I. A COMPARATIVE STUDY OF TRYPTOPHAN METABOLISM, II. PURIFICATION AND PROPERTIES OF PICOLINIC CARBOXYLASE

By

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CHAPTER I

INTRODUCTION

A. Tryptophan Metabolism

1. The Kynurenine-3-Hydroxyanthranilic Acid Pathway

The metabolism of tryptophan has been intensively studied since the discovery of tryptophan by Hopkins and Cole (1) in 1902. At least four reactions of tryptophan are known to be involved in its metabolism: (a) oxidation to formylkynurenine; (b) hydroxylation to 5-hydroxytryptophan; (c) conversion to indole-3-acetic acid; (d) fission to indole, pyruvic acid, and ammonia.

The first compound to be identified as a product of tryptophan metabolism was kynurenic acid. It had been isolated from dog urine by Liebig (2) in 1853 and was shown to be formed from tryptophan in this species by Ellinger (3) in 1904. Following the discovery of kynurenic acid by Liebig, Kotake and Iwao (4) discovered kynurenine as a tryptophan metabolite in rabbit urine following tryptophan administration and Musajo (5) isolated xanthuranic acid from the urine of rats. In 1945, Elvehjem and coworkers (6) reported that niacin-deficient rats resumed growth if tryptophan was added to the diet. Their findings strongly suggested that tryptophan was being converted to niacin. Rosen et al. (7) showed that the urinary excretion of nicotinic acid and its derivatives by rats was increased following tryptophan administration. Similar findings

in man and other species were quickly recorded. In 1947 Beadle et al. (8) reported that a mutant of Neurospora, requiring tryptophan or niacin for growth, could also utilize kynurenine and that it converted excess kynurenine to niacin in the medium. The conversion of L-tryptophan to kynurenine was first observed in vitro by Kotake and Masayama (9), who named the enzyme system responsible "tryptophan pyrrolase."

The details of the conversion of tryptophan to kynurenine were first elucidated by Knox and Mehler (10, 11) using rat liver preparations. However, more detailed studies on tryptophan pyrrolase have been carried out recently. A 1000-fold purified apotryptophan pyrrolase was obtained from rat liver by Greengard and Feigelson (12). This apotryptophan pyrrolase has no catalytic activity unless supplemented with its cofactor, hematin. Protoporphyrin, mesoporphyrin, serotonin, epinephrine and some tryptophan analogs were reported to inhibit tryptophan pyrrolase activity (12, 13). L-Tryptophan, cortisol and chlorpromazine were found to increase tryptophan pyrrolase activity when administered to intact rats (14, 15, 16). However, adrenalectomized or hypophysectomized rats were reported to show a decrease in tryptophan pyrrolase activity (17, 18).

Kynurenine can undergo at least three major reactions: (a) cleavage of the side chain to form anthranilic acid and alanine; (b) transamination to form o-aminobenzoylpyruvic acid which spontaneously cyclizes to form kynurenic acid; (c) hydroxylation to form 3-hydroxykynurenine. The major pathway of kynurenine metabolism in mammals appears to be the conversion to 3-hydroxykynurenine rather than formation of anthranilic acid or kynurenic acid. In vitro demonstration of the hydroxylation of kynurenic was accomplished by de Castro, Price and Brown (19). They

demonstrated a NADPH* dependent, oxygen-requiring enzyme in liver mitochondria which catalyzes the hydroxylation reaction. This system has also been described by Saito et al. (20) and by Kotake et al. (21). The enzyme has been partially purified (22) and thoroughly studied (23, 24).

Kynureninase, which was first observed by Kotake (25), in the presence of pyridoxal phosphate catalyzes the removal of the side chain from kynurenine and 3-hydroxykynurenine at about equal rates to form alanine and anthranilic or 3-hydroxyanthranilic acids, respectively. The products formed by those reactions are two important intermediates in the degradation of the indole nucleus. Anthranilic acid is involved in the aromatic pathway in Pseudomonas fluorescens and 3-hydroxyanthranilic acid is important in the oxidative pathway and in niacin synthesis in mammals and Neurospora. Kynureninases are formed in animal tissues (26, 27), bacteria (28) and Neurospora (29, 30). Pyridoxal phosphate is required for the enzymes from various sources.

The existance of the kynurenine-3-hydroxyanthranilic acid pathway was also confirmed by the use of isotopes. Heidelberger et al. (31) found that tryptophan-β-^{14}C was converted to urinary kynurenine-^{14}C by dogs. Also, they found that tryptophan-β-^{14}C did not give rise to

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* The following abbreviations are used: NADPH, nicotinamide adenine trinucleotide (reduced form); $NADP^+$, nicotinamide adenine trinucleotide (oxidized form); NADH, nicotinamide adenine dinucleotide (reduced form); NAD^+, nicotinamide adenine dinucleotide (oxidized form); 3-OHAA, 3-hydroxyanthranilic acid; Q.A., quinolinic acid; P.A., picolinic acid; N.A., nicotinic acid; Compound I, α-amino-β-carboxylmuconic semialdehyde; Compound II, or α-HMS, α-hydroxymuconic semialdehyde; NMN, nicotinamide mononucleotide; PRPP, 5-phosphoribosyl-1-pyrophosphate; p-CMB, p-chloromercuribenzoate; α-AMA, α-aminomuconic acid; α-HMA, α-hydroxymuconic acid; CoA, Coenzyme A; Tris, Tris-(hydroxymethyl)aminomethane; DEAE-cellulose, diethylaminoethylcellulose; α-KA, α-ketoadipic acid; DHKA, dehydro-α-ketoadipic acid; EDTA, ethylenediamine tetraacetic acid.

labeled N'-methylnicotinamide while tryptophan-3-^{14}C did (31, 32). The pathway for the conversion of tryptophan to niacin was thus confirmed. Using tryptophan labeled with ^{15}N in the indole ring Shayer (33) further corroborated the kynurenine-3-hydroxyanthranilic acid pathway in mammals. Henderson and Hankes (34) found that DL-tryptophan-3a,7a,7-^{14}C was converted to ^{14}C-labeled quinolinic acid and N'-methylnicotinamide in confirmation of the findings of Heidelberger et al. (31). The injection of 3-hydroxyanthranilic acid labeled with ^{14}C in the carboxyl group also produced urinary N'-methylnicotinamide containing-^{14}C (35). The kynurenine-3-hydroxyanthranilic acid pathway of tryptophan metabolism as described above and related reactions are shown in Figure 1.

2. Other Pathways

As mentioned previously at least four reactions of tryptophan are involved in its metabolism and, among these reactions, the kynurenine-3-hydroxyanthranilic acid pathway is most important in the degradation of tryptophan in mammals. Besides the conversion to a vitamin (niacin) as mentioned above, tryptophan is also the precursor of such diverse and biochemically important compounds as a neurohormone (serotonin), a phytohormone (indoleacetic acid), and an eye pigment in Drosophila (ommochrome).

5-Hydroxytryptamine or serotonin first observed by Erspamer as a physiologically active substance in 1954 (36), was isolated and shortly afterward identified by Page, Rapport and their colleagues (37). Thorson et al. (38) showed that human patients with carcinoid formed large amounts of 5-hydroxytryptamine (39). The findings of an enzyme in mammalian kidney (40) which decarboxylates 5-hydroxytryptophan but not tryptophan, 7-hydroxytryptophan, or tyrosine and that the venom glands of the toad

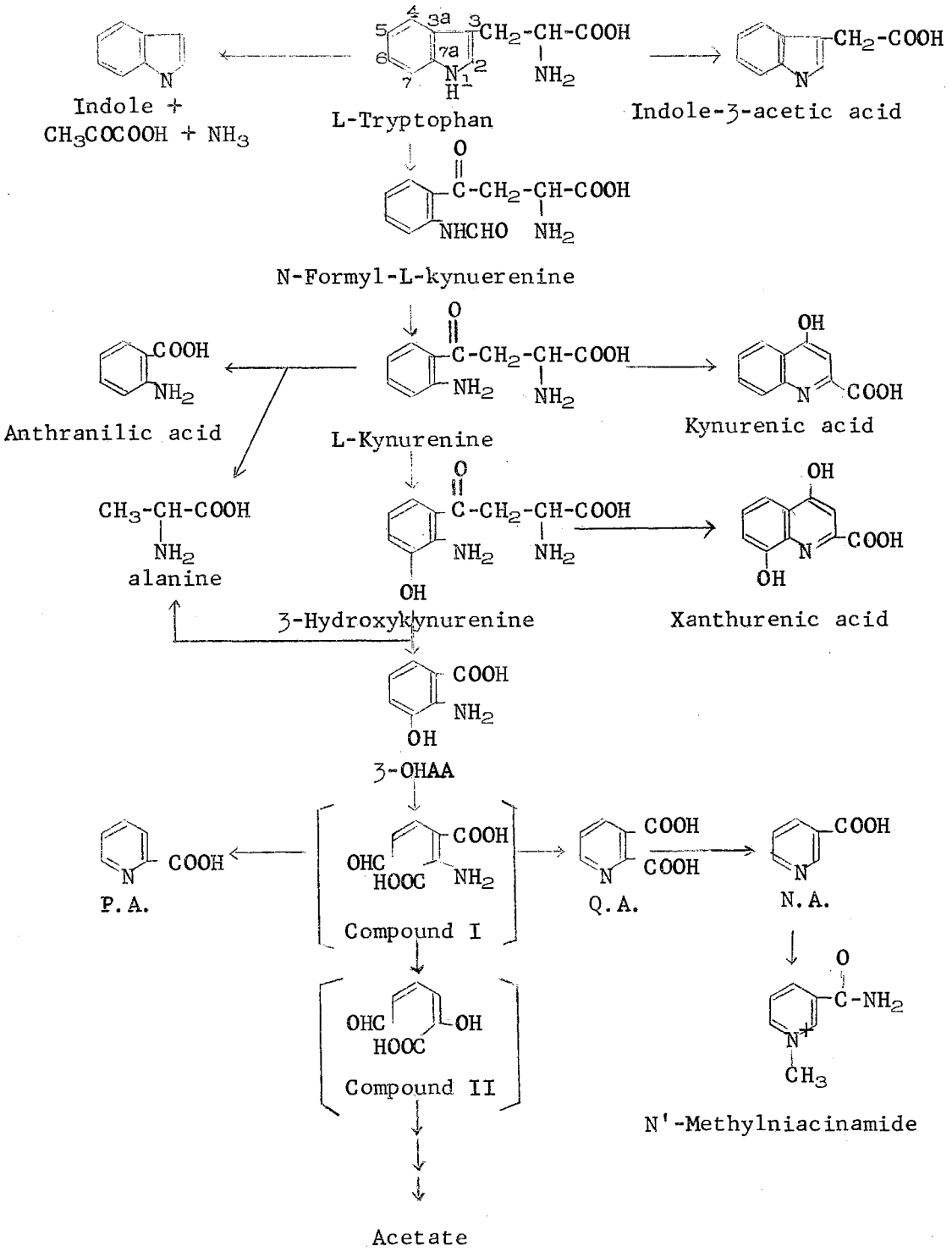


Figure 1. Kynurenine-3-hydroxyanthranilic acid pathway of tryptophan metabolism and related reactions

contain 5-hydroxytryptophan, 5-hydroxytryptamine, and N-methylated derivatives of 5-hydroxytryptamine, but no tryptophan indicate that the conversion of tryptophan to 5-hydroxytryptamine may proceed by hydroxylation of tryptophan followed by decarboxylation of the hydroxylated derivative. The evidence for this pathway is very convincing. It includes the results of both *in vitro* and *in vivo* experiments employing isotopes and has been reviewed (41, 42). Serotonin is degraded by liver and kidney homogenates to 5-hydroxyindoleacetic acid (43) and, 5-hydroxyindoleacetaldehyde was suggested as an intermediate (43). 5-Hydroxyindoleacetic acid appears to be the chief end-product of serotonin metabolism in man (43) and rat (44).

The formation of indole-3-acetic acid from tryptophan in plants is of interest because 3-indole acetic acid (auxin) is a plant growth substance. It was first isolated from human urine and identified by Kögl and coworkers in 1934 (45). In 1935, Thimann (46) found that tryptophan was needed in the medium of *Rhizopus* for auxin production. The enzymes which catalyze the formation of indole acetate from tryptophan in plants were reported in the following years (47, 48, 49). The view that tryptophan is the source of indoleacetic acid in plants (50, 51), in animals (52), and in bacteria (52, 53) has been further confirmed by later experiments. The synthesis of indoleacetic acid from tryptophan seems to be by two reaction sequences. One involves the transamination or oxidative deamination to indolepyruvic acid followed by decarboxylation. The other proceeds by decarboxylation to form tryptamine, then oxidative deamination with monoamine oxidase to give indoleacetaldehyde. Using ^{14}C-labeled tryptophan, Dannenburg and Liverman (51) found that

watermelon slices convert tryptophan to a number of indole derivatives which were identified as indolepyruvic acid, indoleacetic acid, indole-aldehyde, tryptamine and indole acetonitrile. The major route for auxin synthesis in this tissue appears to be through indolepyruvate, though other routes might be important as well (54). In animal tissues and bacteria, the pathway for indoleacetic acid formation also appears to proceed primarily via the transamination route (52).

Ommochromes, the eye pigments found in certain insects, were isolated (55) and tentatively identified as phenoxazine derivatives formed by condensation of two 3-hydroxykynurenine molecules (56). The conversion of tryptophan to the pigments of the eye of Callephora erythrocephala and the molting secretion of Vanessa were demonstrated with isotopes by Butenandt et al. (57, 58).

B. Degradation of 3-Hydroxyanthranilic Acid

1. 3-Hydroxyanthranilic Acid Oxidase

It is known that 3-hydroxyanthranilic acid is a product of tryptophan degradation (59-62) and that it satisfies the nicotinic acid requirements of several organisms (63-68). The major pathway of oxidative degradation of tryptophan is via 3-hydroxyanthranilic acid in many organisms and mammals (69). 3-Hydroxyanthranilic acid oxidase is known to catalyze the oxidation of 3-hydroxyanthranilic acid to a very unstable aliphatic compound, which may either be thespontaneously transformed into quinolinic acid or undergo enzymatic conversion to picolinic acid (70-72). In the early studies of this enzyme by Henderson (73), Schweigert (74) and their coworkers, rat liver slices and homogenates were used. In 1959, a 4-fold purification of this enzyme was obtained by Stevens and

Henderson (75) from the acetone extract of ox liver. Two years later, a 1800-fold purification of this enzyme was reported by Decker et al. (76). However, a few months after Decker's work, a 3000-fold purification of this enzyme was obtained by Iaccarino et al. (77, 78). Recently, a further purification of this enzyme up to 3500-fold had been achieved by Vescia and Prisco (79). The properties of 3-hydroxyanthranilic acid oxidase were thoroughly studied by these workers (75-80) using both partially and highly purified enzyme.

3-Hydroxyanthranilic acid oxidase is a soluble enzyme found in the supernatant fraction of mammalian liver and kidney homogenates. It has been classified as a "phenolytic oxygenase" by Hayaishi et al. (81) since ^{18}O from molecular oxygen, rather than from water, is incorporated into quinolinic acid. Like other phenolytic oxygenases, this enzyme displays a specific requirement for ferrous iron (75, 76, 78, 82). Purified enzyme in the absence of $FeSO_4$ rapidly loses activity on aging. Addition of $FeSO_4$ at 1 mM reverses this effect and results in a slow activation. Reduced glutathione is less effective than $FeSO_4$ in maintaining activity during storage at -20° C (80). Henderson's group found that complete reactivation of the stored enzyme can be achieved after treatment with 0.1 mM ferrous sulfate at pH 3.5 for 5 minutes (75, 80). In 1961, Iaccarino et al. (77) reported that the rate of enzymatic activity is dependent on the partial pressure of oxygen. They also concluded that excess of oxygen inhibited 3-hydroxyanthranilic acid oxidase. However, by using 1 mM Fe^{++} instead of the 0.1 mM Fe^{++} used by Iaccarino et al., Vescia and Prisco (79) reported that a close proportionality was found between oxygen pressure and enzymatic activity.

No inhibition was observed at the highest partial pressures of oxygen.

As is the case with many other enzymes, p-chloromercuribenzoate was reported to inhibit the activity of 3-hydroxyanthranilic acid oxidase at a low concentration (75). However, it was also observed that 3-hydroxyanthranilic acid has a protective effect against p-chloromercuribenzoate (76). Some cations such as Fe^{+++}, Ca^{++} and Cd^{++} and anthranilic acid analogues were also reported to inhibit the enzymatic activity (79). With a 4-fold purified enzyme preparation, Stevens and Henderson (75) observed a substrate inhibition phenomenon at a substrate concentration of 0.15 mM; on the other hand, Decker et al. (76) did not confirm this effect at a substrate concentration as high as 4 mM. However, Vescia and Prisco (79) later showed that inhibition by excess substrate takes place both at pH 6.5 and 8. The K_m value of 3-hydroxyanthranilic acid was found to be 2.1 x 10^{-5} M. The optimal pH of 3-hydroxyanthranilic acid oxidase was found to be 7.5 (76). The highly purified enzyme preparation reveals a typical protein spectrum with a single peak at 280 mμ and with a A_{280} to A_{260} ratio of 1.5 (79).

2. The Products of 3-Hydroxyanthranilic Acid Oxidation

The reaction of 3-hydroxyanthranilic acid oxidase with its substrate, 3-hydroxyanthranilic acid, gives an unstable compound (Compound I; see Fig. 2) which has an absorption maximum at 360 mμ (83, 84). The formation of Compound I results from the oxidative fission of the benzene ring of 3-hydroxyanthranilic acid in the 3-4 position and one mole of oxygen per mole of substrate is required (83). The molar extinction coefficient of Compound I has been reported as 28,300 (83) and 47,500 (85, 86) at 360 mμ in different solvents.

Compound I thus formed is very unstable and is spontaneously converted to quinolinic acid (87). Wiss et al. (71) reported that under

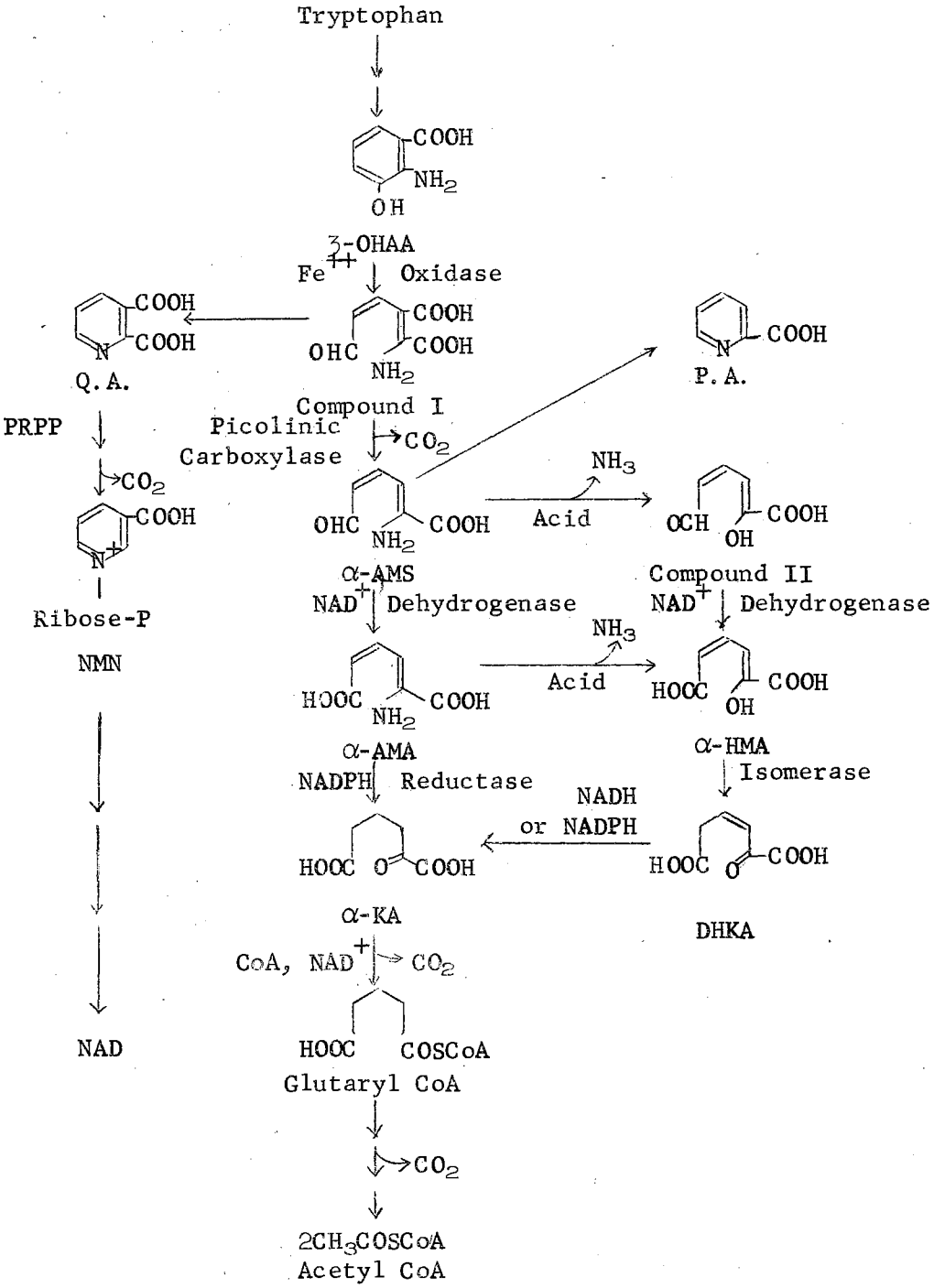


Figure 2. Proposed Metabolic Pathway of 3-Hydroxyanthranilic Acid

acid conditions Compound I undergoes decarboxylation followed by condensation to form picolinic acid. However, Miyake et al. (83) reported, independently, that mineral acids caused a shift in the absorption maximum from 360 mμ to 315 mμ; after neutralization the maximum was found at 375 mμ instead of 360 mμ which suggested that a degradation had taken place to form a new compound. This degradation product, Compound II, was found to react with carbonyl reagents, which indicated the new compound formed has carbonyl characteristics.

The structure of Compound I has not been elucidated. The preparation of derivatives of Compound I by Wiss et al. (86) and the infrared spectra of these derivatives indicated that the structure of Compound I shown in Fig. 2 seems reasonable.

As mentioned previously, Compound I is spontaneously degraded to quinolinic acid (87). Nearly quantitative yields of quinolinate were obtained from rat liver preparations. However, the enzyme which catalyzes the formation of picolinic acid from Compound I was also found in the livers of bovine, guinea pig, pig (79) and other species (89). This enzyme was also shown to be picolinic carboxylase by Mehler (88). The conversion of 3-hydroxyanthranilic acid to niacin has been clearly shown by nutritional (63) and isotopic (35) evidence. However, this conversion had not been demonstrated in vitro, until the recent work by Nishizuka et al. (81) and Gholson et al. (91).

However, the major portion of 3-hydroxyanthranilic acid is not metabolized via picolinic (89, 92), nicotinic (89) or quinolinic (86) acid, but via some other route.

By using tryptophan-3a,7a,7-^{14}C, Henderson and Hankes (34) found that 25 percent of the carbon-14 was expired as $^{14}CO_2$ 12 hours after

injection into a rat. They also showed (93) that 60 percent of the isotope from 3-hydroxyanthranilic acid-carboxyl-^{14}C was expired in 3 hours. With tryptophan-7a-^{14}C, Gholson et al. (94) found that 36 percent of the isotope was expired as $^{14}CO_2$, and 40 to 50 percent was present in the tissues 12 hours after injection. When 3-hydroxyanthranilic acid-1-^{14}C was injected into rats the isotope was found in the methyl carbon of acetate (95). From all of these data, it can be concluded that the main route of tryptophan metabolism to CO_2 is via 3-hydroxyanthranilic acid, and that 3-hydroxyanthranilic acid is oxidized via Compound I to some aliphatic product. In experiments designed to determine the possible metabolic steps between 3-hydroxyanthranilic acid and CO_2, Gholson et al. (96, 97) found that radioactivity from both tryptophan-7a-^{14}C and tritium labeled 3-hydroxyanthranilic acid was found in glutaric acid in the rat. The carbon-14 from tryptophan-7a-^{14}C was found in the carboxyl carbon, again supporting the 3-hydroxyanthranilic acid - Compound I pathway for tryptophan metabolism. The proposed pathway for the formation of glutaric acid from 3-hydroxyanthranilic acid is shown in Figure 2.

Further evidence for the 3-hydroxyanthranilic acid - Compound I pathway was provided by the recent in vitro experiments of Hayaishi's group (98) which provided evidence that Compound I is converted to glutaric acid in cat liver homogenates via Compound II, α-hydroxymuconic acid, α-keto-adipic acid and glutaryl CoA. More recently Hayaishi's group (90) has indicated that α-aminomuconic semialdehyde and α-aminomuconic acid are the true intermediates rather than the corresponding hydroxy compounds. α-HMS dehydrogenase was found to be present in the liver and kidney of rat, cat, beef and chicken. In the presence of NAD^+, but not $NADP^+$, this enzyme

catalyzes the conversion of Compound II to α-hydroxymuconic acid. The reaction may be assayed by observing the decrease of the 375 mμ absorbtion peak of Compound II. α-HMS dehydrogenase is a very unstable enzyme. It loses activity rapidly during storage even at -15° C. p-CMB was found to inhibit its activity. Because of its instability, this enzyme was only partially purified by Hayaishi's group (99) and in this laboratory. α-Aminomuconic acid reductase from cat liver was only partially purified by Hayaishi's group (99) and a detailed study of this enzyme has not yet appeared. However, in order to further elucidate the 3-hydroxyanthranilic acid - Compound I pathway, higher purification and detailed studies of the enzymes mentioned above are necessary.

C. Picolinic Carboxylase

The existance of picolinic carboxylase in mammalian tissue was first reported by Mehler (88) in 1956. In this paper he stated that, ". . . it has been found that in quinolinic acid production only the synthesis of the intermediate (Compound I) is enzyme-catalyzed; the subsequent formation of quinolinic acid is spontaneous. An enzyme which does attack the intermediate has been concentrated from extracts of liver. This enzyme does not make or degrade quinolinic acid, but converts the intermediate to picolinic acid." As mentioned previously, this enzyme was also found to occur in the livers of pigs, guinea pigs (88) and other species (89). Because the activity of this enzyme is low in beef liver, Mehler (88) partially purified picolinic carboxylase from guinea pig liver by using acid treatment, gel absorption and ammonium sulfate fractionation. HCN and p-CMB were reported to inhibit picolinic carboxylase activity (88). The enzymewas reported to exhibit a broad optimum

from pH 6 to 9.5. The product of this enzymatic catalyzed reaction was identified as picolinic acid both from its absorption spectrum and by paper chromatography.

In studying the metabolism of 3-hydroxyanthranilic acid, Decker (102) stated that, "During the purification of the oxidase from beef liver it was often observed that the 360 mμ maximum would decrease more rapidly than that of a non-enzymatic control. This phenomenon was noted in all purification steps but was particularly observed in the latter stages (i.e. pH and gel treatment, 40-65 percent ammonium sulfate precipitation)". He also found that the enzyme activity was inhibitied by 2×10^{-3} M cyanide or 0.035 M ammonium sulfate. Using tritium-labeled 3-hydroxyanthranilic acid he identified the main product of this enzyme reaction to be picolinic acid.

Recently the purification of picolinic acid carboxylase has been reported by Hayaishi's group (99). A 50-fold purification of this enzyme from cat liver was obtained by the Japanese workers. Mg^{++} was reported as a cofactor of this enzyme.

Very little work has been done on the comparative study of the 3-OHAA pathway of tryptophan oxidation. In 1957, Suhadolnik et al. (103) in a paper dealing with the metabolism of 3-hydroxyanthranilic acid in various mammalian species reported that, mouse, pig, and rat liver extracts formed appreciable amounts of quinolinic acid but very little picolinic acid. The picolinic carboxylase activity of mouse, pig and rat liver extract was less than extract from beef or cat liver. A comparative study of the metabolism of tryptophan via the 3-hydroxyanthranilic acid pathway is a part of the present work. The enzymatic activities of 3-hydroxyanthranilic acid oxidase, picolinic carboxylase and α-hydroxymuconic

semialdehyde dehydrogenase in some species of mammals, insects, mollusks, annelids and fishes were assayed. Also, the amount of radioactivity in the CO_2 expired by the various species after injection with tryptophan-5-^{14}C or tryptophan-α-^{14}C was measured. Picolinic carboxylase, an enzyme of the 3-hydroxyanthranilic acid pathway, was also partially purified from beef kidney and its properties were studied.

The objectives of the present investigation were:

1. To determine whether the oxidative metabolism of tryptophan proceeds by the same route in representative species of various animal phyla as it does in mammals.

2. To purify picolinic carboxylase and study its properties in order to obtain more information about the physiological function of this enzyme in vivo.

CHAPTER II

EXPERIMENTAL PROCEDURE

A. Materials

3-Hydroxyanthranilic acid, p-chloromercuribenzoate sodium salt, α,α'-dipyridyl and Tris-(hydroxymethyl) aminomethane were obtained from California Corporation for Biochemical Research. 8-Hydroxyquinoline and o-phenanthroline was obtained from the G. Frederick Smith Chemical Co., DEAE-Cellulose from Sigma Chemical Company, and Sephadex G-25 from Pharmacia, Uppsala, Sweden. DL-Tryptophan-α-^{14}C (100) and 3-hydroxyanthranilic acid-COOH-^{14}C (101) were synthesized in this laboratory. α-Hydroxymuconic semialdehyde was a gift from Dr. L. R. Morgan, Louisiana State University, School of Medicine. Chickens, earthworms, beef liver and kidney were purchased from local sources. Cockroaches and snails were obtained from Dr. C. G. Beames of the Department of Physiology, Oklahoma State University. Fish were obtained from local ponds.

B. Experimental Methods

1. Comparative Study of Tryptophan Metabolism

a. Purification of 3-Hydroxyanthranilic Acid Oxidase

3-Hydroxyanthranilic acid oxidase was purified from beef liver according to the method of H. H. Kang (104) with the following modifications: 1) the acid step was carried out at pH 3.5 at 0° C and in the presence of 1×10^{-3} M $FeSO_4$, 2) calcium phosphate gel treatment was omitted and 3) the enzyme precipitated with ammonium sulfate was dissolved in cold de-

ionized water which contained 1 x 10^{-3} M $FeSO_4$. The purification was carried through to the second ammonium sulfate fractionation step (104) and the oxidase thus obtained was stored at -15° C until used as described.

b. Preparation of Compound I

Compound I was always prepared 10 minutes before use by the following method: one ml of 0.1 M, pH 7, Tris buffer was put into a test tube. 0.5 ml of 3 x 10^{-3} M 3-hydroxyanthranilic acid solution and 0.5 ml of the second ammonium sulfate fraction of oxidase, containing 2.06 mg protein per ml with a specific activity 2470 units per mg protein were then added. The tube was shaken and then allowed to stand at room temperature for about 5 minutes, at which time the absorbency at 360 mμ had ceased to increase. Then the tube was placed in an ice-bath. The Compound I thus obtained was stable enough for assay of picolinic carboxylase for 30 to 60 minutes stored at 0° C.

c. Preparation of Compound II

3-Hydroxyanthranilic acid (15.3 mg) was dissolved in a minimum amount of 2N HCl. Deionized water was then added to about 7 ml and 2N NaOH was added dropwise to neutralize the solution to pH 7 by using pH 6 to 8 range pH paper. Deionized water was again added to make the final volume equal to 10 ml. The concentration of this solution was 1 x 10^{-2} M. The 3-hydroxyanthranilic acid solution thus obtained was then put into a 50 ml beaker which was kept in an ice-bath. Ten ml of the second ammonium sulfate fraction of 3-hydroxyanthranilic acid oxidase (containing 10.3 mg protein per ml with a specific activity equal to 2,470 units per mg protein) were then added to start the reaction. The reaction was carried on with constant stirring by using a magnetic stirrer and 0.1N NaOH was added occasionally to maintain the reaction mixture near pH 7.

The reaction was followed by removing aliquots (about 0.02 ml), diluting to about 10 ml with absolute ethanol and, measuring the fluoresence of 3-hydroxyanthranilic acid with a electronic photofluorometer (Coleman Instruments Inc.). After the complete disappearence of fluoresence, the reaction mixture was adjusted to pH 1 by adding a few drops of 5N HCl and the solution was heated in a boiling water bath for 5 minutes. The solution was then cooled to room temperature under running tap water and 5N NaOH solution was added dropwise to bring it back to neutral pH. Denatured protein was centrifuged down with a clinical centrifuge and discarded. The brown colored solution thus obtained contained Compound II which has two absorption maximum in the ultraviolet at 375 and 265 mμ in 0.1M, pH 7, Tris buffer. The higher absorption peak at 375 mμ is due to the presence of Compound II and the smaller peak at 265 mμ may be due to the presence of quinolinic acid formed spontaneously from Compound I. The Compound II solution thus obtained was used for the assay of α-HMS dehydrogenase activity. Compound II is unstable during storage at -15° C and is converted to an unknown compound having an absorption maximum at 380 mμ. This compound has no activity with α-HMS dehydrogenase. It, therefore, is then necessary to prepare fresh Compound II solution each time before use, or to use a solution stored at -15° C not longer than 2 days in order to assay for α-HMS dehydrogenase.

d. Assays

1) Method for the Assay of 3-Hydroxyanthranilic Acid Oxidase (104)

a) Principle: The formation of Compound I results in an increase of absorbancy at 360 mμ.

b) Procedure: Tris buffer, 2.8 ml of 0.1M, pH 7,

and 0.1 ml of enzyme solution were added to a 3 ml volume cuvette with a light path of 1 cm. The reaction was started by the addition of 0.1 ml 3 x 10^{-3} M 3-hydroxyanthranilic acid solution. The enzymatic activity was measured by following the increase of absorbancy at 360 mμ using the Cary Model 14 or Beckman DB recording spectrophotometer.

c) Definition of Unit and Specific Activity: the rate of the reaction was determined from the initial linear portion (10 to 20 seconds) of the absorbancy versus time curve. One unit of enzyme activity is defined as the amount of enzyme which converts 1 millimicromole (mμMole) of substrate into product per minute. Specific activity is defined as the number of units of enzyme per mg of protein.

d) Determination of Protein: protein was determined both by the Folin-Ciocalteu (105) and 280 mμ/260 mμ method (104) for all the enzymatic assays.

2) Method for the Assay of Picolinic Carboxylase

a) Principle: The decarboxylation of Compound I followed by spontaneous ring closure to form picolinic acid results in a decrease of absorbancy at 360 mμ.

b) Procedure: Potassium phosphate buffer, 2.7 ml of 0.1M, pH 7.5, was put in a 3 ml cuvette and 0.2 ml of Compound I solution prepared as mentioned above was then added. This will usually give an absorbancy at 360 mμ around 1.7 to 1.9. The rate of the spontaneous decrease at 360 mμ was followed until a constant rate was reached. The reaction was then started by the addition of 0.1 ml picolinic carboxylase enzyme solution. The rate of decrease of absorbancy at 360 mμ was followed by using the Beckman DB recording spectrophotometer.

c) Definition of Unit and Specific Activity: The enzyme activity was obtained by subtracting the rate of spontaneous decrease of absorbancy at 360 mμ from the rate of decrease after addition of picolinic carboxylase. One unit of enzyme activity is defined as the amount of enzyme which converts 1 millimicromole (mμMole) of substrate into product per minute. Specific activity is defined as the number of units of enzyme per mg of protein.

3) Method for the Assay of α-HMS Dehydrogenase

a) Principle: The oxidation of α-hydroxymuconic semialdehyde (Compound II) to α-hydroxymuconic acid results in a decrease of absorbancy at 375 mμ.

b) Procedure: Tris buffer, 2.75 ml of 0.1 M, pH 8, was put in a 3 ml volume cuvette. α-HMS dehydrogenase enzyme solution, 0.1 ml, and 0.05 ml of Compound II solution prepared as mentioned above were then added. This amount of Compound II will usually give an absorbancy of about 1.6 to 1.9 at 375 mμ. However, the amount of Compound II and buffer solution added can be varied in order to obtain an approximately equal amount of substrate in the cuvette for each assay. The reaction was started by the addition of 0.1 ml 3×10^{-2} M NAD^+ The rate of decrease of absorbancy at 375 mμ was followed by using the Cary Model 14 or Beckman DB recording spectrophotometer.

c) Definition of Unit and Specific Activity: The rate of the reaction was determined from the linear portion of the absorbancy versus time curve. One unit of enzymatic activity is defined as the amount of enzyme which converts 1 millimicromole (mμMole) of substrate into product per minute. Specific activity is defined as the

number of units of enzyme per mg of protein.

e. Isotopic Measurements

1) Collection of $^{14}CO_2$

Various amounts of ^{14}C-labeled tryptophan as shown in Table II and III were injected intraperitoneally into the animals to be tested as shown in Table II and III separately. The animals were then put into a sealed metabolism chamber as shown in Figure 3. Tube I contained about 30 ml of 1 N of sodium hydroxide solution and tube II and III each contained 5 ml of 1 N sodium hydroxide solution. The CO_2 expired from the animal was trapped in the sodium hydroxide solution in tubes II and III. After 8 hours of collection, the radioactive CO_2 trapped in the sodium hydroxide solution was measured as described below.

2) Measurement of radioactivity

Aliquots (2 ml) of the 1 N sodium hydroxide solution of tubes II and II were transferred separately into a combustion tube and a small amount of carrier sodium carbonate was added to aid the removal of carbon dioxide from the solution. The combustion tube was the connected to a wet combustion system (107) and 5 ml of 10 percent perchloric acid were then added. The CO_2 evolved from the solution was collected into a vacuum ionization chamber and, the radioactivity was measured by using the vibrating reed electrometer (108).

2. Picolinic Carboxylase

a) Enzymatic Assay

The method for assaying picolinic carboxylase was described above.

b) Purification Procedure

All of the following steps were carried out in the cold room at 3° C unless otherwise specified.

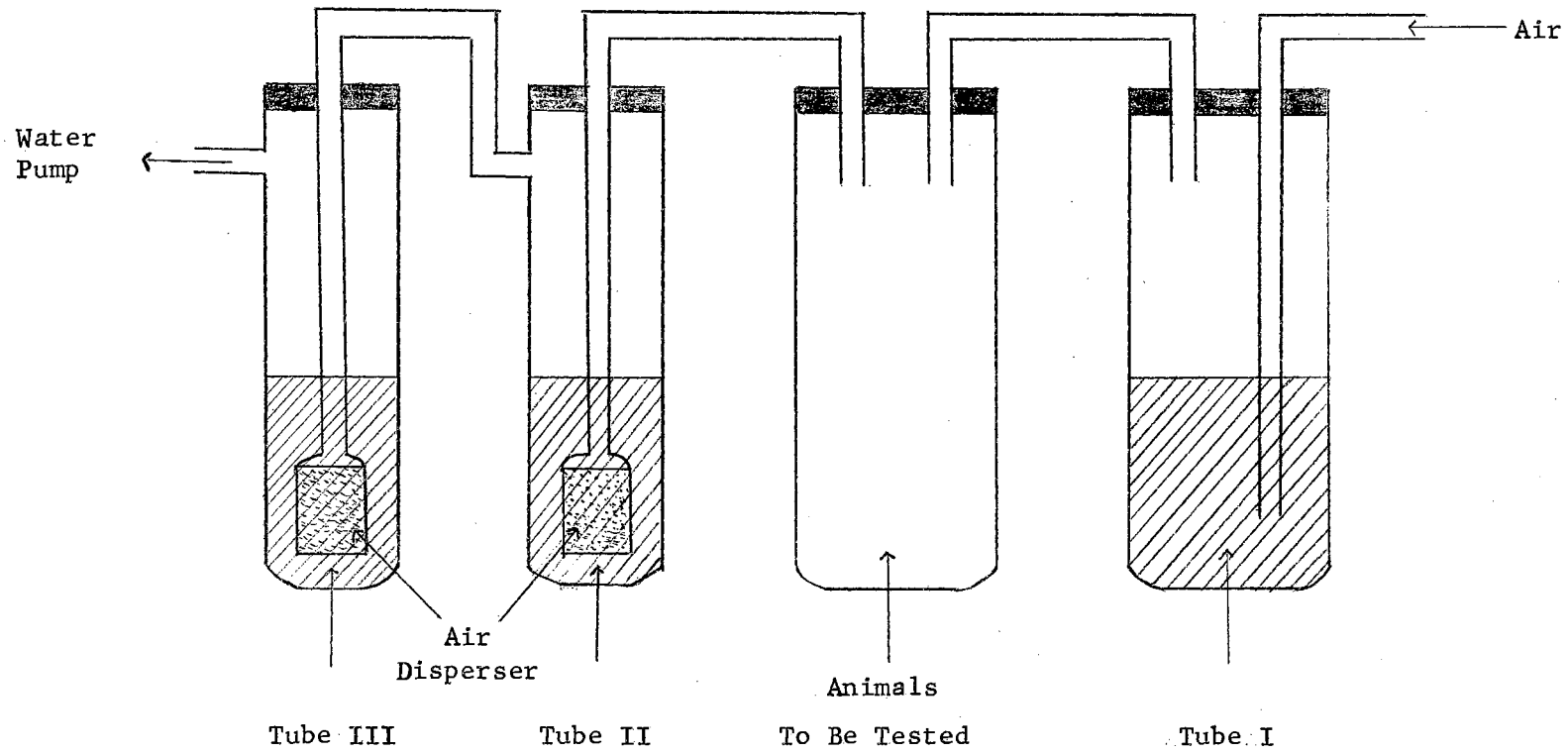


Figure 3. Metabolic chamber for the collection of respiratory CO_2

Step 1. Homogenization: 170 gm of beef kidney purchased from a local slaughter house and stored in a deep freeze (-15° C) were homogenized with four volumes of deionized water (weight per volume) for one minute in a Waring blendor at full speed. The homogenate was centrifuged for 1 hour at 12,800 x g and 0° C. The precipitate was discarded and the supernatant solution retained.

Step 2. Heat Treatment: the supernatant solution was heated in 200 ml portions in a stainless steel beaker with stirring to 65° C in a 3 liter water bath at 85° C and kept at that temperature for 1 minute. Then the solution was cooled down rapidly with stirring to below 10° C in an ice-bath. Denatured protein was removed by centrifuging for 30 minutes at 12,800 x g and 0° C.

Step 3. Acid Treatment: the resulting supernatant solution was adjusted to pH 5 with 2 N hydrochloric acid which was added dropwise with stirring in an ice-bath. After standing for 10 minutes, denatured protein was removed by centrifuging for 30 minutes as before.

Step 4. First Ammonium Sulfate Fractionation: a saturated solution of ammonium sulfate was prepared at 3° C, and concentrated ammonium hydroxide solution was added until the solution was pH 7 as determined by a Beckman pH meter. This saturated ammonium sulfate solution was added slowly with constant stirring to the supernatant obtained from Step 3 until 50 percent saturation (v/v) was reached. The solution was allowed to stand with stirring for 20 minutes, then centrifuged at 12,800 x g for 30 minutes, and the precipitate was discarded. More saturated ammonium sulfate solution was again added slowly with constant stirring until 75 percent saturation was reached, the solution was then allowed to

stand and centrifuged as before. The precipitate was dissolved in 50 ml of cold deionized water.

Step 5: Second Ammonium Sulfate Fractionation: this step was carried out in the same manner as the first ammonium sulfate fractionation, except that the fraction between 35 and 65 percent saturation in ammonium sulfate was collected and dissolved in 10 ml of cold deionized water.

Step 6: Desalting: the second ammonium sulfate fraction was passed through a 2 x 20 cm Sephadex G-25 column, equilibrated in H_2O and eluted with distilled water. The colored protein (red) was easily observed as it passed through the column and the colored fractions were collected.

Step 7: DEAE-Cellulose Chromatography:

a. DEAE-Cellulose Column: About 20 gm of DEAE-cellulose (dry weight) were washed with 1 liter of 1N NaOH solution, followed by 1 liter of 1N HCl solution, then with 1N NaOH solution again, and finally washed with distilled water until it was free from alkali. The acid and base washed DEAE-cellulose was then suspended in 400 ml of 0.2 M, pH 7, potassium phosphate buffer. The pH was adjusted to 7 by the addition of 0.2M KH_2PO_4 solution on a Beckman pH meter with stirring. The neutralized DEAE-cellulose was then washed several times with distilled water. The DEAE-cellulose was then suspended in 100 ml of 0.01 M, pH 7, potassium phosphate buffer, and packed into a 2 cm diameter column until it was 21 cm in height. The column was again washed with several hundred ml of 0.01 M phosphate buffer, pH 7.

b. Elution: Ten ml of desalted enzyme solution from Step 6 (containing 240 mg protein) was placed on the column, and eluted with

100 ml portions of 0.01, 0.05, 0.1 and 0.2 M, pH 7, potassium phosphate buffer. Seventy 5.5-ml fractions were collected with a flow rate of about 1 ml per minute. The absorbancy of each fraction at 280 and 260 mμ was measured. Fractions containing ultraviolet-absorbing material were assayed for enzymatic activity.

CHAPTER III

RESULTS AND DISCUSSION

A. Comparative Study of Tryptophan Metabolism

1. Purification of 3-Hydroxyanthranilic Acid Oxidase

The preparation of highly purified 3-hydroxyanthranilic acid oxidase is one of the most important steps in the study of the enzymatic degradation of 3-hydroxyanthranilic acid. In the present work Compound I and II, which were used as substrates for the assay of picolinic carboxylase, and α-HMS dehydrogenase were prepared enzymatically by the action of 3-hydroxyanthranilic acid oxidase on its substrate, 3-hydroxyanthranilic acid. Therefore, in order to obtain optimum results, a highly purified oxidase was desired. The oxidase was purified as described by H. H. Kang (104) with only slight modifications. The second ammonium sulfate fraction (45 to 65 percent saturation) was used in the present work. Although, this enzyme preparation was usually a colorless protein solution, a very small amount of picolinic carboxylase activity was still present. This phenomena also had been noticed by Decker (102) who reported that it is somewhat difficult to separate these two enzymes. The presence of picolinic carboxylase activity can be detected by observing the increasing rate of "spontaneous" decrease of absorbancy at 360 mμ in the presence of increasing amounts of oxidase. However, the presence of a small amount of picolinic carboxylase in the oxidase will not interfer with the picolinic

carboxylase assay. Thus the second ammonium sulfate fraction of 3-OHAA oxidase was used for the following work.

2. Enzymatic Assays

a. 3-Hydroxyanthranilic Acid Oxidase

The enzymatic oxidation of 3-hydroxyanthranilic acid between carbons 3 and 4 of the benzene ring forms Compound I which has an absorption maximum at 360 mμ at neutral. Because of its high molecular extinction coefficient (ϵ=28,300) at this wavelength, it provides a rapid and easy method for following the enzymatic reaction spectrophotometrically.

Compound I is unstable at room temperature but was found to be stable when stored at 0° C. The molecular extinction coefficient of Compound I at 360 mμ was determined as 28,300 at pH 7.5 by Long et al. (83). In the present study it was found the ϵ remained constant over a pH range of 7 to 9 but decreased to 20,000 at a pH of 5 or 6. 3-OHAA oxidase is assayed at pH 7 and therefore an ϵ of 28,300 was used to calculate the enzymatic activity.

b. Picolinic Carboxylase

The decarboxylation of Compound I by the action of picolinic carboxylase followed by non-enzymatic ring closure to form picolinic acid results in a decrease absorbancy at 360 mμ. Because Compound I is unstable at room temperature, the non-enzymatic spontaneous formation of quinolinic acid will also cause a decrease of absorbancy at 360 mμ. The activity of picolinic carboxylase was therefore obtained by subtracting the rate of decrease of absorbancy at 360 mμ before the addition of picolinic carboxylase from the rate of decrease after the addition of picolinic carboxylase.

c. α-Hydroxymuconic Semialdehyde Dehydrogenase

α-Hydroxymuconic semialdehyde dehydrogenase, which catalyzes the oxidation of α-hydroxymuconic semialdehyde to the corresponding acid in the presence of NAD^+, is a very unstable enzyme. Activity was gradually lost during standing both at 0° and -15° C. $NADP^+$ could not be substituted for NAD as a cofactor for α-HMS dehydrogenase. p-Chloromercuribenzoate was found to inhibit this enzyme.

α-Hydroxymuconic semialdehyde (Compound II or α-HMS), the substrate used for the assay of the dehydrogenase, was prepared by treating Compound I with acid and heat as described previously. The Compound II solution thus obtained has two absorption maxima in the ultraviolet at pH 7, 375 and 265 mμ, as shown in Figure 4. The higher peak at 375 mμ is due to the presence of Compound II, while the smaller peak around 265 mμ is due to the presence of quinolinic acid formed by the non-enzymatic reaction from Compound I. Curve I of Figure 4 shows the absorption spectrum of chemically prepared Compound II, a gift from Dr. L. R. Morgan of Louisiana State University. Both of these compounds prepared by different methods are substrates for α-HMS dehydrogenase.

The molecular extinction coefficient of Compound II in alkaline solution (pH 10.5) at 375 mμ was reported to be 21,000 by Morgan (109). With chemically synthesized Compound II received from Dr. Morgan ε was determined as 22,200 in 0.1 M Tris buffer, pH 10.5, and as 19,600 in 0.1 M Tris buffer at pH 8. The molecular extinction coefficient of Compound II was found to increase gradually when pH was increased, whereas in acidic solution, the 375 mμ peak shifted to 315 mμ. The activity of α-HMS dehydrogenase was calculated by using ε=19,600 at pH 8, which was found to be the optimum pH for the dehydrogenase assay.

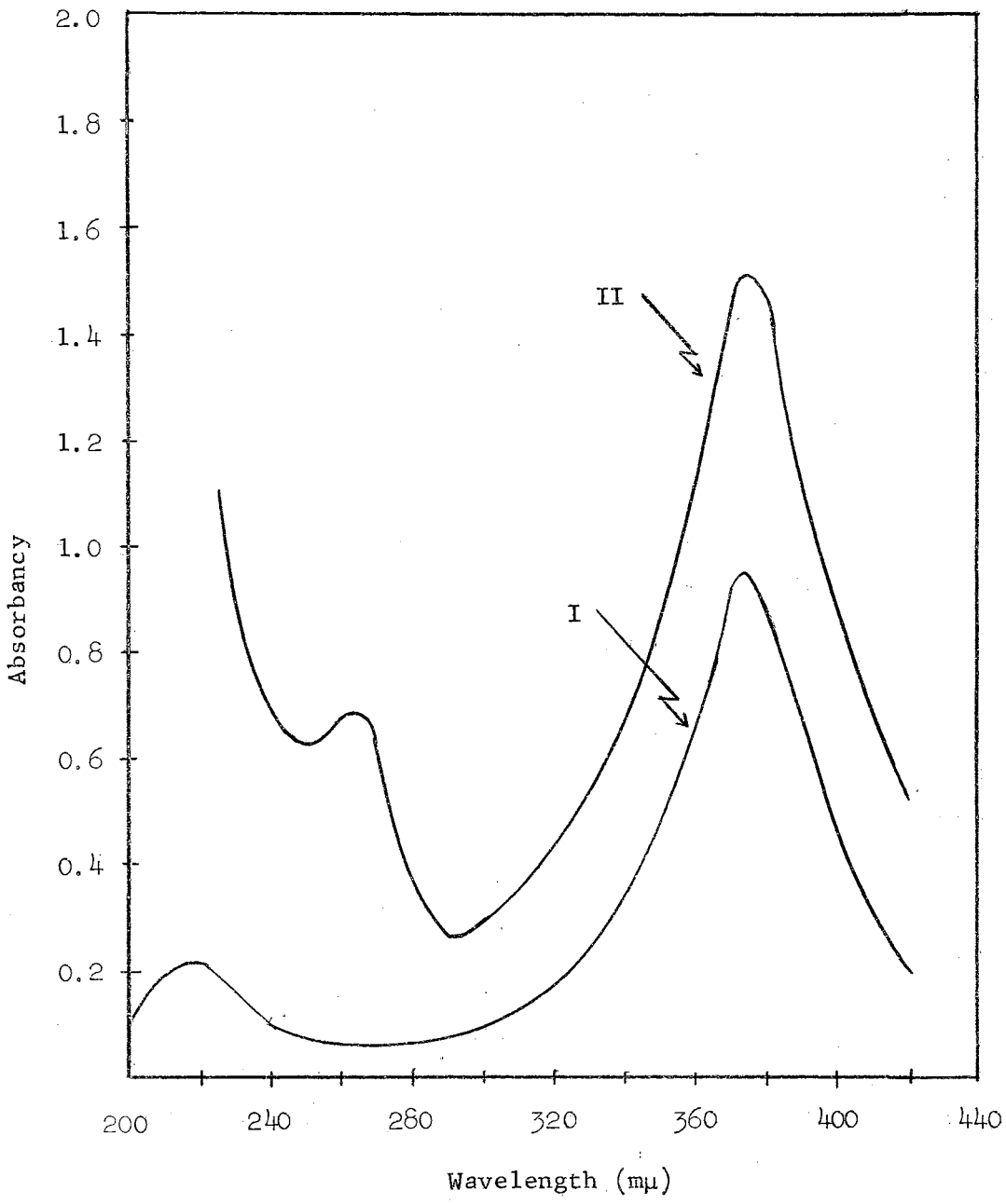


Figure 4: Absorption Spectrum of Chemically and Enzymatically Synthesized α-HMS in 0.1M, pH 7, Tris buffer.

I. Chemically synthesized

II. Enzymatically synthesized

3. In vitro Study of Enzymatic Activities in Different Organisms

Previous in vivo and in vitro studies of tryptophan metabolism in mammals showed that the kynurenine - 3-hydroxyanthranilic acid pathway is the major route for the degradation of tryptophan (69, 73, 74, 77, 79). The oxidation of the benzene ring of tryptophan via 3-hydroxyanthranilic acid, α-hydroxymuconic semialdehyde and α-hydroxymuconic acid to glutaric acid was also suggested by results obtained in Henderson's laboratory (96, 97) in whole animals and in Hayaishi's laboratory (89, 98) by in vitro studies. The comparative study described here was undertaken in order to determine if this pathway is also functioning in other groups of animals besides mammals.

The activities of 3-hydroxyanthranilic acid oxidase, picolinic carboxylase and α-hydroxymuconic semialdehyde dehydrogenase were assayed in ten different species as shown in Table I. All of the three enzymes were found in mammals (rat and cow). However, none of the three enzymatic activities could be found in earthworm, snail, cockroach, grasshopper or tetrahymena. In chicken liver, two of the three enzymes were detected but no picolinic carboxylase activity was observed. However, in fish (bass) liver, high activities of both oxidase and carboxylase were detected. But, rather surprisingly no dehydrogenase was found.

In the mammals tested, all of the three enzyme specific activities were found to be higher in kidney than in liver. In fact in the case of picolinic carboxylase, no activity was detected in rat or beef liver under the assay conditions. The same result was also found in chicken liver. However, according to the reports of Mehler (72) and Decker (102) some picolinic carboxylase activity was found both in rat and beef liver,

TABLE I

ACTIVITIES OF 3-OHAA OXIDASE, PICOLINIC CARBOXYLASE AND α-HMS DEHYDROGENASE PREPARED FROM DIFFERENT ORGANISMS

Enzyme Source	Protein* Concentration (mg/ml)	Specific Activity ‡		
		3-OHAA Oxidase	Picolinic Carboxylase	α-HMS Dehydrogenase
Rat Liver	28.0	127.5	0	8.40
Rat Kidney	21.7	88.7	3.91	11.85
Beef Liver	50.0	15.6	0	6.36
Beef Kidney	28.6	45.5	0.74	3.44
Fish Liver (Bass)	28.6	84.5	2.27	0
Chicken Liver	21.7	21.2	0	1.95
Chicken Heart	4.8	0	0	0
Earthworm	15.6	1.02	0	0
Snail	16.0	0	0	0
Cockroach	25.2	0	0	0
Grasshopper	12.9+	0	0	0
Tetrahymena	3.5	0	0	0
Castor Cotyledon	26.0+	0	0	0

* Determined by method of Folin et al. (105) except as noted+ which were determined by the 280 mμ/260 mμ ratio method (106).
‡ mμmoles/mg Protein/min

although the amount was very small. Therefore, the probable explanation of the present results is that the amount of picolinic carboxylase present in rat and beef liver is so low that it could not be detected under the assay conditions used. However, the present results evidently indicate that picolinic carboxylase in mammals is present mainly in kidney rather than in liver.

The failure to detect activity of any of the three enzymes in snail, cockroach, grasshopper, earthworm and tetrahymena indicates that tryptophan is probably not degradated via 3-hydroxyanthranilic acid to glutaric acid in these organisms. The observation of high activities of both 3-hydroxyanthranilic acid oxidase and picolinic carboxylase but not of α-HMS dehydrogenase in fish liver indicates that tryptophan may not be degraded via the pathway of aliphatic compound formation to CO_2. It is possible that the true intermediate may be α-aminomuconicsemialdehyde rather than α-HMS (99) and that unlike the dehydrogenase from mammalian sources the fish liver dehydrogenase may be specific for the α-amino compound. An unknown special physiological function of picolinic acid and accumulation of picolinic acid in fishes may possibly provide an alternative explanation. However, no final conclusion can be given at present.

4. In vivo Studies of ^{14}C-Labeled Tryptophan Metabolism in Different Species

The results of the enzymatic assays presented in Table I show that no activities of oxidase, carboxylase or dehydrogenase were found in the tissue homogenates of snail, cockroach, earthworm and grasshopper. These findings suggest that the benzene ring of tryptophan is either not degraded in these organisms or is degraded by a pathway different from that found

in mammals. In order to clarify this point tryptophan-5-^{14}C was injected into these different organisms and the expired CO_2 was collected for about eight hours. The radioactivity was counted by the use of a vibrating reed electrometer. The results are shown in Table II.

Consistent with the results obtained from enzymatic assays, tryptophan-5-^{14}C was not oxidized to $^{14}CO_2$ in the grasshopper and cockroach. These results indicate that the benzene ring of tryptophan is not oxidatively degraded in these insects. On the other hand the snail and earthworm which also had no measurable activity of any of the three enzymes examined excreted from 3 to 6 percent of an injected dose of tryptophan-5-^{14}C as $^{14}CO_2$. These results suggest that in these two organisms the benzene ring of tryptophan is oxidized, but by a different pathway than that found in mammals. As would be expected from the presence in chicken liver of the enzymes of the 3-OHAA oxidase pathway, tryptophan-5-^{14}C was oxidized to $^{14}CO_2$ when injected intraperitoneally into a baby chick.

In order to see if the insect species were able to oxidize any portion of the tryptophan molecule as well as to compare the extent of oxidation of the side chain and benzene ring of tryptophan by the other organism another series of experiments was carried out using tryptophan-α-^{14}C (Table III). The percentage of the tryptophan side chain oxidized to CO_2 in the insects was low but measurable indicating that this portion of the molecule is degraded at least to some extent in these organisms. In the other species tested the larger percentage of $^{14}CO_2$ was evolved from the side chain labeled tryptophan than from the ring labeled compound. This result is not surprising since in the known pathways of tryptophan metabolism the side chain is removed before the ring is attacked. It

TABLE II

EXCRETION OF $^{14}CO_2$ FROM DIFFERENT ORGANISMS AFTER INJECTION OF TRYPTOPHAN-5-^{14}C

Organism	Animals Injected	Wt/Animal	Time of $^{14}CO_2$ Collection	Tryptophan Injected		^{14}C Recovered in CO_2	
		gm	hour	mg	mμc	mμc	%
Grasshopper	2	2-3	4.5	0.2	122	0	0
Snail	2	8	4.0	0.2	122	3.42	2.80
	2	8	8.0	0.2	122	4.22	3.46
Earthworm	2	3-4	11.0	0.2	122	7.15	5.85
	2	3-4	8.0	0.2	122	4.79	3.93
Cockroach	2	1-2	8.0	0.2	122	0	0
	2	1-2	8.0	0.28	267	0	0
Chicken	1	106	8.0	15.0	1407	125.6	8.93

TABLE III

EXCRETION OF $^{14}CO_2$ FROM DIFFERENT ORGANISMS AFTER INJECTION OF TRYPTOPHAN-α-^{14}C

Organisms	Animals Injected	Wt/Animal	Tryptophan Injected		^{14}C Recovered in CO_2	
		gm	mg	mμc	mμc	%
Grasshopper	2	2-3	0.06	138.6	0	0
	2	2-3	0.06	138.6	1.23	0.89
	1	2-3	0.03	69.3	0.46	0.66
Snail	2	8	0.06	138.6	2.01	1.45
	2	8	0.20	119.8	9.40	7.80
Earthworm	2	3-4	0.06	138.6	4.36	3.14
	2	3-4	0.06	138.6	7.13	5.15
Cockroach	2	1-2	0.06	138.6	0.91	0.66
	2	1-2	0.20	119.8	1.44	1.20
Chicken	1	63	0.15	347.0	22.43	6.50
	1	95	15.15	347.0	50.30	14.50

$^{14}CO_2$ was collected for 8 hours in every case.

should be noted that increasing the moles of tryptophan injected increased the percent of carbon-14 which was excreted as $^{14}CO_2$ (grasshopper, snail, cockroach, chicken) (Table III). Extreme caution must therefore be observed in interpreting variations in amounts of $^{14}CO_2$ excreted from radioactive compounds unless the moles of compound injected and weight of the test organism are taken into account.

B. Purification and Properties of Picolinic Carboxylase

1. Purification Steps

The enzyme is water soluble and was readily extracted from kidney by homogenizing with four volumes of water. It is stable both to heat (65° C) and acid (pH 5). Heating to 65° C for 1 minute followed by rapid cooling to below 10° C removed about 50 percent protein. A one-fold further purification was obtained by adjusting the protein solution to pH 5 after the heat treatment. Ammonium sulfate fractionation did not give a good purification and recovery. The only purpose of this step was to concentrate the enzyme protein. Passing the ammonium sulfate fraction through a Sephadex G-25 column removed the ammonium sulfate from the enzyme protein and increased the apparent activity by about one fold. DEAE-cellulose chromatography gave a 195-folds purification as compared with the crude homogenate in the tube which had the highest specific activity. The most active four tubes gave an average of 83-fold purification with a specific activity of 52.8 and 20 percent recovery. A summary of the purification sequence and the DEAE-cellulose chromatography diagram is shown on Table IV and Fig. 5.

2. Properties of Picolinic Carboxylase

a. Reaction Catalyzed by Picolinic Carboxylase

TABLE IV

PURIFICATION OF PICOLINIC CARBOXYLASE

Fractions	Volume	Activity	Total Units	Total Protein	Specific Activity	Recovery	Purification
	(ml)	(Units/ml)		(mg)	(Units/mg)	(%)	
Homogenate	590	12.7	7500	11700	0.64	100	1
Heat Treatment	545	12.7	6920	4180	1.66	92.4	2.6
Acid Treatment	530	12.7	6730	2200	3.06	90.0	4.8
First Ammonium Sulfate Fractionation (50 - 75%)	55	57.20	3140	780	4.03	41.9	6.3
Second Ammonium Sulfate Fractionation (35 - 65%)	10	164.0	1640	387	4.25	21.9	6.65
Sephadex Column	12.5	206.4	2580	300	8.60	34.4	13.4
DEAE-Cellulose* Chromatography	22	68.3	1505	28.5	52.80	20	83.0

* The specific activity is the average of the four tubes which give the highest activities (tube No. 28-31). The highest activity in tube 29 is 125 which gives a 195-fold purification.

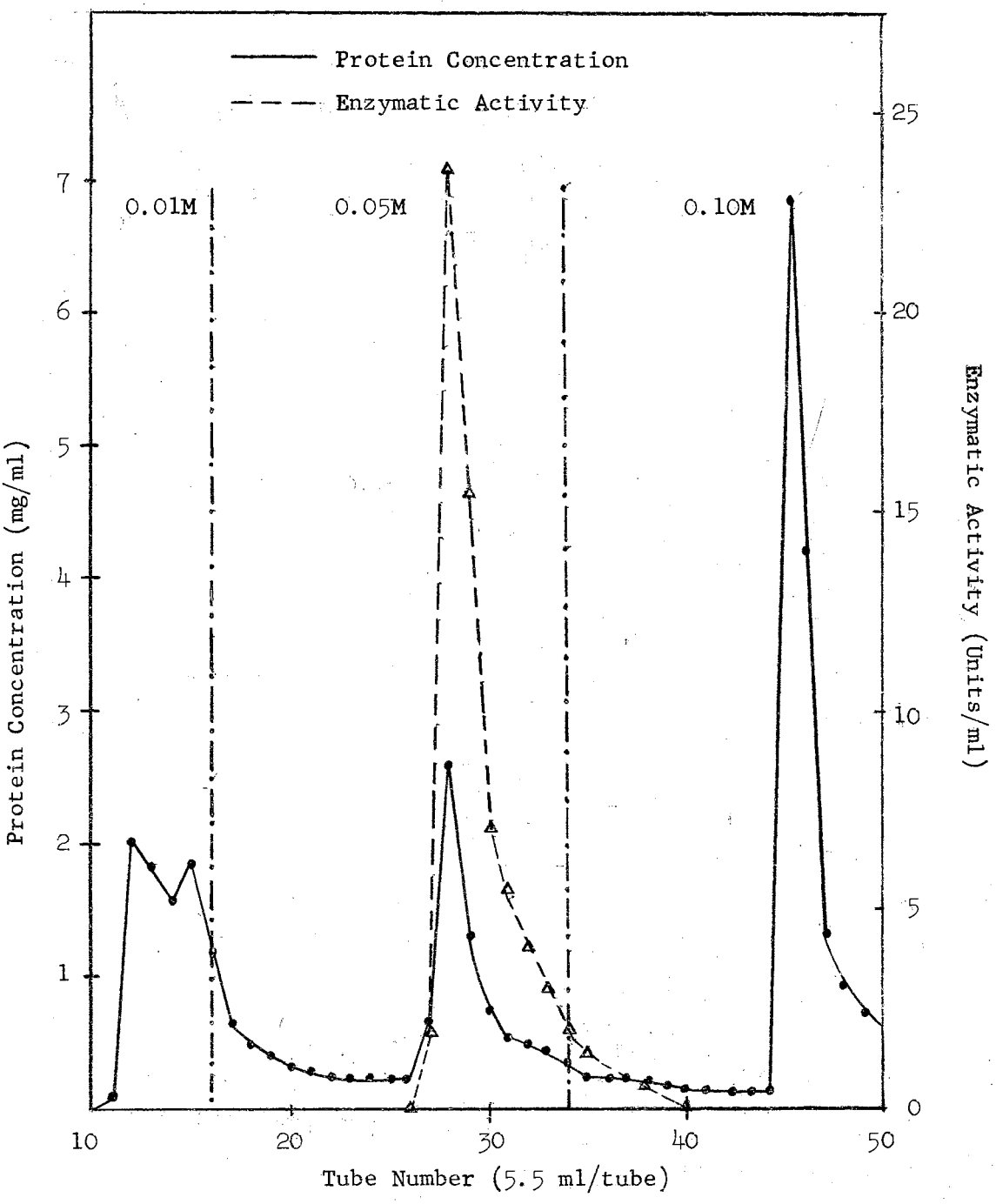
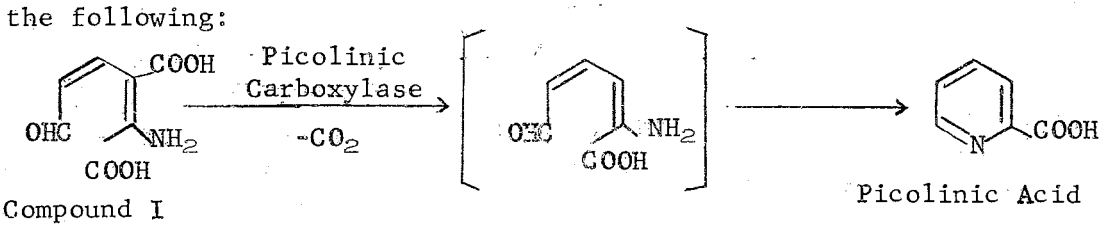


Figure 5: DEAE-Column Chromatography of Picolinic Carboxylase

Picolinic carboxylase catalyzes the β-decarboxylation of Compound I. The decarboxylated Compound I is presumed to form picolinic acid almost instantaneously by a non-enzymatic reaction. The reactions are shown in the following:



Compound I

Picolinic Acid

The removal of the β-carboxyl group of Compound I by the action of picolinic carboxylase was detected by the use of Compound I - β-COOH-^{14}C which was formed by the action of 3-OHAA oxidase on 3-OHAA-COOH-^{14}C. The release of $^{14}CO_2$ from this substrate by the action of picolinic carboxylase as a function of time is shown in Fig. 6. The disappearance of the 360 mμ absorption maximum of Compound I catalyzed by the enzyme, followed by the formation of an absorption maximum at 264 mμ is shown in Fig. 7. This result is the same as that reported by Mehler (88) who identified the 264 mμ absorbing compound which was identified as picolinic acid.

The physiological role of picolinic carboxylase in vivo is not entirely clear. It has been proposed (99) that picolinic carboxylase catalyzes the decarboxylation of the β-carboxyl group of Compound I to form α-amminomuconic semialdehyde which is then oxidized to α-aminomuconic acid by α-HMS dehydrogenase. The dehydrogenase can apparently use either α-HMS or α-AMS as substrate. In the presence of NADPH α-aminomuconic acid is reduced to α-ketoadipic acid which is then further converted to glutaryl CoA. This proposed pathway is shown in Fig. 8. Further work will probably be required to evaluate the role of the α-amino compounds as opposed to the α-hydroxy compounds in this pathway.

b. Effects of Buffer and Ammonium Sulfate

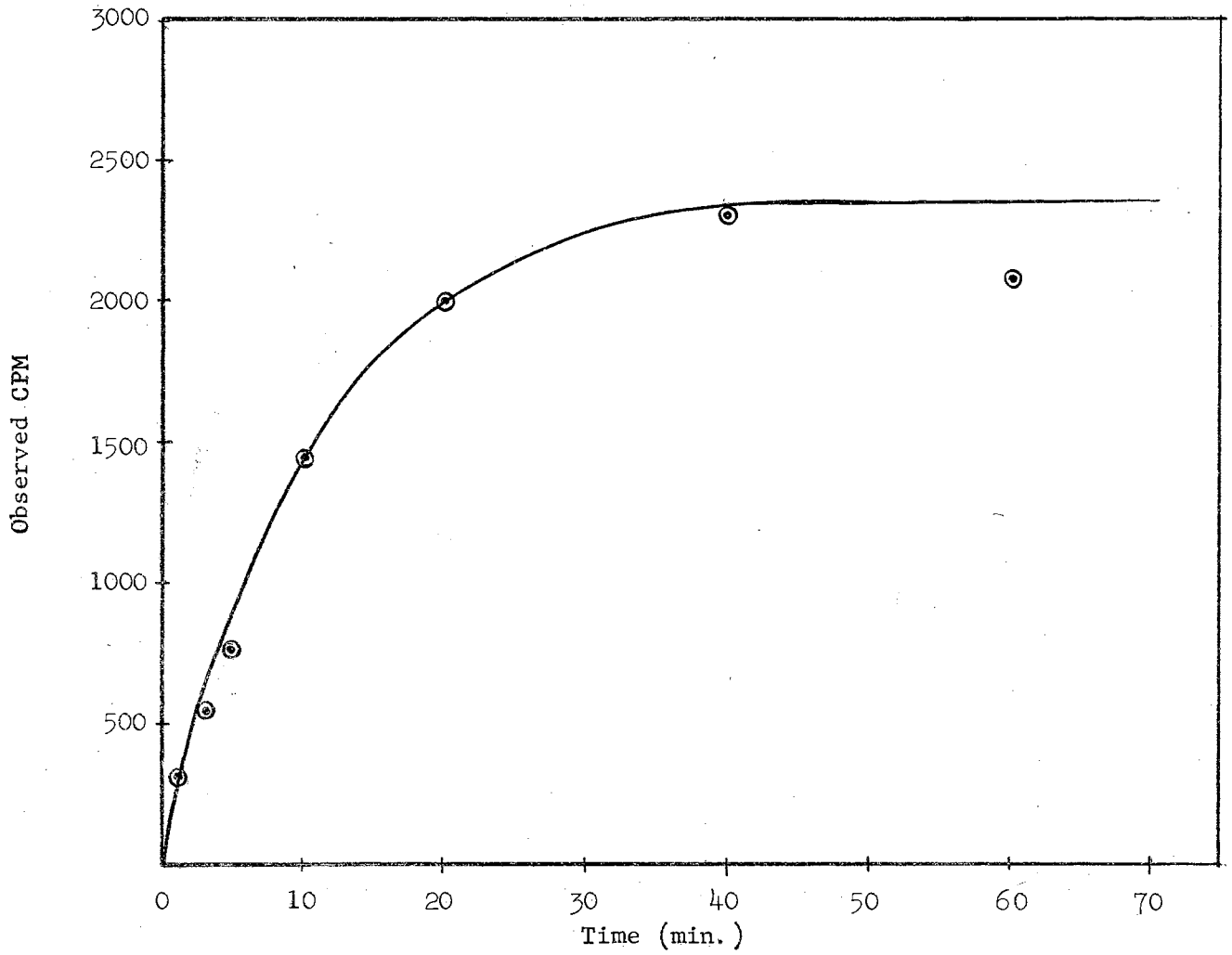


Figure 6. Releasing of $^{14}CO_2$ by the Reaction of Picolinic Carboxylase from Compound I (Room Temperature).

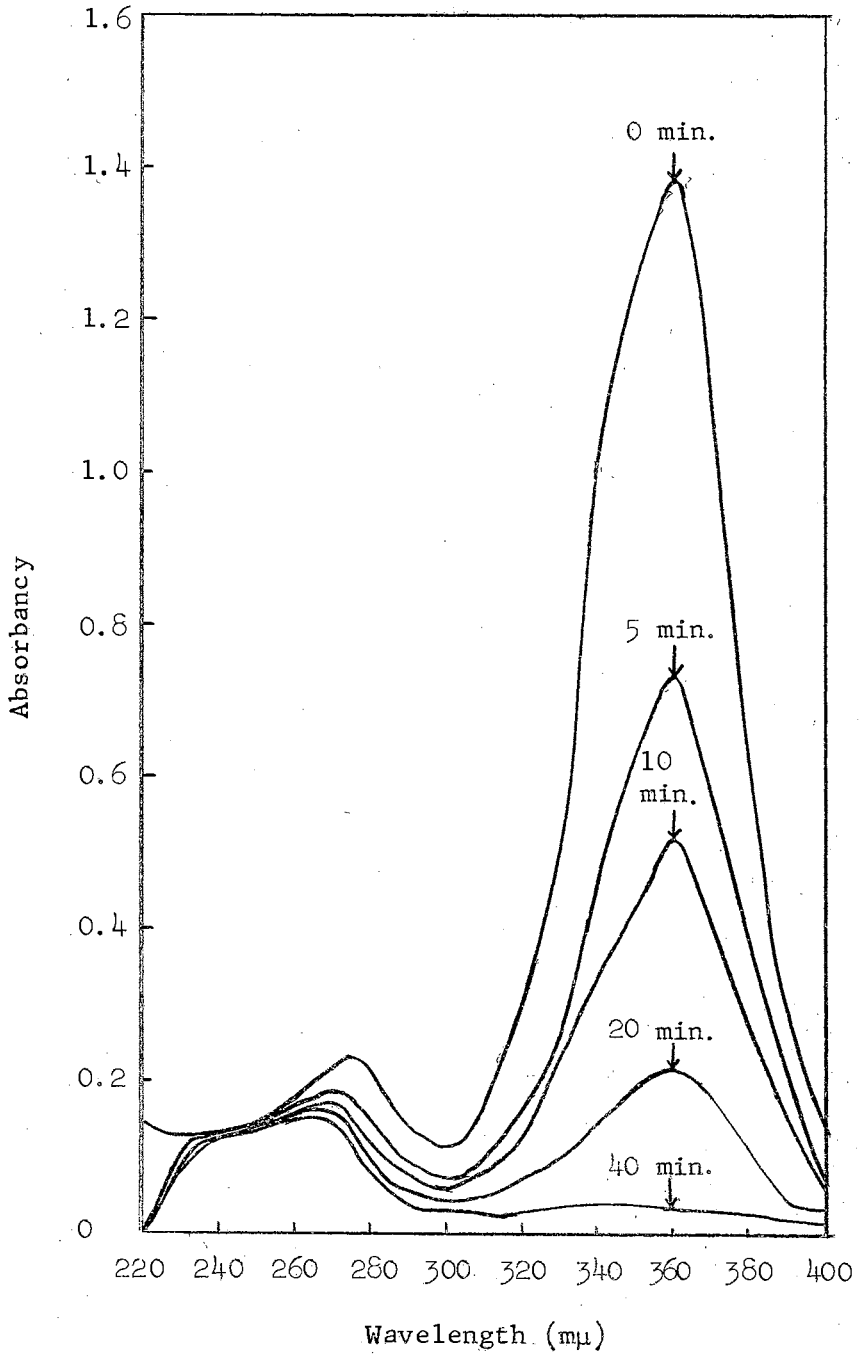


Figure 7. Formation of Picolinic Acid by Picolinic Carboxylase at Room Temperature; pH 7.

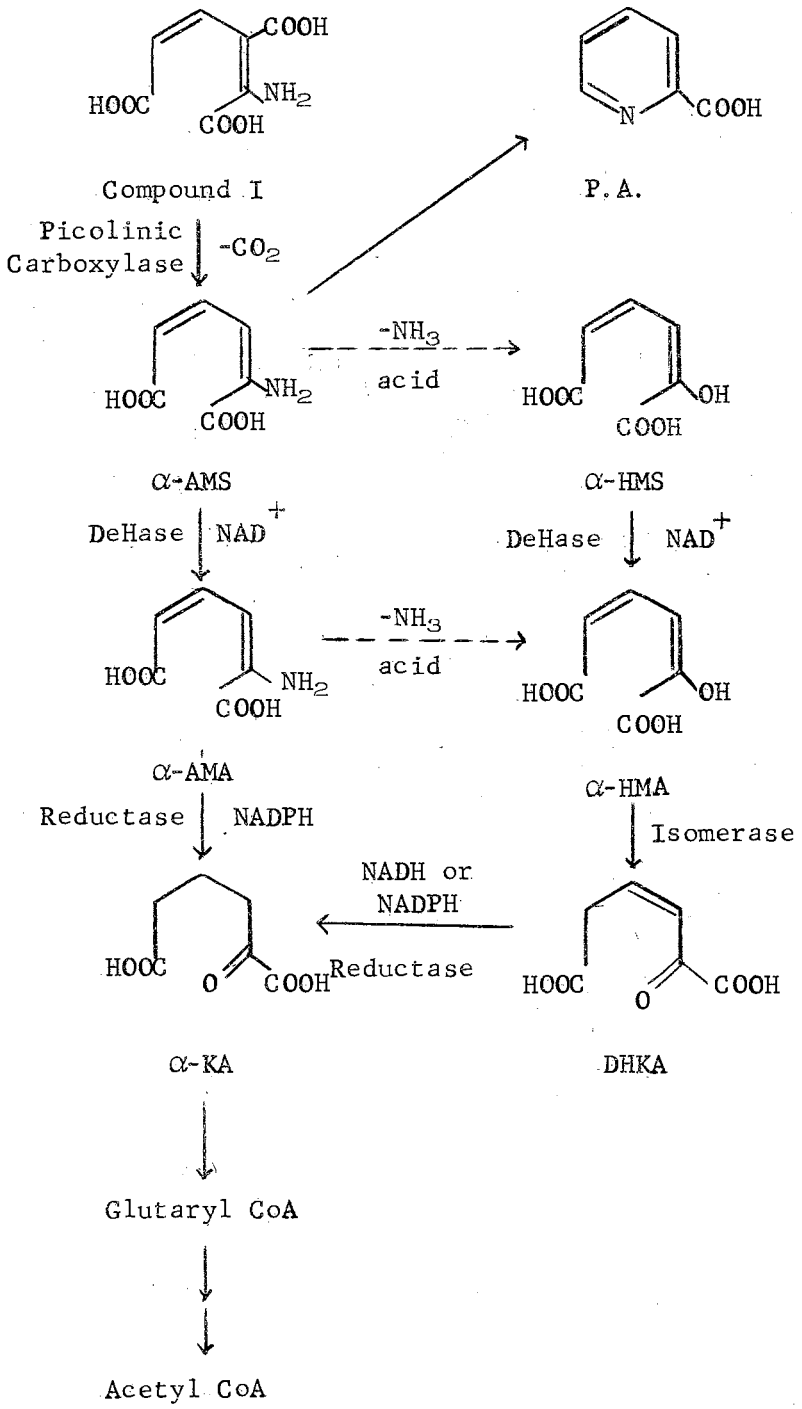


Figure 8. The Proposed Metabolic Pathway of Compound I to Glutaric Acid.

The type of buffer used in the assay for picolinic carboxylase is very important. At the same concentration and pH value, potassium phosphate buffer gives four-times more activity than the Tris buffer used previously. This is shown in Fig. 9.

The inhibition of enzymatic activity by the presence of ammonium sulfate was observed during the purification of this enzyme. Passage of ammonium sulfate fractionated enzyme through a Sephadex column resulted in a doubling of enzymatic activity which appears to be due to the removal of ammonium sulfate. Table V shows the inhibition of enzymatic activity with increasing concentrations of ammonium sulfate under the assay condition. Increasing the concentration up to 0.2 M results in 80 percent loss of activity. Further increase of ammonium sulfate concentration gives no further increase of inhibition.

c. K_m and Optimum pH

Picolinic carboxylase is a rather stable enzyme; stored at -15° C without the presence of any other protective reagents it lost no activity even after months. This enzyme also shows a rather high affinity for its substrate. the K_m value of this enzyme for Compound I was determined to be 4.1×10^{-5} M. A curve which shows the relationship between initial velocity and substrate concentration is presented in Fig. 10 and a Lineweaver-Burk plot is shown in Fig. 11.

Picolinic carboxylase has a rather broad pH optimum. Fig. 12 shows that this enzyme has a maximum activity from pH 7.5 to 8.5. At pH 7 and 9 only a slight decrease in activity was found. However, in acid pH the enzyme loses activity rapidly and no reaction can be detected at pH 5.

d. Effects of Metal Ions, Chelating Compounds and Product on Enzymatic Activity

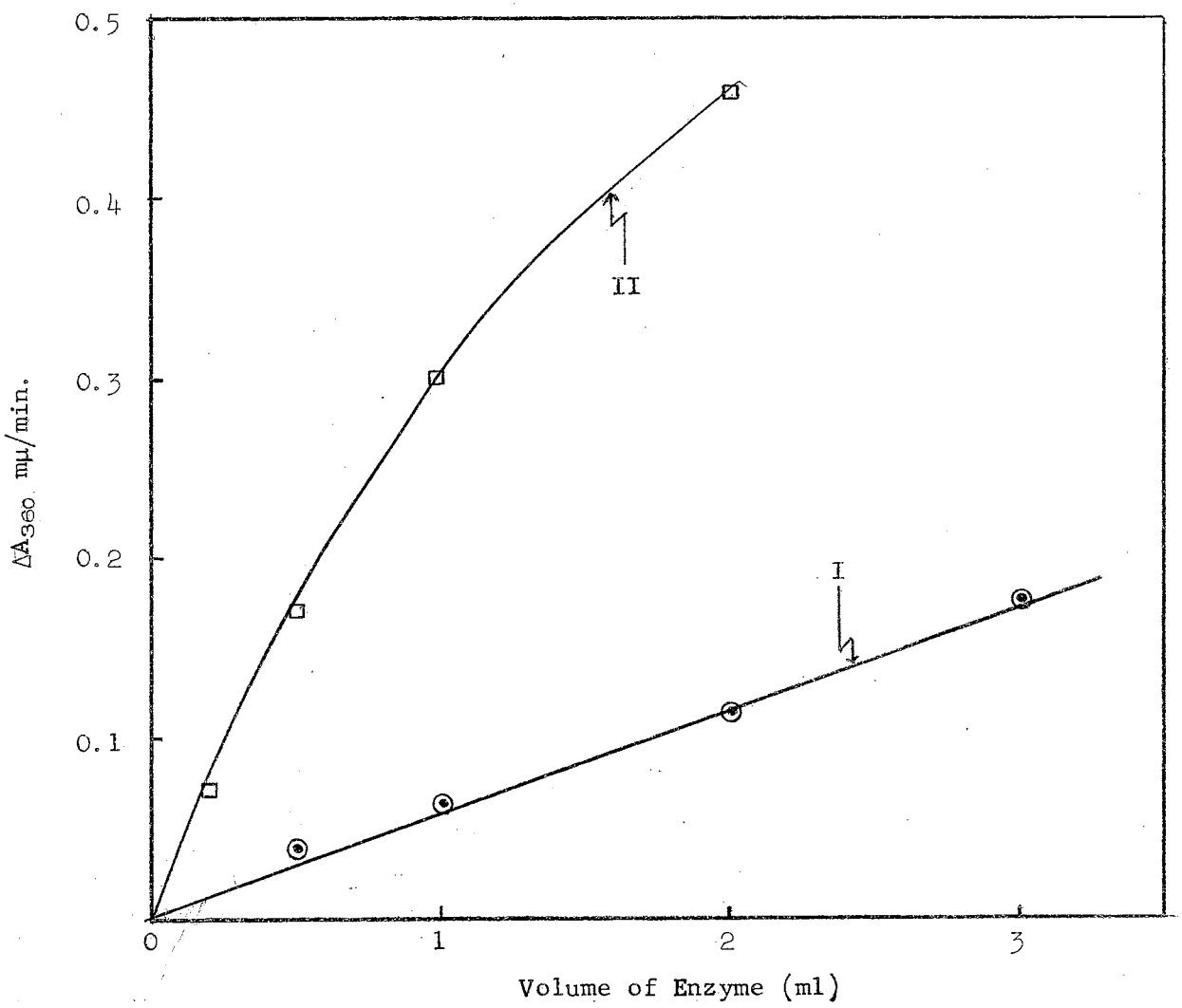


Figure 9. Relationship Between the Enzyme Concentration and Activity.
I. in 0.1M, pH 7, Tris Buffer
II. in 0.1M, pH 7, Potassium Phosphate Buffer

TABLE V

EFFECT OF AMMONIUM SULFATE ON PICOLINIC CARBOXYLASE ACTIVITY

Concentration (mM)	Activity (ΔA/min.)	% Activity
0	0.160	100
1.0	0.145	91
5.0	0.120	75
10.0	0.105	66
50.0	0.080	50
100.0	0.060	38
200.0	0.035	22
300.0	0.035	22

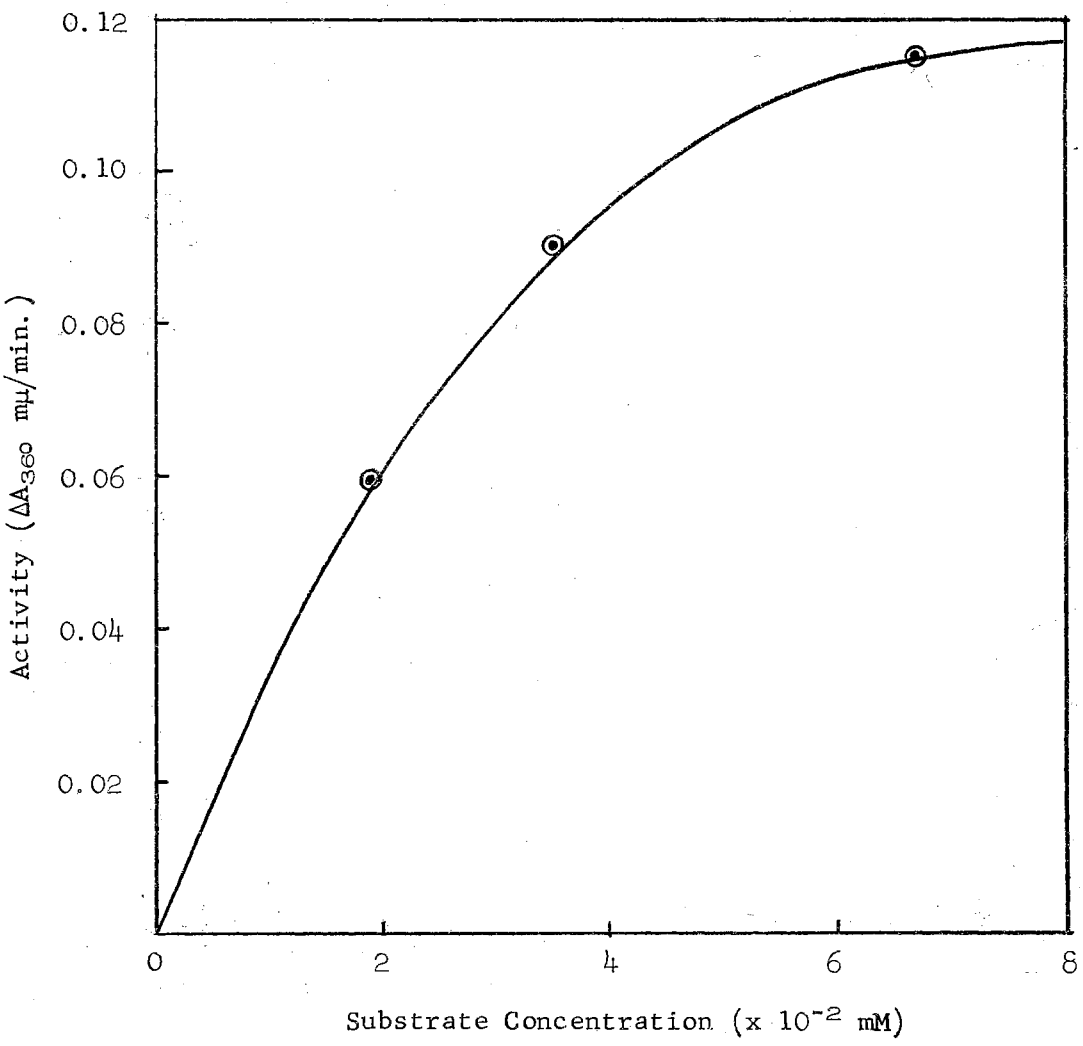


Figure 10. Effect of Increasing Substrate Concentration on the Velocity of Picolinic Carboxylase.

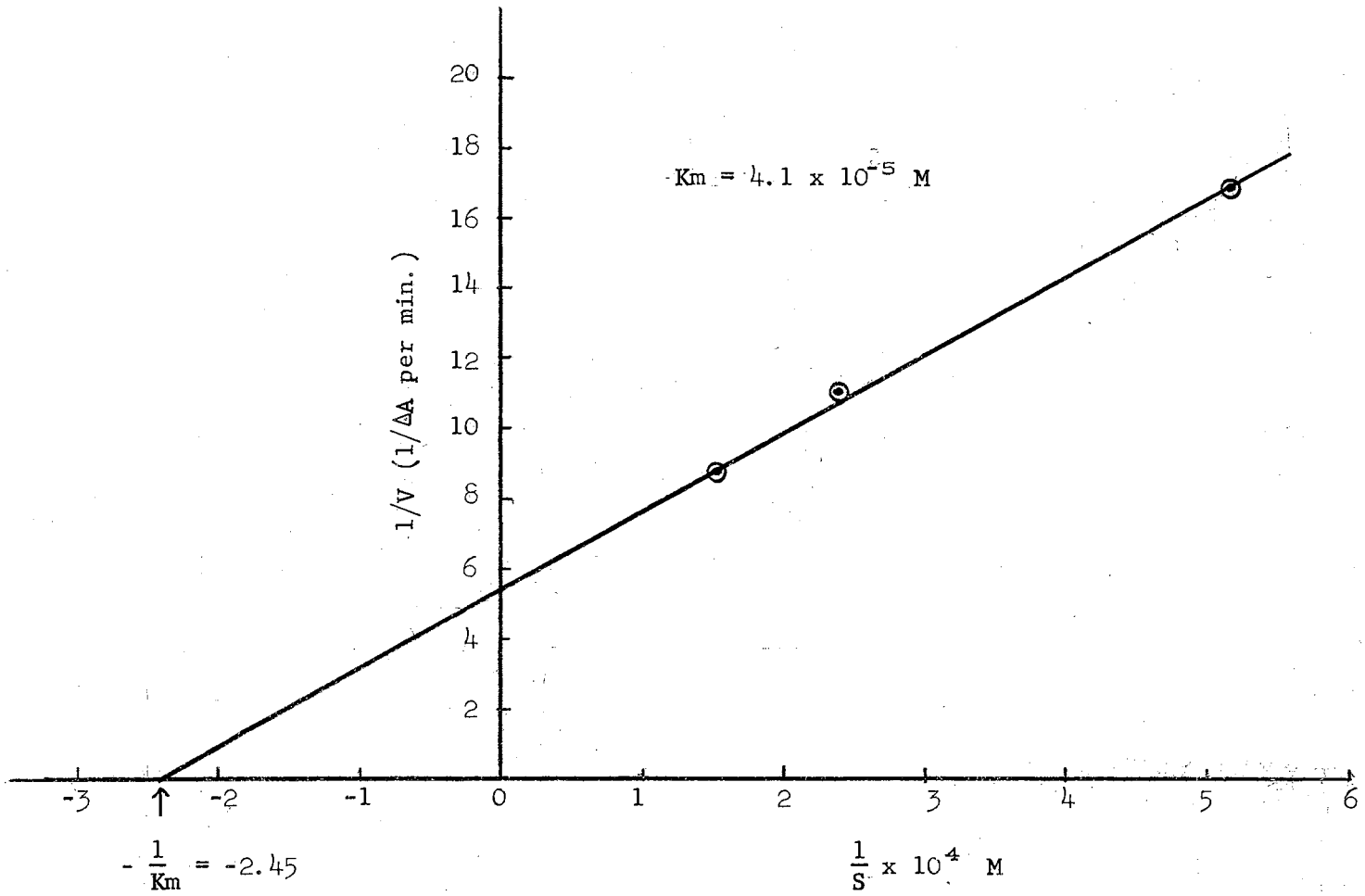


Figure 11. Lineweaver-Burk Plot of Picolinic Carboxylase Versus Compound I.

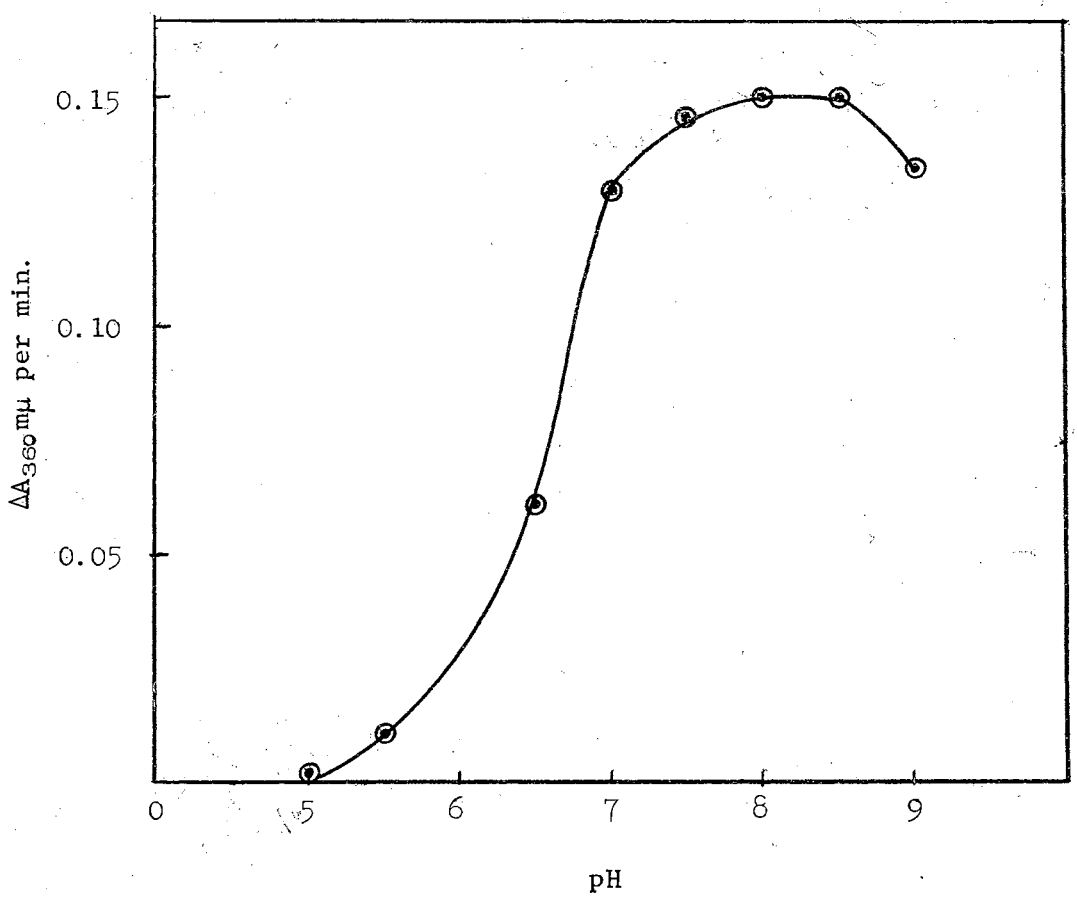


Figure 12. Effect of pH on Enzyme Activity. Measured in 0.1 M Potassium Phosphate buffer.

The effect of several metal ions on the activity of partially purified picolinic carboxylase is shown in Table VI. None of the metal ions tested were found to activate the enzyme. However, Cu^{++} ion at a concentration of 1×10^{-4} M completely inhibited picolinic carboxylase activity. Ca^{++} inhibited about 30 percent at that concentration. Other ions tested neither activated nor inhibited the enzyme. On the other hand, Hg^{++} was found to catalyze a rapid disappearance of Compound I to form an unidentified compound. This compound has an absorption maximum at 265 mμ. This compound has an Rf of 0.15, as detected by U.V. quenching, when chromatographed in butanol:acetic acid:water (4:1:5). In this same solvent system the Rf values for QA and PA are 0.40 and 0.30, respectively. The β-carboxyl group of Compound I is not removed as was demonstrated by the use of Compound I - β-COOH-^{14}C. Mg^{++} was not found to be a cofactor for this enzyme as had been reported by Hayaishi's group (99). Preincubation with several chelating agents (Table VII) for 10 minutes before assay only inhibited activity slightly or not at all. Dialysis against 5×10^{-5} M EDTA at 0° C for 8 hours resulted in only 27 percent loss of total activity. These results indicate that picolinic carboxylase is probably not a metallo-enzyme. The product, picolinic acid, and its analogs quinolinic and nicotinic acids had little or no inhibitory effect at a concentration of 1×10^{-4} Molar. However, p-CMB at a concentration of 1×10^{-4} M completely inhibited the enzymatic activity indicating that picolinic carboxylase is probably a sulfhydryl enzyme.

TABLE VI

EFFECT OF METAL IONS ON ENZYMATIC ACTIVITY

Metals	Concentration (mM)	Activity (ΔA/min.)
None	0	0.15
Fe^{++} ($FeSO_4$)	0.1	0.14
Cu^{++} ($CuSO_4$)	0.1	0
Mg^{++} ($MgCl_2$)	0.1	0.14
Mn^{++} ($MnCl_2$)	0.1	0.14
Co^{++} ($Co(NO_3)_2$)	0.1	0.13
Ca^{++} ($Ca(NO_3)_2$)	0.1	0.10
Hg^{++} ($HgCl_2$)	0.1	?

TABLE VII

EFFECT OF CHELATING AND OTHER COMPOUNDS ON ENZYME ACTIVITY
(Preincubated 10 Minutes Before Assay)

Compounds	Concentration (mM)	Activity (ΔA/min)
None	-	0.16
8-Hydroxyquinoline	0.1	0.12
EDTA	0.033	0.115
α,α'-Dipyridyl	0.1	0.150
o-Phenanthroline	0.1	0.150
p-Chloromercuribenzoate	0.1	0
Picolinic Acid	0.1	0.130
Quinolinic Acid	0.1	0.135
Nicotinic Acid	0.1	0.140

CHAPTER IV

SUMMARY

1. In mammals the benzene ring of tryptophan is oxidized via 3-hydroxyanthranilic acid, α-hydroxymuconic semialdehyde and α-hydroxymuconic acid to glutaric acid. The importance of this pathway of tryptophan metabolism in organisms from several phyla has been investigated by measuring the activities of three enzymes of this pathway: 3-hydroxyanthranilic acid oxidase, picolinic carboxylase and α-hydroxymuconic semialdehyde dehydrogenase. All of these three enzymes were found to occur in mammals, whereas no activity of these three enzymes was found in some organisms such as snail, earthworm, cockroach, grasshopper and tetrahymena. High enzymatic activities of both 3-hydroxyanthranilic acid oxidase and picolinic carboxylase were found in fish liver, but no α-hydroxymuconic semialdehyde dehydrogenase was found. The ability of these various organisms to convert tryptophan-5-^{14}C and tryptophan-α-^{14}C to $^{14}CO_2$ was also examined. No $^{14}CO_2$ was recovered from either cockroach or grasshopper. Three to seven percent was obtained from snail and earthworm. These results indicate that the benzene ring of tryptophan is not oxidatively degraded in the insects studied and that it is degraded in the snail and earthworm by a different pathway than in mammals.

2. Picolinic carboxylase from beef kidney has been purified about 83-fold. Some properties of this enzyme have also been studied.

Picolinic carboxylase is a stable enzyme, for which no cofactor could be demonstrated. High concentrations of ammonium sulfate inhibit its activity. The K_m value for the substrate, Compound I, was determined to be 4.1×10^{-5}M. A broad pH optimum from 7.5 to 8.5 was observed. Cu^{++} ion at a concentration of 1×10^{-4} M completely inhibited enzymatic activity and Ca^{++} at the same concentration produced 30% inhibition. Other divalent metal ions tested neither activate nor inactivate the enzyme. Chelating agents such as 8-hydroxyquinoline, α,α'-dipyridyl and EDTA do not produce a significant inhibition of picolinic carboxylase. However, pCMB, a sulfhydryl inhibitor, completely inhibits the enzymatic reaction at a concentration of 1×10^{-4} M.

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