THE METABOLISM OF 3-HYDROXYANTHRANILIC ACID

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in partial fulfillment of the requirements
for the degree of
DOCTOR OF PHILOSOPHY
May, 1961

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THE METABOLISM OF 3-HYDROXYANTHRANILIC ACID

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ACKNOWLEDGMENTS

The author is sincerely grateful to Dr. L. M. Henderson for his constant encouragement and patient guidance during the course of these studies.

He also wishes to express his appreciation to Dr. Robert Suhadolnik for his assistance and advice in the studies on compound I and to Dr. Franklin Leach for his help and counsel during the enzyme investigations. Acknowledgment is made for the technical assistance of Mr. Hyumg Kang and Mr. Donald Sanders.

He is indebted to the Department of Biochemistry for facilities and financial support during this research.

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ABBREVIATIONS

Abbreviations used throughout the text are: 3-hydroxyanthranilic acid, 3OHAA; diphosphopyridine nucleotide, DPN; triphosphopyridine nucleotide, TPN; flavin mononucleotide, FMN; flavin adenine nucleotide, FAD; pyridoxal phosphate, B_6P; coenzyme A, CoA; adenosine triphosphate, ATP; trichloroacetic acid, TCA; sodium p-chloromercuribenzoate, pCMB; thiamine pyrophosphate, TPP; 2,4-dinitrofluorobenzene, DNFB; diethylaminoethyl cellulose, DEAE Cellulose; nicotinic acid, NA; picolinic acid, PA; quinolinic acid, QA; enzyme, E; and microbiological assay, M.B.A.

I. INTRODUCTION

A. Tryptophan

The detection of an intermediate in the metabolism of tryptophan occurred before the discovery of the amino acid itself when Liebig identified kynurenic acid in 1853 (1). Since the detection of tryptophan in 1902 (2) the knowledge of its metabolic fates has become so extensive and diverse that a comprehensive review of the entire field must be omitted to allow a more thorough survey of the literature pertinent to the research to be reported here. The classic observation by Krehl, _et al._ (3) that tryptophan could serve as a source of niacin in the rat eventually led to the establishment of the main pathway of degradation shown in Fig. 1. Despite the early recognition of the tryptophan-niacin relationship, it has never been possible to demonstrate the formation of niacin _in vitro_. 3-Hydroxyanthranilic acid seems to be one of its immediate precursors, but only limited information has been obtained concerning its metabolism.

B. 3-Hydroxyanthranilic Acid

1. Discovery and Properties

In studies on the tryptophan-niacin relationship in _Neurospora crassa_ Bonner and Beadle (4) isolated five mutant strains of the mold which required niacin, nicotinamide or some related compound for growth. _Neurospora_ strain 4540 accumulated a substance which would replace nicotinic acid for growth of a second strain 39401. This compound was believed to be a direct precursor of niacin. In 1948 Mitchell

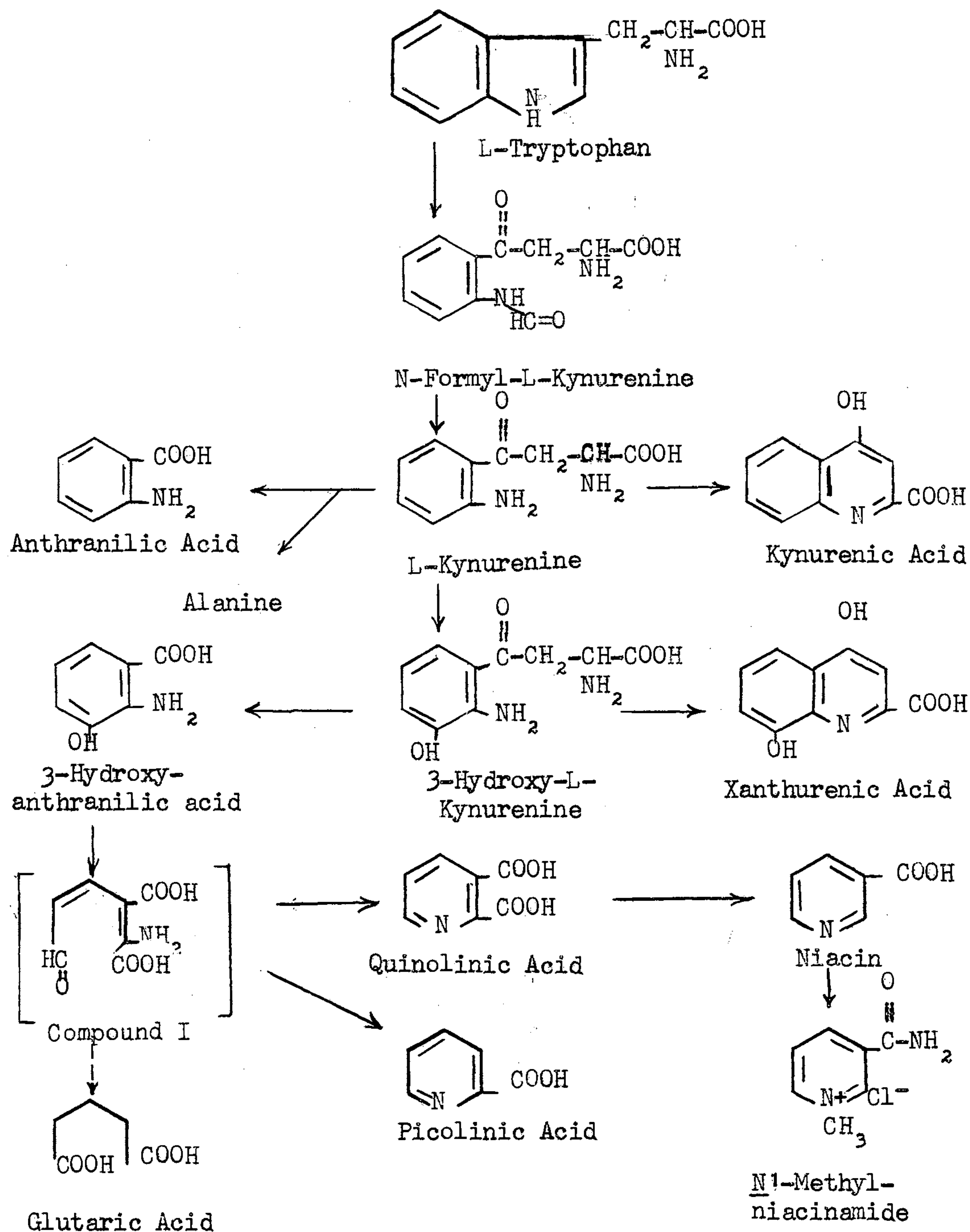


FIGURE 1

TRYPTOPHAN - NIACIN PATHWAY

and Nyc (5) found 3-hydroxyanthranilic acid (3OHAA) to be 70 per cent as effective as nicotinamide in supporting the growth of another Neurospora mutant. Bonner (6) identified this acid as the precursor of niacin in strain 39401. The compound was also found to have a niacin-replacing effect on the growth of rats (7,8) and to cause an increase in excretion of urinary N^1-methylnicotinamide, a metabolite of niacin (9). 3-Hydroxyanthranilic acid fluoresces under the influence of ultraviolet light (10,11) and has absorption maxima at 298 mμ at pH 1 (5) and 317 mμ at pH 7.4 (12). It has been reported to posses carcinogenic properties, causing bladder cancer in a significant percentage of mice (13).

2. 3-Hydroxyanthranilate as a Precursor in the Formation of The Pyridine Carboxylic Acids.

In Vivo

A pyridine carboxylic acid which possessed niacin-replacing activity for the growth of Lactobacillus arabinosus (17-5) when heated with glacial acetic acid was identified as quinolinic acid by Henderson (14,15). When tryptophan was fed or administered by stomach tube to rats there was an increase in quinolinate excretion. It was shown that microflora did not effect this conversion (16). Henderson later reported that 3OHAA as well as tryptophan caused an increase in urinary quinolinic acid. The N^1-methylnicotinamide and niacin content of the urine also increased (17). Isotopic data have since confirmed this metabolic pathway and have shed light on the mechanism of interconversion of these compounds. Shayer and Henderson (18) synthesized tryptophan labeled with deuterium in the

four positions at the benzene ring (C-4,5,6 and 7) and with nitrogen-15 in the indole ring and injected it intraperitoneally into rats. The quinolinic acid isolated from the urine contained both isotopes, but the ratio of deuterium to nitrogen-15 was only half that in the tryptophan indicating that two of the four deuterium atoms were lost in the metabolic degradation. Liefer, et al., (19) suggested that some of the nitrogen of quinolinic acid originated from ammonium ion in the media. When Neurospora strains were grown in the presence of 3OHAA and N^{15}-ammonium chloride, 50 per cent of the nitrogen of quinolinic acid was labeled. Yanofsky and Bonner (20), however, reported that the isotope incorporation was the result of 3OHAA synthesis de novo, since resting cells did not catalyze any incorporation of nitrogen-15 into quinolinic acid. Hankes and Henderson (21) and Hankes and Urivetsky (22) showed that 3OHAA labeled in the carboxyl position with carbon-14, when injected into rats intraperitoneally or subcutaneously, gave rise to N^1-methylnicotinamide and quinolinic acid β-C^{14} in the urine. Wilson and Henderson demonstrated a similar conversion of tritium-labeled 3OHAA to quinolinic and nicotinic acid in the developing chick embryo (23).

It is possible that quinolinic acid is the immediate precursor of niacin. The observations of Mitchell and coworkers (5,7), Bonner (6) and Albert, et al., (9) established 3OHAA as a precursor of niacin in Neurospora and in the rat in vivo. Decker and Henderson (24) demonstrated that this compound can replace niacin for growth in the diets of chicks, hamsters and guinea pigs. Quinolinic acid was thought to be a product of a side reaction of

3OHAA oxidation and was reported not to serve as a source of niacin (4). In 1947, however, it was found that quinolinic acid increased the urinary excretion of $\underline{N}^1$-methynicotinamide when fed to rats and mice (25). Henderson also reported this finding and showed that quinolinic acid would support growth for the rat and Neurospora 4540 (17). The results were later confirmed by Krehl, et al., (26) and Yanofsky, et al., (20). Hankes and Segal (27) demonstrated that tritium-labeled quinolinic acid could be converted to $\underline{N}^1$-methylnicotinamide in the intact rat, and Wilson and Henderson (23) showed a conversion of tritium-labeled quinolinate to nicotinic acid in the chick embryo. A conclusive demonstration of the decarboxylation of quinolinic acid to niacin or $\underline{N}^1$-methynicotinamide has not been reported in vitro.

Formation of niacin from 3OHAA is not the only route of synthesis of the vitamin. Yanofsky (28) showed that tryptophan uniformly labeled with carbon-14 did not give labeled niacin in Escherichia coli or Bacillus subtilis. Henderson, et al., (29) also reported the lack of a tryptophan-niacin relationship in corn and tobacco plants, and Griffith and Byerrum (30) have since shown that the pyridine ring of nicotine, known to arise from nicotinic acid (31), is synthesized from acetate and pyruvate in Nicotiana rustica, L. Also, Ortega and Brown (32) demonstrated that E. coli synthesized nicotinic acid from glycerol. The carbon-14 from glycerol-1, 3-C^{14} was incorporated into the pyridine ring.

Picolinic acid, another pyridinecarboxylic acid, was identified as a metabolite of 3OHAA in vitro by Mehler. Mehler and May injected carboxyl-C^{14} labeled 3OHAA intraperitoneally into rats

and observed that 90 per cent of the isotope was expired as $C^{14}O_2$ within the first 24 hours (34). Since picolinic acid was not metabolized by the rat, they suggested that this acid was the principal metabolite of 3OHAA. Suhadolnik *et al*., however, found that only 3 per cent of the tritium appeared in the urine of the rat as picolinic acid or its conjugates when tritium-labeled 3OHAA was administered intraperitoneally, and 14 per cent of the isotope was found in the quinolinic acid. Neither quinolinic nor picolinic acid was a major product in the metabolism of 3OHAA *in vivo* (35).

In Vitro

Schweigert (36) found that nicotinic acid activity for *Lactobacillus arabinosus* increased 8 per cent in the incubation mixture when 3OHAA was incubated with rat liver slices. Henderson observed that quinolinic acid was decarboxylated under the conditions used by Schweigert to remove the bound nicotinic acid (14). He suggested that 3OHAA was oxidatively cleaved in the 3 and 4 positions of the benzene ring and recyclized to form quinolinic acid and not nicotinic acid. Henderson and Ramasarma (37) later reported a quantitative conversion of 3OHAA to quinolinate in rat liver homogenates. Schweigert and Marquette (38) made the same observation and suggested an oxygen requirement for the reaction. Studies with isotopes have verified this finding (35,39). Bokman and Schweigert (10) found that the enzyme catalyzing the oxidative reaction was present in an acetone powder of liver. Both kidney and liver (40,41) of the rat, pig and bovine contained the enzyme.

Suhadolnik, et al. (35) reported that liver extracts of ten species of mammals contained the oxidase, but there was considerable variation in the amount of this enzyme and the picolinic acid carboxylase present.

The oxidation product of 3OHAA was reported by Bokman and Schweigert (12). As the substrate disappeared a concomitant increase in absorption at 360 mμ was observed. This was shown to be the absorption maximum of the product; the molar extinction coefficient was first reported to be about 20,000. Mineral acid caused a shift in the maximum to 315 mμ. Miyake, et al. (42) and Long, et al. (11) provided further evidence for the participation of this intermediate in the oxidation of 3OHAA. Long and coworkers showed that the disappearance of substrate was accompanied by the appearance of the 360 mμ absorbance and by the uptake of one mole of oxygen per mole of 3OHAA. Quinolinic acid appeared to be the product of the spontaneous decay of the intermediate, since theoretical amounts of quinolinate were produced as the absorption disappeared even after removal of the protein with 90 per cent ethanol. Hayaishi, et al. (43) have shown that the oxidation requires molecular oxygen rather than water, since the isotope from O_2^{18}, not H_2O^{18}, was incorporated into the γ-carboxyl group of quinolinic acid. They classified the enzyme as a "phenolytic oxygenase" (44).

Inhibitors and Cofactors

A dialyzable, heat-stable cofactor for the enzyme was reported by Henderson (45). Long (11) and Miyake (42) reported that this factor was ferrous ion. Stevens (46) and Long have shown this to be an absolute requirement; substitution of other ions often

caused an inhibition of the reaction rather than activation. The enzyme is inhibited by ethylenediaminetetraacetic acid (versene), α, α[1]-dipyridyl and o-phenanthroline (11, 42) probably by chelation with the ferrous ions. Miyake (42), Mehler (33) and Stevens (47) reported that the enzyme was sensitive to sulfhydryl reagents, but that inhibition could be reversed with glutathione. Long and Mehler also showed that high concentrations of cyanide inhibit the enzyme. Auricchio, et al., (48) reported that vitamin K causes a decrease in quinolinic acid formation. The oxidase activity was also depressed in leukemic rats (49) and rats fed 2-acetylaminofluorene (50), a compound implicated as an antagonist of DPN. In the latter two experiments activity could be restored with DPN. It has been suggested on this basis that DPN may be a cofactor as well.

Enzyme Specificity

The work of Stanier, et al. (51) regarding the mechanism of the oxidation of aromatic compounds led to the postulation that 3, 4-dihydroxyanthranilic acid might be an intermediate in the oxidation of 3OHAA. Makino, et al. (52) reported that liver slices formed nicotinic acid when incubated for 24 hours with 3, 4-dihydroxyanthranilic acid. Weinstock, et al. (53) and Hellman and Wiss (54) however, could observe no such conversion to either quinolinic or nicotinic acids by rat or hog liver preparations. It was not utilized by *Xanthomonas pruni* or by the rat for growth; no urinary nicotinic or quinolinic acid was formed in the rat (53). D'Angeli, et al., (55) found that anthranilic acid and the amide of 3OHAA did not possess niacin-replacing activity for the rat or serve as substrates for

the 3OHAA-oxidizing system of liver. Gholson and Henderson (56) reported that 5-hydroxyanthranilic acid-1-C^{14} was not metabolized by the intact rat and was excreted in the urine.

Purification of the Enzyme

Purification of 3OHAA oxidase has been attempted by several workers. Schweigert's group (10) fractionated an extract of acetone powders of liver and found that oxidase activity was in the fraction precipitated at a concentration of ammonium sulfate between 45 and 65 per cent. Long *et al*. (11) and Mehler (33) attempted purification but found the preparations unstable to the conventional methods. Stevens and Henderson (47) obtained a 4-fold purification by a heat step followed by the use of lead subacetate to remove inert proteins. Subsequent ammonium sulfate fractionation resulted in a loss of at least two-thirds of the activity.

3. Product of 3OHAA Oxidation

Much work has been done to characterize the product of oxidation of 3OHAA. The intermediate, referred to as Compound I, detected by its absorption maximum at 360 mμ (11, 12, 42), results from the oxidative fission of the benzene ring of 3OHAA in the 3-4 position and has a half-life of about 40 minutes at 25° C and pH 9.4 (30). The formation of compound I requires one mole of oxygen per mole of substrate (11), as already noted. The molar extinction coefficients have been reported as 28,300 (11) and 47,500 (57,58,59) at 360 mμ in different solvents. Compound I is very unstable to heat but can be stored for several hours at -15° C in 80 per cent ethanol (11). Miyake *et al*. (42) and Long *et al*. (11) reported that the intermediate is unreactive to carbonyl reagents under natural conditions. They

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, reacted with carbonyl reagents, which indicated an oxidative deamination. Subsequent work by Long (60) has shown that one mole of ammonia is released per mole of compound I when acid is added. Mehler (61) also observed that the isotope from carboxyl-C^{14} 3OHAA was lost when compound I was converted to compound II. Wiss et al. (62) reported that acid conditions cause decarboxylation of compound I, but it was not deaminated, so that the condensation product was picolinic acid. Their suggestion that picolinic acid is therefore a non-enzymatic product of acid treatment has been criticized (63) in view of observations of others (33,35) who have noted its enzymatic formation.

Wiss et al. (58) have reported the formation and properties of three derivatives of compound I. In their studies trichloroacetic acid at a final concentration of 5 per cent was used to precipitate the proteins. They reported the formation of a stable 2,4-dinitrophenylhydrazone, a saturated aliphatic product formed by the catalytic reduction of compound I and a dinitrophenyl derivative of the reduced product. The infrared spectra of these derivatives indicated that the structure of compound I is that shown in Fig. 2.

At present the structure of compound I is not known. The conditions for the preparation of derivatives used by Wiss were such that compound II should have been formed. The structure

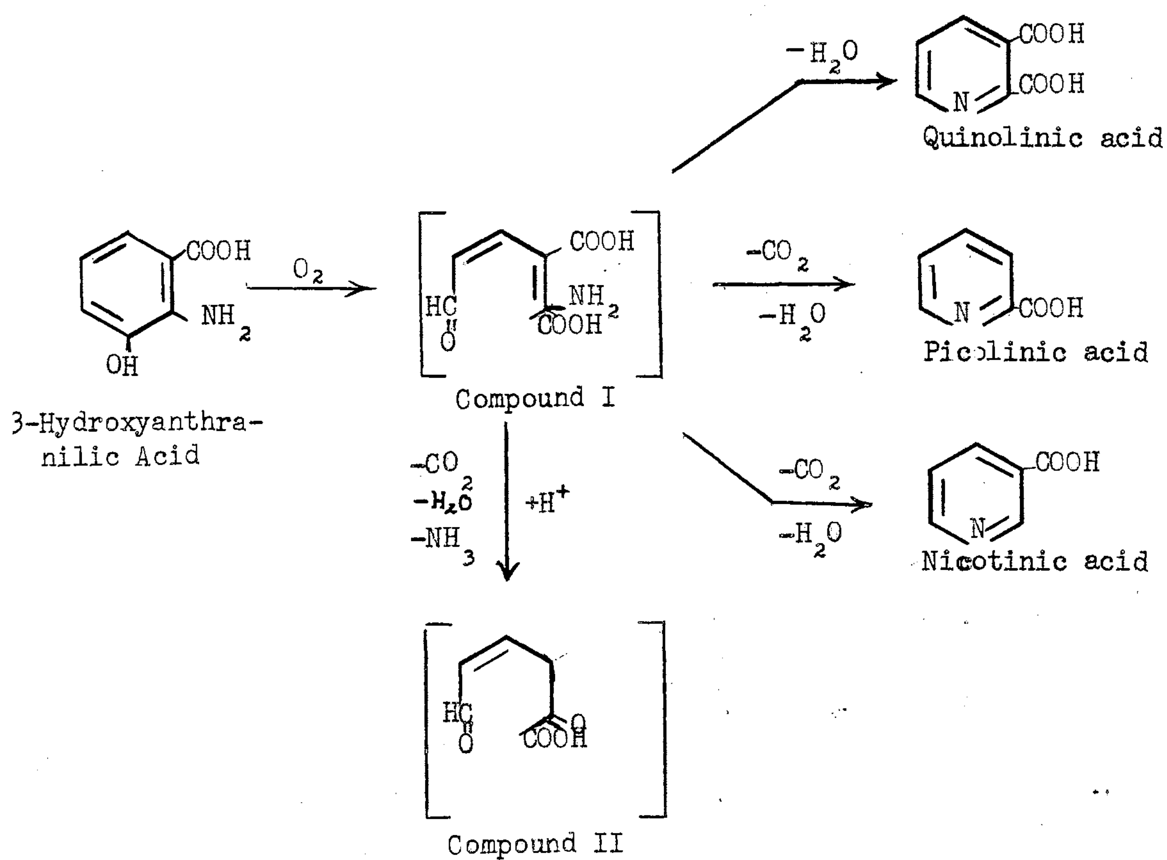


FIGURE 2

METABOLISM OF 3OHAA; DEGRADATION OF COMPOUND I *IN VITRO*

proposed, 1-amino-4-formyl-1,3-butadiene-1,2-dicarboxylic acid (64), seems reasonable. There has been no evidence to support a two-step oxidation to give the products suggested by Miyake et al. (42) and Snell (65). The aldehyde and amino groups of compound I probably undergo a Schiff-base type condensation (66) with the loss of water to give quinolinic acid.

4. Degradation of Compound I

That compound I is spontaneously degraded to quinolinic acid (12, 17) has already been discussed. Nearly quantitative yields of quinolinate are obtained from rat liver preparations. The livers from bovine, guinea pig, pig (33) and other species (35) contain a cyanide-sensitive enzyme in the soluble fraction. Mehler (33) showed that this is a decarboxylase requiring no known cofactors and catalyzing the formation of picolinic acid from compound I. The formation of nicotinic acid has not been demonstrated in vitro, but the growth studies and urinary excretion of metabolites of niacin already mentioned support its formation in vivo. In the intact animal, however, the pyridinecarboxylic acids never account for more than 20 per cent of the degraded 3OHAA (35). Neither picolinic acid (34,35) nor nicotinic acid (34) is metabolized. When 0.02 mmoles per day of quinolinic acid was injected into rats, 80 per cent was excreted unchanged (17). The data indicate that the major portion of 3OHAA was metabolized via some other route. Acid was shown to degrade compound I to compound II, which is presumably 1-hydroxy-4-formyl-1,3-butadiene-1-carboxylic acid. This same compound appears to be produced as the first oxidation

product of catechol by an unidentified gram-negative organism (67). Such a route of degradation in mammals has been suggested (68) but has not been shown.

Henderson and Hankes (69) reported that 25 per cent of the carbon-14 from tryptophan-3a, 7, 7a-C^{14} was expired as $C^{14}O_2$ in 12 hours. They also showed (21) that 60 per cent of the isotope from carboxyl-C^{14} labeled 3OHAA was expired in 3 hours. Gholson et al. (70) reported that 36 per cent of the isotope from tryptophan-7a-C^{14} was expired as $C^{14}O_2$, and 40 to 50 per cent was present in the tissues 12 hours after the tryptophan was injected. The labeling pattern of isolated amino acids suggested that carbon-7a of tryptophan is catabolized via the carboxyl of acetate. This observation was confirmed when an acetate trapping technique with cyclohexylalanine was used (71). When 3OHAA-1-C^{14} was injected into rats the isotope was found in the methyl carbon of acetate (56). The interpretation of these data was that the main route of tryptophan metabolism to CO_2 is via 3OHAA, and that 3OHAA is oxidized via compound I to some aliphatic product, not through the pyridine-carboxylic acids.

Consideration of the possible metabolic sequences between 3OHAA and CO_2 suggest a number of carboxylic acids as intermediates. Gholson et al. (72,73) tested some of these by the metabolite overloading technique in the rat with both tryptophan-7a-C^{14} and tritium-labeled 3OHAA. Glutaric acid was found to be labeled in both cases. The carbon-14 from the tryptophan was found in the carboxyl carbon, again supporting the 3OHAA-compound I pathway for tryptophan metabolism.

No other intermediates have been established. Figure 3 shows a hypothetical pathway for the formation of glutaric acid from 3OHAA (73).

No conversion of 3OHAA to compounds other than picolinate and quinolinate has been demonstrated *in vitro*. In order for further metabolism to take place, an oxidation or deamination must occur to prevent ring closure.

Other Metabolic Paths of 3OHAA Degradation.

Though degradation of 3OHAA to CO_2 *via* the 3OHAA oxidase-catalyzed reaction is most probable, there is a second possibility. Viollier and Sullman (74) have reported the presence of an enzyme in the particulate matter of liver which metabolizes 3OHAA to a red compound. This product has never been characterized.

5. Conclusion

The complete metabolism of 3OHAA to CO_2 is yet to be elucidated; indeed, the first step of oxidation has not been clearly defined. The oxidase has never been extensively purified, and this fact has made characterization of the product more difficult. The formation of niacin has yet to be demonstrated unequivocally *in vitro*. It is apparent that extensive studies *in vitro* are necessary to provide many of the answers.

The purpose of the studies to be described was 3-fold: (1) to purify the enzyme and define the reaction in which 3OHAA is oxidized to compound I, (2) to characterize the oxidation product, and (3) to find a system to study the metabolism of compound I *in vitro*.

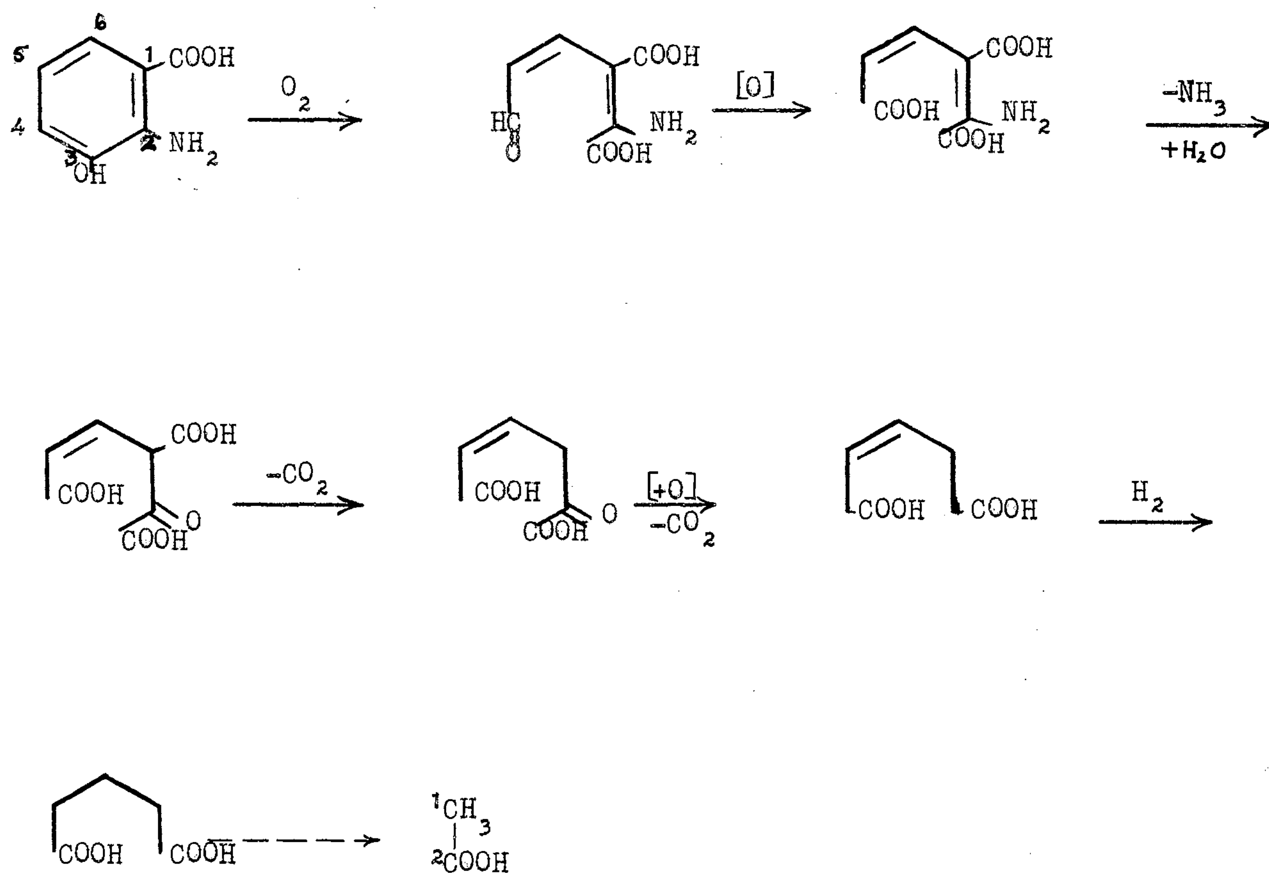


FIGURE 3

HYPOTHETICAL PATHWAY FOR THE FORMATION

OF

GLUTARIC ACID FROM 3OHAA (67).

II. METHODS AND MATERIALS

A. Materials

3-Hydroxyanthranilic acid (3OHAA) was obtained from Hoffman-La Roche. Reduced glutathione, cysteine-HCl, quinolinic acid and nicotinic acid were obtained from Nutritional Biochemicals Corp. Tris (2-amino-2-(hydroxymethyl)-1,3-Propanediol) and sodium p-chloromercuribenzoate (pCMB) were purchased from Sigma. Picolinic acid, picrolonic acid, semicarbazide-HCl and thiosemicarbazide HCl were obtained from Eastman Kodak Co. All salts used in these studies were of C. P. grade. Certain preparations of ammonium sulfate were recrystallized from 0.002 M Tris-0.003 M Versene buffer. Saturated ammonium sulfate at 3° C was prepared and adjusted to pH 8.0 with concentrated ammonium hydroxide. This salt solution was diluted one to ten for reading of pH.

Tritium-labeled 3OHAA was prepared by the method of Wilzbach (75) and recrystallized to constant specific activity (700 μc/mmole). Paper chromatography in Partridge solvent system (76) showed only one radioactive spot. 3-Hydroxyanthranilate-1-C^{14} and the carboxyl-C^{14} labeled form were gifts from Dr. L. V. Hankes, Brookhaven National Laboratories. Lactobacillus plantarum (formerly L. arabinosus 17-5) (ATCC no. 8014) and all reagents for microbioassay were kindly supplied by Dr. R. J. Sirny, Oklahoma State University. Water used for enzyme purification was prepared by redistilling steam condensate,

passing it through a deionizing column and redistilling a third time in glass. This was found to be necessary because the deionized water contained some unidentified inhibitor.

B. Preparation of Acetone Powders, Extracts and Homogenates

Liver extracts were prepared from acetone powders. Rat livers were obtained and used immediately in the early studies, but this species was not satisfactory later because of the large amounts of tissue needed in enzyme purification. Fresh beef liver was purchased for this purpose.

Acetone powders were prepared by homogenizing the liver tissue with 10 volumes of cold acetone in a Waring blendor for 2 minutes at full speed. This mixture was then filtered with suction on a Buchner funnel and resuspended in three volumes of acetone. The Waring blendor was used to aid in breaking the tissue pad. The preparation was again filtered under vacuum. The pad was broken up by rubbing between the hands in the cold and allowed to dry on large filter paper. After the powder was passed through a No. 40 mesh screen to remove the fibrous material it was dried over paraffin and P_2O_5 *in vacuo*. The product was stored at -15°C *in vacuo* until used. The extracts were prepared by adding 9 volumes of glass-distilled water to the powder and stirring. The mixture was allowed to stand one hour at room temperature and centrifuged at 2°C and 10,000 xg for ten minutes.

Homogenates were prepared for purification studies in a one to five w/v ratio of fresh tissue to water. Homogenization was accomplished at 5°C in a Waring blendor for one minute at

full speed. The suspension was passed through two layers of cheese cloth to remove large unbroken particles. Homogenates used in studies on the degradation of compound I were prepared in a similar manner except that a Potter-Elvehjem homogenizer was used.

C. Quantitative Methods.

1. Enzyme Assays

Specific methods for assays of the enzymic reactions are described in the individual sections.

2. Protein Determination

Protein was determined by the trichloroacetic acid-turbidity method with the procedure of Stadtman, *et al*. (77) and by 280 mμ absorption (78).

3. Microbiological Assay for Quinolinic and Nicotinic Acids

Quinolinic acid was assayed as nicotinic acid. The quinolinate was chemically decarboxylated by autoclaving with glacial acetic acid. A microbiological assay was done by the modified method of Snell and Wright (79) using *Lactobacillus plantarum*. Growth was measured by titrating the acid produced after a 72-hour incubation at 37°C.

4. Isotopic Analyses

Paper chromatograms of radioactive materials were analyzed by a Radiological Service Co. Automatic Windowless Chromatogram Scanner. Quantitative assays for the isolated quinolinic, picolinic and nicotinic acids were made by the isotope dilution technique (35). Tritium-labeled compounds were decomposed by the zinc fusion method (80), and carbon-14 labeled compounds were degraded by the wet

combustion method of Van Slyke (81). Radioactivity was measured with the Cary model 31 vibrating reed electrometer. The Packard Tricarb liquid scintillation counter was used for analysis of isolated glutaric acid. Five-tenths ml. aliquots of the eluted fractions from the silica gel column were added to 10 ml. of scintillation fluid[1] (82) and the preparations were counted for 5 minutes.

[1] 60 % toluene, 40% absolute ethanol, 0.4% diphenyloxazole and 0.02% POPOP.

III. EXPERIMENTAL AND RESULTS

A. 3-Hydroxyanthranilic Acid Oxidase

Purification of 3OHAA oxidase was undertaken to obtain information concerning the properties of the enzyme and the product of the reaction it catalyzes. Stevens and Henderson (47) obtained a 4-fold purification of the oxidase by a heat step, a pH step and treatment with lead subacetate. When attempts were made to fractionate the enzyme with ammonium sulfate it was found that a substantial amount of activity was lost.

The data reported in paragraphs 1 to 5 were obtained by Dr. F. R. Leach and were confirmed by the author.

1. Effect of Ammonium Sulfate on Activity

The loss in activity when salt fractionation was attempted suggested that the ammonium sulfate was inhibiting or denaturing the enzyme. An experiment was designed to test the effect of the salt on enzyme activity. All enzyme assays were made by spectrophotometric measurement of the rate of formation of compound I at 360 mμ. Reactions were incubated in cuvettes with a one cm light path. A control consisted of all components before the addition of substrate. Optical density readings were taken at 15-second intervals for 2 minutes on the Beckman model D.U. spectrophotometer. The reaction mixtures contained 0.3 μmole of ferrous sulfate and 0.1 ml of a 20 per cent heated and centrifuged preparation of enzyme. Saturated ammonium sulfate (0.1 ml) was added at the same time as substrate (curve 1, Fig. 4), 2 minutes after substrate (curve 2) and 2 minutes before substrate (curve 3). It was

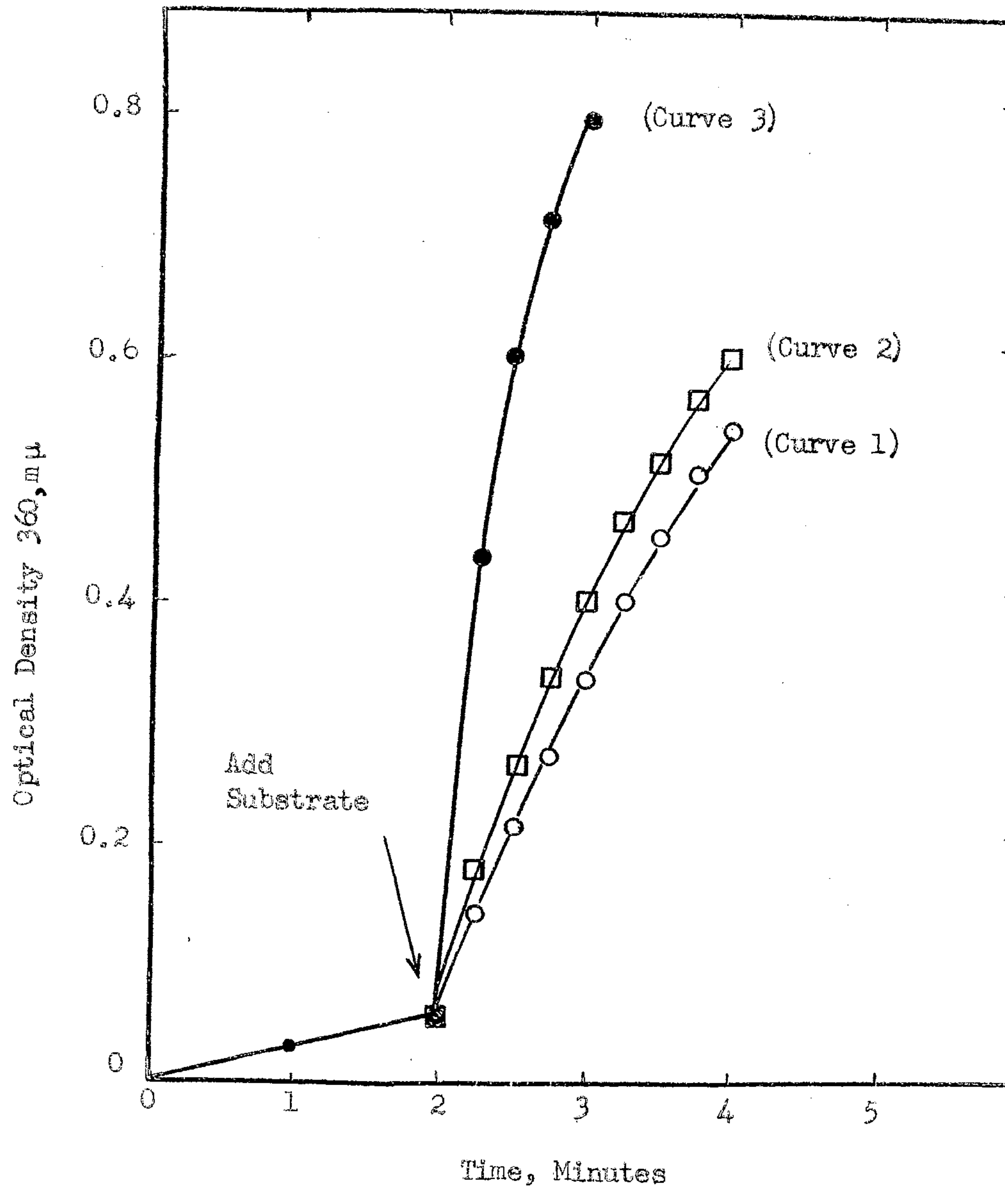


FIGURE 4

THE EFFECT OF AMMONIUM SULFATE ON 3OHAA OXIDASE ACTIVITY

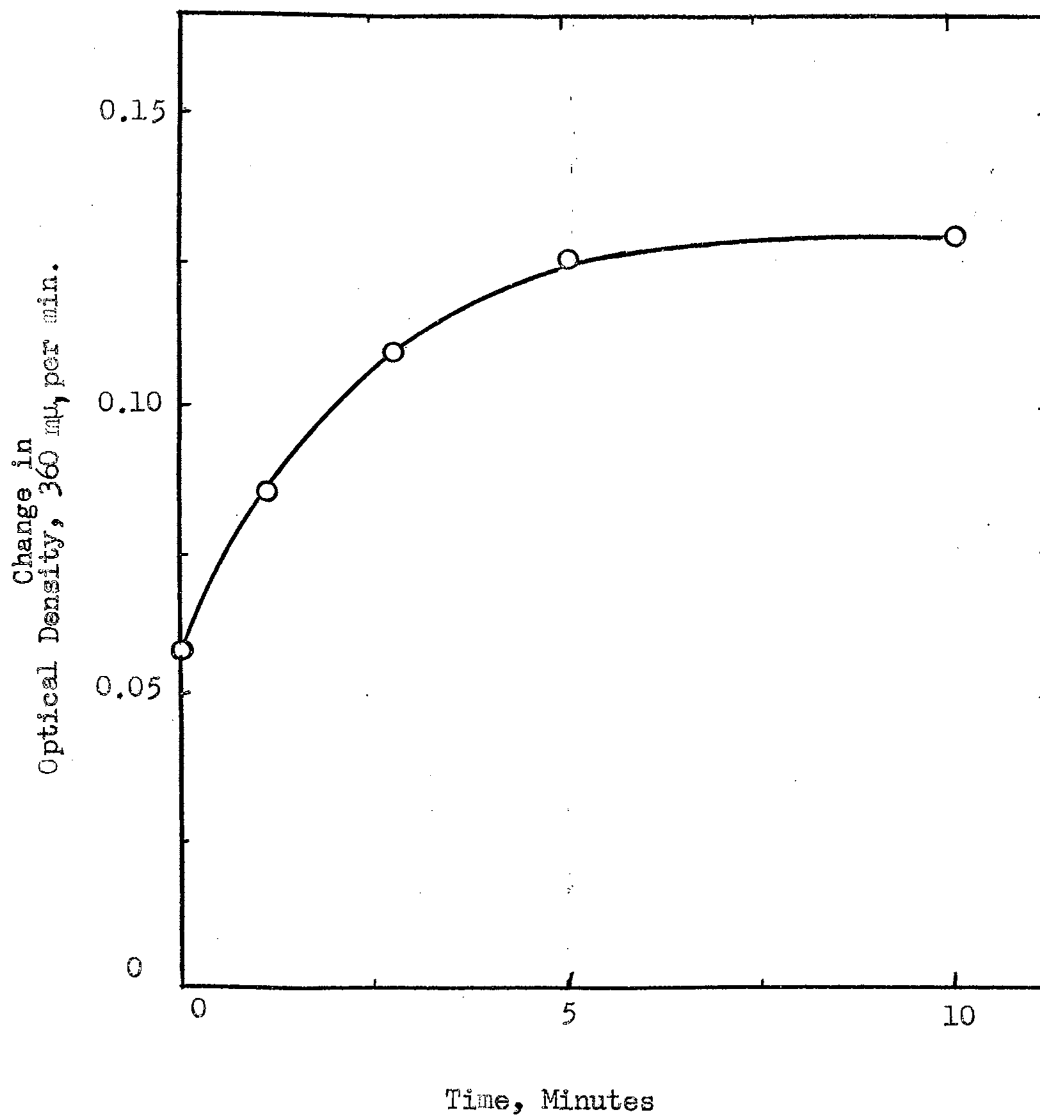
observed that the salt had no effect on the absorption maximum or extinction coefficient of compound I when added after the reaction had proceeded 2 minutes. The addition of the salt with substrate caused only a slight decrease in activity but not enough to account for the loss in activity found during purification. When the enzyme, ferrous ion and ammonium sulfate were preincubated 2 minutes before addition of substrate, there was an activation rather than inhibition.

2. Effect of Time on the Activation by Ammonium Sulfate and Ferrous Ions.

Preincubation time required for activation of the heated enzyme by ferrous sulfate and ammonium sulfate was determined. As is shown in Fig. 5 maximum activity was attained after 5-minutes exposure of the enzyme to these components. This study was repeated after the concentration of ammonium sulfate for best activity was determined and after the enzyme was purified. An identical curve was obtained.

3. Concentration of Ammonium Sulfate for Maximum Activity.

The effect of changing the amount of ammonium sulfate on the reaction rate during the 5-minute preincubation is shown in Fig. 6. Maximum activation occurs when the solution is approximately 0.035 molar with respect to this salt. At higher concentrations there is a decrease in the activity. This value is not absolute and varies somewhat with the particular preparation, the stage of purification and the age of the preparation. In two cases it was observed that the salt caused an inhibition of activity.



Time, Minutes

FIGURE 5

EFFECT OF TIME OF PREINCUBATION OF ENZYME

WITH

AMMONIUM SULFATE AND FERROUS ION ON ACTIVITY

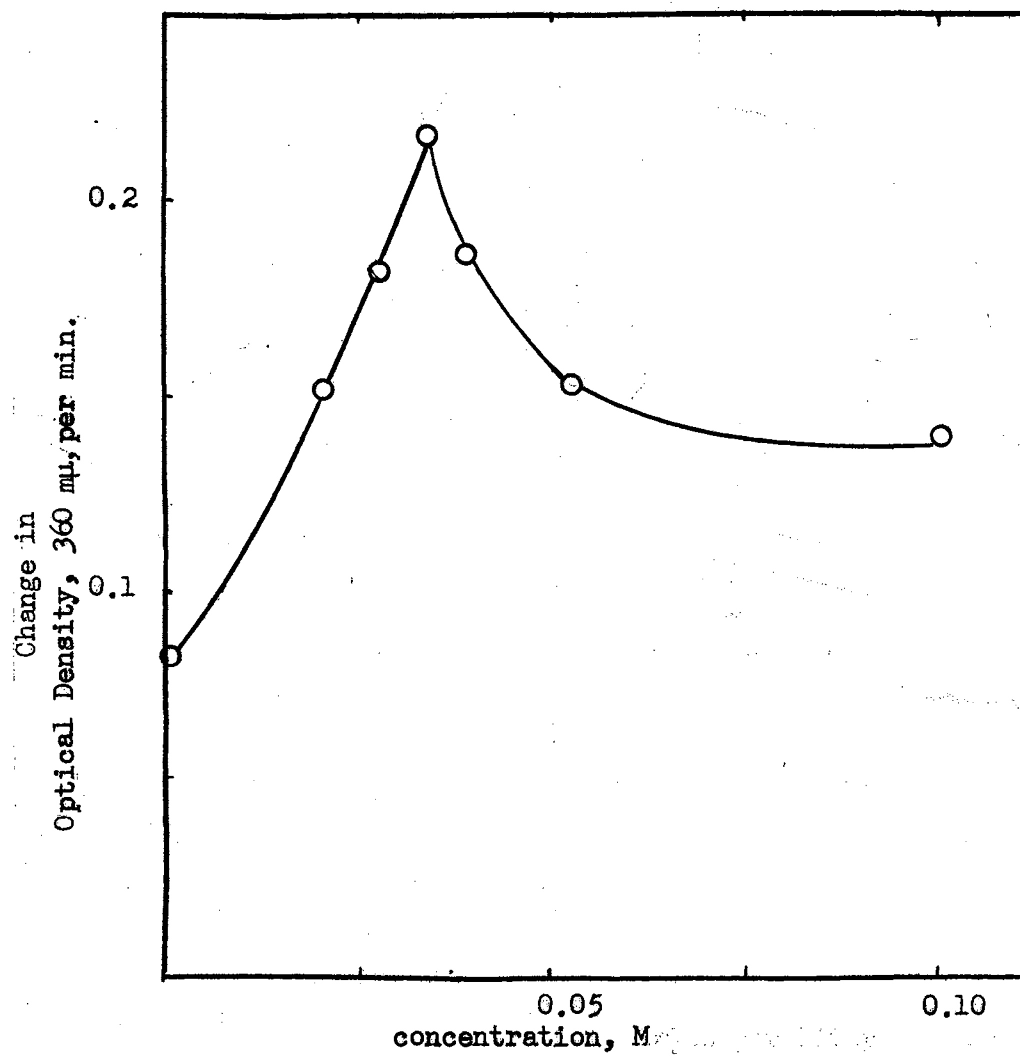


FIGURE 6

CONCENTRATION OF AMMONIUM SULFATE FOR MAXIMUM ACTIVATION

4. Effect of other Ions.

Other salts were tested to determine the specificity of this activation on the preparation. It was found that other salts having neither ammonium or sulfate in common gave this activation as well. Further, Triton-X-100, a non-ionic detergent caused stimulation at this state of purification. Thus it may be considered that activation is the result of the ionic effect of the salt solutions and to the surfactive properties of the detergent. It should be noted, however, (Table I) that the ionic strength for maximum activation with each salt was different. Also, Triton-X-100 failed to activate in more pure preparations.

5. Sequence of Addition of Ferrous Sulfate and Ammonium Sulfate.

Strehler and Comier (83) in a study of bacterial luminescence used a method for the determination of the order of interaction of various factors in multicomponent systems. This suggested a similar study for the determination of the activation sequence of the oxidase. This consisted of comparing the enzymatic activities resulting when each component is added last to the preincubation mixture containing the other components. If the component that reacts first with the enzyme is added first, activation of the enzyme should begin before the addition of the other component. This fact will then be reflected in an increase in the enzymatic activity when substrate is added. Conversely, if the component that reacts last is added first, no activation will occur until the first component is added, and there will be less activity. Figure 7 depicts the results of this study.

TABLE I

EFFECT OF OTHER SALTS AND A DETERGENT ON OXIDASE ACTIVITY

Salt Added	Concentration, M	Reaction Rate, Change in O.D. per min.
----	----	.062
$(NH_4)_2SO_4$	.048	.216
$MgCl_2$	.048	.138
Na_2SO_4	.048	.186
NaCl	.096	.124
Triton-X-100	0.06%	.202

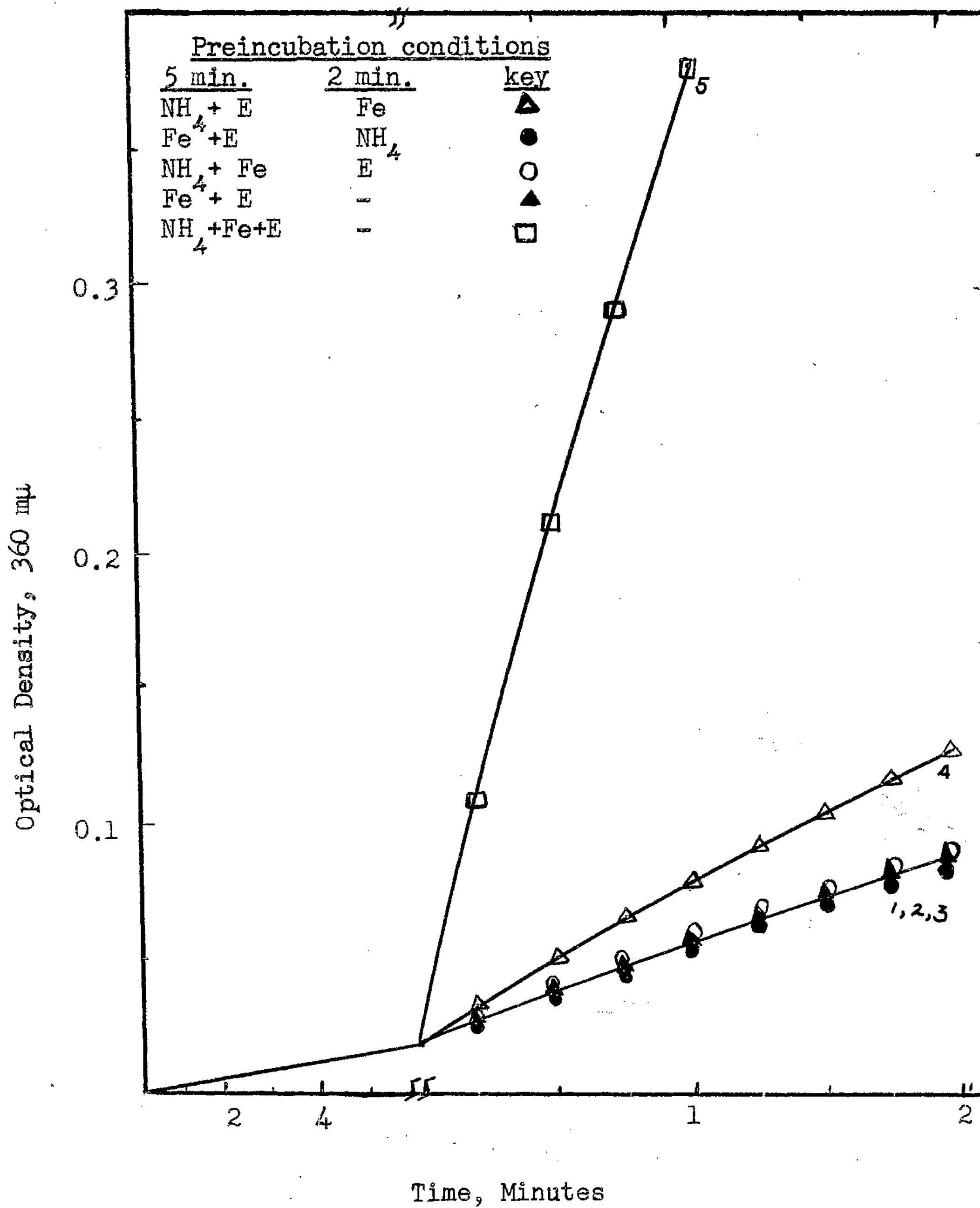


FIGURE 7

EFFECT OF SEQUENCE OF ADDITION OF AMMONIUM SULFATE

AND FERROUS SULFATE ON ACTIVITY

When ammonium sulfate was added 3 minutes after ferrous ion there was the least amount of activity (curve 1). When ammonium sulfate and ferrous ions were incubated together for 3 minutes and the enzyme added for two minutes there was only a slight increase indicating that the two salts did not interact to produce a required intermediate in the absence of the enzyme (curve 2). When the enzyme was preincubated with ferrous ion there was no increase in rate above curves 1 and 2 showing a requirement for ammonium sulfate (curve 3). Enzyme preincubated 3 minutes with ammonium sulfate before addition of ferrous sulfate showed a slight increase in activity over the others. This suggested that the primary activation was an ion effect before addition of ferrous ions. Though this difference is small several experiments have given the same result. Curve 5 shows an incubation with both components added to the enzyme 5 minutes before substrate.

6. Activation Studies

In the course of purification other factors in addition to glutathione, ferrous ion and ammonium sulfate have been observed to produce remarkable activation or restoration of activity. Figure 8 shows the effect of acid on the activity and specific activity of the enzyme. An homogenate was centrifuged at 105,000 xg for 30 minutes, heated to 55°C for 5 minutes, cooled and recentrifuged. Concentrated hydrochloric acid was added slowly with rapid stirring to the enzyme until the solution was pH 3.5. The resulting preparation was allowed to stand at room temperature for the period of study. Five-ml aliquots were removed and neutralized at suitable time intervals, centrifuged and assayed for activity. Protein was

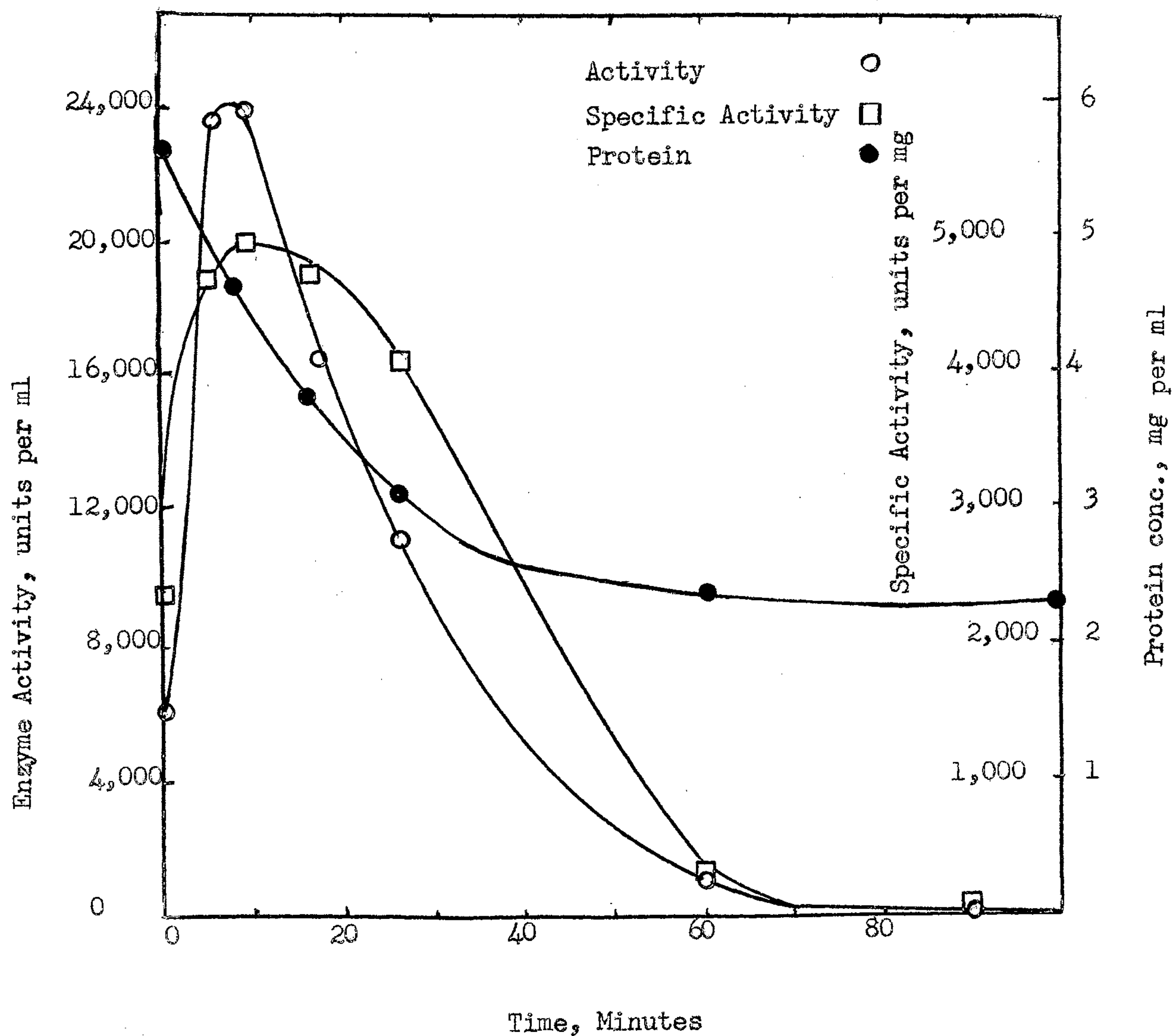


FIGURE 8

EFFECT OF ACID-EXPOSURE TIME ON SPECIFIC ACTIVITY

estimated by measurement at 280 mμ (78). It was found that immediately after the addition of acid there was a 2-fold increase in activity. The specific activity reached maximum values after 10 minutes. Longer exposure to the acid resulted in a rapid decrease in enzyme activity. This pH adjustment step was incorporated into the purification sequence to be described.

Alkaline conditions were also found to give a stimulation of enzyme activity equal to or greater than that observed by acid treatment. There was a restoration of activity when the ammonium sulfate-precipitated enzyme that had lost its activity was exposed to alkaline pHs. Enzyme purified through the calcium phosphate gel stage (described below) was used in a study to determine the optimum conditions for alkaline activation. Five ml samples were adjusted to the desired pH with 0.1 and 1.0 N sodium hydroxide. The aliquots were then refrigerated for 5 hours and assayed at pH 7.0. Figure 9 shows that maximum activation over this time period occurred at pH 10.0. A pH of 6.0 caused a complete loss of activity during this time. It was noted that though there was a large stimulation at alkaline pH the enzyme was no more stable than at pH 7.0.

7. Stability Studies

It was obvious from the activation studies that stability of this enzyme and activation were not necessarily related. Both acidic and basic conditions stimulated enzyme activity, but neither was suitable for maintaining this activity. A series of conditions for storage of the enzyme was tested. A sample of the calcium phosphate gel-treated enzyme was divided into five 10-ml aliquots.

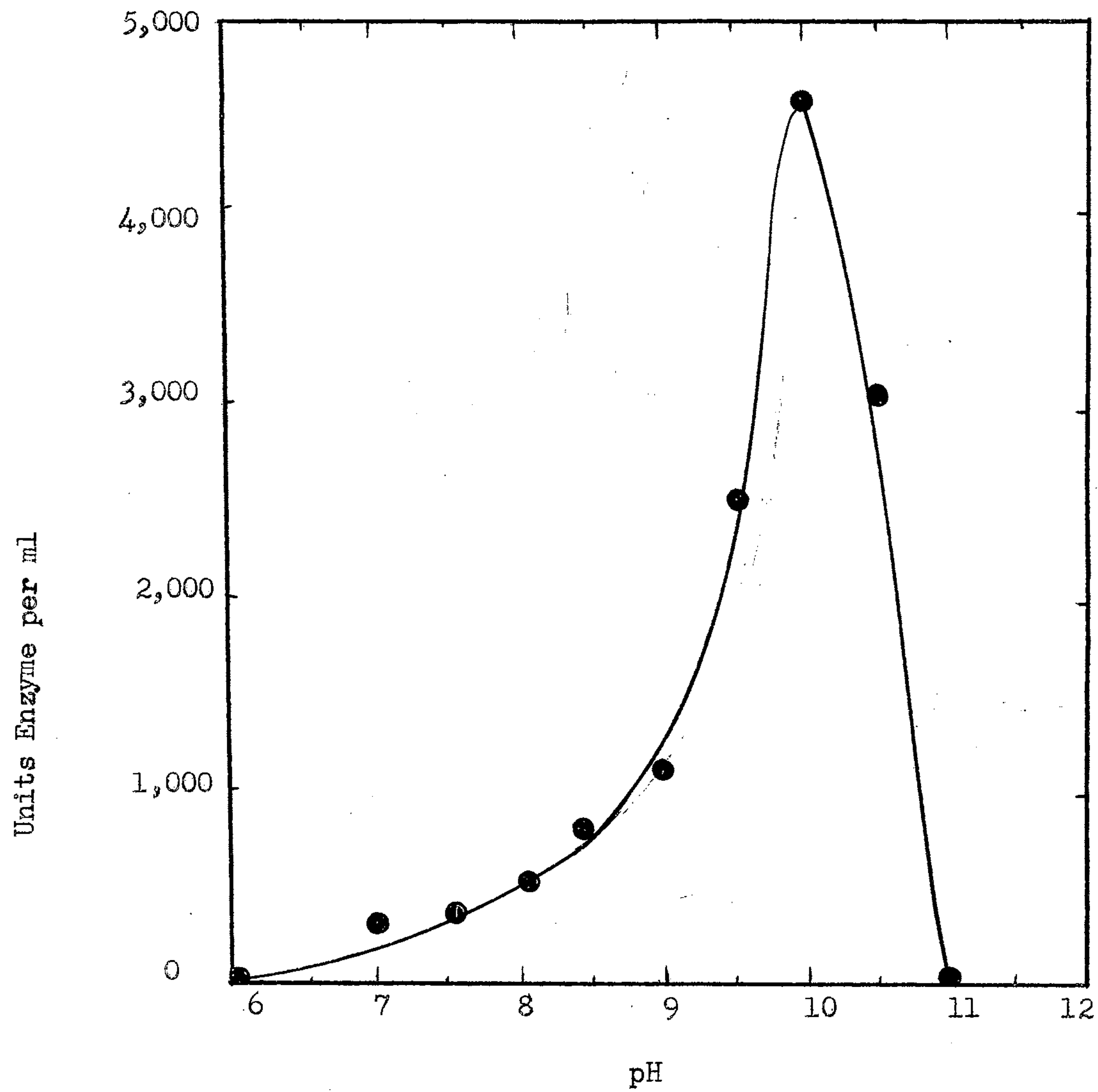


FIGURE 9

EFFECT OF ALKALINE pH ON ACTIVITY

TABLE II

STORAGE CONDITIONS FOR AMMONIUM SULFATE-PRECIPITATED ENXYME

Treatment	Time, hours	Activity, units/ml	Per cent of control
Control, refrigerated	0	718	100
Control, refrigerated	24	424	59
Control, refrigerated	96	92	13
N_2, cysteine, refrigerated	24	602	85
N_2, cysteine, refrigerated	96	556	78
Frozen	12	820	114
Frozen	96	738	103
Lyophilized, frozen	72	646	90
Lyophilized, refrigerated	72	408	57

These were stored as shown in Table II for various lengths of time and were assayed. The enzyme that was frozen had the best retention of activity. Cysteine caused a good retention of activity, but glutathione would stabilize somewhat better. In subsequent studies storage of an ammonium sulfate-fractionated enzyme by freezing and with 10^{-3} M glutathione for two months not only gave retention of activity but caused nearly 2-fold increase.

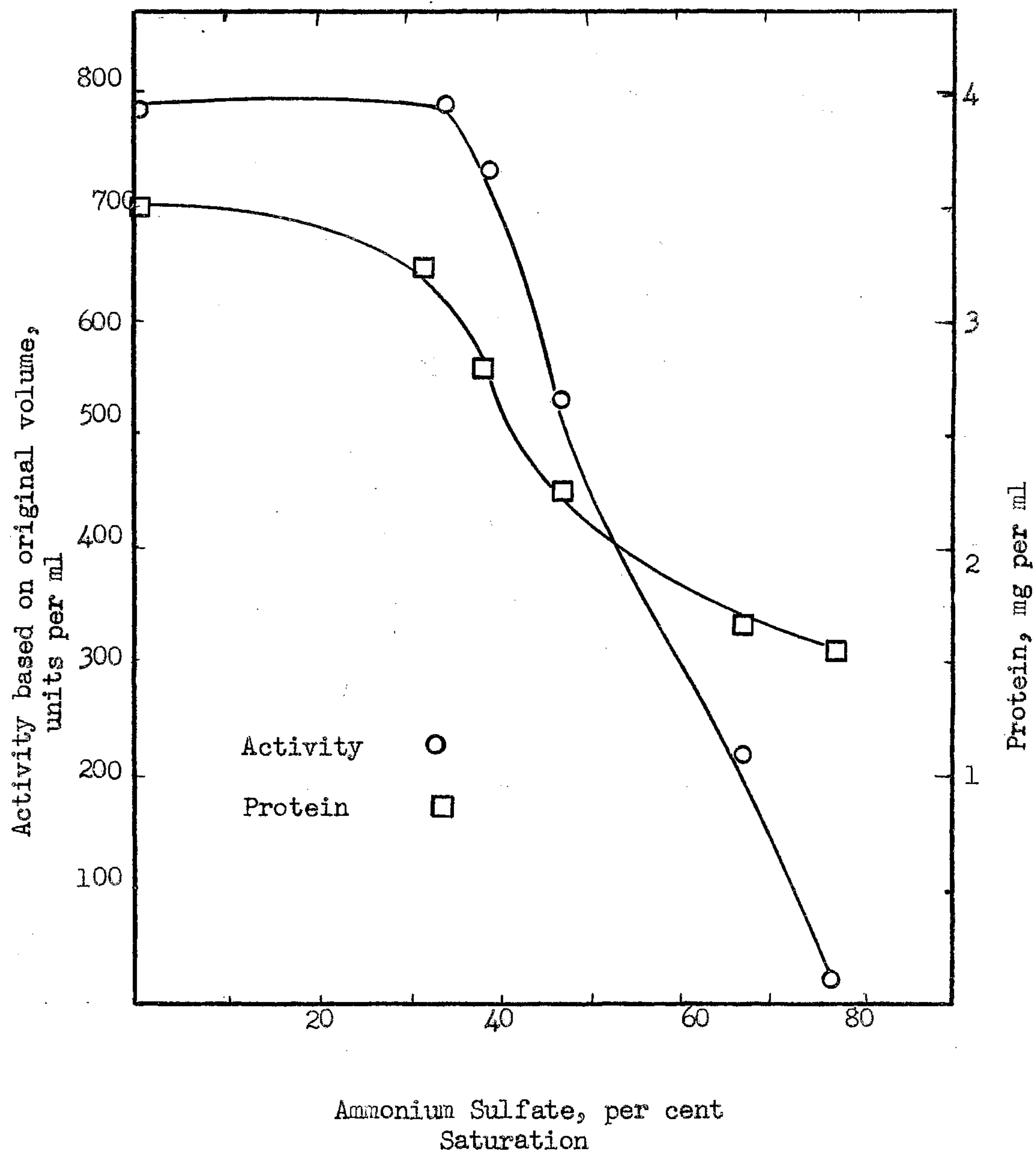
8. Purification Sequence

Several workers (11,33,42) reported the oxidase to be unstable to conventional purification techniques, but in view of the results of the activation and stability studies further purification was made possible. All enzyme purification steps were performed at 3-5 C° unless otherwise specified. Fractions were assayed for enzymatic activity spectrophotometrically. Assay systems contained 0.3 μmole ferrous sulfate, 3. μmole of glutathione, 105 μmole of ammonium sulfate and enzyme in 0.01 M Tris buffer, pH 7.0, in 3.0 ml upon addition of 0.1 μmole of 3OHAA. An enzyme concentration to produce an optical density change of 0.400 in two minutes was most desirable; substrate concentration does not become limiting during this period, and experimental error in making readings is minimized. One unit is that amount of enzyme producing an optical density change of 0.010 per minute under the conditions of the assay described above.

The 20 per cent homogenate was centrifuged in the Spinco preparative centrifuge at 105,000 xg (30 rotor) for 30 minutes, and the precipitate was discarded. The supernatant was heated

in stainless steel beakers with constant stirring for 5 minutes at 55°C. The heated fractions were cooled immediately and centrifuged at 25,000 xg for 30 minutes in a Lourdes "Superspeed" refrigerated centrifuge to remove denatured protein. The supernatant was acidified to pH 3.5-3.7 with 2 N hydrochloric acid and allowed to stand at room temperature for 10 minutes before neutralization with 4 N sodium hydroxide. The preparation was again cooled to 3°C and centrifuged at 25,000 xg. It is notable that treatment by acid brought the total activity to a relatively constant value in most preparations.

The supernatant solution was treated with 10 ml calcium phosphate gel (84) (25.8 mg per ml.) per 100 ml of enzyme solution at 5°C for 25 minutes with continued stirring. The mixture was centrifuged for 20 minutes at 20,000 xg, and the gel residue was discarded. The enzyme solution was then fractionated with cold saturated ammonium sulfate at or above pH 7.0. The bulk of the enzyme is precipitated between 45 and 65 per cent saturation, (Fig. 10) but the fraction between 50 and 60 per cent was taken to eliminate as much inert protein as possible. The precipitate was dissolved in 0.1 the original volume of 0.01 M Tris buffer, pH 7.0 to give a solution containing 6 to 8 mg of protein per ml. This stage of purification has been the one of highest specific activity and has been stored frozen with 10^{-3} M glutathione for two months with no loss in activity. The average specific activity was 11,000 following the steps described above with certain preparations as high as 21,000. The extent of purification was somewhat variable since the specific activities of the homogenates differed from one preparation to another.



Ammonium Sulfate, per cent
Saturation

FIGURE 10

AMMONIUM SULFATE FRACTIONATION;

PRECIPITATION OF ACTIVE ENZYME FROM SOLUTION

Further purification has not been successful because of the instability of the enzyme to dialysis required to remove excess ammonium sulfate. The use of DEAE cellulose columns in further purification has been attempted. The dissolved ammonium sulfate fraction of enzyme was placed on a column 2 x 34 cm and eluted with water. Two ml fractions were collected and assayed. It was found that most of the protein was eluted in the first 16 ml and that activity corresponded with this protein peak, (Fig. 11). It is probable that this does not mean the enzyme is pure but, rather, that the ammonium sulfate existing in solution caused stripping of the column. When the enzyme preparation was dialyzed for 3 hours before column treatment all protein stayed on the column and was eluted with 0.1 M phosphate buffer, pH 7.1. However, two-thirds of the activity was lost by the dialysis and column treatment and could not be restored. Numerous dialysis studies have been undertaken with the ammonium sulfate fraction of enzyme, but no suitable conditions have yet been found which can be used to remove the salts without losing activity. In addition to those steps described, negative adsorption on Norite A (3g per 100 ml of enzyme) for 25 minutes was used in earlier purification, but subsequent use of this step was no longer successful in increasing specific activity. The reason for this is not known. Variation of pH during adsorption had no effect on the ability of the charcoal to remove foreign proteins.

A summary of a typical purification is shown in Table III.

9. Measure of Purity

Starch Gel Electrophoresis

The enzyme was subjected to zone electrophoresis using

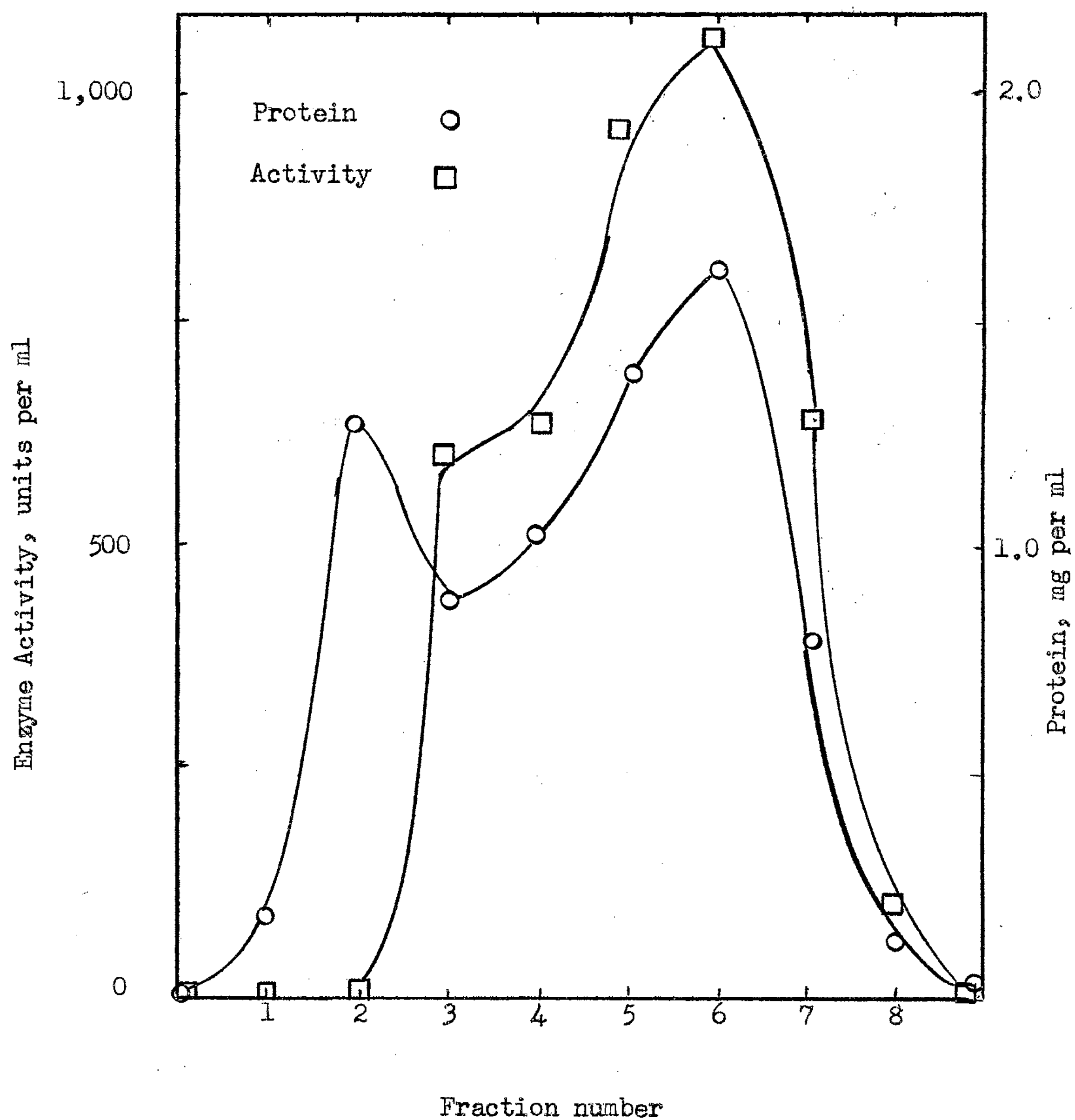


FIGURE 11

ELUTION OF PURIFIED ENZYME FRACTION FROM DEAE CELLULOSE

TABLE III

PURIFICATION SEQUENCE OF 3OHAA OXIDASE

	Vol., ml	Activity, units/ml	Total Units	Protein, mg/ml	Sp. Act., units/mg	Degree of Purification	Yield
nogenate	870	1,220	10.6 x 10^5	70	17.4	1	100
pernatant	600	2,060	12.4	15	137.0	7.9	117
ated	510	3,520	18.0	8.5	414	23.8	169
ld-Treated	400	12,240	49.0	4.3	2,850	163.5	462
-Treated	420	10,000	42.0	2.5	4,000	230	396
$I_4)_2SO_4$ ecipitated	100	96,000	96.0	2.7	35,555	2,040	907

the starch gel method of Smithies (85) to determine the approximate per cent purity of the ammonium sulfate fraction. Potato starch gel (7 per cent w/v) was prepared in 0.03 M Tris buffer, pH 8.5. Blocks were prepared by pouring the hot gel into trays, 1 x 4 x 36 cm. Two-tenths ml of enzyme (one per cent solution) was absorbed on a strip of filter paper and inserted into a slit in the gel. Electrophoresis was conducted for 14 hours at 3° C in 0.03 M Tris at 125 volts. The gel was removed from the tray and cut into 4 sections lengthwise. Two sections of the gel were stained with saturated Amido Black 10-B protein dye in methanol : water : glacial acetic acid (50:50:10 v/v). 3-Hydroxyanthranilic acid was applied to one of the unstained sections to determine the location of the oxidase. The experimental procedure was as follows: Whatman no. 1 paper pretreated in a solution of 0.1 mM ferrous sulfate, 1.0 mM glutathione and 0.035 M ammonium sulfate was applied to the gel. The strip was removed after 5 minutes and a second strip of Whatman no. 1 paper, treated with 1.0 mM 3OHAA and partially dried, was applied. After 30 minutes the paper was removed. The distribution of 3OHAA in the gel was examined with an ultraviolet Mineralite (long wavelength). Fluorescence of the 3OHAA transferred from the paper to the starch gel was observed except at one spot which corresponded to the protein band detected by the dye. It appeared that this area contained active oxidase which metabolized the substrate. Attempts to elute the oxidase from the remaining gel strip by freezing and thawing and by extraction with ammonium sulfate and 0.1 M phosphate buffer were unsuccessful. The use of this method as a purification step was not promising.

Amido Black dye treatment showed that there were at least four protein components. These components did not move more than 5 cm from the origin during the 14-hour period suggesting that the isoelectric point may be near pH 8.5. From the size of the individual protein components it was estimated that the enzyme could not be more than 25 per cent pure at this stage of purification.

Ultracentrifugation

A sample of the purified enzyme was examined in the ultracentrifuge at the University of Illinois. The enzyme fraction was prepared for ultracentrifugation by dialysis for one hour at pH 7.5 against 0.02 M sodium phosphate. The time-sequence photographs taken during ultracentrifugation showed a broad protein band. A dark residue settled to the bottom. It cannot be determined whether these results are characteristic of the native or denatured protein since the effect of dialysis is not known (Fig. 12).

10. Enzyme Properties

Km values of the enzyme for 3OHAA were determined in four experiments with different preparations of the ammonium sulfate-fractionated enzyme. All results compared favorably, and the average value was 2.08×10^{-5} (Fig 13). At higher concentrations (Fig. 14) it was observed that the substrate did not inhibit as was previously reported with crude preparations (46).

The ferrous ion concentration required for maximum activation differed slightly from that reported by Stevens (47). An "apparent" Km for ferrous ion was found to be 2.0×10^{-5}. An increase in enzyme activity was observed with an increase of ferrous ion greater

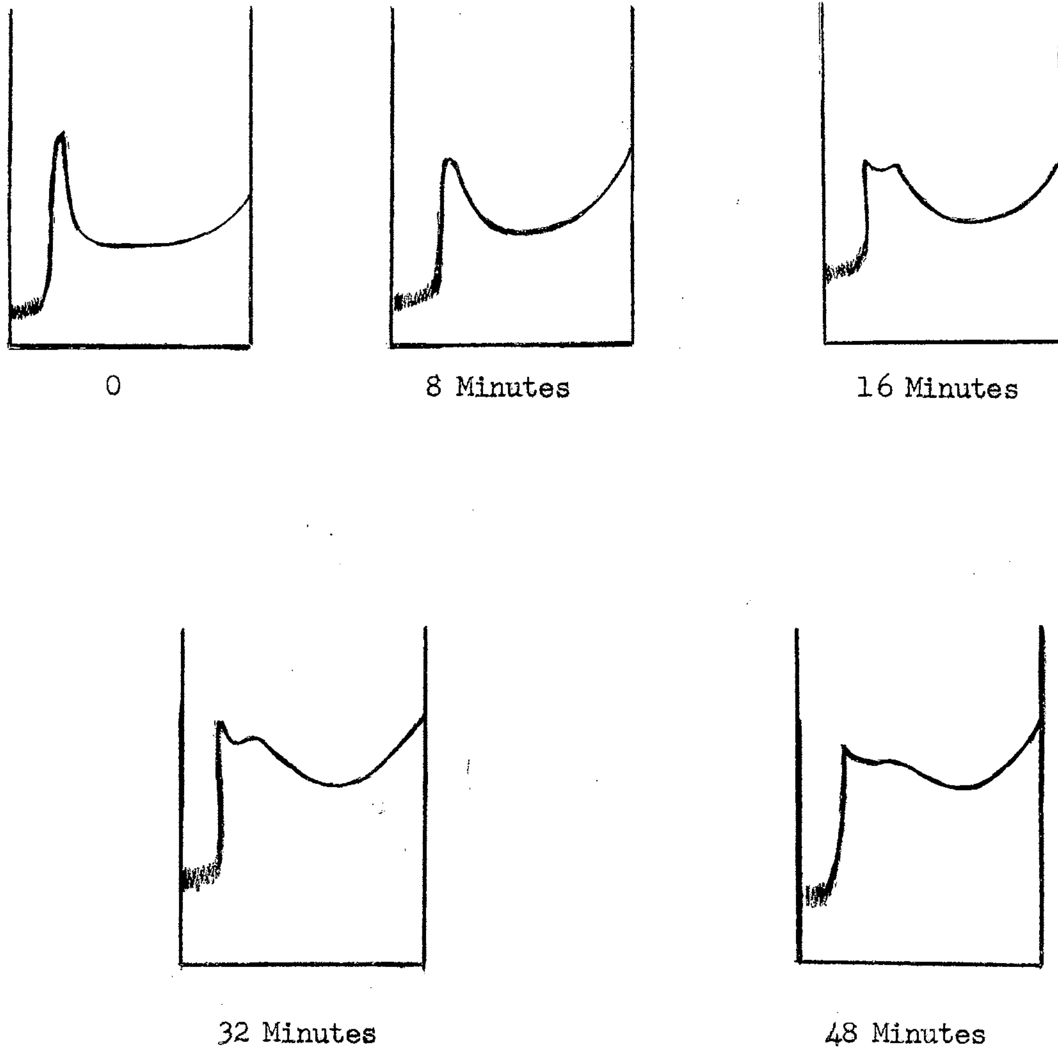


FIGURE 12

ULTRACENTRIFUGE PATTERN OF ENZYME

PURIFIED THROUGH AMMONIUM SULFATE FRACTIONATION

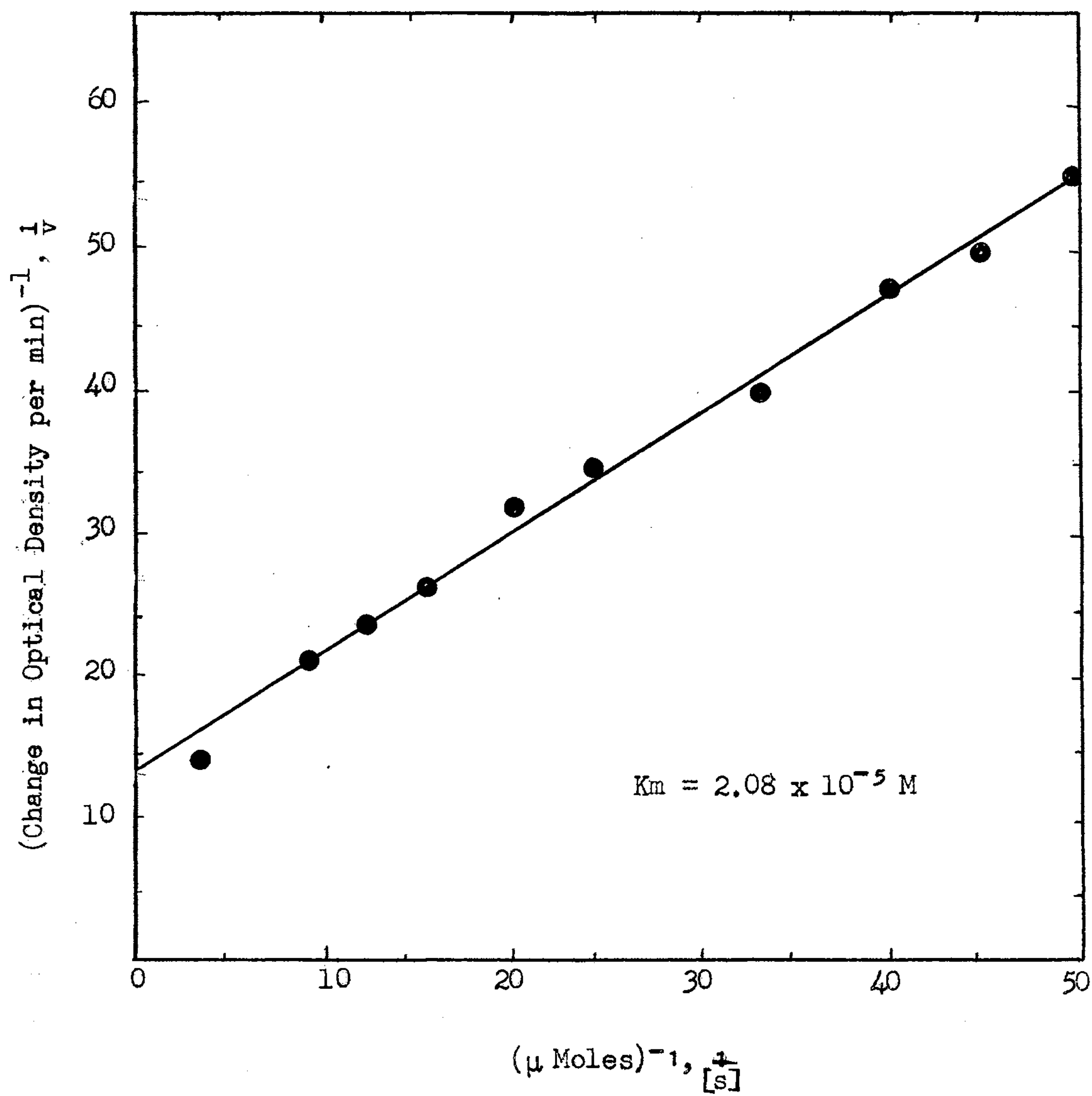
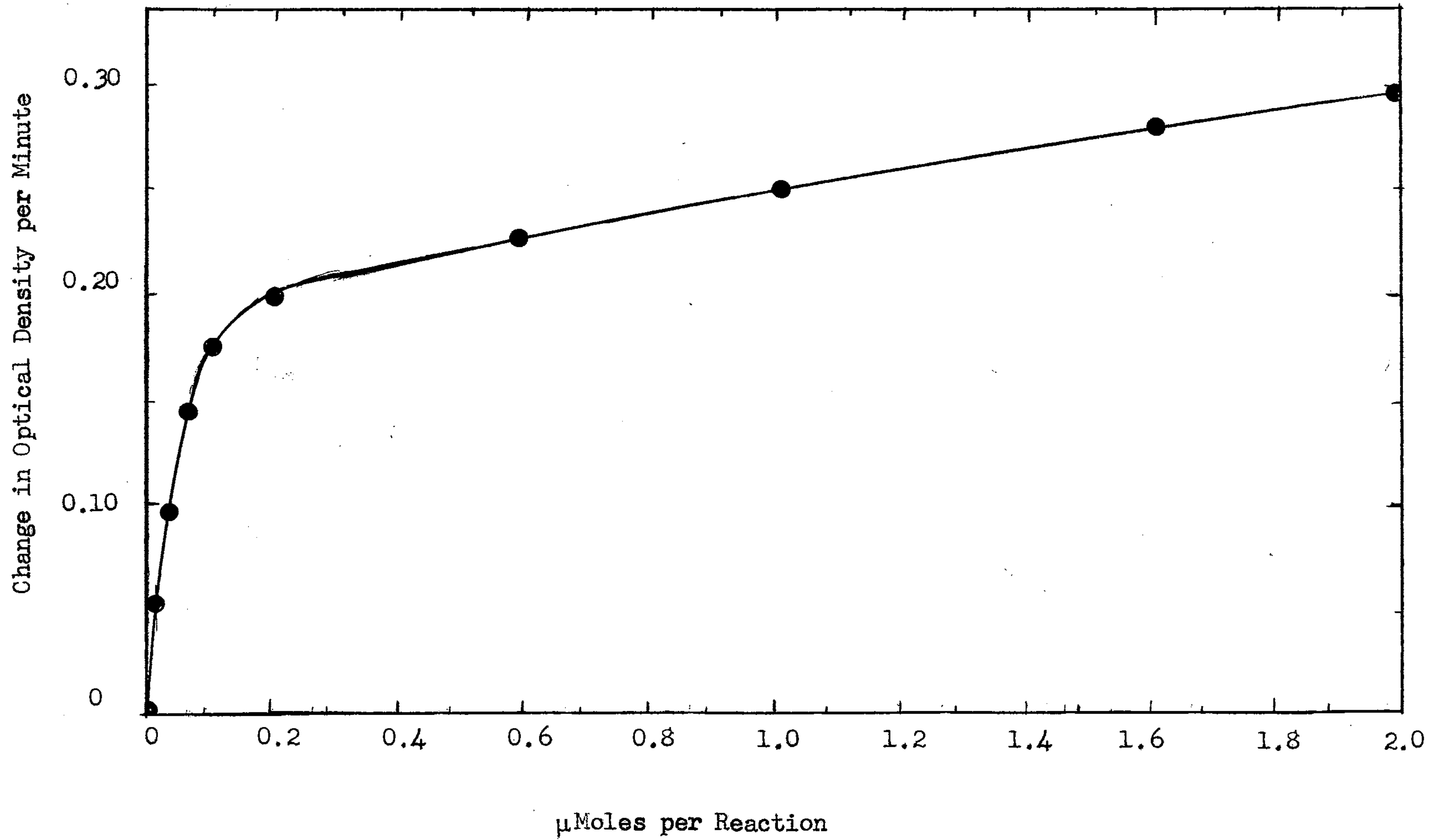


FIGURE 13

DETERMINATION OF Km OF ENZYME FOR 3OHAA

FIGURE 14

EFFECT OF 3OHAA CONCENTRATION ON THE RATE OF COMPOUND I FORMATION



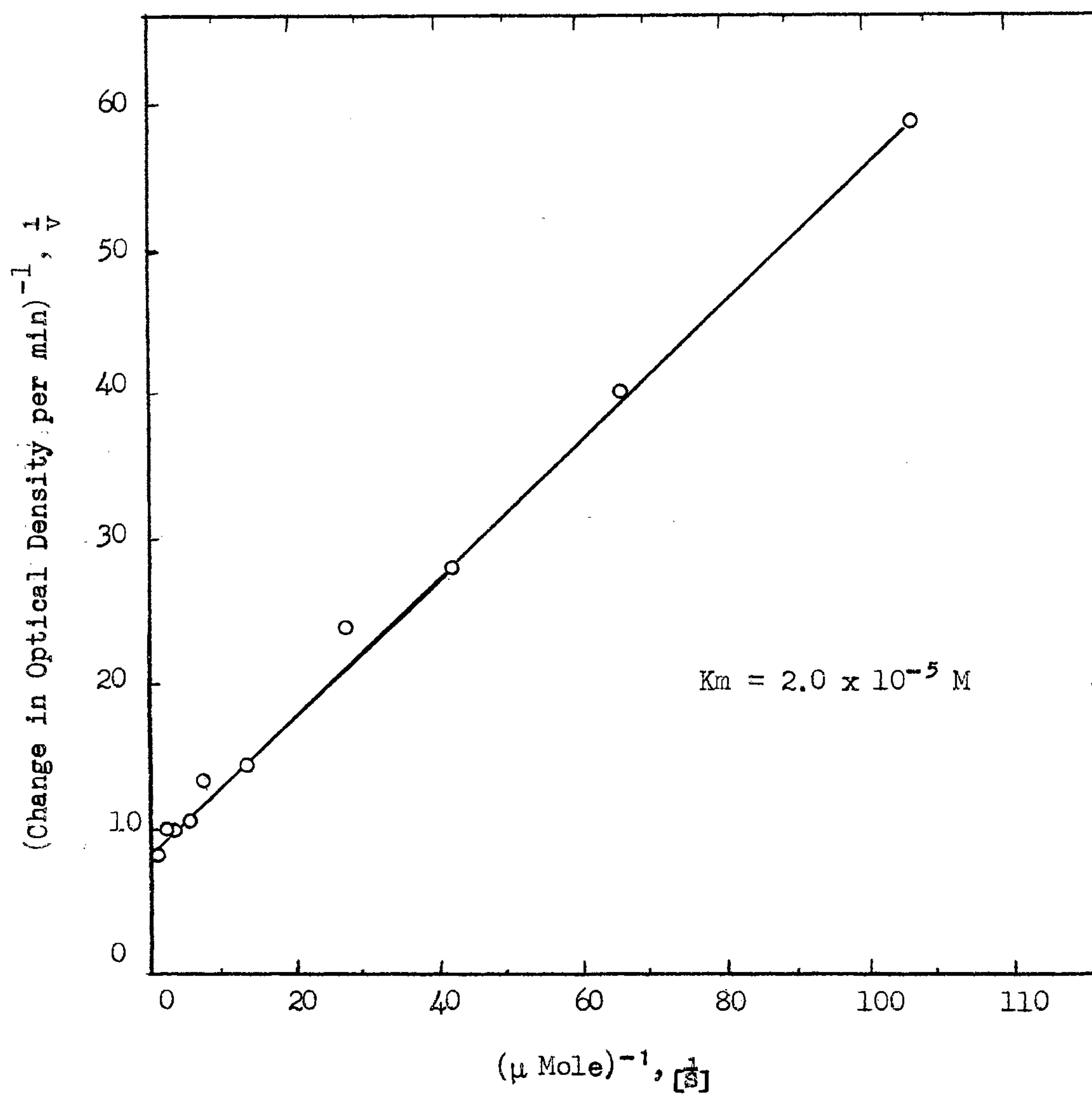


FIGURE 15

DETERMINATION OF Km OF ENZYME FOR FERROUS SULFATE

than 10^{-4} M but this activity was not on the linear portion of the curve obtained with more dilute solutions (Fig. 15).

11. Possible Function of the Sulfhydryl Group on the Enzyme.

The sulfhydryl group is necessary for activity of the enzyme. The question to be answered is, does this functional group participate directly in catalysis or is it involved in maintaining the structure of the enzyme. Suda and Tokuyama (86) have used a method to suggest the function of the sulfhydryl group in the two enzymes, homogentisicase and pyrocatechase. This involved the sequential addition of p - choromercuribenzoate (pCMB) and substrate to the enzyme. A similar procedure was followed with 3OHAA oxidase. The results of the experiment are shown in Fig. 17. The enzyme was preincubated in the usual manner but without glutathione. The two compounds, pCMB and 3OHAA, were added in sequence 15 seconds apart. When 3OHAA was added first or at the same time as pCMB, there was 25 per cent inhibition, but when pCMB was added first there was 68 per cent decrease in activity. This could be explained if the sulfhdryl group is at or near the active site of the enzyme. The pCMB and 3OHAA compete for this position.

12. Picolinic Acid Carboxylase in Beef Liver

During the purification of the oxidase from beef liver it was often observed that the 360 mμ maximum would decrease more rapidly than a non-enzymatic control. This phenomenon was noted in all purification steps but was particularly observed in the latter stages (ie. pH and gel treatment, 40-65 per cent ammonium sulfate precipitation). The addition of 2 x 10^{-3} M cyanide or 0.035 M

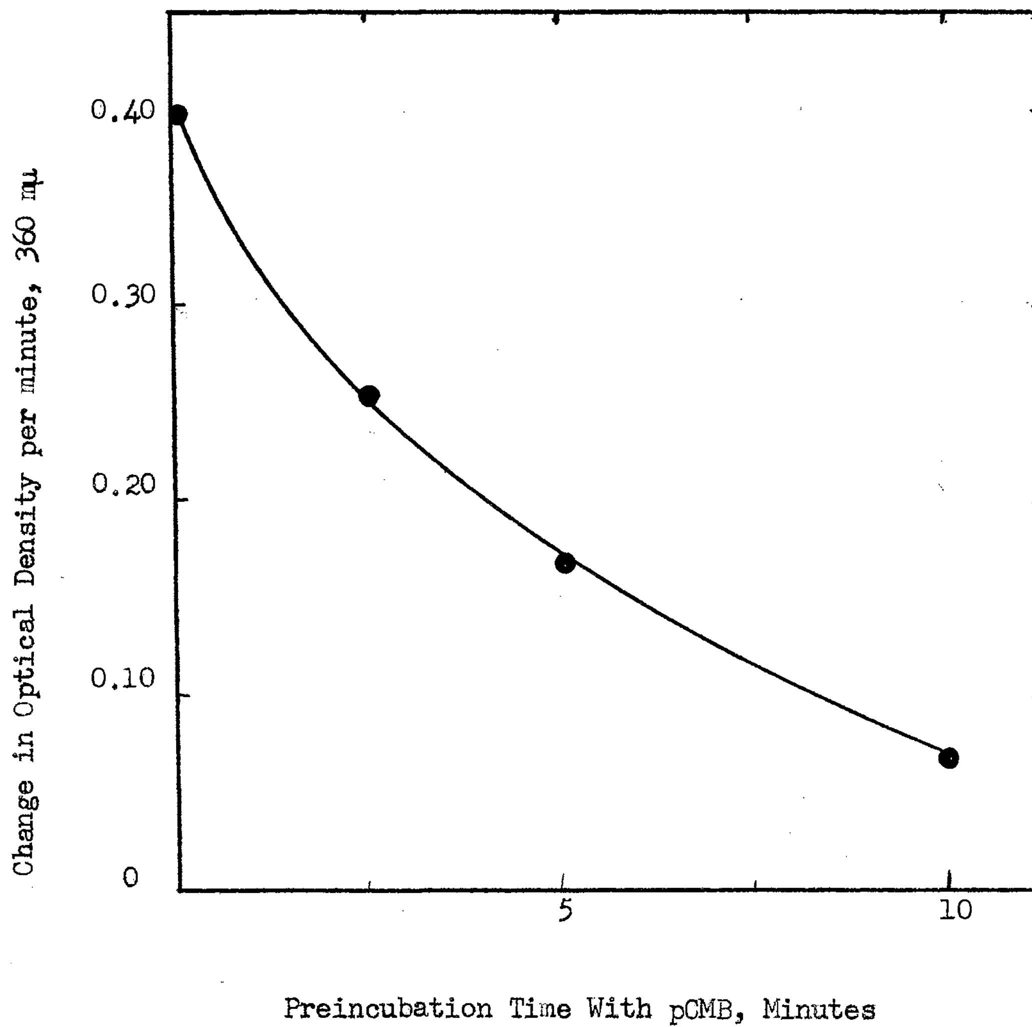


FIGURE 16

EFFECT OF TIME OF pCMB TREATMENT ON ENZYME ACTIVITY

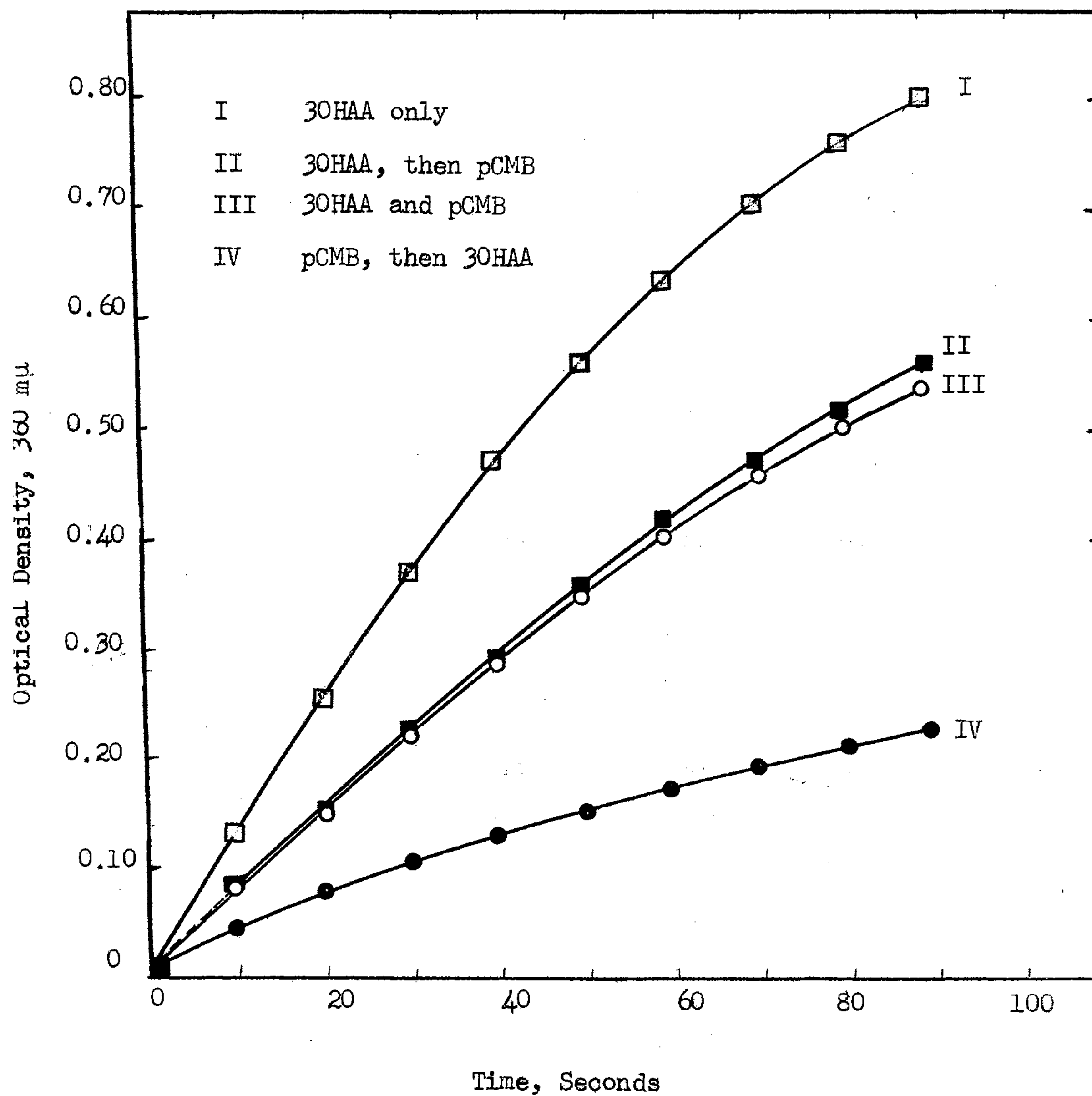


FIGURE 17

EFFECT OF ADDITION OF 3OHAA AND pCMB IN SEQUENCE

ammonium sulfate caused inhibition of the disappearance of compound I. When the enzymatic oxidation of 2 μmole of 3OHAA was observed in the Warburg respirometer it was found that considerable gas was given off that slowly disappeared. If the gas were CO_2 it was probably being absorbed by the potassium hydroxide in the center well. A radioisotopic tracer study with tritium-labeled 3OHAA was done to determine the nature of the products. The substrate was treated with an excess of the enzyme preparation to give compound I. When the absorption at 360 mμ reached a maximum, the reaction was accurately divided into two equal portions. To the first portion was added four volumes of cold absolute ethanol. The reaction in the second portion was allowed to continue 15 minutes at which time all of the compound I had disappeared. Four volumes of ethanol were added to this portion. The two preparations were centrifuged 15 minutes at 20,000 xg to remove the protein, and 100 mg of quinolinic acid and 100 mg of picolinic acid were added. The samples were concentrated nearly to dryness on a rotary evaporator, then diluted to 10 ml and placed on Dowex 50-(H)- W x 8 columns 2 x 35 cm.

The two acids were eluted from the columns with 0.1N/H Cl in 10 ml aliquots. The quinolinic acid, found in fractions 4 to 8, was recrystallized twice from 50 per cent ethanol; the picolinic acid in fractions 38 to 50 was sublimed twice at 105°C _in vacuo._ Melting points were the same as the authentic acids. Samples were weighed, combusted and counted on the vibrating reed electrometer. Table IV summarizes the results. The major product of the enzymatic degradation of compound I was picolinic acid. The enzyme present in

TABLE IV

DETERMINATION OF THE AMOUNT OF QUINOLINIC AND

PICOLINIC ACIDS PRODUCED BY BEEF LIVER

	Control incubated 2 min.		Test incubated 15 min.	
	mμc	per cent	mμc	per cent
inolinic acid	138	57	37	13.6
colinic acid	104	43	235	86.4
total	242	---	272	----

purification of the oxidase was picolinic carboxylase. It is interesting that such rigorous treatment as acid, heat and calcium phosphate gel did not resolve these two closely related enzymes. The enzymes could be separated by careful ammonium sulfate fractionation. The 50 to 60 per cent salt fraction was essentially free of picolinate carboxylase activity.

B. Studies on The Structure of Compound I

The structure of the enzymatic oxidation product of 3OHAA, compound I, has not been conclusively established. Wiss and Bettendorf (58) have reported the only isolation of derivatives of compound I, but the conditions for removal of proteins in their work were such that compound I might have been converted to compound II, a product shown to react with carbonyl reagents (11). Wiss later reported that acid conditions convert compound I to picolinic acid (62). The experiments to be described were done to determine whether the methods used by Wiss would, in fact, destroy compound I or convert it to picolinic acid, and to prepare stable derivatives of this intermediate.

1. Method of Assay

The enzymatic reaction for the formation of compound I was routinely followed by measuring the rate of disappearance of fluorescence of 3OHAA. Reaction mixtures for the oxidation of 2 - 3 μmoles of 3OHAA contained 0.3 μmole ferrous sulfate and 0.5 ml of a 10 per cent rat liver acetone powder extract in Krebs-Ringer phosphate buffer, pH 7.0. The volume was adjusted so that addition of substrate resulted in a total volume of 3.0 ml. One-tenth ml aliquots were removed at various time intervals and transferred to 9.9 ml of absolute ethanol. Fluorescence was measured in the Coleman Photofluorometer with B-1 and PC-1 filters against a standard of quinine sulfate. A unit of oxidase activity was defined as the amount of enzyme necessary to oxidize 1 μmole of 3OHAA per minute.

2. Effect of Trichloroacetic Acid on Compound I

a. Measurement of Quinolinic and Picolinic acids

Twenty-three μc (8 μmoles) of tritium-labeled 3OHAA was oxidized to compound I, and the preparation was immediately divided into two fractions. Four volumes of cold absolute ethanol were added to one portion; the other was cooled to 3°C and 3 ml of 50 per cent trichloroacetic acid (TCA) added. The denatured proteins were removed from both by centrifuging at 9,000 xg for 3 minutes at 0°C. The TCA-treated preparation (5 per cent) was neutralized after both solutions had been allowed to stand at 0°C for 1 hour. Both reactions were warmed to 50°C to allow complete conversion to the pyridine carboxylic acids. One hundred mg. of carrier picolinic and quinolinic acids were added, both solutions were reduced in volume, and after adsorption on Dowex-50-H 1.5 x 30 cm, the columns were developed with 0.1 N HCl (35). The two acids were identified by the ferrous ammonium sulfate spot test (87). The quinolinic acid from the ethanol-treated reaction was eluted in the fractions between 40 to 80 ml. Probably because of the high salt content the acids in the TCA-treated mixture passed through the column with no separation. Rechromatography of the acids on a larger column (1.5 x 48 cm) and elution of quinolinic acid with water resolved the mixture. The quinolinate was found in the fractions collected between 10 and 30 ml, and the picolinic acid was eluted by 160 to 240 ml of 0.1 N HCl. Quinolinic acid was recrystallized from 50 per cent ethanol, and the picolinic acid was sublimed at 100°C *in vacuo*. Samples were weighed, combusted and counted on the vibrating reed electrometer. The results

contained in Table V show that picolinic acid is not a product of TCA treatment of compound I, contrary to the report of Wiss (62). It is also evident that compound I must have been destroyed, since its condensation product, quinolinic acid, is found to account for only 7 per cent of the total isotope under these conditions. It was concluded that TCA was not satisfactory for removal of proteins from preparations of compound I.

b. Structure of Compound II

Long *et al.* (11) and Miyake *et al.* (42) reported that compound I is unstable to mineral acids and is converted to a second product, compound II, with the loss of ammonia. Compound II was probably the major product in the experiment described above. An experiment designed to determine whether compound I loses the γ-carboxyl group as well as the amino group when treated with acid, was done. 3-Hydroxyanthranilic acid-1-C^{14} and -carboxyl-C^{14} were used as substrates. This experiment was done in view of the unpublished results of Benassi and Henderson that the carboxyl-labeled compound I loses its radioactivity when treated with carbonyl reagents in acid solution. 3-Hydroxyanthranilic acid containing 49 mμc of carbon-14 in each of the two positions was oxidized to compound I with an acetone powder extract of liver, and each reaction mixture was divided into two equal portions. One portion was treated with 2 N HCl to pH 1.0. Water was added to the other. After 5 minutes the acidified portions were neutralized, and all mixtures were treated with four volumes of ethanol. Aliquots were removed for spectrophotometric measurements at 360 and 375 mμ.

TABLE V

EFFECT OF 5 PER CENT TRICHLOROACETIC ACID ON COMPOUND I

Treatment	Tritium in:	QA per cent	PA per cent	Total per cent
Ethanol, pH 7		54.0	3.4	59.0
TCA, pH 1		7.0	3.7	10.7

The remainders were dried in vacuo, combusted and counted. The results in Table VI show that acid treatment of carboxyl-labeled compound I caused a loss of 75 per cent of the radioactivity. Isotope from ring-labeled compound I was quantitatively recovered. It is apparent that compound I is decarboxylated as well as deaminated in its conversion to compound II. Recovery of the isotope from compound II formed from 3OHAA-1-C^{14} rules out the formation of a volatile product. A recent report by Mehler (60) has verified this observation.

3. Preparation of Derivatives of Compound I.

a. Catalytic Reduction of Compound I

In view of the fact that TCA destroys compound I, the method of preparation of derivatives by catalytic reduction attempted by Wiss (58) was repeated using cold ethanol to precipitate proteins and stop the enzymatic reactions. Seventy mμc of 3OHAA-1-C^{14} was oxidized to compound I as described in section B-1. Four volumes of cold ethanol were added, and the mixture was centrifuged at 10,000 xg for 5 minutes at -15°C. A portion was allowed to stand at room temperature such that quinolinic acid-3-C^{14} was formed. The remaining supernatant was immediately reduced at 0°C under 3 atmospheres of hydrogen pressure with PtO_2 as catalyst. Reduction of the quinolinic acid -3-C^{14} under the same conditions as well as quinolinate HCl-3-C^{14} in absolute ethanol was also done. The three resulting preparations were dried in vacuo and chromatographed on Whatman no. 1 paper using two different solvents, butanol:pyridine:water (6:4:3, v/v) and the Partridge system (76) butanol:glacial acetic acid:water, (5:1:4, v/v). Radioactivity was detected by the windowless chromatogram scanner. Results of the experiment are contained in

TABLE VI

EFFECT OF ACID ON THE CARBOXYL GROUP OF COMPOUND I

$3OHAA\text{-}1\text{-}C^{14}$	O.D. of Aliquots 360 mμ	375 mμ	Radioactivity d/s	Per cent Total in Acid
Before acid	.505	.097	574	---
After acid	.380	.421	573	100
$3OHAA\text{-}C^{14}OOH$				
Before acid	.485	.095	596	---
After acid	.298	.325	151	25.3

TABLE VII

CHROMATOGRAMS OF REDUCED COMPOUND I, QUINOLINIC ACID AND QUINOLINIC ACID HYDROCHLORIDE

	Rf Values	
	Butanol:pyridine:water	Butanol:acetic acid:water
Quinolinic acid-3-C^{14}	0.1	0.1
Reduced compound I-3-C^{14}	0.85	0.95
Reduced quinolinic acid-3-C^{14} (2,3-piperidinedicarboxylic acid)	0.82	0.95

Table VII. Quinolinic acid in both systems has an Rf of 0.1. Under the conditions used for the reduction of compound I, quinolinic acid was not reduced. The reduction of compound I in 80 per cent ethanol at pH 7.0 produced one compound with an Rf the same as that obtained when quinolinic acid-3-C^{14} was reduced as the hydrochloride. Since it has been reported that catalytic reduction of quinolinic acid--HCl resulted in the formation of cis and trans 2,3-piperidinedicarboxylic acid (88) these results suggested that compound I was reduced to this compound under neutral conditions (Fig. 18).

To verify this possibility an isotope dilution study was made. 2,3-Piperidinedicarboxylic acid was prepared as follows: 2.0 g of quinolinic acid was dissolved in 175 ml absolute ethanol and the solution saturated with dry hydrogen chloride gas. This was reduced under 50 pounds pressure of hydrogen gas using 0.5 g PtO_2 at 3° C for 14 hours. The product was filtered, taken to dryness on the rotary evaporator and kept in vacuo over NaOH pellets and P_2O_5 overnight. The dried yellow residue was dissolved in 25 ml of hot acetone-water (1:1). Darco G-60 was added, and the solution was heated 3 minutes and filtered. The volume was readjusted to 25 ml by adding water, and then acetone was added until the volume was 50 ml. White needle-like crystals formed on standing overnight at 3° C. The product was recrystallized from acetone-water and melted at 239° C, leaving no residue. The pH was adjusted to 3.5 and the recrystallized compound melted at 227° C. No gas formation due to carbon dioxide was observed near 180° C. This indicated that no quinolinic acid was present. These melting points (239° and 227° C) are in agreement with those of the cis isomer of

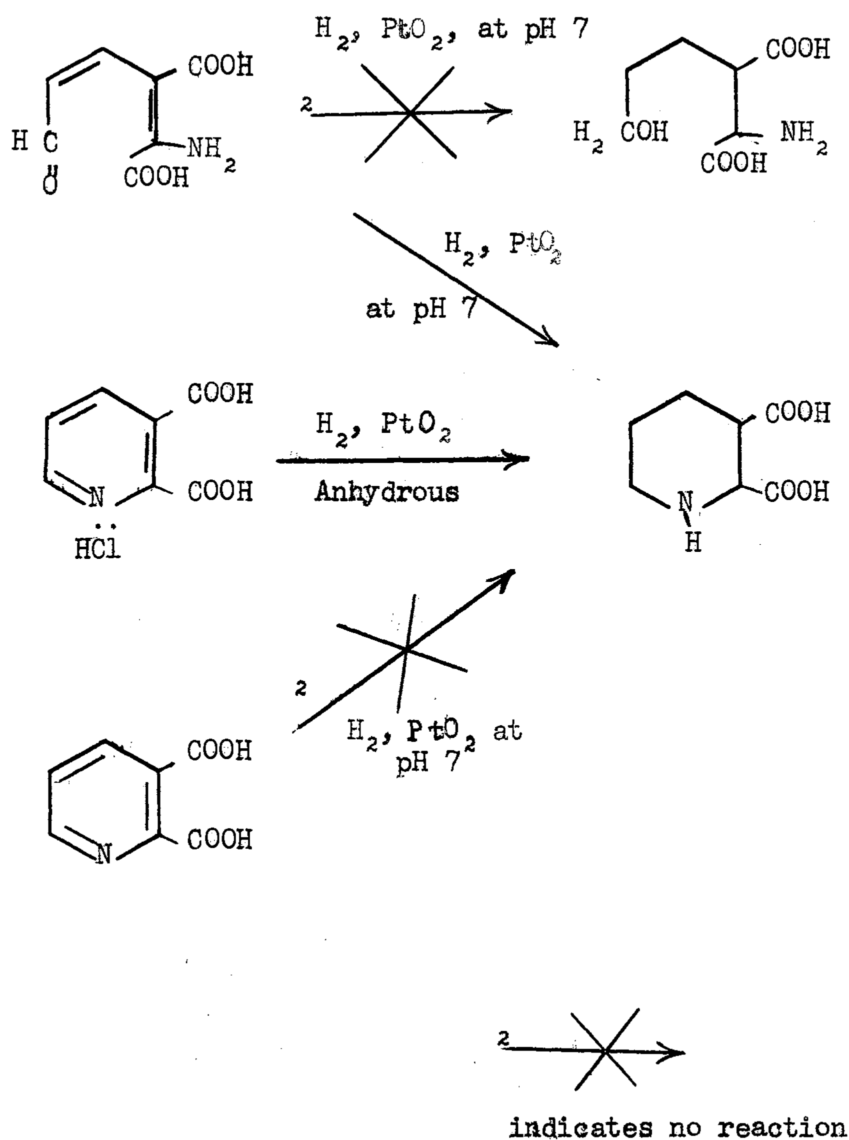


FIGURE 18

PRODUCTS OF REDUCTION OF COMPOUND I

piperidine dicarboxylic acid and its hydrochloride, respectively (88).

One-hundred-ninety mμc of 3OHAA-1-C^{14} was oxidized to compound I, and the mixture was treated with ethanol as before; two-thirds of this was allowed to condense to quinolinic acid, and one-third was reduced under 3 atmospheres of hydrogen with PtO_2 for 12 hours at 0°C and pH 7.0. The quinolinic acid -3-C^{14} was divided into two parts. One portion was reduced catalytically in anhydrous ethanol and hydrogen chloride. An aliquot from each of the three preparations was removed for microbioassay to determine quinolinic acid. One-hundred mg of carrier cis piperidine dicarboxylic acid was added to these solutions and the diluted product recrystallized. Samples were weighed, combusted and counted. The results in Table VIII show that 30 per cent of the reduced quinolinic acid-3-C^{14} was cis piperidine dicarboxylic acid. None of the quinolinic acid-3-C^{14} was recrystallized upon the addition of unlabeled piperidine dicarboxylic acid. Twenty-one per cent of compound I was reduced to form a product which gave cis piperidine dicarboxylic acid. Microbioassay indicated that 50 per cent of the compound I had been converted to quinolinic acid before it was reduced. If half of the material converted to piperidine dicarboxylic acid were in the trans form and were not isolated, this would mean that over 80 per cent of the cis isomer was recovered. By this same reasoning 60 per cent of the quinolinic acid HCl-3-C^{14} may have been reduced to the cis isomer. A sample (100 μg) of isolated piperidine dicarboxylic acid-C^{14} chromatographed on paper with butanol: pyridine: water, (6:4:3, v/v) had a small but well defined spot at Rf of 0.82 confirming the fact that the major

TABLE VIII

ISOTOPE DILUTION STUDIES WITH PIPERIDINE-2,3-

DICARBOXYLIC ACID

Reactant	Reducing conditions	Radiochemical yield, % cis-piperidine dicarboxylic acid
Quinolinate-3-C^{14}	$H_2 \cdot PtO_2$	~30
	dry HCl	
Quinolinate-3-C^{14}	$H_2 \cdot PtO_2$	0
	80% EtOH, pH 7.0	
Compound I-C^{14}	$H_2 \cdot PtO_2$	21.3
	80% EtOH, pH 7.0	

radioactive spots obtained in previous chromatograms were cis 2,3-piperidinedicarboxylic acid.

To determine whether an aliphatic amino dicarboxylic acid was also formed upon reduction of compound I, 2,4-dinitrofluorobenzene (DNFB) was added to a reduced mixture of the intermediate. This reagent was also added to quinolinic and 2,3-piperidinedicarboxylic acids as controls. Approximately 27 mμc of reduced compound I and quinolinic acid and 2.0 mg of the crystallized piperidinedicarboxylic acid was made basic with sodium carbonate (pH 8.0) and treated with 5 ml of ethanolic DNFB (0.1 per cent w/v). The reaction mixtures were shaken two hours and taken to dryness *in vacuo*. The residues were washed five times with 5 ml of ether, and the flasks were extracted with 1.0 ml of ethyl acetate and 1.0 m. of water to remove any DNFB derivatives. The ethyl acetate and water extracts were dried, redissolved in 2 or 3 drops of ethyl acetate, combined and placed on a paper chromatogram. The solvent used was butanol: NH_3 (1:1, v/v). The ammonia was a 1 per cent aqueous solution. The results in Table IX show that none of the radioactivity coincided with the yellow spots of the dinitrophenyl (DNP) derivatives. These results imply that there were no aliphatic products formed by reduction of compound I. It is apparent that the reagent did react with several compounds, probably amino acids, in the extract. This indicates that the experimental conditions were suitable for reaction of the reagent with amino compounds. The Rf for radioactivity from 2,3-piperidinedicarboxylic acid and reduced compound I again coincided at 0.92.

Preparation of an aldehyde--or amino--condensation product was

TABLE IX

DINITROPHENYL DERIVATIVES OF THE PRODUCTS OF REDUCTION

Reactants	Rf Values (Butanol:NH_3) C^{14}	DNP Derivatives (Yellow Spot)
DNFB + QA-3-C^{14}	0.1	0.56
DNFB + reduced Compound I	0.94	0.27, 0.53, 0.70
DNFB + piperidine-2,3-dicarboxylic acid	0.91	0.25, 0.53, 0.70

attempted by reducing carboxyl-C^{14} labeled compound I in the presence of acetaldehyde and of methylamine. Three separate preparations of compound I from carboxyl-C^{14} 3OHAA were made. The first was reduced as described above at pH 7.0, the second after the addition of a 100-fold molar excess of acetaldehyde and the third, with a 100-fold excess of methylamine. The products were chromatographed in the butanol: pyridine: water solvent and counted with the chromatogram scanner. No well-defined derivative with either reactant could be found. Compound I was, instead, again converted to piperidinedicarboxylic acid. A broad spot of an Rf of about 0.3 was found for the acetaldehyde reaction in one experiment but was lost on rechromatography and could not be repeated.

b. Reaction of Compound I with Metal Ions

Attempts were made to prepare a dioxolane derivative with the postulated aldehyde group. When 2 µmoles of compound I was treated with 20 µmoles of ethylene glycol and 100 µg of ferric chloride a mixture resulted having the spectral absorption shown in Fig. 19. There was nearly complete disappearance of the characteristic absorbance of 360 mµ. But when compound I was treated with ferric chloride alone an identical curve resulted. Figure 20 shows a time-rate study of the disappearance of compound I. It can be seen that the rate of decay is very rapid when the compound is treated with ferric ion, while the spontaneous decay rate of the untreated mixture is in accord with that reported for compound I (11). When aluminum chloride was added to the intermediate a similar response was observed. Table X shows the result of a microbioassay of the products of these reactions. Fifty per cent of the compound I treated

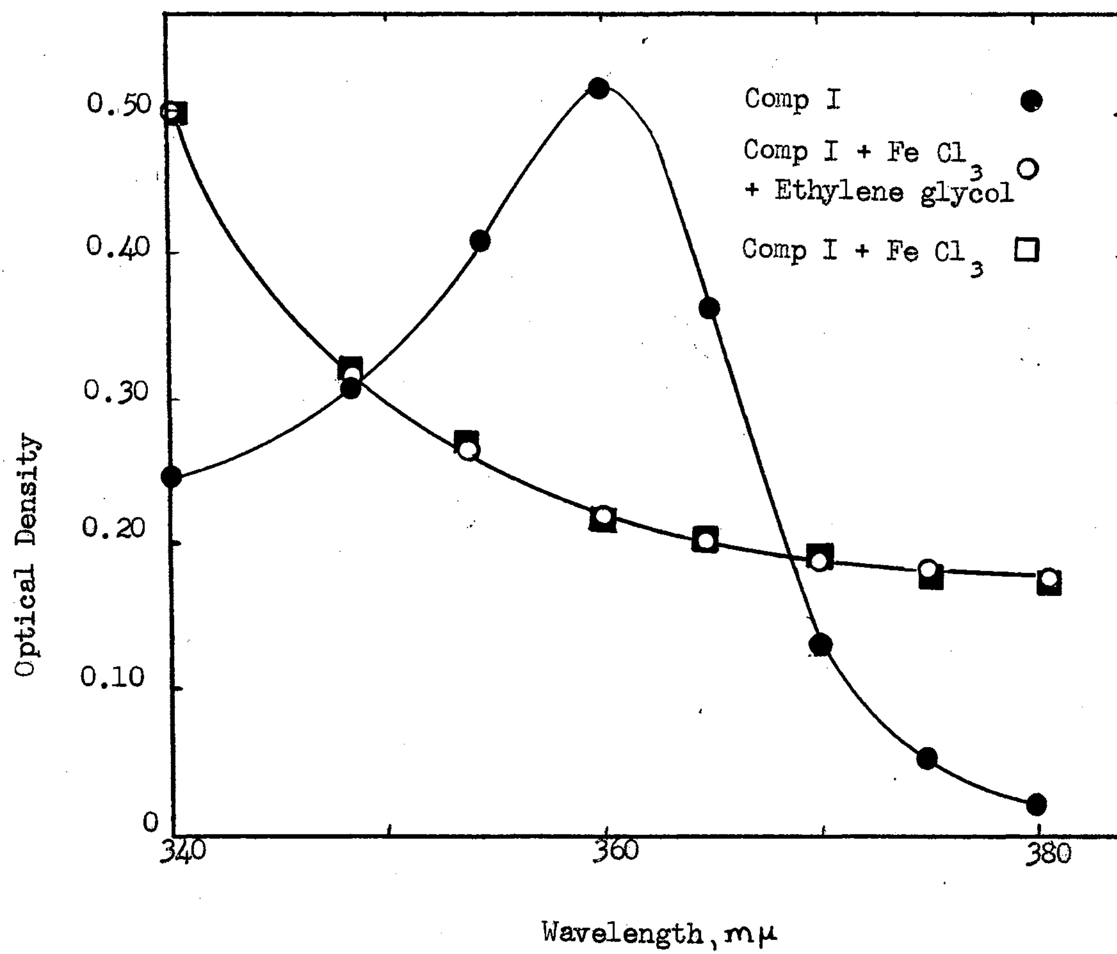


FIGURE 19

ABSORPTION SPECTRUM OF THE PRODUCT OF COMPOUND I AND FERRIC CHLORIDE

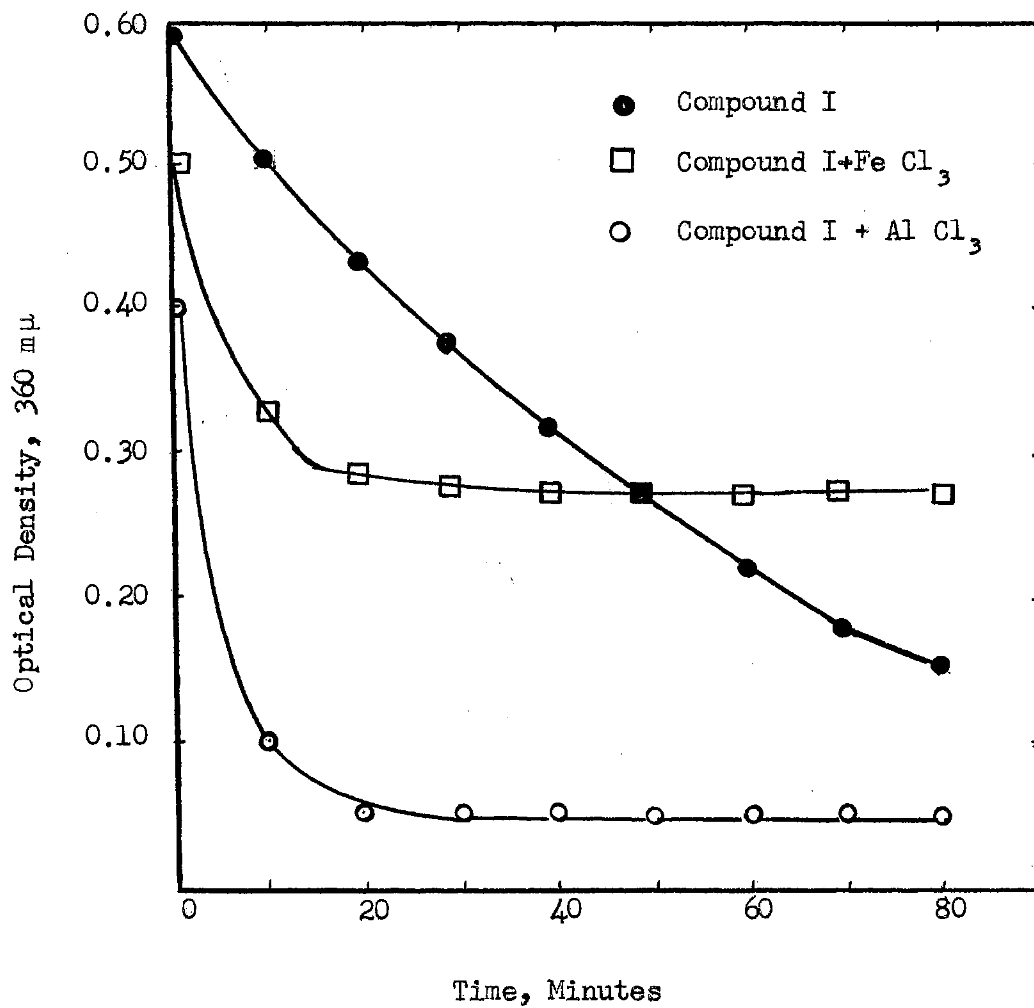


FIGURE 20

THE EFFECT OF FERRIC CHLORIDE AND ALUMINUM CHLORIDE

ON THE

RATE OF COMPOUND I DISAPPEARANCE

TABLE X

MICROBIOASSAY FOR QUINOLINIC ACID (AS NICOTINIC ACID) AFTER ADDITION OF ALUMINUM CHLORIDE AND FERRIC CHLORIDE

Reaction Before Assay	μg Quinolinate/ml	Per Cent of Total
Compound I → QA	19.6	100
Compound I + $AlCl_3$	8.55	43.7
Compound I + $FeCl_3$	11.6	59.2
QA + $FeCl_3$	19.6	100

with the metal ions was not converted to quinolinic acid. The ions did not catalyze ring closure to quinolinate nor did they decarboxylate compound I or quinolinate to give niacin. Either the ions chelated with compound I preventing ring closure or they prevented quinolinic acid from being assayed as niacin. A control in which ferric chloride was added to a known amount of quinolinic acid resulted in a nearly quantitative assay for niacin after decarboxylation of quinolinate. Therefore, the ferric chloride did not prevent decarboxylation or inhibit bacterial growth in the study. It was thus concluded that a derivative or chelate of compound I was formed with the ions.

A paper chromatogram of the product of the metal-compound I reaction was prepared. One μmole of tritium-labeled 3OHAA (700 mμc) was oxidized to compound I and the alcoholic solution was immediately treated with aluminum chloride. The solution was taken to dryness *in vacuo*, applied to a paper strip and developed in the butanol: pyridine: water solvent. Three radioactive spots were found at Rf's of 0.1, 0.41, and 0.51. The areas under the curves were approximately one-fourth, one-fourth and one-half of the total radioactivity, respectively. Studies are presently being made to determine the nature of the derivative. It is possible that the complex will facilitate the formation of other derivatives of the aldehyde or amino groups.

c. Derivatives of Compound I Produced in a Purified System

When a more purified enzyme preparation was obtained in later studies, derivatives of the aldehyde and amino group of compound I were attempted assuming that there would be less interference from

extraneous substances. Four µmole of 3OHAA was oxidized to compound I using a minimum amount of enzyme. A total of 2.5 mg of protein was needed for complete oxidation, but most of this was removed by the addition of the ethanol. This was approximately one-twentieth of the protein used in the experiments previously described. The rate of disappearance of compound I was followed spectrophotometrically. The carbonyl reagents thiosemicarbazide (TSC) and semicarbazide (SC) and the amine reagent picrolonic acid (POA) were added to an aliquot of compound I in a 40:1 molar ratio of reagent to substrate at pH 7.0. After 5 minutes nearly all of the absorbance at 360 mµ had disappeared in the thiosemicarbazide and semicarbazide reactions indicating a complete disappearance of compound I. The optical density of the picrolonic acid-treated reaction approached infinity because of the absorbance of the reagent itself at this wave length.

Radioisotopic tracers were used to study these products and to verify the formation of derivatives. Carboxyl-C^{14} 3OHAA was used to prepare compound I. If compound II or picolinic acid were formed in the reaction, the labeled carboxyl group would be lost, and no interfering degradation products would be detected. Only quinolinate and compound I derivatives would be likely to be detected by radioactivity on a chromatogram. Four µmole (600 mµc) of carboxyl-C^{14} compound I was prepared as before and divided into four parts. All reagents were added in the molar ratio of 10:1. Solvents were evaporated _in vacuo_, and the residue was chromatographed in butanol: pyridine: water and counted. In Table XI are recorded the results of this study. Three very distinct radioactive areas on the chromatogram resulted

TABLE XI

CHROMATOGRAMS OF DERIVATIVES FORMED FROM

γ-CARBOXYL-C14 COMPOUND I

Reactant	Rf Values of Radioactivity Butanol:pyridine:water
Compound I QA	0.29
TSC + Compound I	0.29, 0.68, 0.85
SC + Compound I	0.42
POA + Compound I	0.48, 0.84

when compound I was treated with thiosemicarbazide. Semicarbazide caused considerable smearing of the radioactivity. In both cases the major product was quinolinic acid (Rf= 0.29), but it was shown that compound I reacted with these carbonyl reagents to give a product(s) which retained the labeled carboxyl carbon. Treatment with picrolonic acid resulted in two radioactive areas, but it is not known whether these are derivatives of quinolinic acid or compound I.

d. Other Attempts to Prepare Derivatives

The spectrophotometric detection of reactions with other reagents was attempted. The neutral reagents listed below were added to alcoholic solutions of compound I prepared by oxidation with an acetone powder extract, and aliquots were removed and diluted with water for readings. These preparations were compared with the same amount of untreated compound I and the reagent alone as controls. Pyridoxamine, pyridoxal, acetaldehyde, Tollens reagent and maleic anhydride failed to change the absorption maximum of compound I.

C. Metabolism of Compound I.

The observations of Gholson et al. (72, 73) that tritium-labeled 3OHAA gave rise to glutarate and acetate *in vivo* led to the postulation that the main route of tryptophan degradation is *via* 3OHAA to compound I. The two known products of compound I degradation *in vitro*, quinolinic and picolinic acids, have been shown to be minor products *in vivo* (35), and neither is metabolized to an appreciable extent in the whole animal (17, 34, 35). For these reasons it was believed that some other degradation product of compound I might be formed in the whole animal. The degradation of compound I *in vitro* and *in vivo* was investigated in an attempt to find an aliphatic route of metabolism.

1. Methods

Isolation and measurement of radioactive glutaric acid was done by the method of Gholson et al. (73). The Packard Tricarb liquid scintillation counter was used for analysis of the radioactivity.

Quinolinic acid was assayed microbiologically as nicotinic acid by the procedure described under Methods.

Oxygen uptake was measured with the Warburg respirometer. The various enzyme preparations were pre-incubated as described below for 10 minutes during which time the endogenous rates were determined. One or two μmoles of substrate were added from the side arm, and readings were taken at suitable time intervals. Twenty per cent potassium hydroxide was used in the center well with a filter paper wick to absorb the CO_2 liberated during incubation.

Pre-gassing with oxygen was not necessary, but rapid shaking during the reaction was important to provide sufficient exposure to the oxygen for this amount of substrate.

Disappearance of compound I was followed by spectrophotometric measurement of the rate of decrease in the absorption at 360 mμ. Aliquots of the reaction mixture for determination were removed at 3-minute intervals and diluted to 3 ml in cuvettes with a one cm light path before measurement in the Beckman D.U. spectrophotometer. A control preparation of compound I produced in a reaction catalyzed by a rat liver acetone powder extract was used to determine the spontaneous decay rate of the intermediate. A unit of activity of the enzyme which metabolizes compound I was defined as that amount necessary to produce an optical density decrease of 0.001 per minute greater than the spontaneous rate of decay.

2. Preliminary Observations

Three isotope dilution studies were made to determine if small amounts of glutaric acid could be formed from 3OHAA *in vitro*.

(1) Rat liver slices were prepared with a Stadie-Riggs microtome. Five hundred mg of tissue 5 mm in thickness was incubated in 4 ml of Krebs-Ringer phosphate buffer, pH 7.1. Three μc (700 μg) of tritium-labeled 3OHAA was added, and the mixture shaken for 1 hour on a Dubnoff metabolic incubator at 37°C and 140 cycles per minute. The contents were homogenized with a Potter-Elvehjem homogenizer, and the proteins were precipitated with four volumes of ethanol. One hundred mg of glutaric acid was added as carrier, the ethanol was evaporated *in vacuo* and the residue was passed through

a Dowex 50- (H) column 2 x 20 cm to remove amino acids, proteins and quinolinic and picolinic acids. The eluant was concentrated to 5 ml _in vacuo_, acidified and chromatographed on silicic acid (73). The glutaric acid was detected by titration with 0.07 N sodium hydroxide and was assayed for radioactivity.

(2) Three μc of tritium-labeled 3OHAA was incubated with liver slices in the presence of 0.3 μmoles of ferrous sulfate, 1 μmole of DPN, 60 μmoles ATP and 20 mg of glutaric acid. The latter component was added to inhibit metabolism of labeled glutarate. After 1 hour of incubation the reaction was treated as above, and the glutarate was isolated and counted.

(3) Three μc of tritium-labeled substrate was incubated with 500 mg of kidney tissue as slices and 0.3 μmole ferrous sulfate, 1 μmole DPN, 60 μmoles of ATP and 20 mg of glutarate. The incubation was allowed to proceed for 1 hour, then treated as above; the glutarate was isolated and counted. None of the glutarate was found to be labeled in any experiment.

3. Metabolism of Compound I _In Vivo_

An attempt was made to observe the conversion of compound I to glutarate in the intact rat. If this intermediate were on the pathway from 3OHAA to glutarate, it might be possible to detect the reaction by the metabolite overloading technique. The major difficulty was to obtain enough isotopic compound I to effectively label the glutarate but in a small enough volume to be injected into a rat. This was overcome when 3OHAA oxidase was prepared in concentrated form (Section A). Ten μmoles (7.0 μc) of tritium-labeled

3OHAA was incubated with 1.5 ml of enzyme purified through the ammonium sulfate-fractionated step. The enzyme was preincubated 5 minutes with 0.3 μmole of ferrous sulfate and 3 μmole of glutathione before addition of substrate. The final solution of 4.0 ml was stirred rapidly during the 3 minutes necessary for the disappearance of 3OHAA. An aliquot of the mixture was diluted with water, and the absorbance at 360 mμ was measured. The yield of compound I was 88 per cent of the theoretical amount. The entire mixture containing 8 mg of protein was injected intraperitoneally into a 200 g. rat. Carrier glutarate (300 mg) was injected immediately. The rat urine was collected over a 24-hour period and purified. The glutarate was isolated, counted and found to be unlabeled.

4. Compound I Metabolism *In Vitro*

a. Measurement of Compound I Metabolism by Oxygen Uptake

The oxidation of 3OHAA to compound I requires one mole of oxygen per mole of substrate. It seemed feasible that a system for the oxidation of compound I or one of its aliphatic degradation products could be detected by observing oxygen uptake in addition to that required for 3OHAA oxidation. Table XII contains typical results obtained in a series of Warburg respirometric studies. Two μmole of substrate was used in all experiments. Liver slices weighing approximately 300 mg and 5 mm in thickness were prepared with a Stadie-Riggs microtome. Five-tenths ml of a 10 per cent homogenate in Krebs-Ringer phosphate buffer, pH 7.0 was used when called for in the reaction. Red blood cells were prepared from rat blood obtained by heart puncture; blood was withdrawn in a syringe containing oxalate and centrifuged.

TABLE XII

OXYGEN UPTAKE UPON THE OXIDATION OF 3OHAA CATALYZED BY SEVERAL PREPARATIONS

Tissue Preparations and Additions	μmole O_2 per μmole substrate
Acetone powder extract	0.93
Acetone powder extract + mitochondria	0.91
Acetone powder extract + mitochondria + ATP	0.89
Acetone powder extract + Red Blood Cells	1.15
Homogenate + ATP, FAD, DPN	1.02
Liver slices	2.35

The supernatant was decanted, and the cells were washed once in physiological saline. The centrifuged, packed cells were suspended in an equal volume of saline and 0.25 ml was added to the Warburg vessels. Liver mitochondria were prepared in 0.25 M sucrose, 0.002 M Tris buffer and 0.003 M versene by the method of Schneider (89). When red blood cells and mitochondria were used, 0.5 ml of a 10 per cent acetone powder of liver was added as a source of the oxidase.

All results except those with the slices were essentially the same. Nearly one mole of oxygen was consumed per mole of substrate. Addition of cofactors affected the controls and the test reactions in the same manner so that a particular requirement could not be detected. Liver slices consistently catalyzed the uptake of greater amounts of oxygen than the theoretical values for 3OHAA oxidation, but the addition of cofactors could not be observed to increase the uptake. Variations were frequent in the assays probably because of the high amount of endogenous substrates in the crude preparations.

b. Spectrophotometric Detection of the Metabolism of Compound I.

It was decided that a more accurate measurement of compound I metabolism could be obtained by measuring the rate of disappearance of the absorption maximum at 360 mμ. Ten per cent fresh rat liver and kidney homogenates were prepared in 0.9 per cent potassium chloride solution with the Potter -Elvehjem homogenizer. Compound I was produced by incubating 2 μmoles of 3OHAA with 0.5 ml of the acetone powder extract and Krebs-Ringer buffer in a total volume

of 3.0 ml. When the substrate had disappeared as determined by fluorometric assay, 0.1-ml aliquots from the incubation were added to three cuvettes, one with water, a second with 0.05 ml of liver homogenate and the third with 0.05 ml of kidney homogenate. Figure 21 shows that the rate of decomposition of compound I is the same with or without the liver homogenate. The compound I incubated with the kidney homogenate, however, disappeared considerably faster. It was assumed this was an enzymatic degradation because of the heat-labile nature of the catalyst. When a portion of the kidney homogenate was heated nearly to boiling for one minute all activity was lost.

One-tenth μmole of each of the following components was added to individual incubation mixtures before addition of compound I to determine whether the newly-found enzyme required a cofactor and, therefore, to define the type of reaction: flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), diphosphopyridine nucleotide, (DPN) triphosphopyridine nucleotide (TPN), thiamine pyrophosphate (TPP), pyridoxal phosphate (B_6P) and coenzyme A (Co A). Both liver and kidney homogenates were tested. It is seen from Fig. 22 and Fig. 23 that none of the cofactors had any effect with either liver or kidney tissue. The half-life of compound I in the presence of the kidney with or without the cofactors was, again, noticeably less than in the presence of the extract or liver homogenate.

c. Product of Metabolism of Compound I in Kidney

The quinolinic acid produced in reactions catalyzed by

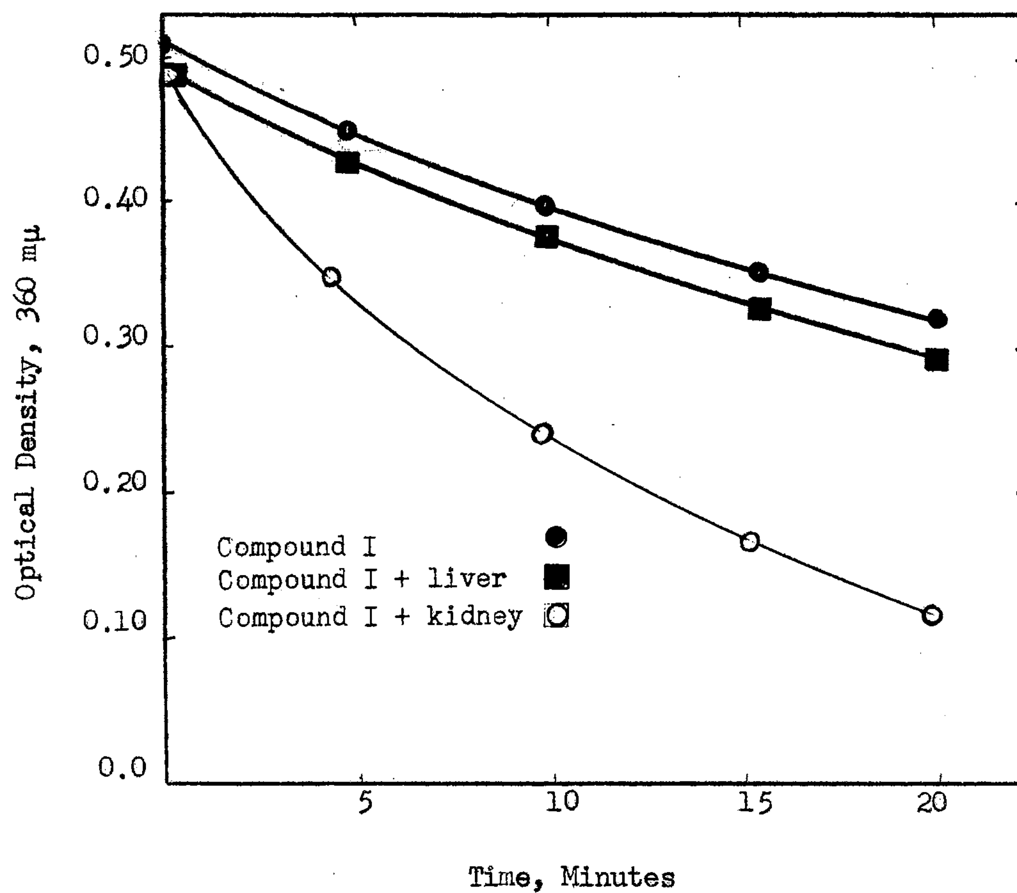
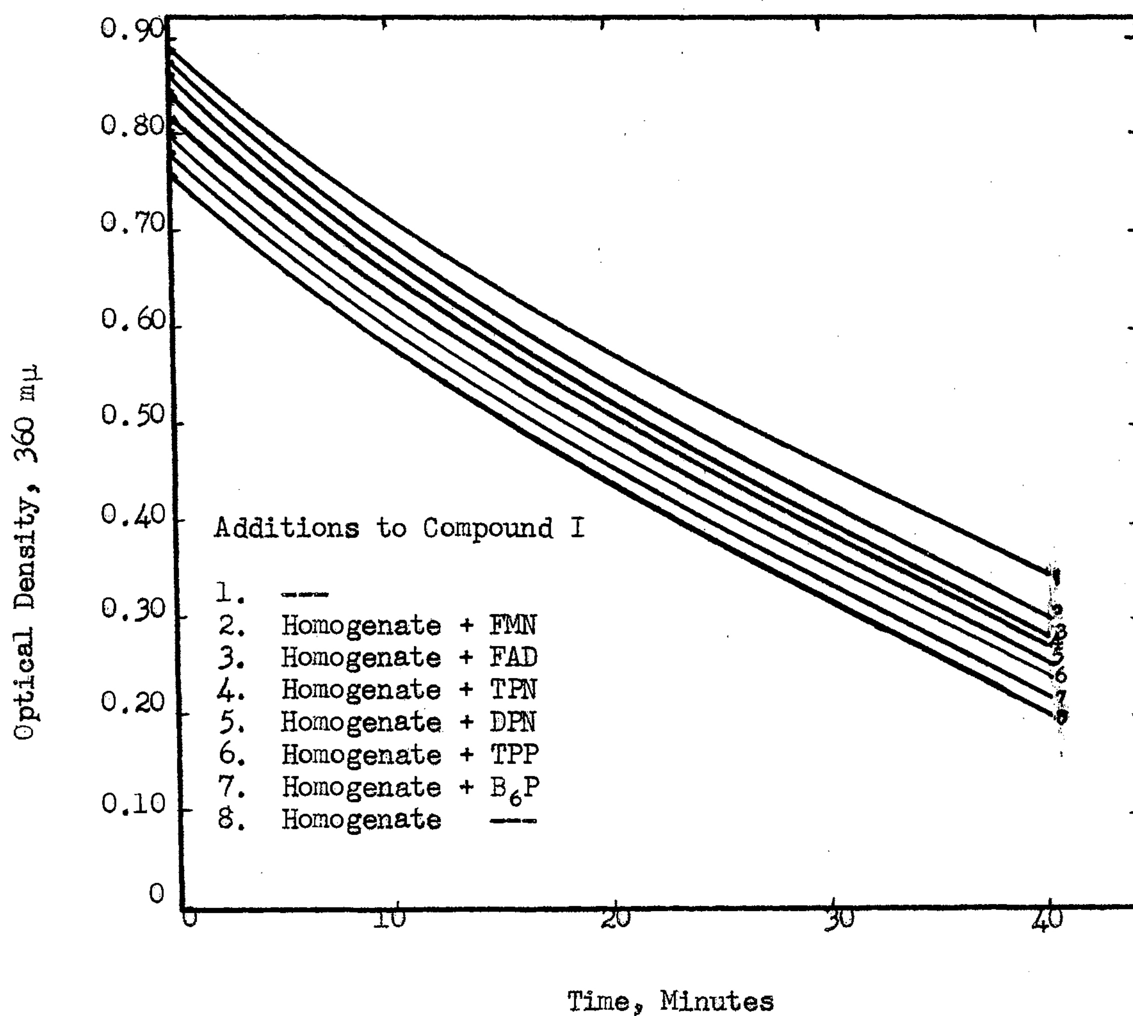


FIGURE 21

RATE OF COMPOUND I DISAPPEARANCE

WHEN

INCUBATED WITH DIFFERENT TISSUE PREPARATIONS



Time, Minutes

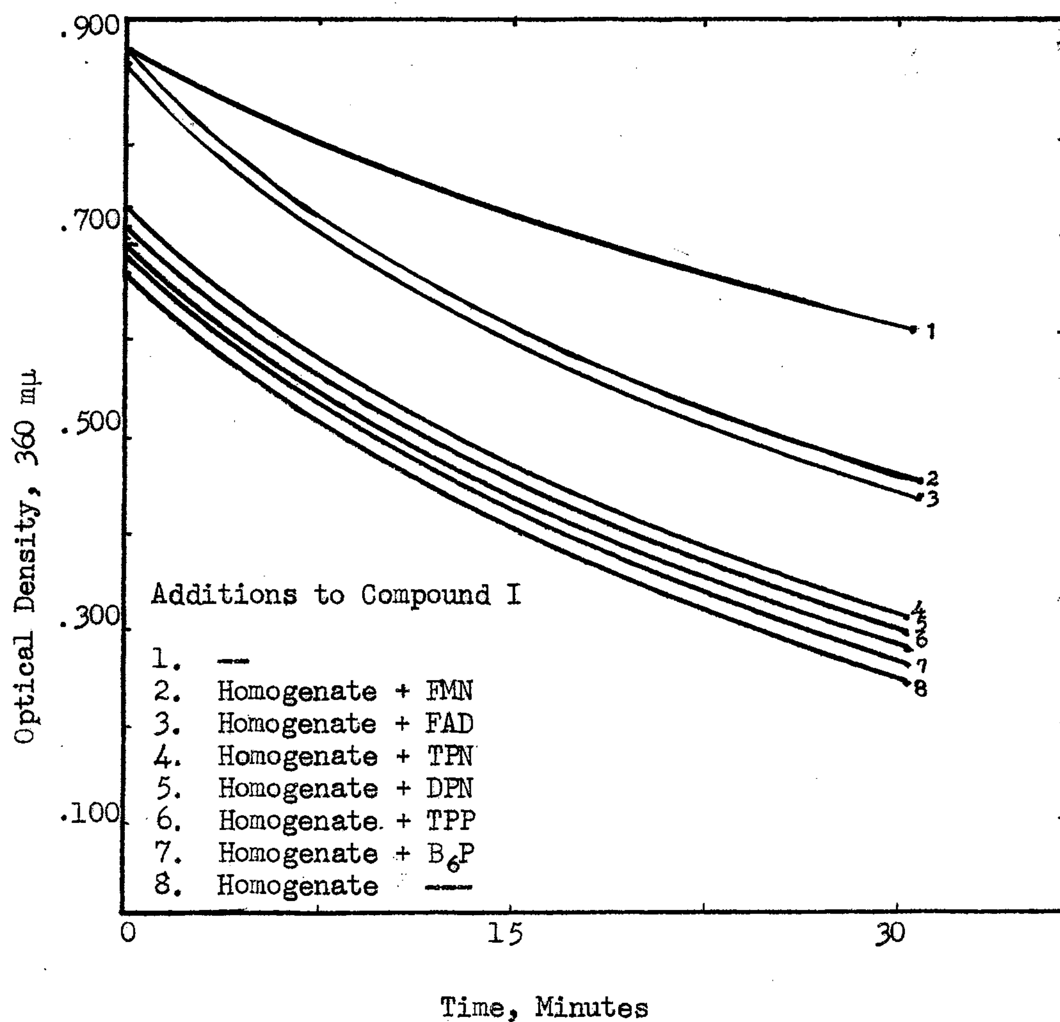
Readings were taken at 5 minute intervals.

FIGURE 22

RATE OF COMPOUND I DISAPPEARANCE

WHEN

INCUBATED WITH A LIVER HOMOGENATE AND COFACTORS



Time, Minutes

Readings were taken at 5 minute intervals.

FIGURE 23

RATE OF COMPOUND I DISAPPEARANCE

WHEN

INCUBATED WITH A KIDNEY HOMOGENATE AND COFACTORS

liver extracts and homogenates and by kidney homogenates was assayed microbiologically. The kidney as well as the liver contains 3OHAA oxidase (40, 41) so that compound I was produced in situ for the three preparations examined. Two μmole of 3OHAA was incubated with 0.5 ml of 10 per cent preparations in 3 ml of Krebs-Ringer buffer for one hour. The preparations from the acetone powder extract, liver homogenate and kidney homogenate contained 89, 78 and 32 per cent of the theoretical amount of quinolinic acid, respectively (Table XIII). These data confirmed the presence of an enzyme in the kidney which degraded compound I to a product or intermediate other than quinolinic acid or nicotinic acid.

The enzyme of the kidney preparation metabolizing compound I was found in the 21,000 xg supernatant of the homogenate and was sensitive to cyanide at 10^{-3} M. An isotope dilution study was made to determine if the product of this reaction were picolinic acid. Tritium-labeled 3OHAA was converted to compound I using a liver acetone powder extract. The incubation mixture was divided into two portions; 0.5 ml of the kidney supernatant was added to one part. After one hour of incubation the reactions were treated with four volumes of absolute ethanol. An aliquot was removed from each for microbiological assay. One hundred mg of quinolinic, picolinic and nicotinic acids were added as carriers, reisolated, purified and assayed for radioactivity as described in section B. The results of this study are contained in Table XIV. No nicotinate was found. A substantial amount of the isotope in the kidney-treated preparation was isolated as picolinic acid. Much of the

TABLE XIII

MICROBIOASSAY FOR QUINOLINIC (NICOTINIC) ACID PRODUCED

IN THREE ENZYME PREPARATIONS

Preparation	Per cent Quinolinic Acid (of theoretical)
Rat liver acetone powder Extract (10 per cent)	89
Liver homogenate (10 per cent)	78
Kidney homogenate (10 per cent)	32

TABLE XIV

ISOTOPE DILUTION STUDY OF THE AMOUNT OF PYRIDINECARBOXYLIC ACIDS PRODUCED IN A RAT LIVER ACETONE POWDER EXTRACT AND A KIDNEY HOMOGENATE

	Acetone Powder Ext.		Kidney Homogenate	
Per cent of Substrate Appearing as:	Isotope	M.B.A.	Isotope	M.B.A.
Quinolinic Acid	31.0	87.0	15.0	39.0
Picolinic Acid	2.3	[3]5.8	21.0	[3]53.0
Nicotinic Acid	0	----	0	----
Product Accounted for	33.3	92.8	36.0	92.0

[3]Correction for loss of isotope based on comparison of isotope in quinolinic acid *vs.* growth activity of decarboxylated quinolinate = 2.5.

tritium was unaccounted for, but this was probably lost by exchange. It is notable that, though the isotope dilution method indicated only 31 per cent quinolinic acid, microbioassay showed that there was 87 per cent of the acid produced in the liver-catalyzed reaction. Similarly, the microbioassay demonstrated that the reaction incubated with the kidney supernatant contained two and one-half times more quinolinic acid as found by isotope dilution. If the value obtained by isotope dilution for picolinic acid is increased by two and one-half times to correct for the exchange of tritium, there would be a yield of 53 per cent picolinic acid in the kidney incubation, and 92 per cent of the product would be accounted for. It is possible that the major product of compound I degradation in the kidney preparation is picolinic acid.

d. Metabolism of Compound I as Determined by Microbiological Assay for Quinolinic Acid.

It was apparent from the previous observations that a more sensitive method for the identification of an enzyme system that degrades compound I was necessary because of the high endogenous rates obtained in the Warburg studies, the isotope loss in tracer studies and the slight or no change in rate of compound I degradation determined spectrophotometrically. A final difference in the quantity of quinolinic acid between a control and a test reaction with a cofactor should be easier to detect than the change in the rate of formation of this product at this early stage of investigation. The amount of quinolinic acid produced in a reaction catalyzed by a rat liver acetone powder extract or rat liver homogenate was taken

as standard; a decrease in quinolinate produced in an incubation with a cofactor was taken as indication of enzyme activity. Table XV summarizes the results of three different experiments. Twenty per cent rat liver homogenates were incubated with 2 μmoles of 3OHAA in Krebs-Ringer buffer. Endogenous niacin activity was determined in an aliquot of the reaction mixture removed before addition of substrate. The cofactors shown were added to different reaction mixtures prior to substrate addition. After incubation with shaking at 140 cycles per minute for 2 hours at 37°C, the reactions were stopped by addition of four volumes of ethanol. An aliquot from each and aliquots from the controls were decarboxylated to niacin for microbiological assay by autoclaving with glacial acetic acid. Column 2 of Table XV shows the average of the data of two experiments designed to determine the result of combining the cofactors most effective in reducing the quinolinic acid yield in the study reported in column 1. When FMN and DPN were added separately there were 19 and 13 per cent respectively less quinolinate produced compared to control flasks. When the cofactors were combined in an incubation there was 35 per cent less quinolinate than in the control homogenate showing a complementary effect. Experiments are presently being done to confirm this observation.

TABLE XV

EFFECT OF VARIOUS COFACTORS ON THE AMOUNT OF QUINOLINIC ACID PRODUCED WITH LIVER HOMOGENATES, AS DETERMINED BY MICROBIOLOGICAL ASSAY

Incubation Mixture	Cofactor Concentration, mM	Experiment 1 Per cent Quinolinate	Experiment 2 Per cent Quinolinate
Acetone Powder Extract	---	82	---
Homogenate	---	76.5	75.0
Homogenate + DPN	10^{-3}	66.0	66.5
Homogenate + TPN	10^{-3}	77.0	---
Homogenate + FAD	10^{-3}	67.0	---
Homogenate + FMN	10^{-3}	61.0	58.0
Homogenate + B_6P	10^{-3}	73.7	---
Homogenate +FMN + DPN	10^{-3}	---	49.0

IV. DISCUSSION

A. 3-Hydroxyanthranilic Acid Oxidase

The study of the purification of 3OHAA oxidase has uncovered several unusual characteristics. Despite earlier reports to the contrary, the enzyme is very stable. It can be heated to 55°C for five minutes. It can be stored for two months with no loss in activity. It is not only stable to acid and base treatment for short periods of time but is activated by these treatments. The presence of an ionic environment also causes a stimulation in activity. Ferrous ions are required for maximum activity, and glutathione will restore and maintain activity in aged preparations provided the enzyme is preincubated with ferrous ions before assay.

Two basic questions are apparent: (1) what is the mechanism of action of the activators, (2) what are the functional groups of the so-called "active site"? Some of the observations made suggest answers to these questions.

Each activator could affect the enzyme in a different way and in sequence resulting in several different forms of the enzyme. However, it has not been established for certain that any two of these activators do not have the same function. There is evidence for a preferred sequence of addition. It seems likely that the ionic environment affects the enzyme before iron is added.

When the enzyme has been purified and activated with acid and base the ionic effect of addition of ammonium sulfate is not always observed. The acid or base activation is therefore probably related in some way to the salt activation. The primary difference between ammonium sulfate and acid activation is that the acid has a more permanent, holding effect; after neutralization the enzyme is still activated. Ammonium sulfate must be added to the enzyme 5 minutes before assay to obtain maximum activity.

Treatment with base not only activates the crude enzyme but also restores activity that has been lost through aging of the acid-treated or ammonium sulfate fractions of the enzyme. It would seem that acid and base are functioning in a different manner. The alkali could restore some lost configuration (or negativity) to the inactive enzyme.

After preparation of several batches of the enzyme it became obvious that the amount of oxidase activity in homogenates differed widely from one preparation to another. In all cases treatment with acid produced a marked stimulation, and the activity per gram of tissue was of the same order in each preparation. If the crude enzyme were "masked" in some manner by intramolecular folding by a peptide or as a pro-enzyme in an intermolecular complex. then acid would cause a partial denaturation; the groups of forces could be considered as unstable to acid and would be released during treatment.

The activation by ferrous ion and glutathione are probably not occurring in the same manner as acid, base or salt

activation. No other activator will replace it (46). In all stages of purification ferrous ion is required for maximum activity. However, if substrate is added before ferrous ion preincubation, the enzyme is not activated by the ion. This suggests that the ferrous ion is a cofactor bound to the enzyme at or near the active state.

Glutathione does not activate at all times. Fresh preparations of enzyme often have as much activity without reduced glutathione addition. This is not surprising since sulfhydryl groups of proteins are likely to be slowly oxidized at pH 7.0. It is conceivable that another functional group involved in catalysis is a sulfhydryl group. The fact that 3OHAA has a protecting influence on the enzyme in the presence of pCMB indicates that the sulfhydryl group could be at the active site. It has been reported that high concentrations of glutathione (10^{-2}M) inhibit the enzyme. If some disulfide bonds were essential in maintaining the configuration of the protein this would also be expected.

All of the activators affect the "active site". However, to limit the definition of active site to two or three peptides bonds which are influenced by these activators seems incorrect. Most likely the secondary and tertiary structures of the enzyme play as much of a role as the actual binding sites in its activity and specificity.

A possible scheme to explain the activation discussed is shown in Fig. 24. An ionic environment partially exposes the active site, while acid and base affect the enzyme in a similar but more extreme manner causing it to hold its configuration.

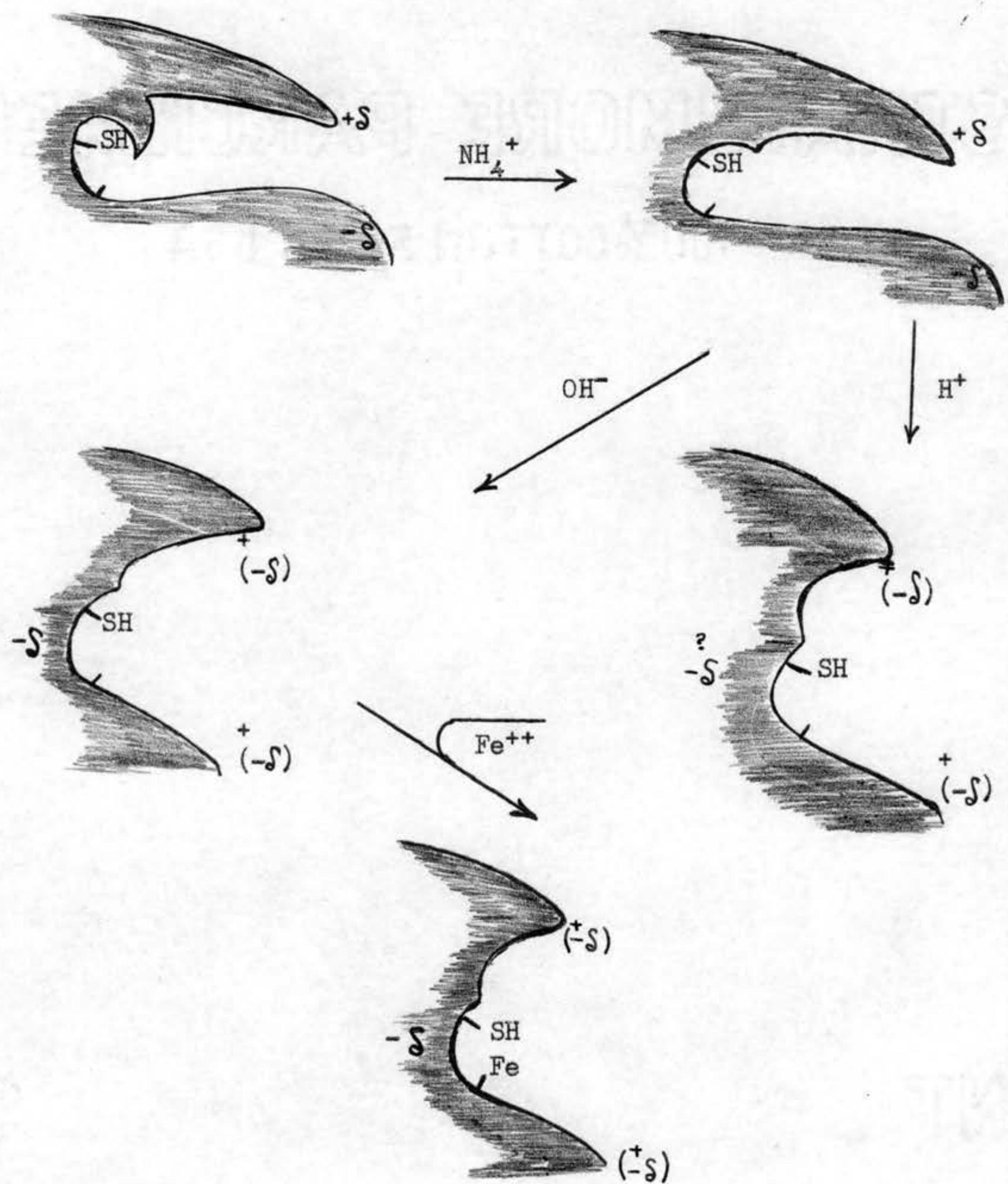


FIGURE 24

PROPOSED SCHEME OF 3OHAA OXIDASE ACTIVATION

The base also restores an internal negativity necessary for binding of iron. Ferrous ion is then bound, and the enzyme is in its most active state.

There are at least 31 forms theoretically possible when all five activating factors are considered. Many of these are active. Until more information is obtained concerning the effects of the activators these forms cannot be eliminated.

B. Structure of Compound I

Compound I is probably formed from the oxidative cleavage of 3OHAA in the 3-4 position of the benzene nucleus giving rise to the structure shown in Fig. 2. Molecular oxygen is required for this oxidation. There is no evidence to support a two-step oxidation as suggested by Miyake *et al*. (42) and Snell (65). Also, until now, little direct evidence to support the structure of compound I as shown has been presented. In order for quinolinic acid to result from 3OHAA oxidation this structure seems likely. There are several reasons why characterization of this compound has been difficult: (1) only small amounts of product could be formed very easily; that which could be produced in an enzyme-catalyzed reaction has been a minor component of the mixture as a whole, (2) the product is unstable even under the best conditions and particularly in acid, which is usually favorable for carbonyl reactions, (3) the molecule might be sterically hindered because of the unsaturation, and the close proximity of the postulated aldehyde and amino groups could prevent the formation of derivatives.

Attempts to reduce the molecule and thereby lessen the

steric effect have been shown, instead, to catalyze the condensation between the aldehyde and amine as well. It is probable that reduction of the double bonds occurs, at least in part, before condensation of the carbonyl and amino groups. If condensation occurred first quinolinic acid would be formed. This acid was found not to be reduced at pH 7.0 in 80 per cent ethanol in the conditions used for these reduction experiments. However the fact that aldehydes and amines do react under conditions of reduction is in accord with several reports (66,90).

The observations of Wiss and Bettendorf (58) that compound I reacts with carbonyl reagents are questioned here in view of the effect of acid (i.e., TCA) on the intermediate. The conditions for the precipitation of proteins in their studies were such that compound I would be converted to compound II. Long (60) reported that a deamination occurs upon treatment of compound I with acid resulting in a product which is sensitive to carbonyl reagents. Studies here and elsewhere (60) indicate that compound I is also decarboxylated by acid, perhaps making compound II less hindered.

Wiss (62) later reported that picolinic acid results from acid treatment rather than enzymatically, as shown by Mehler (33) and others (35). In an isotope tracer experiment this acid conversion to picolinic acid could not be confirmed. These studies were done using tritium-labeled substrate. It should be noted that possibly some of the isotope is lost when compound I is formed. As was observed in section 3 a microbioassay showed that there was

twice as much acid formed from compound I as the isotope data would suggest because of the exchange of tritium with the medium. This fact must be considered when quantitative determinations with this isotope are made.

There were four reagents which gave derivatives of compound I: (1) metal ions, (2) thiosemicarbazide, (3) semicarbazide and (4) picrolonic acid. The complex or chelate formed with the metal ions does not provide evidence concerning any specific functional group. It may facilitate the formation of other derivatives, however, by preventing the spontaneous decay of compound I to quinolinic acid. Semicarbazide and picrolonic acid appeared to react with compound I, but the most encouraging results were obtained with thiosemicarbazide. All reagents formed compounds which gave radioactive spots on a chromatogram different from that of quinolinic acid. Since carboxyl-labeled compound I was used these were not derivatives of compound II, a product known to react with carbonyl reagents. The results can then be interpreted to mean that true derivatives of compound I were formed under the conditions of this experiment.

C. Metabolism of Compound I

The observations of Gholson, *et al*. (72, 73) that tryptophan-7a-C^{14} and tritium-labeled 3OHAA give rise to labeled glutarate in the intact rat strongly suggest that the main route of 3OHAA metabolism is *via* compound I. The pyridinecarboxylic acids are not metabolized appreciably so that it is probable that compound I is degraded to some aliphatic product before ring closure. The fact that compound I is converted to picolinic and quinolinic acids *in vitro* indicates

that the enzymic oxidation or deamination is slow or inhibited and ring closure occurs first.

The preliminary experiments did not reveal an enzyme system which converts compound I to glutarate in vitro. The tritium-labeled intermediate did not label glutarate in vivo. Neither experiment provided substantial evidence against its role as an intermediate, however. Compound I can condense to quinolinate before further metabolism can occur, and exogenous compound I may not equilibrate with that formed endogenously.

There are undoubtably numerous enzymatic reactions between compound I and glutarate. Before products can be identified it is necessary to detect a system for the metabolism of compound I. The tissue sources for this system were the liver and kidney since both contain 3OHAA oxidase activity. Three methods of assay were employed. Oxygen uptake measurements are ineffective under the conditions employed because of the high endogenous rates observed and because it is not known that the first step is an oxidative one. Nevertheless, if oxidation were observed in the preparation of compound I it would be apparent that metabolism of compound I were occurring regardless of which reaction were oxidative.
The use of a spectrohotometric assay to measure the rate of disappearance of the absorption maximum at 360 mμ is difficult because, until the system and its requirements can be defined, the enzymatic rate will be so slight that it will not be distinguishable from the spontaneous rate or from picolinic carboxylase activity. Nevertheless, it is a quick assay that may be useful in later studies.

The best method found in these investigations was the measure of the final amount of quinolinic acid; the faster the rate of enzymatic decomposition of compound I, the less quinolinate will be found. If addition of a cofactor affects the amount of quinolinic acid formed, then the structure of a second product might be proposed based on the nature of the reaction in which the particular co-enzyme participates. The fact that diphosphopyridine nucleotide and flavin mononucleotide caused a 35 per cent decrease in quinolinate suggests that there are two reactions, an oxidation and a deamination, occurring, since the cofactors are commonly required for these reactions.

The detection of picolinic carboxylase activity in rat kidney recalls the question of its function. Picolinic acid is a minor excretory product in rat and this compound is not metabolized. One can only postulate that this enzyme produces an intermediate that is attacked by other enzymes in the aliphatic degradation to glutarate, oxidizing or deaminating it. In view of these above data, the sequence of reactions below seems quite logical:

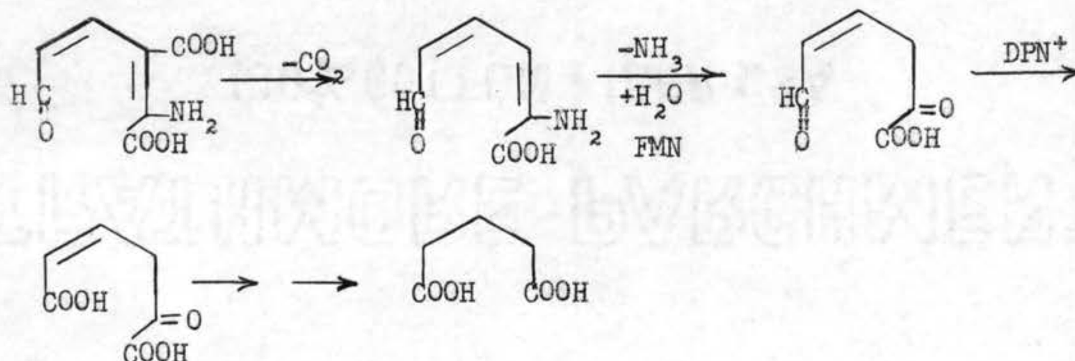


FIGURE 25

PROPOSED PATHWAY OF COMPOUND I METABOLISM

The above studies are essentially preliminary investigations to determine a system for study of the further metabolism of compound I and to obtain a suitable method of assay.

A loss of tritium has been repeatedly observed throughout the entire studies when tritium-labeled 3OHAA is converted to quinolinic acid. This loss is probably the result of an exchange of the tritium from labile positions on compound I with the medium. 3-Hydroxyanthranilic acid is labeled in three positions on the ring by the Wilzbach method,(75) but there is no evidence that uniform labeling occurs; in fact, because of the influence of the various functional groups one would not expect uniform labeling. Exchange could occur from only one position after oxidation of substrate, but still, a loss of more (or less) than one-third of the isotope might be observed. Because of this loss in radioactivity, whatever the reason may be, tritium-labeled 3OHAA cannot be used as a quantitative tool without correction being made for the loss.

V. SUMMARY

The metabolic fate of 3-hydroxyanthranilic acid was studied. The specific activity of the enzyme that catalyzes oxidation of the benzene ring was increased 2,000 fold. During fractionation of the enzyme there was approximately a 200-fold removal of protein and a 10-fold stimulation in activity by acid and base. An ionic environment, (ammonium sulfate), ferrous ions and glutathione are required for maximum activity. An ordered reaction sequence of ammonium sulfate and ferrous ions with the enzyme has been demonstrated; in this the ammonium sulfate appeared to react first.

The product of the oxidation of 3-hydroxyanthranilic acid, compound I, was shown to be decarboxylated and deaminated in the presence of trichloroacetic acid. When the purified enzyme was used to prepare compound I and when alcohol was employed for the precipitation of proteins, the intermediate was observed to react with semicarbazide, thiosemicarbazide and picrolonic acid, indicating the presence of a carbonyl and an amino group on the molecule.

An enzyme in rat kidney was identified which catalyzed the disappearance of compound I. This enzyme was identified as picolinic carboxylase by isotopic tracer studies. Addition of the cofactors diphosphopyridine nucleotide and flavin mononucleotide to rat liver homogenates caused a decrease in the final concentration

of quinolinic acid. This suggests that the cofactors are stimulating the action of other enzymes which metabolize compound I.

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