peak of maximum absorbance were pooled and evaporated to dryness in vacuo at 60°C. After an unsuccessful attempt to remove all the water from the residue with evaporation over P_2O_5 in vacuo, the residue was redissolved in more water and lyophilized to dryness. The dried extract was dissolved in a minimum of absolute ethanol. The few crystals obtained were dissolved in 0.2N HCl and chromatographed beside standard homarine using the solvent systems mentioned above.

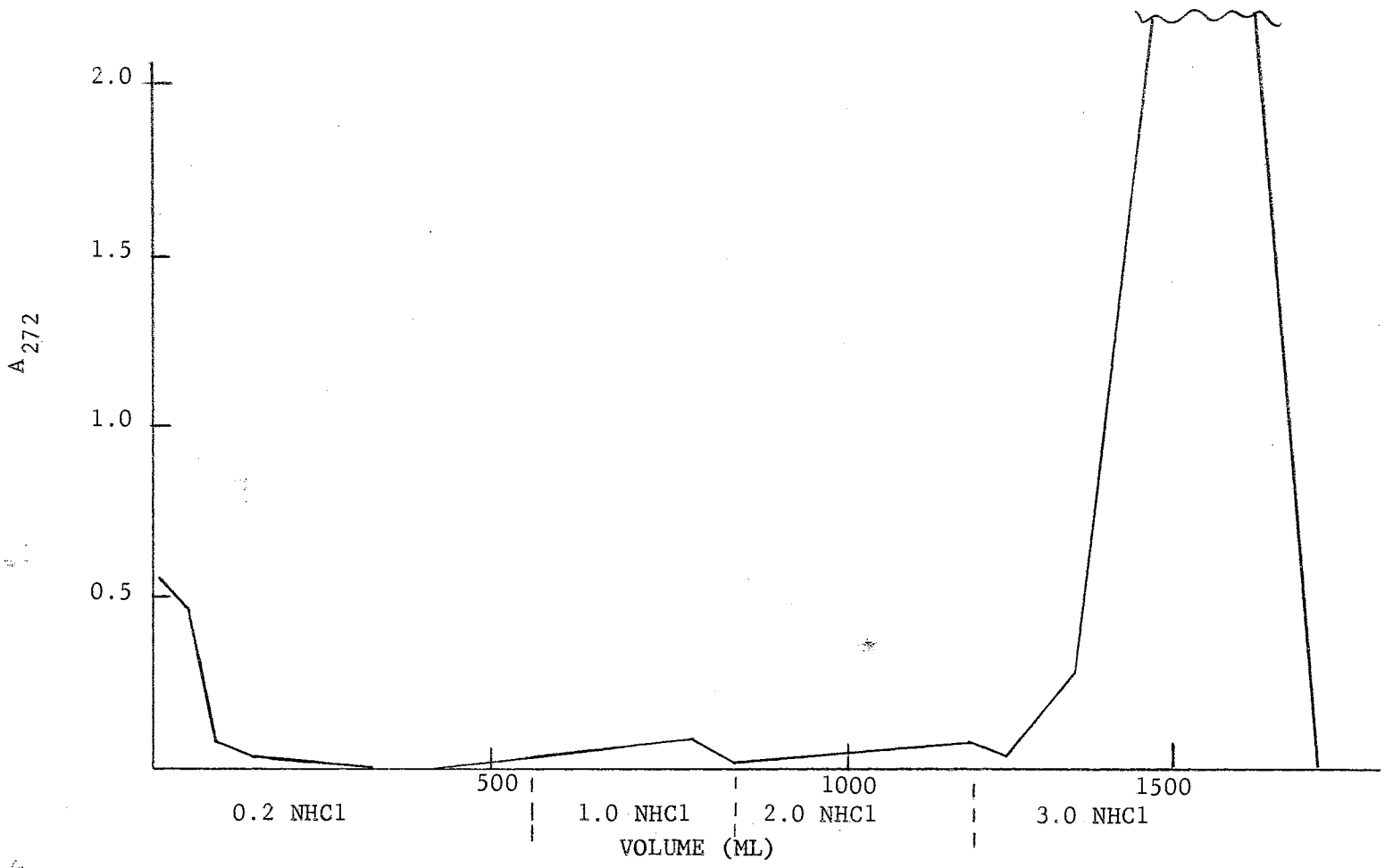

Figure 3. Elution of Standard Homarine from Dowex 50W-X8 in the H^+ Form

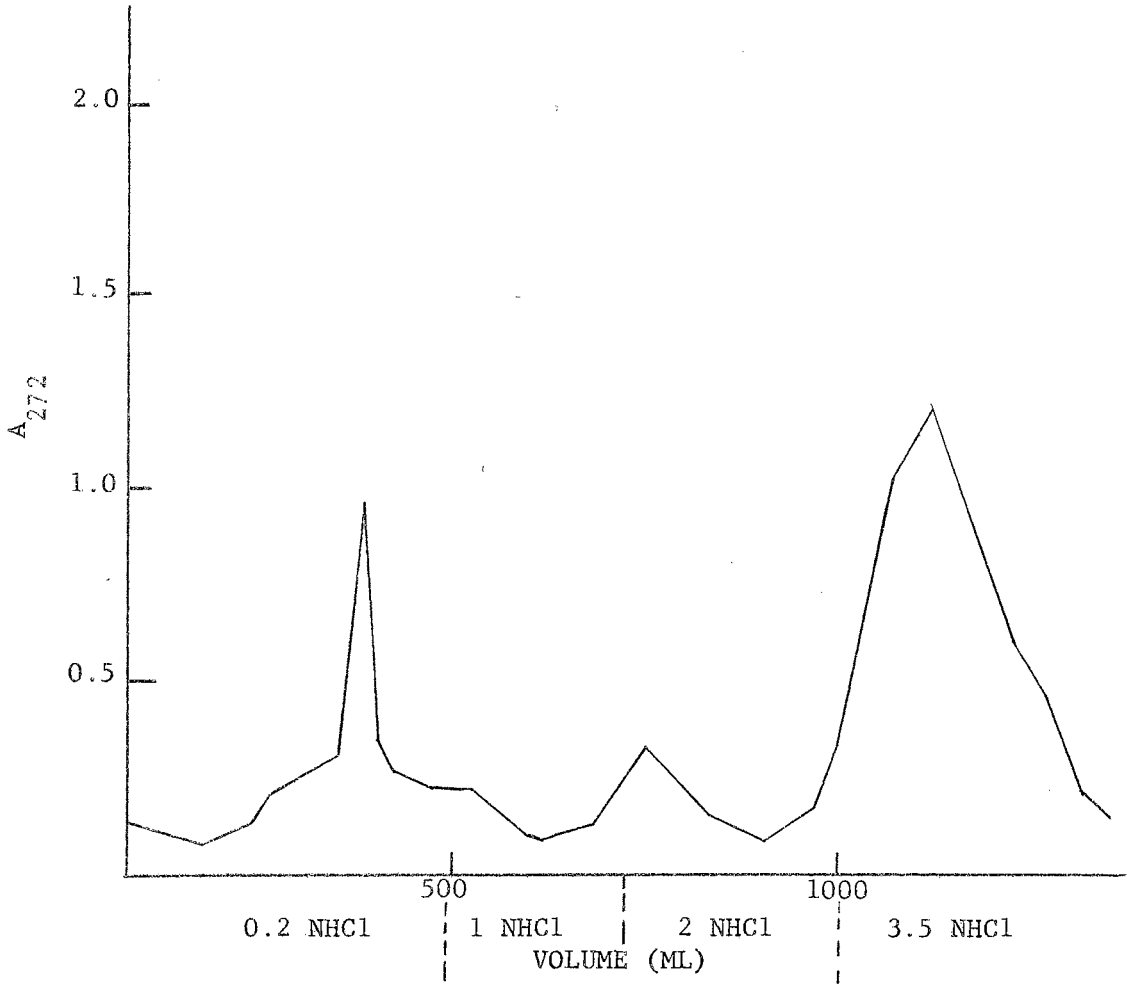

Figure 4. Elution of Lobster Extract from Dowex 50W-X8 in the H^+ Form

b. Injection of Tryptophan-^{14}C

The tail muscle tissue of a living lobster was injected with 15 μc of DL-tryptophan-benzene-U-^{14}C. The lobster was put in a dissicator which contained 100 ml of artificial sea water, and the respiratory CO_2 was collected in 100 ml portions of 1N NaOH. The NaOH was changed every 12 hours for 48 hours. The radioactivity of the CO_2 released into an ionization chamber by the addition of perchloric acid to the NaOH was counted. After 48 hours, 0.26 percent of the total radioactivity injected was found in the CO_2.

At the end of 48 hours, the lobster was sacrificed by putting the lobster in a closed chamber containing dry ice (CO_2), and 106 gm of muscle tissue from the tail and two chela were extracted as previously described, except that the denatured protein was washed twice with 100 ml portions of 5 percent perchloric acid. After removal of the $KClO_4$ from the pooled neutralized extracts and adjustment to pH 4.5 with HCl, the absorbancy and counts/minute of an aliquot were measured.

Before the extract was chromatographed on the Dowex 50W-X8 column, 150 mg of standard homarine was added to the extract as carrier. The solution was then stirred 10 minutes on the Mag-Mix with 50 gm of Norite A (57) which had been pretreated with 20 percent acetic acid by boiling 20 minutes and washing five times with distilled water, followed by oven drying.

After stirring the lobster extract with Norite A, the suspension was centrifuged at 10,000 x g for ten minutes. The charcoal was then eluted three times with 500 ml portions of 100 percent ethanol:water:NH_4OH (50:49:1, v/v/v) according to Kalckar (58) and two times with 500 ml portions of five percent phenol. The five eluates were evaporated to

dryness and dissolved in 45 ml of 0.2N HCl. Then 25 mg of carrier homarine HCl was added.

The solution was then put on the Dowex 50W-X8 resin column and eluted with 250 ml 0.2N HCl, 250 ml 1N HCl, 250 ml 2N HCl, and about 700 ml 3.5N HCl. The ultraviolet absorbance at 272 mμ was read against a distilled water blank, and 1:9 dilutions were made of each fraction and the absorbance checked again. The radioactivity of each fraction was measured by liquid scintillation counting.

The counts/minute and absorbance of each fraction were plotted versus fraction number in Figure 5.

Eluant included in fractions 111-159 (peak 1) and in fractions 70-110 were pooled separately, evaporated in vacuo, redissolved in distilled water, and lyophilized to dryness. The residue from fractions 111-159 was recrystallized from absolute alcohol and filtered. Twenty milligrams were dissolved in 0.2N HCl. The residue from the evaporation of fractions 70-110 was washed with absolute alcohol, and 50 mg were dissolved in 0.2N HCl. The two solutions were chromatographed beside standard homarine in 0.2N HCl as shown in Table V.

TABLE V

PAPER CHROMATOGRAPHY OF FRACTIONS 7-110 AND FRACTIONS 111-159

Solvent System	R_f		
	Standard Homarine·HCl	Fractions 70-110	Fractions 111-159
n-Butanol:Acetic Acid:H_2O (73:10:17, v/v/v)	0.20	0.20	0.20
95% Ethanol:NH_4OH (95:5, v/v)	0.37	0.51	0.37

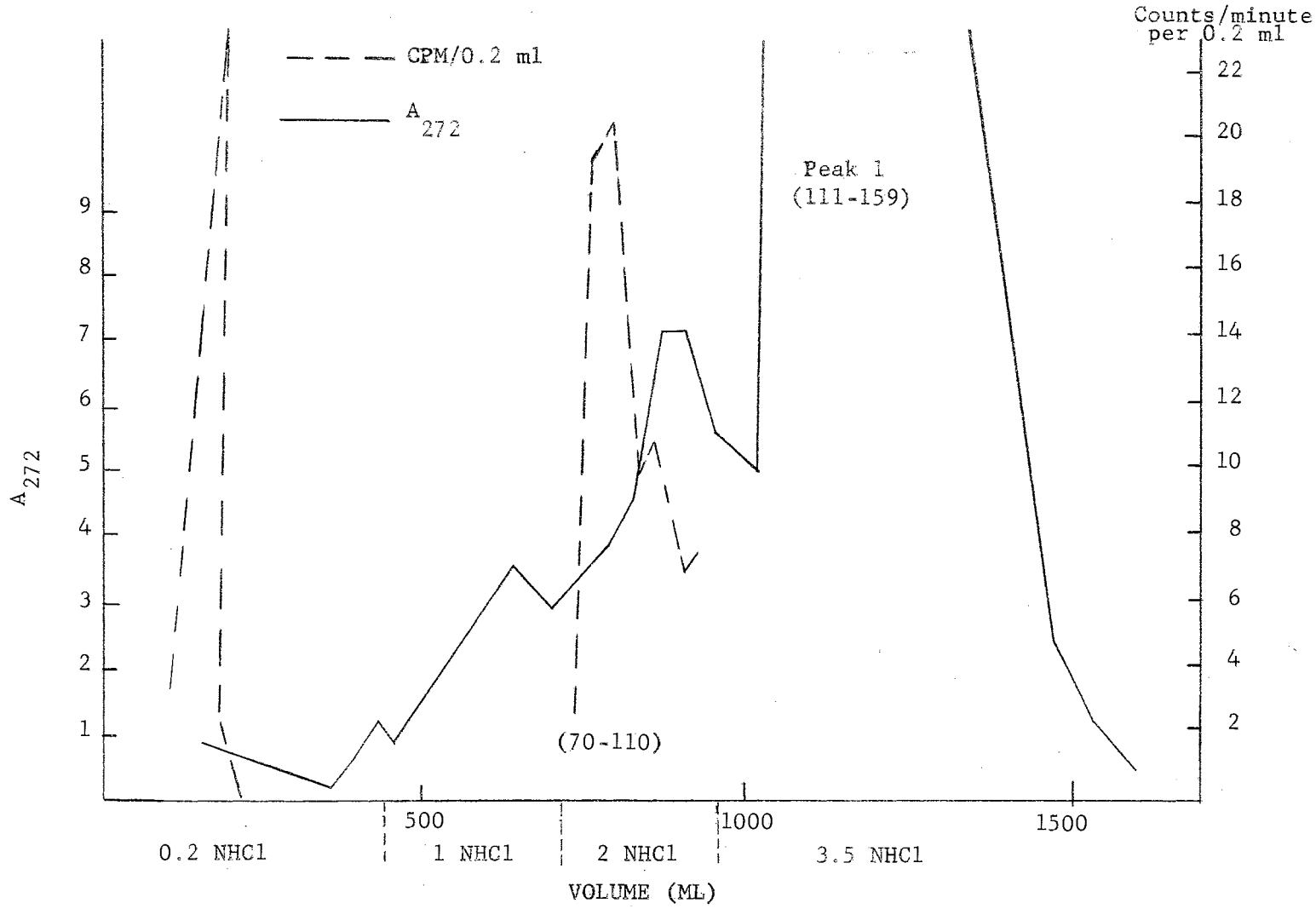

Figure 5. Elution of Distilled Water Extract of Lobster Muscle Injected with 15 μc Tryptophan-^{14}C from Dowex 50W-X8 in the H^+ Form

This HCl solution of fractions 111-159 was diluted 2,000 times and was scanned over a range of 320 mμ to 220 mμ on the Cary 14 Recording Spectrophotometer as shown in Figure 6.

The melting range of crystals from fractions 111-159 was found by use of the Kofler Micro Melting Point Apparatus.

c. Injection of ^{14}C-Labeled Homarine

In the same manner as previously described, 3.3 μc of homarine $CH_3-^{14}C$ were injected into a living lobster. After 48 hours, 0.28 percent of the total radioactivity injected was found in the respiratory CO_2. The lobster was then sacrificed and all soft tissues were homogenized and extracted as previously described, except in this case, the extract was not treated with charcoal.

After concentrating the neutralized perchloric acid extract in vacuo, at about 50°C, the solution was put on a Dowex 1-X8 column which had been cycled with 1N HCl and 2N NaOH to the OH^- form according to Leonard and MacDonald (69). The resin was eluted with distilled water, homarine passing through unretarded as reported by these workers (69). The radioactive fractions were pooled, condensed, and put on a Dowex 50W-X8 ion exchange column in the H^+ form. Ten milliliter fractions were collected at a rate of 4 seconds per drop or about 1 ml per minute. The resin was eluted with 280 ml of 0.2N HCl, 350 ml 1N HCl, 350 ml 2N HCl, and 1000 ml 3.5N HCl.

The fractions for the radioactive peaks shown in Figure 7 were respectively pooled and condensed in vacuo, and the nearly dry residue was dissolved in absolute ethanol. The radioactive solutions were chromatographed beside standard homarine dissolved in absolute ethanol and standard N-methyl-pyridine in absolute ethanol in three solvent

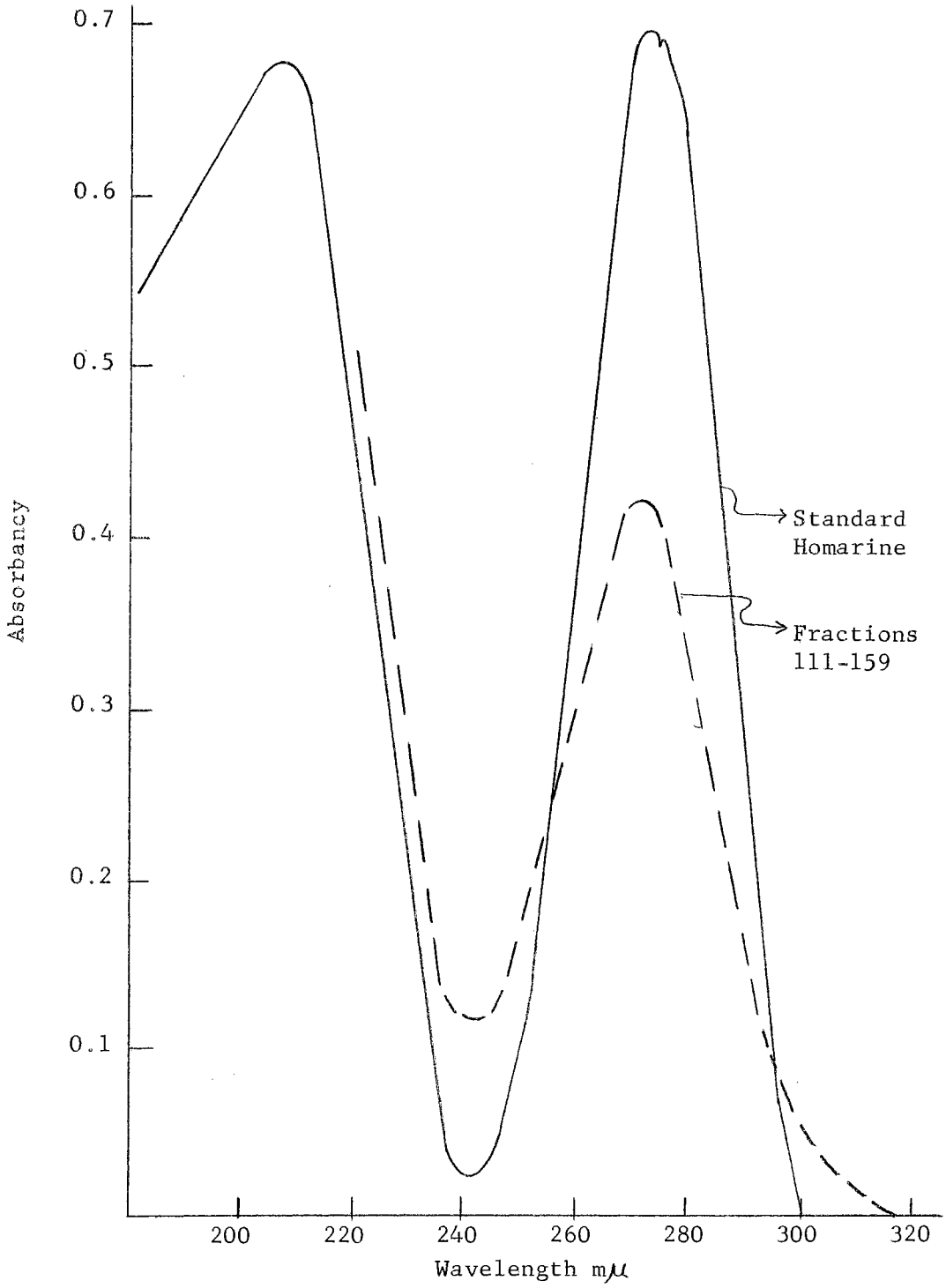

Figure 6. Ultraviolet Spectra from 220 mμ -320 mμ of Standard Homarine and Fractions 111-159 from the Tryptophan-^{14}C Injection Experiment

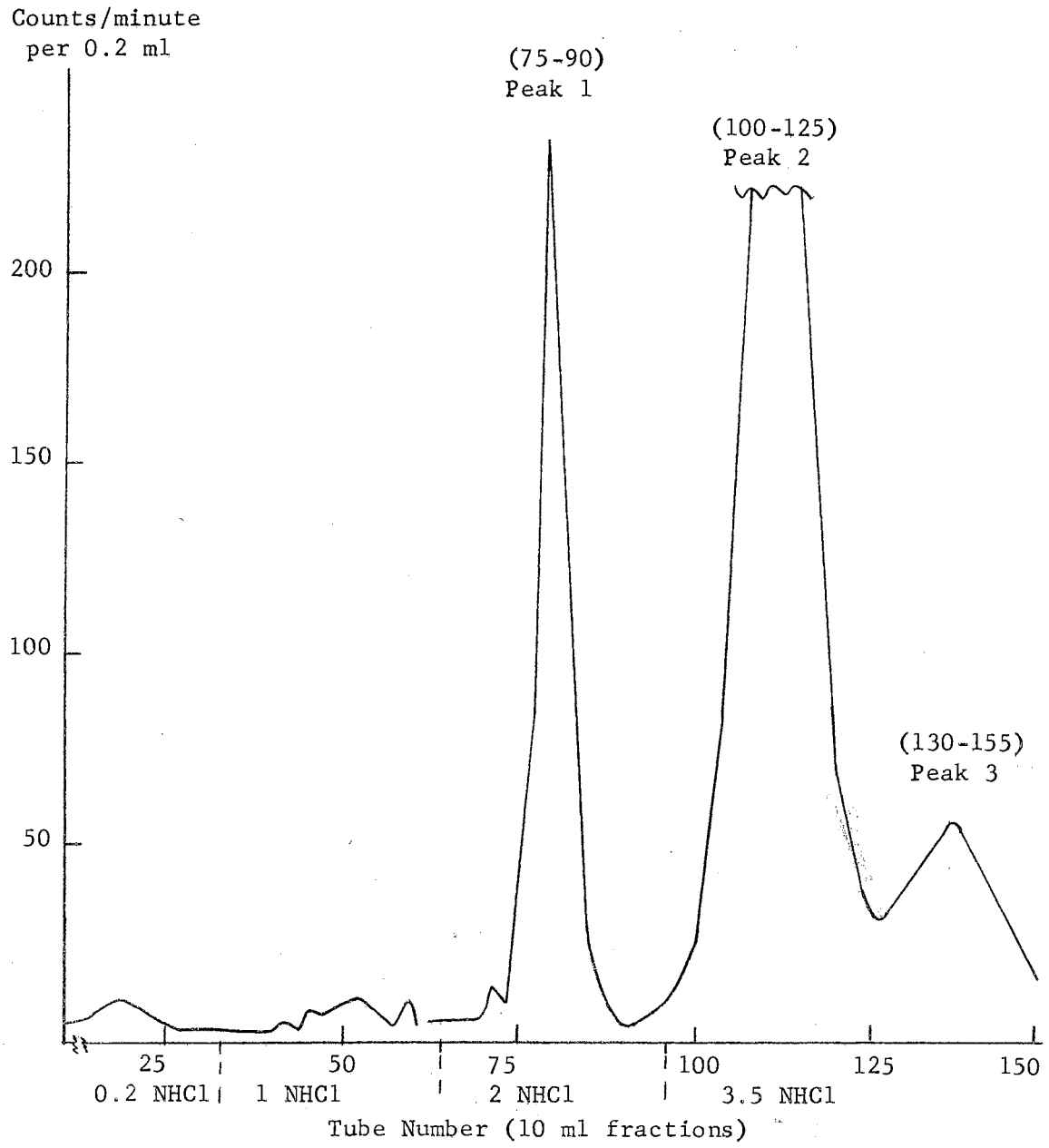

Figure 7. Elution of Distilled Water Extract of Lobster Muscle Injected with 3.3μc Homarine-^{14}C from Dowex 50W-X8 in the H^+ Form

systems as shown in Table VI. In addition, portions of peaks 1 and 3 were hydrolyzed in HCl by mixing $2\frac{1}{2}$ ml sample and $2\frac{1}{2}$ ml concentrated HCl in glass tubes and autoclaving for 2 hours. The hydrolysates were also chromatographed beside standard homarine and standard N-methyl-pyridine by descending paper chromatography as also shown in Table VI.

d. Injection of ^{14}C-Labeled N-Methylpyridine

The tail muscle of a living lobster was injected with 2.9 μc of N-methylpyridine-CH_3-^{14}C. The respiratory CO_2 contained no radioactivity after 48 hours of collection. At this time, the lobster was sacrificed, and all the soft tissues of the lobster excluding the intestines were extracted in the same manner as for the lobster injected with homarine-^{14}C. The extract was column chromatographed on Dowex 1-X8 and Dowex 50W-X8 ion exchange columns as before. Ten milliliter fractions were collected from both ion exchange columns, and the radioactivity of each fraction was counted by liquid scintillation. The results of the Dowex 50W-X8 elution are shown in Figure 8.

The peak fractions were pooled, condensed, and chromatographed beside standard homarine and standard N-methylpyridine in the two solvent systems shown in Table VII on page 39. The paper chromatographic strips were then cut into 1 cm sections and the radioactivity of each was measured. The cpm of each section was measured by use of the Tri-Carb Liquid Scintillation Spectrometer, where each section was put in a vial with 10 ml of toluene-ethanol liquid scintillation fluid. Figure 9 on page 40 is a plot of counts per minute versus distance along the chromatogram. A and B were chromatographed in 95% ethanol:NH_4OH (95:5, v/v); C and D in n-butanol:acetic acid:H_2O (73:10:17, v/v/v).

TABLE VI

PAPER CHROMATOGRAPHY OF PEAKS 1, 2, AND 3 IN HOMARINE·HCl-^{14}C INJECTION EXPERIMENT

	n-Butanol Acetic Acid:H_2O (73:10:17, v/v/v)	95% Ethanol: NH_4OH (95:5, v/v)	tert-Butanol: NH_4OH:H_2O (60:30:10, v/v/v)
Compound	R_f's	R_f's	R_f's
Std. Hom.	0.27	0.41	0.74
Peak 1	0.14, 0.17	0.01, 0.06, 0.28	0.69, 0.84
Std. N-Me-Pyridine	0.37	0.52	0.61
	0.15, 0.20	0.05, 0.29	0.76
Peak 1	0.15, 0.20	0.05, 0.29	
Std. Hom.	0.34	0.44	0.75
Peak 2	0.32, 0.47	0.43	0.74
Std. Hom.	0.25	0.43	0.71
Peak 3	0.17, 0.34, 0.02	0.06, 0.09	0.34, 0.67
Std. N-Me-Pyridine	0.30	0.59	?
	0.03, 0.15, 0.35	0.05, 0.09	0.52
Peak 3			
Std. Hom. Hydrolyzed	0.25	0.50	
Peak 1	0.08, 0.13, 0.3	0.03, 0.12, 0.54	
Std. N-Me-P Hydrolyzed	0.23	0.67	
Peak 1	0.30, 0.51	0.64, 0.02, 0.17	
Std. Hom. Hydrolyzed	0.27	0.59	
Peak 3	0.03, 0.34	0.04, 0.13	
Std. N-Me-P Hydrolyzed	0.30	0.66	
Peak 3	0.031, 0.17	0.10, 0.16, 0.50	

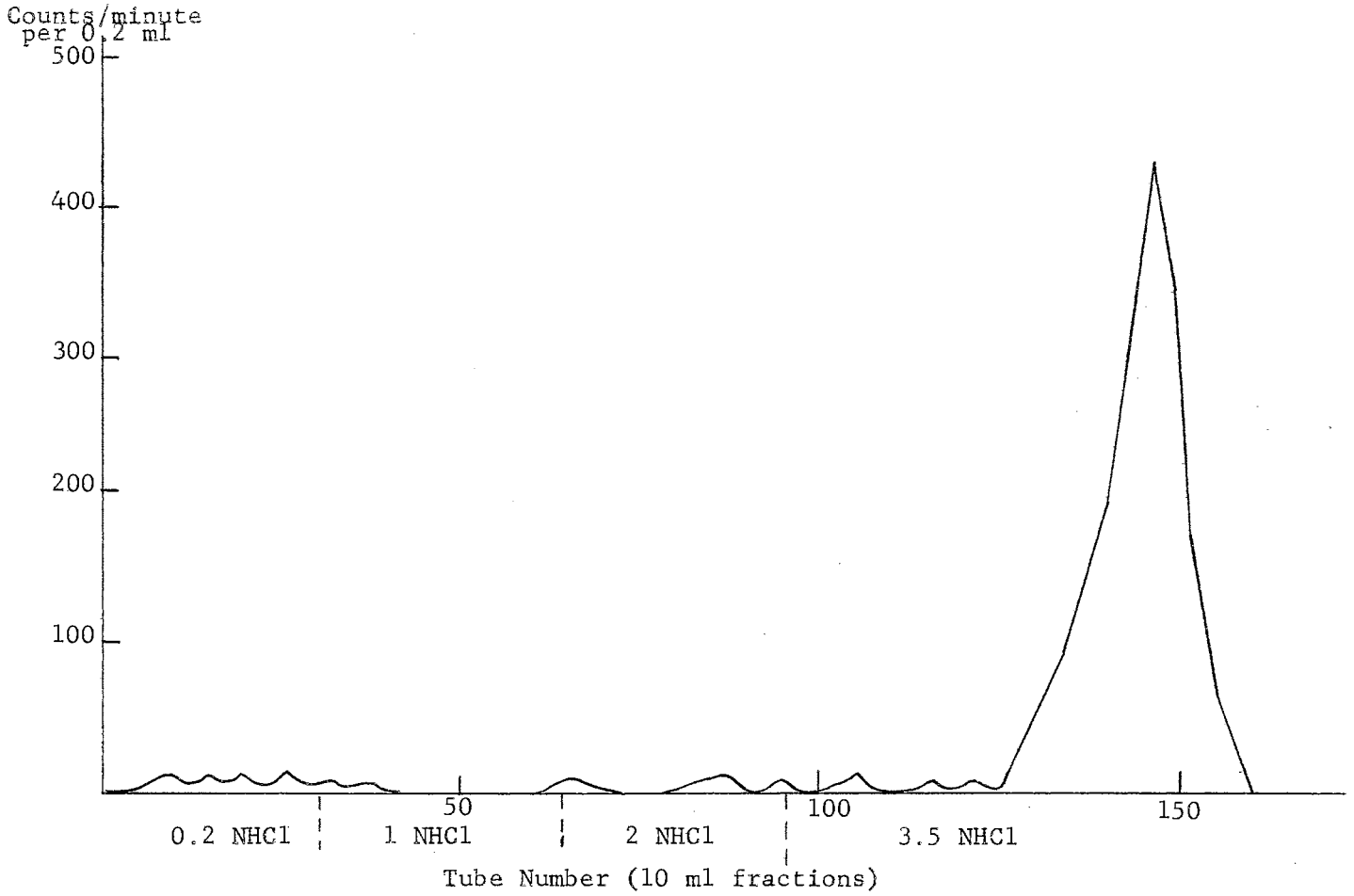

Figure 8. Elution of Distilled Water Extract of Lobster Tissue Injected with 2.9 μc of N-methylpyridine-^{14}C from Dowex 50W-X8 in the H^+ Form

TABLE VII

PAPER CHROMATOGRAPHY OF RADIOACTIVE PEAK IN N-METHYLPYRIDINE-^{14}C INJECTION EXPERIMENT

Solvent System	Standard Homarine	Std. N-Methyl-Pyridine	Peak
n-Butanol:Acetic Acid:H_2O (73:10:17, v/v/v)	0.29	0.33	0.25 0.23
95% Ethanol:NH_4OH (95:5, v/v)	0.69	0.75	0.54, 0.63 0.51, 0.63

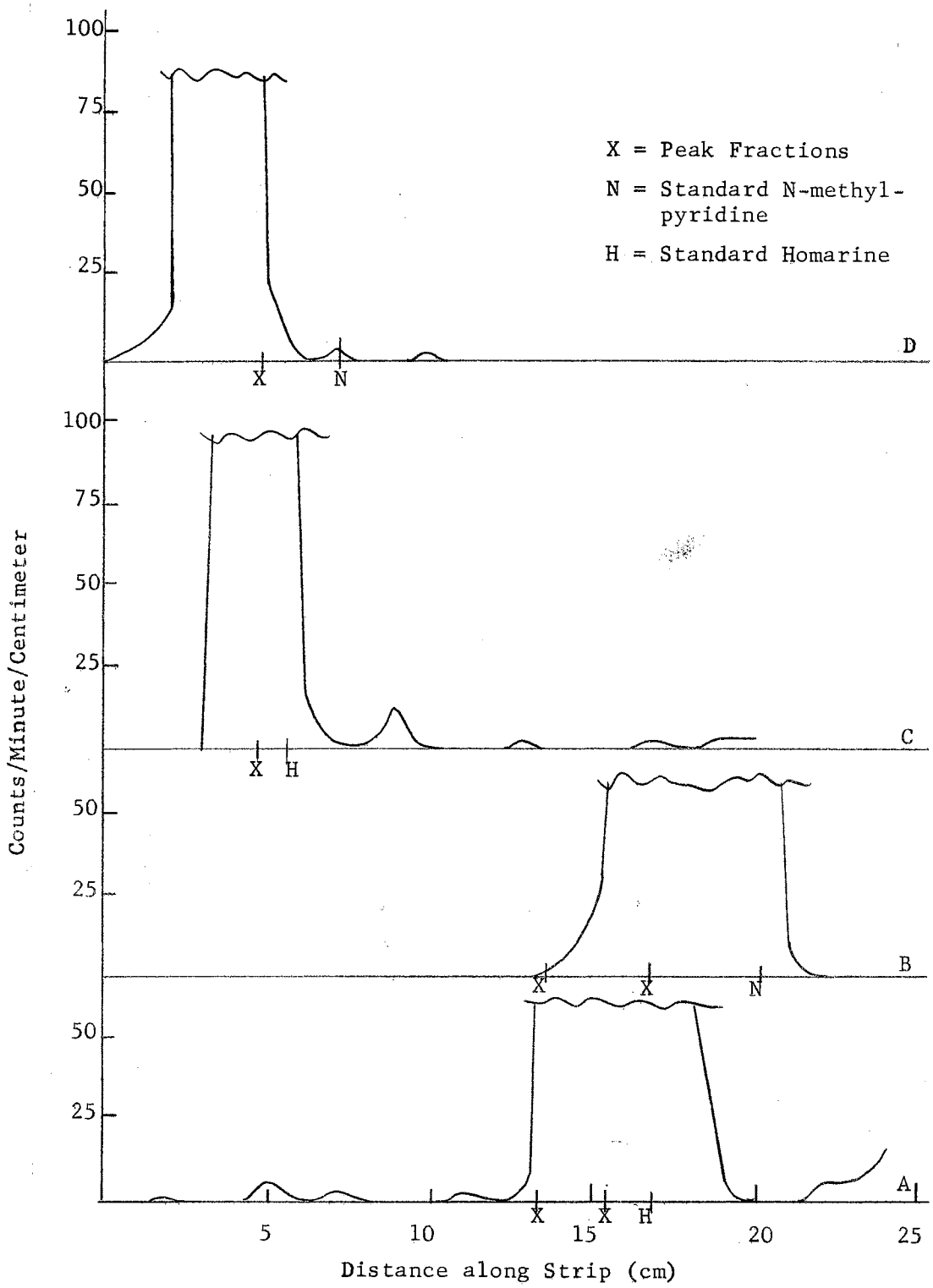

Figure 9. Paper Chromatography of Radioactive Peak Isolated by Ion Exchange Chromatography of the Extract of Lobster Tissue Injected with N-Methyl-pyridine-^{14}C

CHAPTER IV

RESULTS AND DISCUSSION

A. Quinolinic Acid-Nicotinic Acid Relationship in Corn

1. Absorption of Labeled Compound by Corn Seedlings

After nine days of germination, the unabsorbed solution in each flask was pooled with the water used to rinse the seedlings for each respective flask. An aliquot from each was counted on the Tri-Carb Liquid Scintillation Counter. The results are shown in Table II. The unabsorbed activity represented less than 20 percent of the total activity administered to each flask--an absorption of approximately 2 μc per flask.

2. Elution of Alcohol Extract

The results of the measurement of the counts per minute and absorbance of the eluant from the alcohol extract are plotted as a function of fraction number in Figure 1. Four major radioactive peaks resulted, three of which corresponded to peaks of high optical density at 260 mμ.

a. Determination of Peak 1

The first peak was identified as trigonelline on the basis of 1) recrystallization to a constant specific activity (99, 84, 88, 75, and 80 mμc/m mole) of a mixture of the unknown picrate and standard trigonelline picrate, and 2) the melting range of trigonelline picrate which was found to be 240-245°C which agreed with Henderson, et al. (53).

b. Determination of Peaks 2, 3, and 4

Solutions of Peaks 2, 3, and 4 were chromatographed in the five solvent systems shown in Table III. Table III also lists the R_f values, obtained. On the basis of R_f values, peak 2 is unidentified, peak 3 contains nicotinic acid and a small amount of diphosphopyridine nucleotide, and peak 4 is quinolinic acid.

3. Elution of Water Extract of Corn

The results of the measurement of counts per minute and optical density of the eluant from the water extract are plotted as a function of fraction number in Figure 2.

a. Determination of Peak 1

The fractions included in peak 1, as shown in Figure 2, were pooled and subjected to Dowex 50-X8 column chromatography to remove DPN^+ and other cations (the column was eluted with increasing concentrations of HCl with the trigonelline eluting off with 2N HCl). The radioactive fractions were then pooled and concentrated to a small volume. The radioactive peak was determined to be trigonelline on the basis of a positive test for quaternary nitrogen compounds. Upon addition of unknown solution to $AuCl_3$ in 1% HCl and to KI_3 in 3N H_2SO_4 (16, 17) a fine needle precipitate and a plate precipitate, respectively, resulted.

b. Determination of Peaks 2, 3, 4 and 5

The fractions included in peak 5 were pooled and rinsed through a Dowex 50 column which was in the hydrogen form. This step was taken to remove excess ammonium formate which was used to elute peak 5 from Dowex 1.

Then the fractions included in peaks 2, 3, 4, and 5 were condensed and chromatographed beside standard quinolinic acid, nicotinic acid, and

nicotinic acid mononucleotide in the five solvent systems shown in Table IV.

On the basis of R_f values, peak 2 was determined to be nicotinic acid and peak 5 was determined to be quinolinic acid. Peaks 2 and 3 were not identified.

From the results of these experiments, it is concluded that quinolinic acid is a precursor of both nicotinic acid and trigonelline in corn.

Further evidence for the intermediate role played by quinolinic acid was provided by Andreoli et al. (9) who found that quinolinic acid is a precursor of nicotinic acid mononucleotide in E. coli and by Hadwiger et al. (39) and Yang et al. (104, 103) who found that quinolinic acid is a precursor of ricinine in castor plants and of nicotine in tobacco plants. However, it has been shown that nicotinic acid is not a free intermediate in the conversion of quinolinic acid to nicotinic acid mononucleotide in E. coli (9) and of quinolinic acid to nicotinic acid mononucleotide and ricinine in castor plants (39, 105).

It now appears that the exact relationship of C-3 and C-4 compounds such as glycerol and succinate to quinolinic acid and the exact relationship of quinolinic acid to nicotinic acid in bacteria and higher plants is as yet unknown.

B. Some Studies on the Metabolism of Homarine in the Lobster

1. Detection of Homarine

Gasteiger et al. (27) found homarine to be eluted from Dowex 50-X8 by 2N HCl. However, it was found in this study to be eluted from Dowex 50W-X8 resin by 3N HCl and not 2N HCl as shown by the maximum 272 mμ absorbance in the 3N HCl fractions as shown in Figure 3.

The crystals obtained from the maximum absorbing fractions gave the same R_f value (0.49) as standard homarine in descending paper chromatography.

Isolation of homarine from lobster was accomplished by eluting the deproteinized lobster extract from Dowex 50W-X8 (Figure 4) with 3.5N HCl instead of 3N HCl in an attempt to remove the homarine in a sharp peak.

An absolute ethanol solution of the crystals obtained from the eluant of maximum optical density was paper chromatographed beside standard homarine resulting in R_f values of 0.31 and 0.30 for the standard and extracted compounds respectively.

2. Injection of Tryptophan-^{14}C

Tryptophan-^{14}C was injected into the tail muscle of a lobster in an attempt to determine whether or not tryptophan is a precursor of homarine. Collection of the respiratory CO_2 from the lobster after injection yielded only 0.26 percent of the total radioactivity.

The chela and tail muscles were extracted because Gasteiger et al. (27) reported that chela muscles contain a high percent of homarine--4.4 mg homarine per gm wet weight.

The deproteinized muscle extract contained 1,353,750 cpm and 34 mg homarine before treatment with charcoal. After addition of 150 mg homarine as carrier, and Norite A treatment the counts per minute was zero and the homarine content 0.5 mg. The charcoal was then eluted until the homarine eluting from the charcoal was negligible. Of the counts per minute present in the extract before charcoal treatment, 74 percent was recovered by elution; and of the homarine present before charcoal treatment, 15 percent was recovered by elution.

The elution of the charcoal treated extract from Dowex 50W-X8 with HCl resulted in two radioactive peaks (Figure 5) neither of which corresponded to homarine. Table V shows the results of paper chromatography of fractions 70-110 and fractions 111-159 beside standard homarine in two solvent systems. The non-radioactive peak (fractions 111-159) had the same R_f values as homarine while the radioactive peak (fractions 70-110) had an R_f value different from that of homarine. This non-radioactive peak had an absorbance maximum of 270 mμ and a minimum at 242 mμ while standard homarine had a maximum at 272 mμ and a minimum at 242 mμ as shown in Figure 6. Also, the melting range of this peak was found to be 145°-157°C while that of homarine was found to be 150°-155°C. Hence, the homarine isolated was not radioactive; and it is therefore concluded that tryptophan is not a precursor of homarine in the lobster.

3. Injection of Homarine-^{14}C

Homarine-CH_3-^{14}C was injected into the tail muscle of a living lobster in order to determine whether or not homarine is a precursor of N-methylpyridine. In this case, the respiratory CO_2 contained only 0.28 percent of the total radioactivity injected.

The deproteinized tissue extract was not treated with charcoal as in the tryptophan experiment but was column chromatographed on Dowex 1-X8 in the OH^- form before eluting from Dowex 50W-X8. The compound was not retarded by the anion exchanger as was observed by Leonard and MacDonald (69); however, it was eluted from the cation exchanger only by 3-3.5N HCl. On the other hand, Leonard and MacDonald (69) reported homarine to have appeared in the eluate after the passage of three bed-volumes of distilled water. The progress of the eluate was followed by

ultra-violet absorption at 272 mμ and counts per minute on the Tri Carb Liquid Scintillation Counter. Figure 7 shows the three radioactive peaks isolated from the Dowex 50W-X8 elution, and Table VI shows the results of paper chromatography of the radioactive peaks and their hydrolysates. The following conclusions were drawn from the R_f values. Peak 1 is neither homarine nor N-methylpyridine; peak 2 is homarine; and peak 3 is neither homarine nor N-methylpyridine. The hydrolysate of peak 1 may contain either or both homarine and N-methylpyridine, and the hydrolysate of peak 3 contains neither homarine nor N-methylpyridine. More solvent systems should be employed before definite conclusions concerning the hydrolysis products of Peak 1 can be reached. It is concluded on the basis of the available information that homarine is not a direct precursor of N-methylpyridine.

4. Injection of N-methylpyridine-^{14}C

N-methylpyridine-CH_3-^{14}C was injected into a living lobster to determine whether or not it is a precursor of homarine. It appears that N-methylpyridine is not catabolized since there was no radioactive respiratory carbon dioxide detected within 48 hours after injection.

Only one radioactive peak as shown in Figure 8 was isolated from Dowex 50W-X8, and it corresponds more closely to homarine than to N-methylpyridine on the basis of R_f values in two solvent systems (Table VII) and the position of the radioactive peaks on the paper chromatographs (Figure 9). However, it is necessary to chromatogram this isolated substance in additional solvent systems before definite conclusions can be reached. Also crystals should be isolated and the melting point determined, and derivatives should be prepared.

Gasteiger et al. (27) reported that most homarine was found in the largest invertebrates and in the metabolically most active tissues. On this basis Haake and Mantecon (38) postulated that the poorly developed circulatory systems of the larger invertebrates have a sink to store CO_2 during periods of activity and that homarine functions as this sink by the carboxylation of the N-methylpyridinium ion. They further postulated that homarine may be involved in the "active transport of ions by passage of the dipolar but neutral homarine out of cells and return of the positive N-methylpyridinium ion into cells."

However, the results of the present study indicate that N-methylpyridine is not a product of the decarboxylation of homarine. However, it is tentatively concluded that homarine is a product of the carboxylation of N-methylpyridine.

CHAPTER V

SUMMARY

A. Quinolinic Acid-Nicotinic Acid Relationship in Corn

Corn seedlings were grown in a solution containing quinolinic acid-2,3,7,8-^{14}C for four days. Twenty seedlings were then extracted with boiling 95% ethanol, the extract was chromatographed on Dowex 1-X8 in the formate form and eluted with increasing concentrations of formic acid and finally ammonium formate. The radioactive peaks isolated were determined to be trigonelline, nicotinic acid, diphosphopyridine nucleotide, quinolinic acid, and an unknown substance.

Fifteen additional seedlings were extracted with cold distilled water, and the extract was chromatographed on Dowex 1-formate as before. The radioactive peaks isolated were identified as nicotinic acid, quinolinic acid, trigonelline, and two unknown compounds. It was therefore concluded that quinolinic acid does act as a precursor of nicotinic acid and trigonelline in corn.

B. Some Studies on the Metabolism of Homarine in the Lobster

Homarine was isolated from lobster tissue by extraction with distilled water followed by elution of the deproteinized lobster extract from Dowex 50W-X8 with increasing concentrations of HCl. Homarine was detected by absorbance at 272 m .

First, an experiment was carried out to determine whether or not tryptophan is a precursor of homarine in the lobster. A live lobster

was injected with DL-tryptophan-benzene-U-^{14}C and the homarine isolated. It was found completely inactive, indicating that tryptophan is not a precursor of N-methylpicolinic acid in the lobster.

Next, an experiment was done to determine whether or not homarine is a precursor of N-methylpyridine. Homarine-CH_3-^{14}C was injected into a live lobster and the radioactive peaks from column chromatography isolated. N-methylpyridine was not found to be present as a radioactive product; hence it was concluded that homarine is not a precursor of N-methylpyridine in the lobster.

Finally, an experiment was conducted to determine whether or not N-methylpyridine is a precursor of homarine. A live lobster was injected with N-methylpyridine-CH_3-^{14}C and the one radioactive peak isolated from column chromatography. In paper chromatography, this peak corresponded more closely to homarine than to N-methylpyridine. Hence, it is tentatively concluded that N-methylpyridine is a precursor of homarine in the lobster.

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VITA

Sue Ellen Badiei

Candidate for the Degree of

Master of Science

Thesis: STUDIES ON THE BIOSYNTHESIS OF SOME PYRIDINE CARBOXYLIC ACIDS

Major Field: Chemistry

Biographical:

Personal Data: Born October 10, 1941, in Ogden, Utah, to Joseph H. and Yerda M. Robertson.

Education: Attended grade school and high school in Reno, Nevada, graduated June, 1959; received the Bachelor of Science degree with a major in Chemistry from Oklahoma State University, Stillwater, Oklahoma, in August, 1963; completed the requirements for the Master of Science degree in July, 1968.

Professional Experience: Employed by the Department of Biochemistry summer, 1963. Served as graduate research assistant in the Department of Biochemistry, Oklahoma State University from September, 1963, to July, 1964.