# PURIFICATION AND PROPERTIES OF 3-HYDROXY-ANTHRANILIC ACID OXIDASE

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PURIFICATION AND PROPERTIES OF 3-HYDROXY-

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# INTRODUCTION

3-Hydroxyanthranilic acid oxidase is of special interest in the study of tryptophan metabolism. The reaction involving this enzyme is believed to be one of the most important and least known steps in the metabolic scheme of tryptophan. Nutritional studies and *in vivo* isotope work have established that the major pathway of tryptophan to niacin or carbon dioxide is *via* the reaction catalyzed by this enzyme.

The *in vitro* oxidation of 3-hydroxyanthranilic acid has been studied by a number of workers. The unstable primary oxidation product, "Compound I," has been found to undergo spontaneous non-enzymatic reaction, forming quinolinic acid. However, no *in vitro* system has been found which would explain the observed *in vivo* conversion of 3-hydroxyanthranilic acid to either niacin or to an aliphatic degradation product. The present work was undertaken to provide a purified enzyme in the hope that it would facilitate *in vitro* studies of the fate of 3-hydroxyanthranilic acid, and also help elucidate the chemical structure of Compound I.

Apart from these considerations, the enzyme is of special interest since it is a member of a new class of metalloenzymes which catalyze the oxidative rupture of the aromatic rings of certain phenolic compounds at the carbon bond adjacent to the phenolic group. Little is known about this interesting reaction, and so far the chemistry of ring-splitting reactions has been studied using crude enzyme preparations.

# CHAPTER I

## REVIEW OF LITERATURE

### A. 3-Hydroxyanthranilic Acid

#### 1. Discovery and Properties

Bonner and Beadle (1) observed that a substance accumulated in Neurospora strain 4540 as a product of tryptophan metabolism and found that the compound had a niacin-replacing activity for certain strains of Neurospora. This substance was identified as 3-hydroxyanthranilic acid by Bonner (2) and by Mitchell and Nyc (3) who also synthesized the compound (4). It also has niacin-replacing activity in rats (5). 3-Hydroxyanthranilic acid fluoresces under ultra-violet light (6, 7), and has absorption maxima at 298 mμ at pH 1 (3) and at 317 mμ at pH 7.4 (8). The compound has been reported to have carcinogenic properties in rats (9).

#### 2. Metabolism

3-Hydroxyanthranilic acid occupies an important position in the scheme of tryptophan metabolism since it is believed to be an obligatory intermediate in the biosynthesis of niacin from tryptophan (10). Also the major pathway of oxidative degradation of tryptoplan is via 3-hydroxyanthranilic acid in many organisms and mammals (11). These pathways have been established mainly through in vivo isotope work. However, not all of the reactions have been satisfactorily demonstrated by in vitro experiments.

3-Hydroxyanthranilic acid labeled in the carboxyl position with $C^{14}$ gave rise to N'-methylnicotinamide and quinolinic acid-$C^{14}$ in the urine of rats (12, 13), and tritium atoms from 3-hydroxyanthranilic acid were incorporated into quinolinic and nicotinic acids in the developing chick embryo (14). A tryptophan-niacin relationship has also been found in Xanthomonas pruni (15), but not in Bacillus subtilis or Escherichia coli (16). Neither corn nor tobacco plants have this relationship (17). Recent experiments employing isotopes indicate that the major portion of tryptophan and 3-hydroxyanthranilic acid are oxidized via a 5-carbon dicarboxylic acid such as glutaconyl coenzyme A, then to acetoacetyl coenzyme A and acetyl coenzyme A and ultimately to carbon dioxide (11, 18).

3. The Product of 3-Hydroxyanthranilic Acid Oxidation

Spectrophotometric evidence for the formation of an intermediate in the conversion of 3-hydroxyanthranilic acid into quinolinic acid was first presented by Bokman and Schweigert, who observed the appearance of a product characterized by a strong absorption maximum at 360 mμ (8, 19). This intermediate is spontaneously converted into quinolinic acid (7), or enzymatically converted into picolinic acid (20).

Long et al., who also have reported the detection of the intermediate (7), demonstrated that two atoms of oxygen are used for each molecule of 3-hydroxyanthranilic acid and that the labeled indole nitrogen atom of tryptophan was incorporated into the pyridine ring of quinolinic acid (21). This evidence indicates that the amino nitrogen of 3-hydroxyanthranilic acid was incorporated into the pyridine ring by a process involving ring rupture between carbons 3 and 4, followed by ring closure forming the quinolinic acid.

Miyake et al. (19) and Long et al. (17) independently observed that another compound, termed Compound II, with an absorption maximum at 375 mμ in neutral solution, was formed when a solution of Compound I was acidified. This change involves the loss of ammonia (22) and carbon dioxide from the carboxyl position of 3-hydroxyanthranilic acid (23). Wiss et al. (24) have reported the formation of a 2,4-dinitrophenylhydrazine derivative of Compound I. However, this supposed derivative of Compound I has been found to be that of Compound II (25). These reactions involving Compound I are shown in Figure 1.

B. 3-Hydroxyanthranilic Acid Oxidase

1. Purification

In early studies by Henderson (26), Schweigert (27) and their coworkers, rat liver slices and homogenates were used for enzyme studies. Later Schweigert's group (6) found that acetone-dried powders of rat liver were an active source of the enzyme, and that the enzyme in an extract of acetone powder could be precipitated at a concentration of ammonium sulfate between 45 and 65 per cent saturation (19). These early attempts, however, were not too successful (7, 28), except for the four-fold purification achieved by Stevens and Henderson (29) by removing foreign proteins using heat and lead subacetate. Recently, however, Italian workers (30) reported a substantial purification which is comparable to that achieved in this laboratory (31).

Purification attempts by Mehler were hampered by inactivation of the enzyme during fractionation using acidic ammonium sulfate (28). Italian workers have used a small amount of ferrous ion and mercaptoethanol in all stages of purification to prevent inactivation. The enzyme is quite stable to heat and acid treatments but can be activated

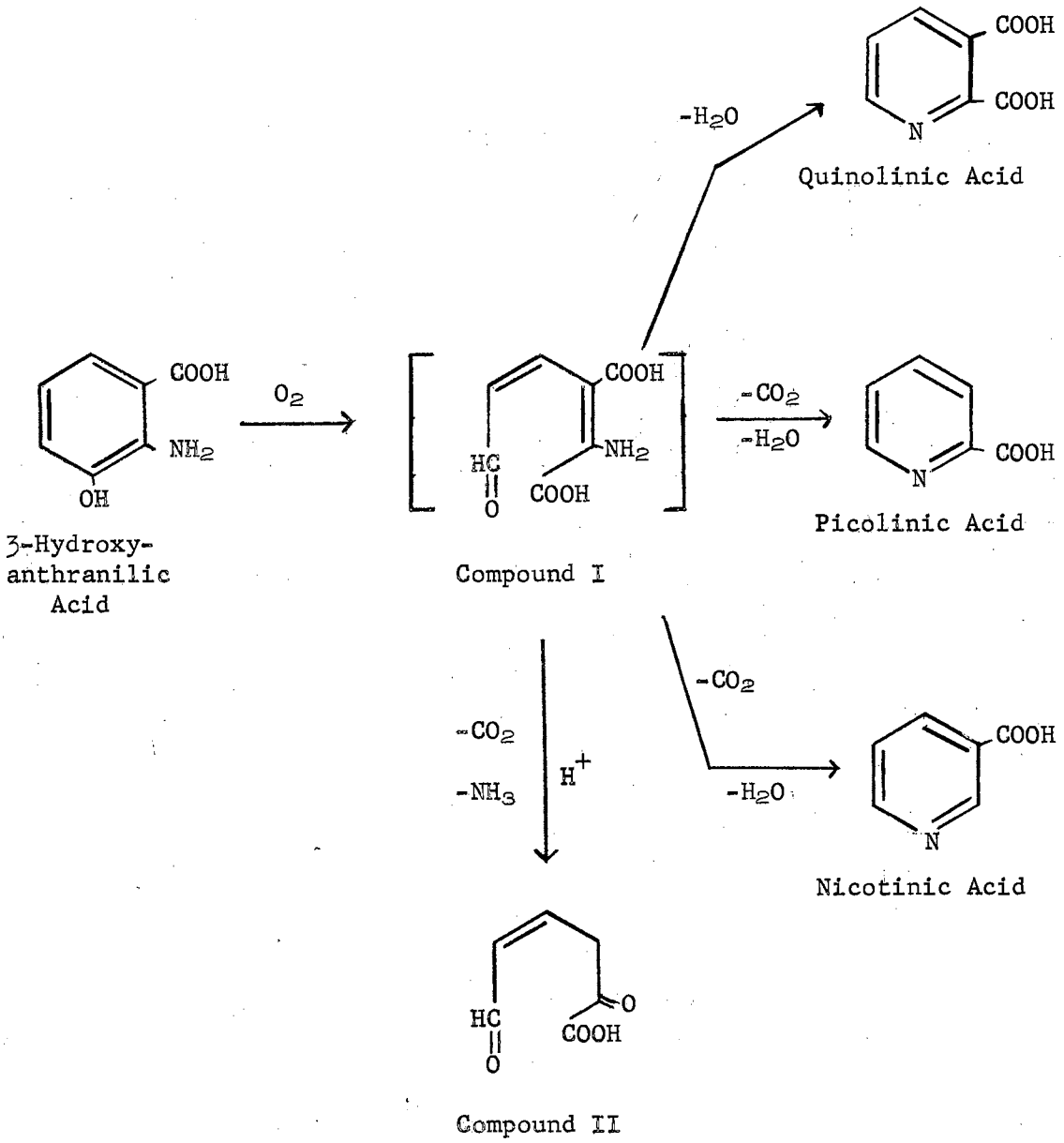



Figure 1. Reactions of Compound I

by acid, heat, neutral ammonium sulfate solution, and reduced glutathione under certain conditions. The utilization of these properties have made it possible to obtain approximately 2,000-fold increase in specific activity in this laboratory.

2. Properties of 3-Hydroxyanthranilic Acid Oxidase

a. Aromatic Ring-Splitting Oxidases: 3-Hydroxyanthranilic acid oxidase is a water-soluble enzyme found in liver and kidney of mammals. The enzyme has been classified as a "phenolytic oxygenase" by Hayaishi et al. (32) since $O^{18}$ from molecular oxygen, but not water, is incorporated into quinolinic acid.

Other members of this group of enzymes are homogentisic acid oxidase, pyrocatechase, and protocatechuic acid oxidase (33). These enzymes catalyze the oxidative cleavage of the aromatic ring in a position adjacent to an hydroxyl group. All four oxidases could be activated by ferrous ion and inhibited by metal-binding agents (33). Homogentisic acid oxidase, 3-hydroxyanthranilic acid oxidase, and protocatecuic acid oxidase were inhibited by p-chloromercuribenzoate, and inhibition was reversed by reduced glutathione. Knox found that the inhibition of homogentisic acid oxidase by p-chloromercuribenzoate was prevented by the addition of either ferrous ion or reduced glutathione (34). This observation led him to suggest that the iron was linked to the enzyme through its sulfhydryl group.

b. Activation of Enzyme: L o n g et al. found ferrous ion to be a cofactor of the enzyme ( 7). They demonstrated that only ferrous ion could restore the activity of the dialyzed enzyme, and that α-α'-dipyridyl and o-phenanthroline inhibited the enzyme reaction. Stevens and Henderson reported that the greatest activation was

achieved after the enzyme had been incubated with ferrous ion and reduced glutathione at pH 3.2 or 3.3 (29).

An insight into the mechanism of the ferrous ion activation phenomenon was provided when Decker et al. found that the presence of small amounts of any neutral salts or ammonium sulfate had considerable activating effect, and suggested that the increase in ionic strength first changed the protein configuration so that the ferrous ion binding became more effective (31, 35). Consistent with this hypothesis was the finding that the enzyme is acted on by ammonium sulfate first, then with ferrous ion.

Various workers have found that many thiol compounds activate the enzyme (19, 28, 29).

c. Inhibition of Enzyme: As in the case of many other enzymes, the inhibitory effect by sulfhydryl reagents suggests an involvement of the sulfhydryl group in an essential part of the enzyme (18, 28, 29). But it is noted that only p-chloromercuribenzoate is really effective at a low concentration (29), and that 3-hydroxyanthranilic acid has a protective effect against p-chloromercuribenzoate, a phenomena not frequently observed in other enzymes (31). As expected, the enzyme is also readily inhibited by various chelating agents. Sodium cyanide did not significantly affect activity when tested at $10^{-3}$ M (29). Decker observed that the enzyme lost activity when fractionated with saturated ammonium sulfate solution or when dialyzed against water (35).

d. Kinetic Properties: Using a four-fold purified enzyme preparation, Stevens and Henderson (29) found the Km for 3-hydroxyanthranilic acid to be $2.6 \times 10^{-5}$ M. More recently Iaccarino et al.,

using a more purified enzyme, have reported a Km value of $7 \times 10^{-6}$ M, and found that the plotting 1/s against 1/v did not give a straight line (30). The smallest concentration of 3-hydroxyanthranilic acid used was about $2.2 \times 10^{-6}$ M. However Decker, using approximately fifty-fold purified enzyme preparation, was able to obtain a linear relationship between 1/s and 1/v, and found that the Km for 3-hydroxyanthranilic acid and the apparent Km for ferrous ion were $2 \times 10^{-5}$ M (31). The range of concentrations employed for 3-hydroxyanthranilic acid was between $6.7 \times 10^{-5}$ M and $6.7 \times 10^{-6}$ M.

# CHAPTER II

# EXPERIMENTAL

## A. Materials

3-Hydroxyanthranilic acid was obtained from Hoffmann-La Roche. Reduced glutathione and anion exchange cellulose (DEAE-SF, 0.4 milliequivalents per gram) were obtained from California Corporation for Biochemical Research. Tris (2-amino-2-(hydroxymethyl)-1,3-propanediol) and p-chloromercuribenzoate were purchased from Sigma Chemical Company. Sephadex G-25 was from Pharmacia, Uppsala, Sweden. Inorganic salts of reagent grade were used. Calcium phosphate gel was prepared by the method of Keilin and Hartree (36). Glass-distilled water was used. Beef liver was purchased from a local market.

## B. Methods

### 1. Enzyme Assay

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

b. Reagents: $6 \times 10^{-3}$ M $FeSO_4$; $3 \times 10^{-3}$ M 3-hydroxyanthranilic acid, pH 7.0; 1 M tris buffer, pH 7.0; $4 \times 10^{-2}$ M HCl; enzyme preparation.

c. Procedure: 2.5 ml of glass-distilled water, 0.1 ml of the $FeSO_4$ solution, 0.03 ml of HCl solution (since the amount of the hydrochloric acid solution which gives the maximum enzyme activity depends upon protein concentration, ionic strength, and also on the

history of the enzyme, the exact amount should be determined by trying 0.02, 0.03, 0.04, 0.05 and 0.06 ml. of hydrochloric acid solution) and 0.1 ml. of the enzyme solution are mixed in a cuvette with a 1.0 cm. light path, and incubated for 3 minutes at room temperature.

Then 0.2 ml. of tris buffer is added to the cuvette, mixed, and after 30 seconds 0.1 ml. of the 3-hydroxyanthranilic acid solution is added to start the reaction. The reaction rate was determined by measuring the rate of increase in absorption at 360 mμ using the Cary 14 recording spectrophotometer.

d. Definition of Unit and Specific Activity: The rate of the reaction was determined from the linear portion of the optical density versus time curve. One unit of enzyme is that amount giving an increase in optical density of 0.001 per minute under the above conditions. Specific activity is the number of units of enzyme per mg. of protein. Protein was determined by the trichloroacetic acid method of Stadtman et al. (37) with 12 per cent of trichloroacetic acid, and also by measuring the optical density at 280 mμ.

2. Purification Procedure

The following description is that of Decker (31, 38) except for a few minor modifications.

Step 1. Homogenization: All steps were performed in cold room at 3° C unless otherwise specified. Fresh beef liver was homogenized in five volumes of glass-distilled water (weight per volume) for one minute in a Waring blender. The homogenate was filtered through two layers of cheesecloth, and centrifuged for 1 hour at 10,000 x g (5.75 inch head of a Serval SS-4 Centrifuge was used), and the supernatant

was saved. Higher speed centrifugation is recommended for more efficient removal of inert proteins when the volume is not very large.

Step 2. Heat Treatment: The supernatant solution was heated in 1000 ml. portions with stirring at 55° C for 5 minutes in a large glass beaker and then cooled in an ice bath. Denatured protein was removed by centrifuging for 30 minutes at 10,000 x g.

Step 3. Acid Treatment: The resulting supernatant solution was made 2 x $10^{-4}$ M in ferrous ion by adding solid ferrous sulfate, then was adjusted to pH 3.5 with 2 N hydrochloric acid which was added slowly with stirring. The acidified solution remained at room temperature for 30 minutes. After neutralization with 2 N sodium hydroxide, the mixture was centrifuged at 10,000 x g for 30 minutes.

Step 4. Calcium Phosphate Gel Treatment: The supernatant solution was treated with one-tenth of its volume of calcium phosphate gel (26 mg. per ml.) for 20 minutes with constant stirring. Centrifugation at 10,000 x g for 30 minutes sedimented the gel which was discarded.

Step 5. First Ammonium Sulfate Fractionation: A saturated solution of ammonium sulfate was prepared at 3° C, and concentrated ammonium hydroxide solution was added until the solution was pH 7.0 as determined by pH indicator paper. This saturated ammonium sulfate solution was added slowly with constant stirring to the enzyme-containing solution until 40 per cent saturation was reached. The solution was let stand for 20 minutes, then centrifuged at 10,000 x g for 20 minutes, and the precipitant was discarded. More saturated ammonium sulfate solution was added slowly with constant stirring until 65 per cent saturation was reached, and centrifuged as described above. The precipitate was dissolved in neutral buffer, which is 0.01 M in tris and 0.001 M in reduced

glutathione to yield a protein concentration of 8 mg. per ml.

Step 6. Second Ammonium Sulfate Fractionation: Further purification was achieved by fractionating it between 45 and 65 per cent saturation of ammonium sulfate. This step was carried out in the same manner as the first ammonium sulfate fractionation. The final precipitate was redissolved in 0.01 M, pH 7.0 tris buffer (also 0.001 M in reduced glutathione) to yield a desired protein concentration, and stored at -20° C.

3. Chromatographic Procedure

a. Desalting: The fraction from the second ammonium sulfate fraction was passed through a Sephadex column (G-25) of 4.5 x 14 cm, and eluted with distilled water. The colored protein was easily observed passing through the column and was collected.

b. DEAE-Cellulose Column: Five g. of DEAE-cellulose (dry weight) was recycled by washing with about 300 ml. of 1 N HCl solution, then with 1 N NaOH solution. The material was finally washed with distilled water until it is free from alkali, and suspended in 200 ml. of water. The fine slurry of the ion exchanger was poured on a column of 1.1 cm. in diameter, and allowed to settle down by opening the outlet. The column was again washed with 50 to 100 ml. of water. The height of the column was 28 cm.

c. Elution: The column was charged with desalted enzyme solution (100 mg. of protein in 15 ml. of water). A gradient elution was carried out from 560 ml. of 0.002 M ammonium sulfate solution to 400 ml. of 0.03 M ammonium sulfate solution (Figure 2). An initial flow rate of about 3.5 ml. per 10 minute did not vary greatly during the course of the operation. Two hundred fractions of average 3.5 ml.

per fraction were usually collected. The optical density at 280 mμ of each fraction was measured. Fractions containing light-absorbing material were assayed for enzyme activity.

# CHAPTER III

## RESULTS AND DISCUSSION

### A. Purification of 3-Hydroxyanthranilic Acid Oxidase

#### 1. Enzyme Assay

It was imperative to have a good enzyme assay method during the purification of 3-hydroxyanthranilic acid oxidase. Some of the past problems may illustrate the point. It was found that only little enzyme activity was left after ammonium sulfate fractionation, or after dialysis against water, which was necessary for the chromatographic separation. Thus it was concluded that the enzyme was too unstable and that some other way must be found to prevent the inactivation. However, the fact is that the enzyme was not denatured at all and that only the assay method detected only a small fraction of the enzyme present. As will be mentioned later, 3-hydroxyanthranilic acid oxidase must be incubated with ferrous ion at an optimum incubation pH of approximately 3.5 before starting the enzyme reaction.

The rate obtained this way represents probably the maximum enzyme activity. Obtaining the maximum reaction rate after acidic incubation is important for the same reason that the initial reaction rate should be taken in an enzyme assay.

2. Purification Steps

The enzyme was readily extracted into water during the homogenization. A 1.8-fold purification was obtained by the heat step. The precipitation of foreign proteins by the pH step resulted in a 3.2-fold purification over the previous step. The best results were obtained when a small amount of ferrous ion ($2 \times 10^{-4}$ M) was dissolved into the solution before adjusting the pH of solution to 3.5. At this low pH the enzyme becomes very sensitive, and the added ferrous ion not only activates the enzyme but also stabilizes it. It was observed that when the enzyme was not fully activated at this stage substantial amount of enzyme activity was lost during the next fractionation, the gel step which gave a 1.4-fold purification. Specific activity of about 30,000 was obtained by the first ammonium sulfate fractionation. The second salt fractionation removed much of the colored substances, and the specific activity was about 60,000, which was equivalent to approximately 50-fold increase in the specific activity over that of the homogenate.

3. Chromatography of Enzyme

Further purification was achieved by chromatography of the enzyme, and this method seems promising for future work. So far the best results were obtained using DEAE-cellulose (diethylaminoethanol exchange groups on a Solk-Floc cellulose lattice) for column material and ammonium sulfate solution as the eluant.

In one experiment, 100 mg of desalted protein (Sephadex treated) from the second ammonium sulfate fractionation was applied to a column 28 cm in height and eluted with a concave gradient from 560 ml of 0.002 M ammonium sulfate solution to 400 ml of 0.03 M ammonium sulfate solution. Two hundred fractions averaging 3.5 ml were collected. The 96th tube had

highest specific activity of 264,000 units per mg protein and contained 3.6 per cent of total enzyme activity. Fifteen tubes from the 90th to the 104th tubes contained 40 per cent of total enzyme activity with average specific activity of 190,000 (Figure 2). This was equivalent to about 3.4-fold purification over the second ammonium sulfate fractionation with 40 per cent recovery of enzyme, or about 166-fold purification over the homogenate. The summary of purification sequence is shown in Table I.

All assays for enzyme activity were performed by incubating the enzyme with ferrous sulfate at pH 3.5 for 3 minutes prior to the addition of substrate. When so activated the homogenate has about 65 times the specific activity previously reported, and therefore, the various "activations" reported in the previous scheme of purification are not demonstrated in this table.

B. Ferrous Ion and 3-Hydroxyanthranilic Acid Oxidase

1. Possible roles of ferrous ion in the enzyme reaction

As with the other three known members of the phenolytic oxidases, 3-hydroxyanthranilic acid oxidase is activated by ferrous ion. However, nothing definite is known about the mechanisms involving the ferrous ion for any of these enzymes. One speculation is that the metal ion is involved in activating the oxygen molecule. This may occur since the stable bond between the two oxygen atoms must be broken for oxygen to oxidize a compound (39). Another possibility is that the metal is acting as a binding link between the enzyme and substrates.

2. Activation of the enzyme

Although the 3-hydroxyanthranilic acid oxidase is a stable enzyme in that it is not easily denatured by heat (55° C), acid (pH 3.5), dilution, or aging, the active enzyme can be readily changed to an inactive form.

Figure 2

Chromatography of 3-hydroxyanthranilic acid oxidase on DEAE-cellulose. One hundred mg of protein (specific activity 55,500) was applied and eluted with a concave gradient from 560 ml of 0.002 M $(NH_4)_2SO_4$ to 400 ml of 0.03 M $(NH_4)_2SO_4$. The fractions contained an average of 3.5 ml. Protein was determined by absorption at 280 mμ (--------): 0.1 ml from each tube was assayed for the enzyme activity (———).

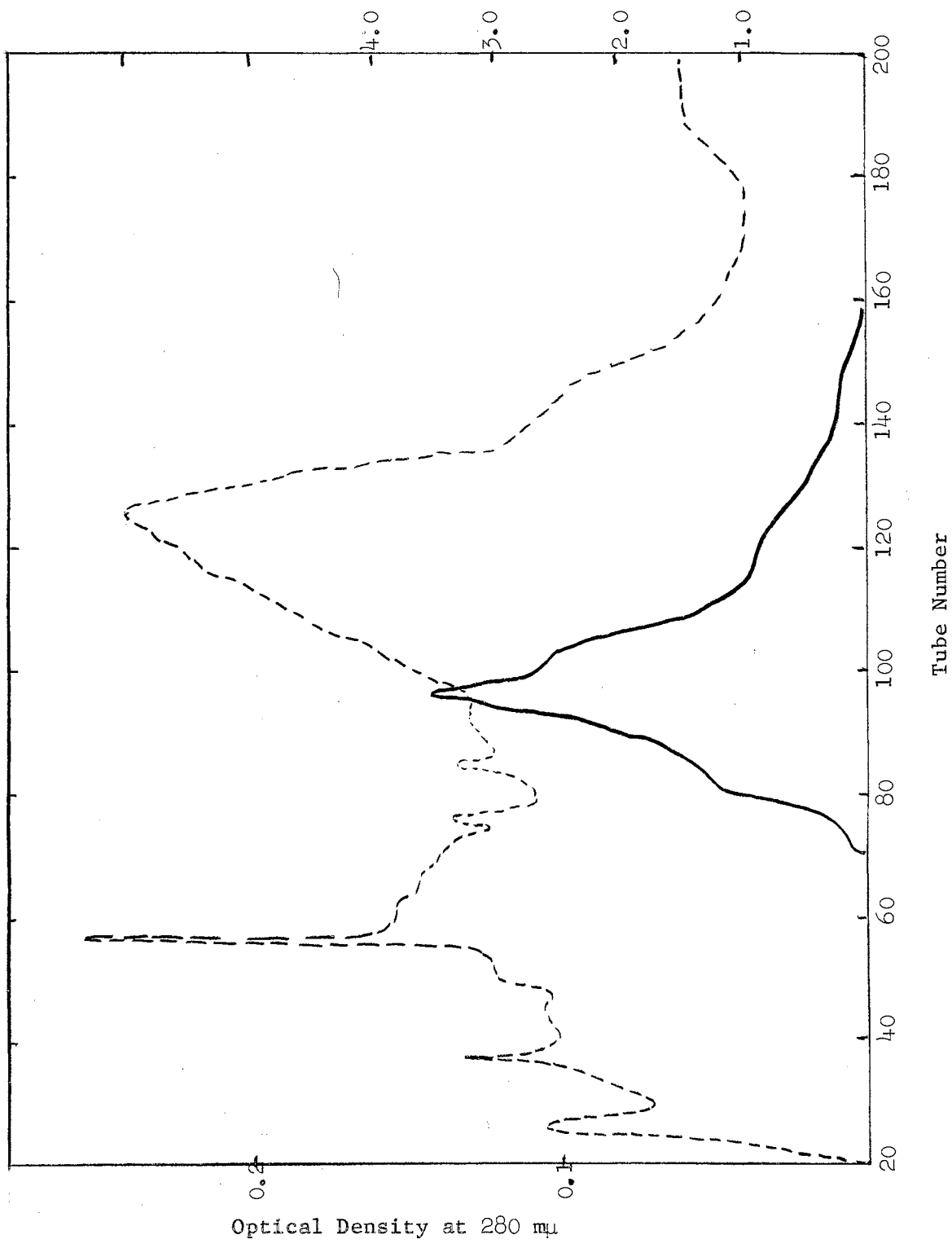

Enzyme Activity, △O.D./Minute at 360 mμ
4.0
3.0
2.0
1.0
200
180
160
140
120
100
80
60
40
20
Tube Number
0.4
0.2
Optical Density at 280 mμ

TABLE I

PURIFICATION OF 3-HYDROXYANTHRANILIC ACID OXIDASE

| | Vol. (ml) | Activity ($\times 10^4$) (units/ml) | Total Units ($\times 10^6$) | Protein (mg/ml) | Specific Activity (units/mg) | Yield (%) | Purification |
|---|---|---|---|---|---|---|---|
| Homogenate | 2,800 | 8.0 | 224 | 70 | 1,140 | 100 | 1 |
| Supernatant Solution | 2,200 | 8.0 | 176 | 35 | 2,280 | 79 | 2 |
| Heated | 2,000 | 7.8 | 156 | 19 | 4,100 | 69 | 3.6 |
| Acid Treated | 1,950 | 7.8 | 152 | 6.0 | 13,000 | 68 | 11.4 |
| Gel Treated | 2,150 | 1.4 | 137 | 4.0 | 16,000 | 67 | 14 |
| First Ammonium Sulfate PPT (40-65%) | 195 | 35 | 68 | 15 | 23,000 | 33 | 24 |
| Second Ammonium Sulfate PPT (45-65%) | 110 | 50 | 55 | 9.0 | 55,500 | 27 | 49 |
| DEAE-Cellulose* Fractionation | 45 | 0.42 | 2.2 | 0.26 | 190,000 | 11 | 166 |

*One hundred mg out of 990 mg of protein from the second ammonium sulfate fraction was used.

This change into the inactive form is probably due to the loss or oxidation of the enzyme-bound ferrous ion, and the enzyme can be reactivated by incubating it with ferrous ion under various conditions as shown in Figure 3. The best incubation conditions were pH 3.5, or 55° C, or a high ionic strength (0.033 M in ammonium sulfate at pH 7.0). The low pH incubation resulted in the highest activity. The heat-treated enzyme which lost most of its activity after 5 days at 4° C could be reactivated by repeating the heating procedure with fresh ferrous sulfate.

The dissociation of the ferrous ion from the protein does not seem likely in view of data to be discussed in the studies with chelating agents. In one experiment, the activity of an inactive enzyme preparation could be partially restored (20%) by incubating the enzyme with reduced glutathione ($2 \times 10^{-3}$ M) alone, indicating that the bound ferric ion was reduced by incubating it with ferrous ion at pH 3.5 in the absence of reduced glutathione. These facts may be explained as follows:

$$\text{Enzyme-S-Fe}^{+} \xrightarrow{\text{aging}} \text{Enzyme-S-Fe}^{++} \xrightarrow{\text{GSH}} \text{Enzyme-S-Fe}^{+} + \text{GSSG}$$

$$\text{Enzyme-S-Fe}^{++} \xrightarrow{\text{Fe}^{++},\ \text{pH } 3.5} \text{Enzyme-S-Fe}^{+} + \text{Fe}^{+++}$$

Iron may be linked to the enzyme through thiol-metal bond, as will be discussed in the studies with p-chloromercuribenzoate.

Figure 4 shows that the optimum pH of incubation with iron was about 3.5 with $2 \times 10^{-4}$ M $FeSO_4$ as described on page 9. When acetic acid was used in place of HCl to adjust the pH the observed enzyme activity was only about half of that obtained using HCl, probably due to the formation of metal-acetate complex.

Figure 5 shows the pH-curve for the enzyme reaction, where the pH of the solution was adjusted with a buffer (the final concentrations

Figure 3

Effect of pH, heat, and ammonium sulfate on the enzyme activity. The enzyme activity (0.04 ml of heat treated preparation) was determined at pH 7.0 at room temperature after 5 minutes incubation with $2 \times 10^{-4}$ M $FeSO_4$. Hydrochloric acid (0.04 N) was used to adjust the incubation pH to 3.5, and hot water-bath was employed to heat the enzyme preparation for 5 minutes. The concentration of ammonium sulfate was 0.033 M.

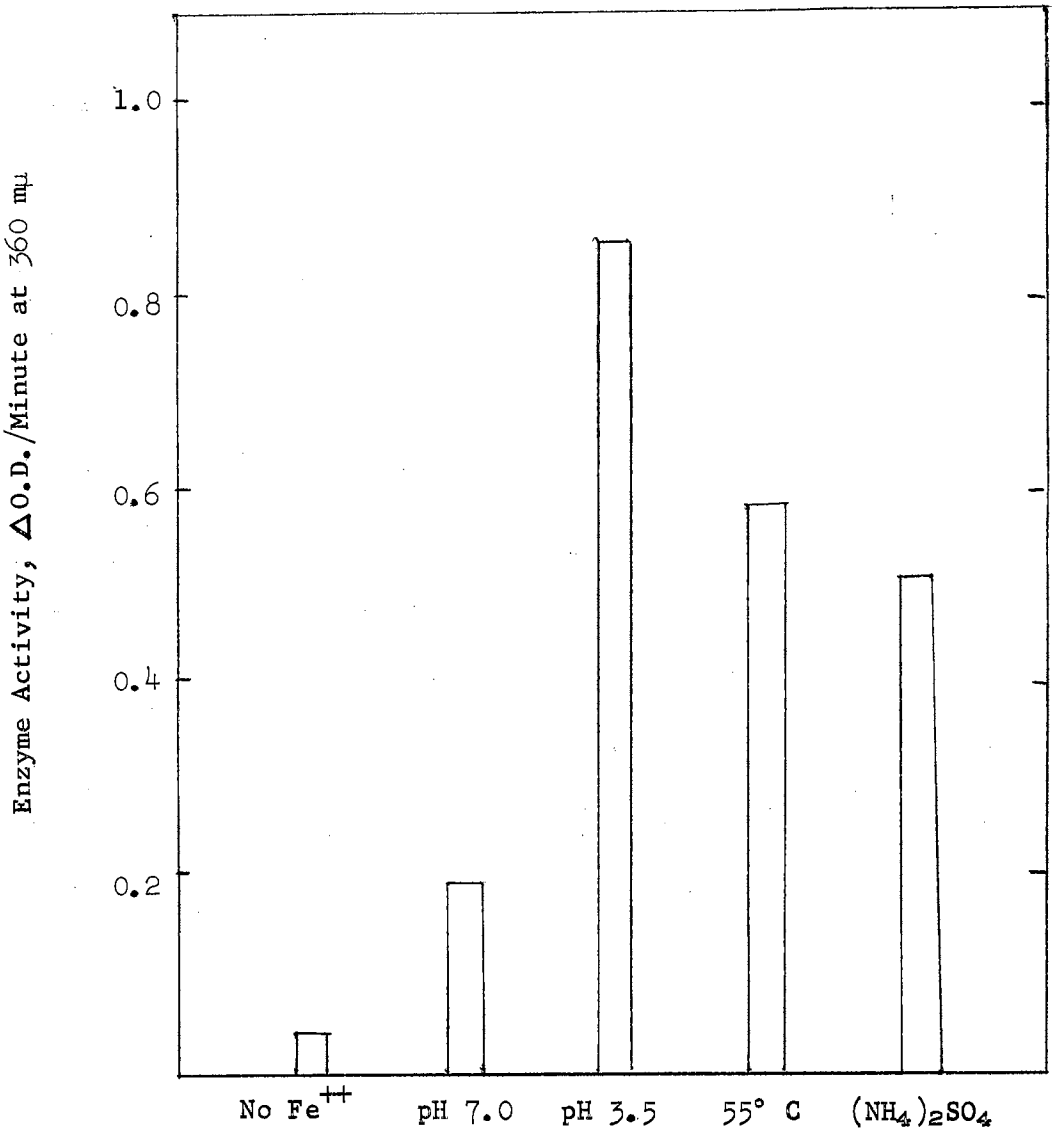

Enzyme Activity, ΔO.D./Minute at 360 mμ
1.0
0.8
0.6
0.4
0.2
No Fe++
pH 7.0
pH 3.5
55° C
(NH4)2SO4

Figure 4

Effect of pH on enzyme incubation with $2 \times 10^{-4}$ M $FeSO_4$. Aliquots of enzyme (100 μg of protein purified through the ammonium sulfate fraction step) in 2.6 ml of glass distilled water were adjusted to various pH values by adding 0.04 N HCl, and then after 5 minutes at room temperature 0.3 ml of a buffer composed of 0.1 M tris, 0.3 M $(NH_4)_2SO_4$, and 0.1 M sodium acetate was added 30 seconds before the addition of substrate. The final pH was 7.7.

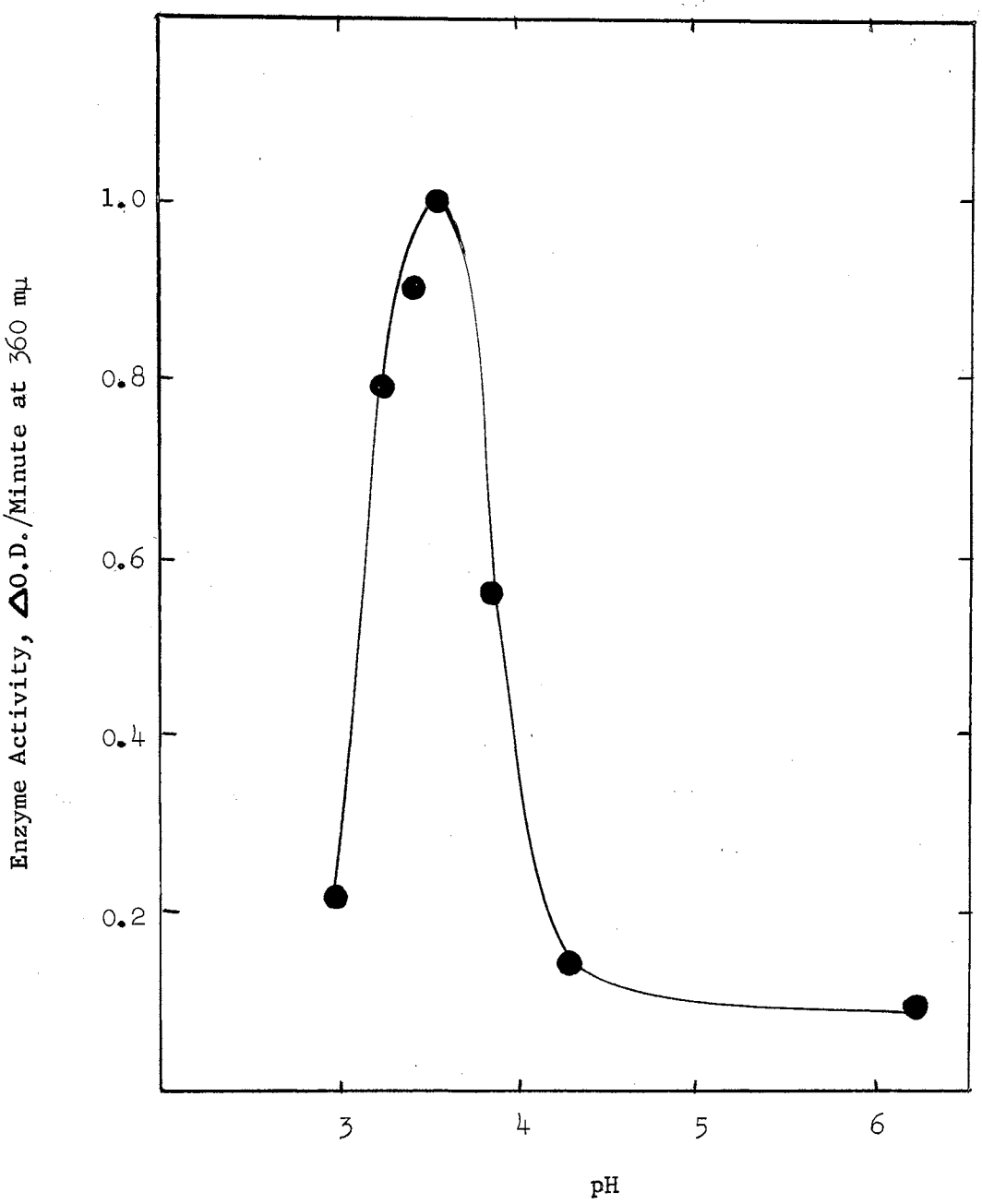
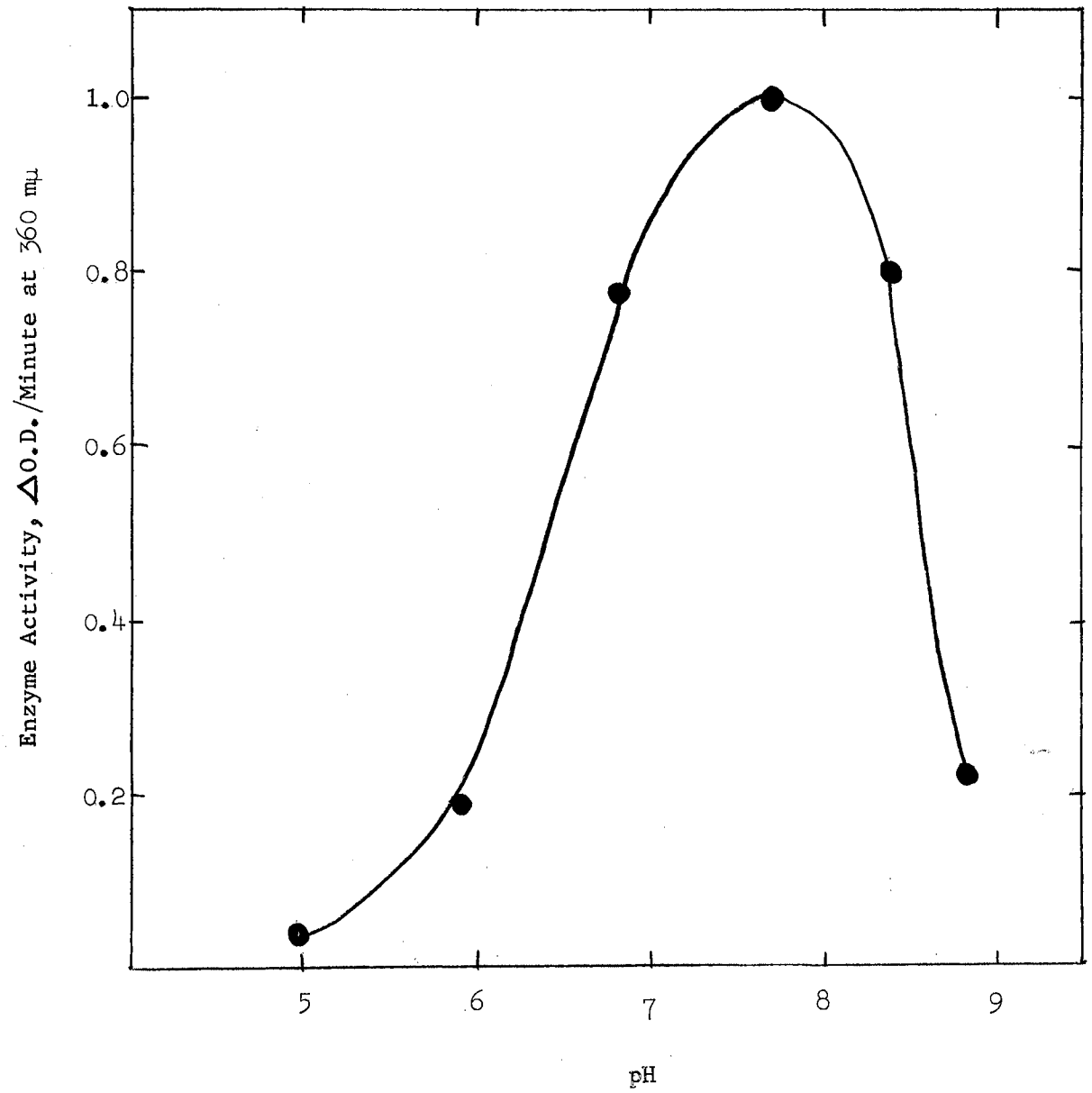

Enzyme Activity, ΔO.D./Minute at 360 mμ
1.0
0.8
0.6
0.4
0.2
3
4
5
6
pH

Figure 5

Effect of pH on 3-hydroxyanthranilic acid oxidase activity. An aliquot of the ammonium sulfate fractionated enzyme solution was incubated for 5 minutes at pH 3.6 and then adjusted to the indicated pH values with a buffer mixture composed of 0.01 M tris, 0.03 M $(NH_4)_2SO_4$ and 0.01 M sodium acetate which had previously been adjusted with HCl and NaOH as needed.


Enzyme Activity, ΔO.D./Minute at 360 mμ
1.0
0.8
0.6
0.4
0.2
5
6
7
8
9
pH

were 0.01 M in tris, 0.03 M in ammonium sulfate, 0.01 M in sodium acetate) with an optimum at pH 7.5. However, the optimum pH was about 7.0 when only tris buffer was used (0.033 M final concentration).

Figure 6 shows that the optimum pH for activation was changed from 3.5 to 4.5 as the ionic strength of the medium was increased by making it 0.033 M in ammonium sulfate. Therefore, it seems likely that both pH and the ionic environment function in a similar manner in transforming the enzyme to the active form.

3. Further studies on the enzyme activation

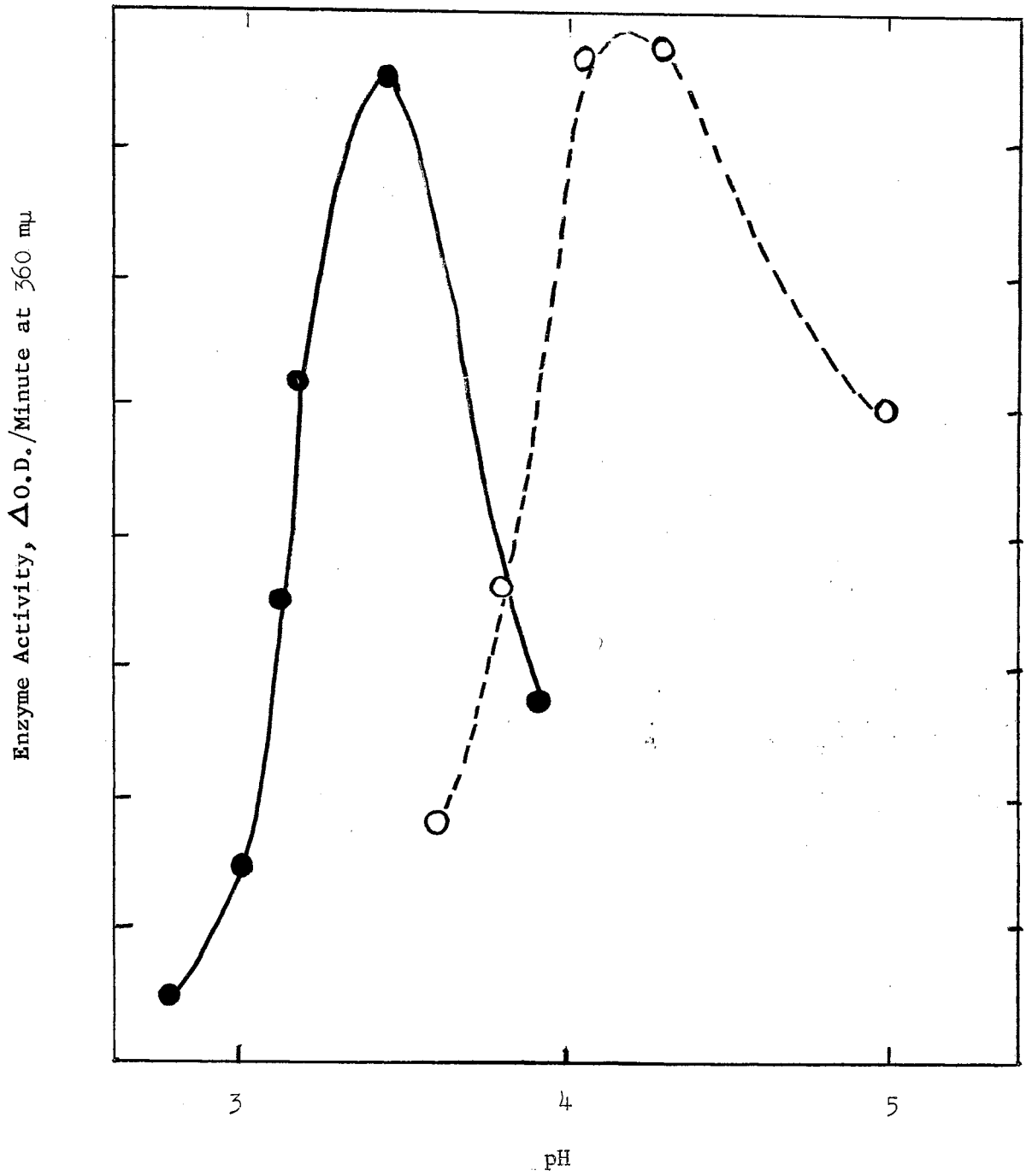
a. Kinetic studies: The observed Michaelis-Menten relationship between the enzyme and ferrous ion (40) is consistent with the view that ferrous ion combines with the enzyme in the ratio of one ferrous ion to one active center to form a metal-enzyme complex. This interpretation is valid only when it is known that equilibrium is established during the incubation of the enzyme with the ferrous ion (40). The apparent Michaelis constant for ferrous ion was found to be $2 \times 10^{-5}$ M (35), and this value is directly related to the true dissociation constant of enzyme-metal complex (41). The derivation of the relationship is done assuming that only the enzyme-metal complex is able to combine with 3-hydroxyanthranilic acid. Also the place of oxygen in the kinetic relationship is unknown. Thus the graphically-determined constant (Km) for the ferrous ion is related to its true dissociation constant $K_A$ as follows:

$$K_m = \frac{K_A}{1 + \frac{S}{K_s}}$$

where S is concentration of the substrate and $K_s$ is dissociation constant for the enzyme-metal substrate complex. Although the true dissociation

Figure 6

Shift of optimum pH of incubation with ferrous ion due to the addition of 0.033 M ammonium sulfate. An aliquot of the ammonium sulfate fractionated enzyme solution was incubated for 3 minutes at the indicated pH and then adjusted to pH 6.8 with 1 M, pH 7.0 tris buffer (●—●). When incubated with 0.033 M ammonium sulfate (O----O) the rate was measured at pH 7.7 (optimum pH) by adjusting the pH with 1 M, pH 7.8 tris.


Enzyme Activity, ΔO.D./Minute at 360 mμ
3
4
5
pH

constant for the enzyme-ferrous ion complex is larger than the graphically determined value of $2 \times 10^{5}$ M, the apparent constant is directly related to the true constant.

From Figure 7 it will be noted that although the maximum velocity of the enzyme reaction was a function of incubation pH, the Michaelis constant for the ferrous ion was not dependent on the pH of the incubation. Thus the maximum velocity after pH 3.5 incubation was 4.6 times higher than the maximum velocity obtained after pH 6.3 incubation. The Michaelis constants were $2 \times 10^{-5}$ M in both cases. Since the $V_{max}$ (maximum initial velocity) is a function of the number of active sites, and the Michaelis constant is not governed by the number of such sites, it can be concluded that the acid activation and probably as well as the heat and salt activation were the result of an increase in the number of active sites of enzyme.

The same value of Michaelis constants for ferrous ion under various incubation conditions indicate that there is only one form of active enzyme-metal complex. In contrast to this, the inactive form of the enzyme may exist in more than one form, probably in different protein configurations. Thus, the salt-activation was not as effective as acid-activation, being unable to act on more forms of inactive enzyme existing in various configurational forms.

b. Effect of chelating agents on the enzyme: Table II shows the effect of $\alpha,\alpha'$-dipyridyl on the enzyme activity after incubation at pH 7.0 and pH 3.5. Incubation of the enzyme with the chelating agent at the concentration of $1 \times 10^{-3}$ M at pH 3.5 for 3 minutes resulted in complete loss of enzyme activity while only 38 per cent of activity was lost after the incubation of the enzyme with the chelating

Figure 7

Lineweaver-Burk plots for ferrous ion and 3-hydroxyanthranilic acid oxidase. An aliquot of ammonium sulfate fractionated enzyme (25 μg) was incubated with the indicated amount of ferrous ion at pH 3.5, 0.02 M sodium acetate (O----O) or at pH 6.3, 0.02 M sodium acetate (●——●) for 3 minutes. After incubation the pH of the medium was adjusted to 6.8 with 1 M, pH 7.0 tris for the pH 3.5 acetate solution, or with pH 8.9, 0.05 M tris for the pH 6.3 acetate solution.

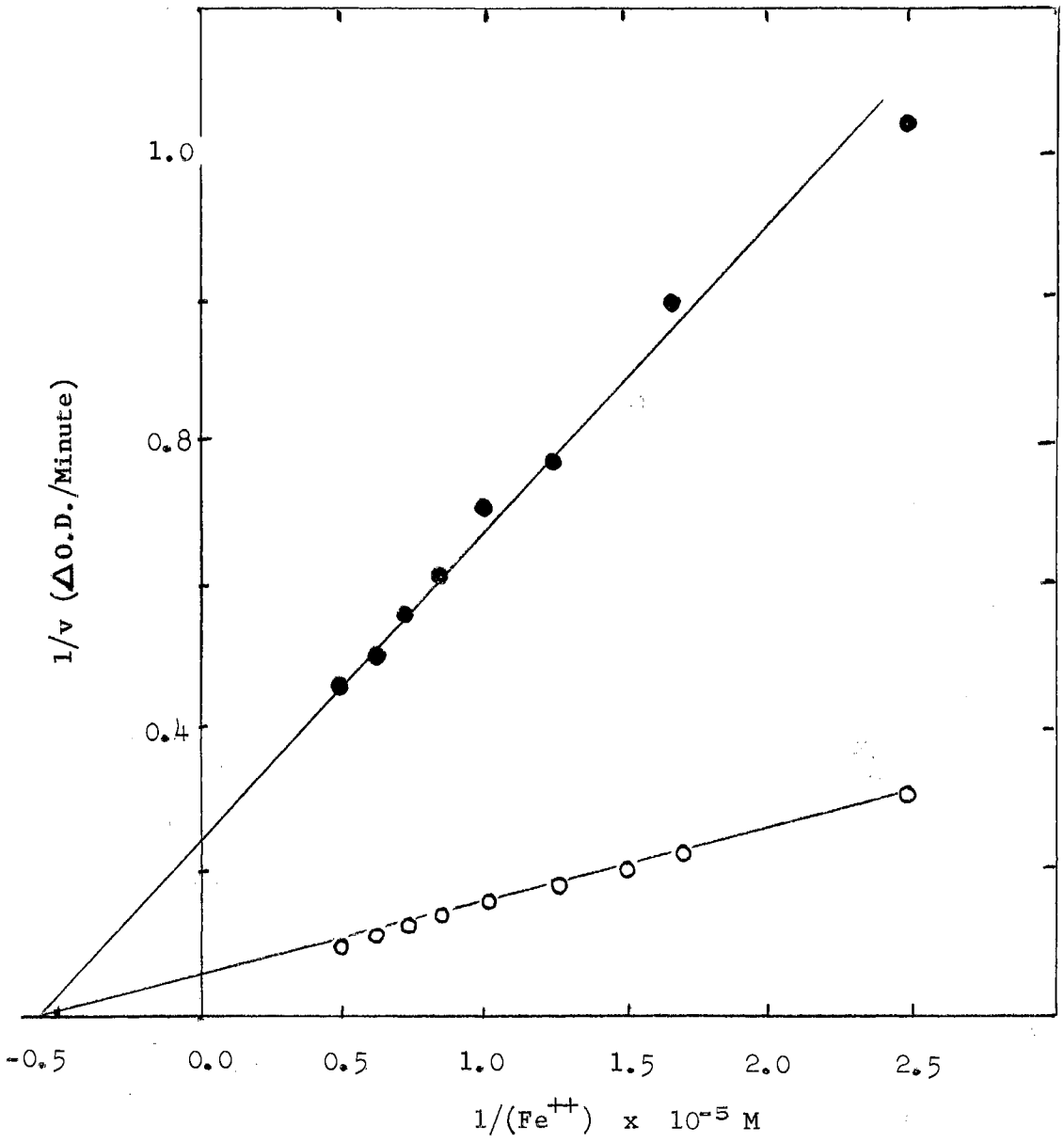

1.0
0.8
0.4
1/v (ΔO.D./Minute)
-0.5
0.0
0.5
1.0
1.5
2.0
2.5
1/(Fe++) x 10-5 M

TABLE II

EFFECT OF α-α'-DIPYRIDYL ON THE ENZYME AT pH 7.0 AND pH 3.5

| Incubation Time (minutes) | Enzyme Activity After Incubation At pH 7.0 (Δ O.D./min) | Enzyme Activity After Incubation At pH 3.5 (Δ O.D./min) |
|---|---|---|
| 0.0 | 3.20 | 3.20 |
| 0.5 | | 2.00 |
| 1.0 | | 1.10 |
| 1.5 | | 0.70 |
| 2.0 | | 0.30 |
| 3.0 | 2.70 | |
| 5.0 | 2.50 | |
| 10 | 2.30 | |
| 20 | 2.00 | |

agent at pH 7.0 for 30 minutes. Generally similar results were obtained using ethylenediaminetetraacetic acid or o-phenanthraline. Thus pH 3.5 was not only the optimum pH for enzyme activation by the activator but it is also an effective pH to inhibit the enzyme by the chelating agent. Probably, here, the low pH has caused a change in protein configuration for an easier entrance or departure of ferrous ion to or from the protein. In Figure 8 are shown these speculations on the effect of pH on the enzyme.

C. The Enzyme and Sulfhydryl Group

1. Activation by Reduced Glutathione

A purified enzyme preparation was kept with and without reduced glutathione (GSH) for 4 days at -20° C, and the results comparing the activity of the two samples are shown in Table III. The activities of the two enzyme preparations were same as the original activity of 4 days earlier when the rates were measured after incubating the enzyme with ferrous ion at pH 3.5, indicating that no enzyme denaturation had occurred during the storage. However, when the rates were measured without incubation with ferrous ion, the preparation kept with GSH was much more active than that of the preparation without GSH. It seems here that the major effect of GSH upon the enzyme was to prevent the oxidation of the enzyme-bound ferrous ion.

2. Inhibition of the Enzyme by p-Chloromercuribenzoate

The conclusion that an enzyme is a "sulfhydryl enzyme" has usually been based on the analytical demonstration of sulfhydryl groups in a protein and the reversible inactivation of the enzyme by reagents known to combine these groups (42). Although no analytical demonstration has been possible due to the purity of the enzyme preparation,

Figure 8

Possible scheme for representing the effect of pH on ferrous ion activation and on α-α'-dipyridyl inhibition of the enzyme. See the text for details.

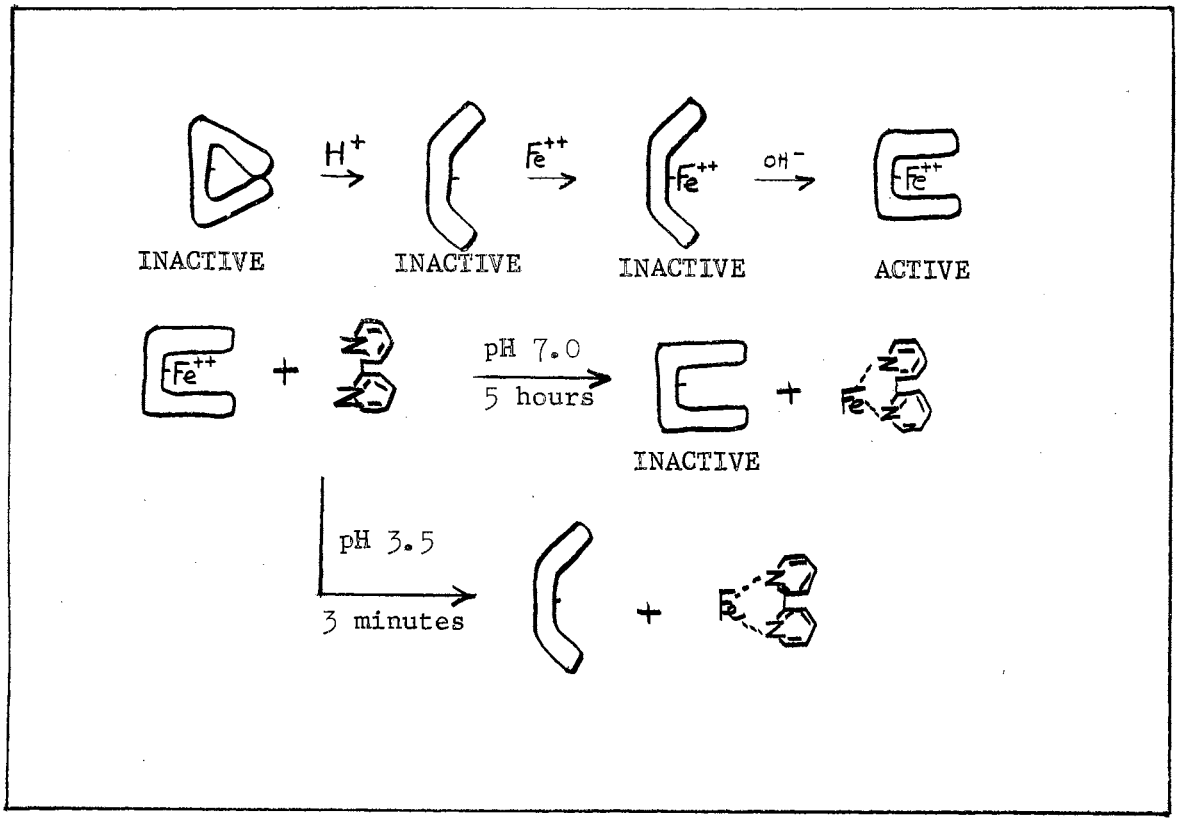

H+
Fe++
Fe++
OH-
Fe++
INACTIVE
INACTIVE
INACTIVE
ACTIVE
Fe++
pH 7.0
5 hours
INACTIVE
pH 3.5
3 minutes
Fe

TABLE III

PARTIAL PROTECTION FROM PCMB INHIBITION BY FERROUS ION

| | Enzyme Activity $\Delta$ O.D./minute |
|---|---|
| Enzyme + Fe, pH 3.5 (3 min)* pH 7.0, tris | 7.2 |
| Enzyme + PCMB (1 min) Fe, pH 3.5 (2 min) pH 7.0, tris | 0.0 |
| Enzyme + Fe, pH 3.5 (3 min) PCMB (3 min) pH 7.0, tris | 3.0 |
| Enzyme + Fe, pH 3.5 (3 min) PCMB (1 min) pH 7.0, tris | 4.0 |

Concentration of PCMB = $4 \times 10^{-6}$ M

Concentration of $Fe^{++}$ = $2 \times 10^{-4}$ M

Concentration of tris = 0.033 M

*Incubation time

enzyme inhibition by sulfhydryl reagents, particularly by p-chloromercuribenzoate (PCMB), and its reversal by thiol compounds have been well demonstrated (30, 32). However, these two criteria do not differentiate between at least two possible types of participation of sulfhydryl groups in enzyme activity: (a) as sites of attachment or attack on the substrate or cofactor, and (b) as a component of peptide chains involved in the maintenance of secondary or tertiary structure. The study of the effect of the substrate or cofactor on the p-chloromercuribenzoate inhibition constitutes an approach to demonstrate whether substrate or cofactor and p-chloromercuribenzoate combine at the same enzymatic locus. The behavior of inhibition, when the concentration of 3-hydroxyanthranilic acid or ferrous ions were varied, are shown in Figures 9 and 10, respectively. The inhibition by p-chloromercuribenzoate is competitive with 3-hydroxyanthranilic acid but not with ferrous ion. On the basis of "classical" kinetics, the inhibition of p-chloromercuribenzoate thus does not appear to be competitive with ferrous ion. However, ferrous ion had protective effect on the p-chloromercuribenzoate inhibition, as shown in Table III. The irreversibility of p-chloromercuribenzoate-sulfur reaction could produce such noncompetitive behavior with respect to ferrous ion, even though both p-chloromercuribenzoate and ferrous ion were competing for the same site (42).

At least two metalloproteins are known where ferrous ions are linked to the proteins through thiol-metal linkage. Klotz et al. have recently established that two ferrous ions are attached to hemerythrin, an oxygen-carrying protein found primarily among the sipunculid worms, through thiol-metal linkage as follows (43):

Figure 9

Kinetic study of the effect of 3-hydroxyanthranilic acid concentration on the enzyme activity (●) and the inhibition of this reaction by p-chloromercuribenzoate (○). The usual assay procedure, as described on page 9, was employed except the variation of the substrate concentration. When PCMB was used, it was introduced into cuvette at the same time with the substrate. Ammonium sulfate fractionated enzyme was used.

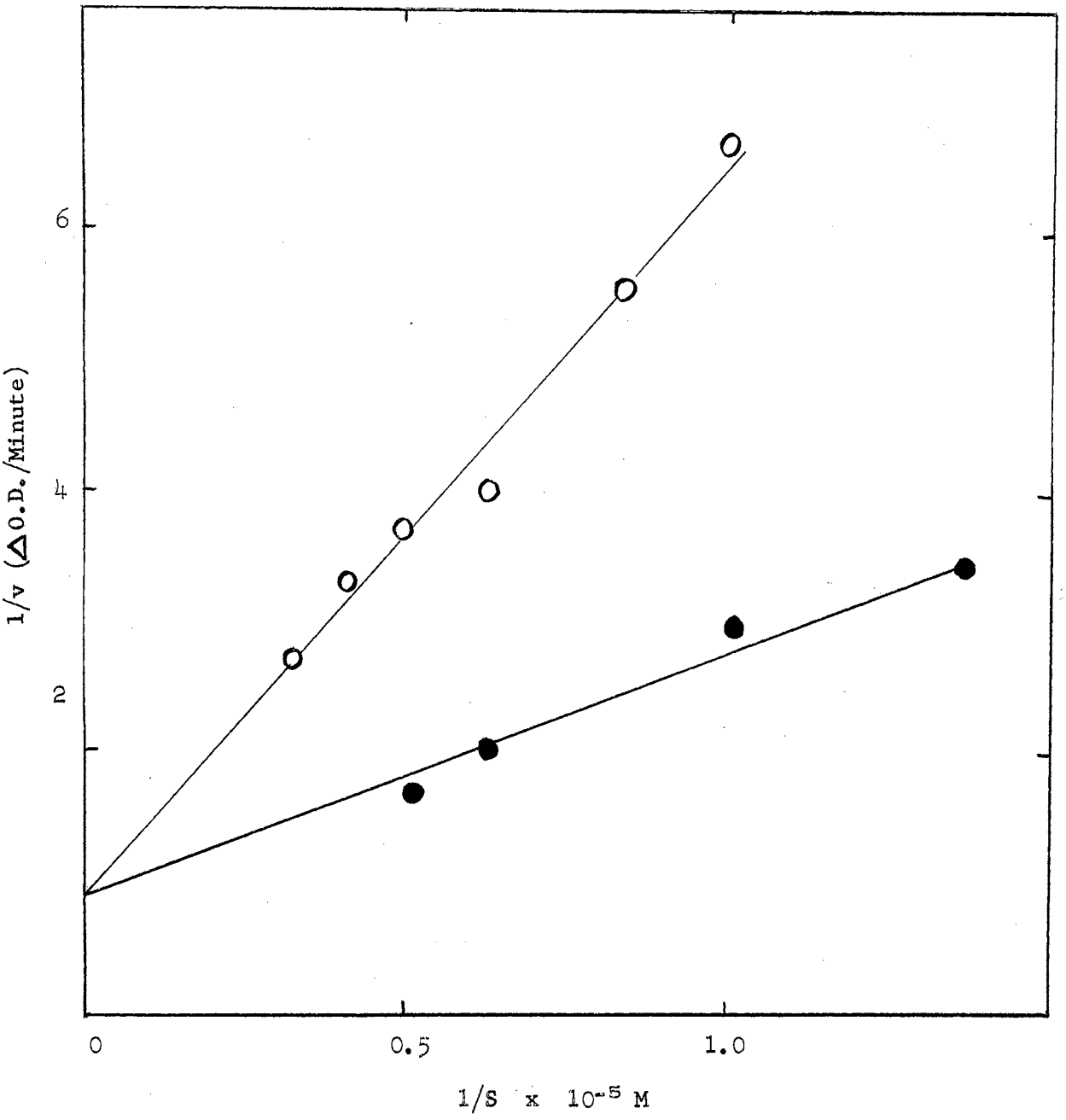
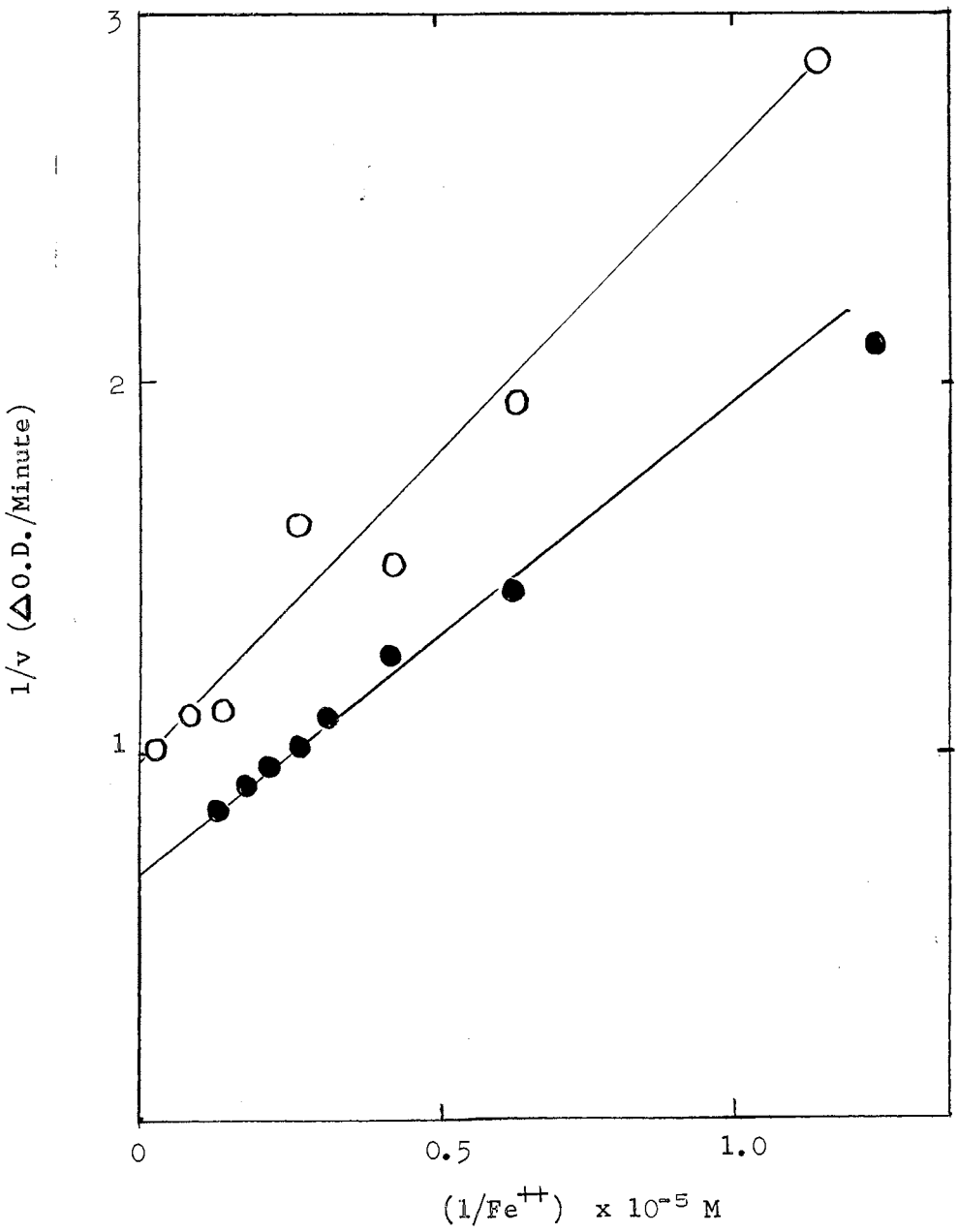
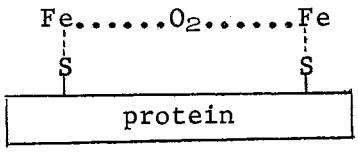

6
4
2
1/v (ΔO.D./Minute)
0
0.5
1.0
1/S x 10⁻⁵ M

Figure 10

Kinetic study of the effect of ferrous ion concentration on the velocity of 3-hydroxyanthranilic acid oxidation by the enzyme (●) and the inhibition of this reaction by $1.6 \times 10^{-6}$ M p-chloromercuribenzoate (○). The usual assay procedure, as described on page 9, was employed except the variation of the concentration of ferrous ion. When PCMB was used, it was introduced into cuvette at the same time with ferrous ion. Ammonium sulfate fractionated enzyme was used.


3
2
1
0
0.5
1.0
1/v (ΔO.D./Minute)
(1/Fe++) x 10-5 M




The second metalloprotein is ferritin, which was found to lose ferrous ion when treated with p-chloromercuribenzoate (44).

In view of the above findings, it is tempting to say that ferrous ion in 3-hydroxyanthranilic acid oxidase is linked to the protein through thiol-metal bond. Thus we can observe the kinetic evidence of the competition between 3-hydroxyanthranilic acid and p-chloromercuribenzoate since p-chloromercuribenzoate would less readily combine with sulfur by displacing ferrous ion when 3-hydroxyanthranilic acid is attached to or near ferrous ion.

An attempt was made to titrate the sulfhydryl groups of the enzyme with PCMB by Boyer's method (45) based on the increase in absorbancy at 250 mμ on formation of the mercaptide bond. Figure 11 shows the result as PCMB was added to a purified enzyme solution (2.2 mg protein/ml). Although the observed increase in optical density was not due to PCMB itself, no definite conclusion could be drawn due to the inhomogeniety of the enzyme solution.

D. Studies on the Progress Curves of the Enzyme Reaction

The enzyme activity which has been so far discussed refers to only initial rate. In this section, the enzyme activity after the initial stage will be discussed.

When a small amount of an active enzyme preparation (7 μg protein) was introduced into a cuvette containing only tris buffer (0.01 M, pH 7.0) and the reaction was started by adding 3OHAA, the velocity fell significantly with time (curve 3, Figure 12). However, this

Figure 11

Absorption spectra of the enzyme (ammonium sulfate fractionated preparation, 2 mg protein in 3.0 ml, light path of 1 cm) before (-----) or after (——) 42 minutes of incubation with 2 x $10^{-5}$ M p-chloromercuribenzoate at pH 7.0 tris (0.033 M). The blank contained the same amount of PCMB.

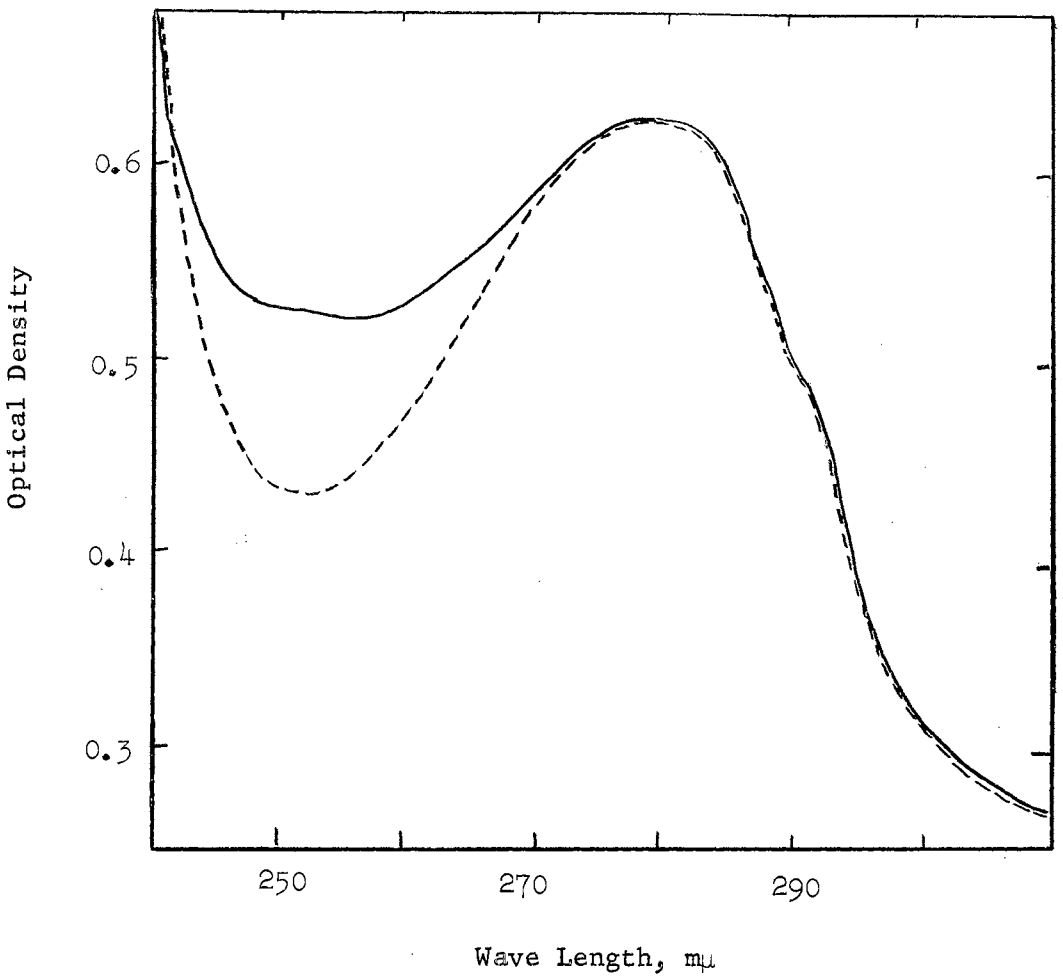

Optical Density
0.6
0.5
0.4
0.3
250
270
290
Wave Length, mμ

Figure 12

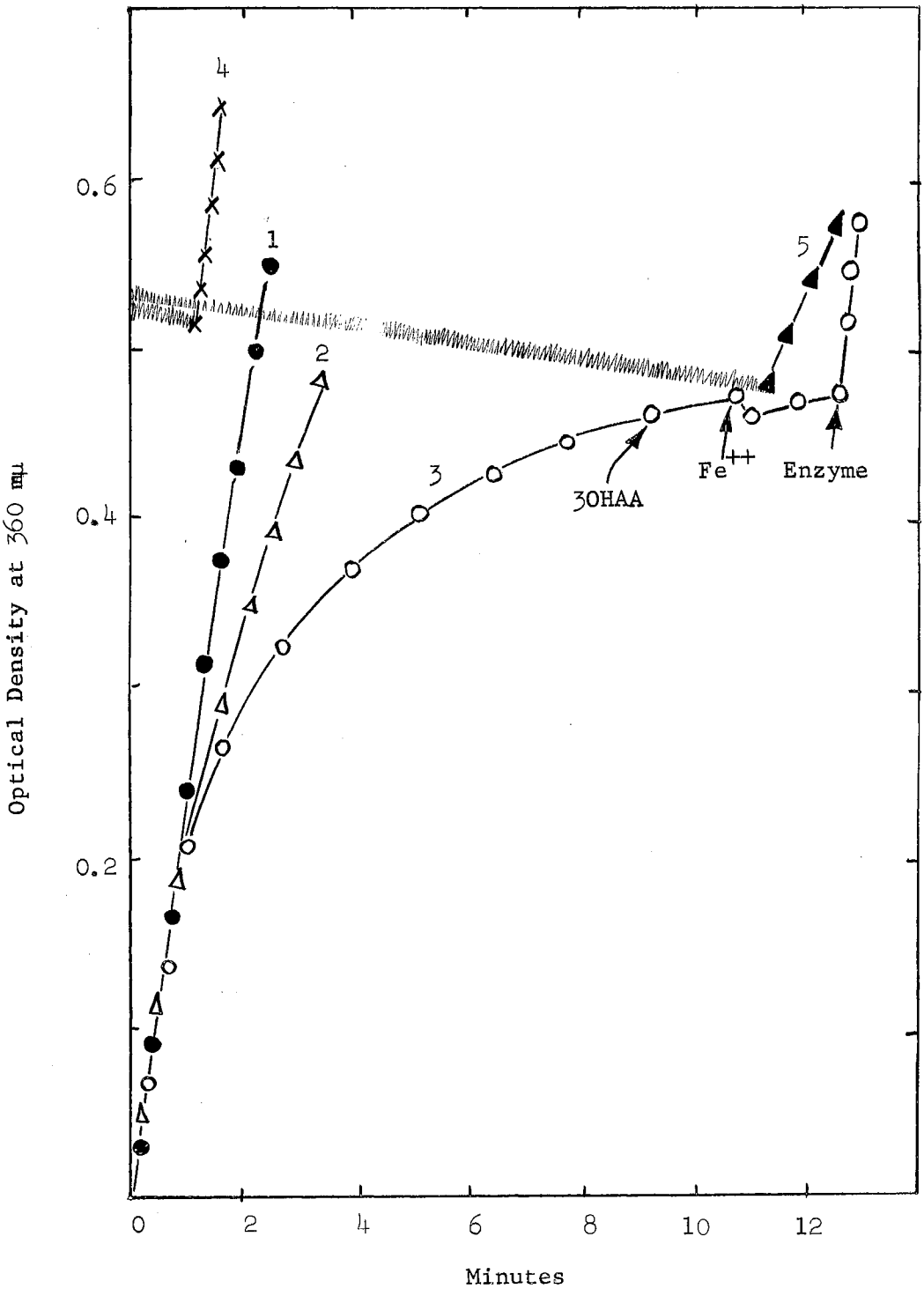
Effect on the progress curve of the enzyme reaction by ferrous ion, reduced glutathione, and Compound I. In all cases 7 μg of ammonium sulfate fractionated enzyme was used in cuvette containing 0.01 M, pH 7.0 tris.

Curve 1, ferrous sulfate ($2 \times 10^{-4}$ M) was introduced into the cuvette just prior to the addition of 3-hydroxyanthranilic acid ($2 \times 10^{-4}$ M).

Curve 2, reduced glutathione ($1 \times 10^{-3}$ M) was added at the beginning of the reaction.

Curve 3, the second additions of 3OHAA, enzyme and the first addition of ferrous sulfate were done as indicated in the figure.

Also the enzyme rates were measured after the enzyme was incubated with Compound I for 1 minute (curve 4) and after 12 minutes (curve 5). See the text for details.


4
1
2
3
5
0.6
0.4
0.2
0
2
4
6
8
10
12
Minutes
Optical Density at 360 mμ
3OHAA
Fe++
Enzyme

decrease in the rate could be prevented when the reaction was started in a cuvette containing $2 \times 10^{-4}$ M ferrous ion (curve 1) or $1 \times 10^{-3}$ M reduced glutathione (curve 2). The fall of the rate was not caused by the lack of either 3OHAA or oxygen, since the addition of more 3OHAA did not increase the rate (curve 3), but the addition of more enzyme increased the rate.

Another possibility that the enzyme-bound ferrous ion was lost upon dilution was not likely. If this were the case, reduced glutathione (GSH) could not prevent the decrease in rate. The curve 3 shows that addition of ferrous ion (or GSH) after the initial stage could not restore the activity, indicating that ferrous ion (or GSH) in the curve 1 (or curve 2) was not reactivating the inactivated enzyme but was preventing the inactivation, possibly from the product of the reaction. It was found that when the enzyme was incubated with compound I in a cuvette (O.D. was 1.7) for 12 minutes the enzyme had only 40 per cent of its original activity. It seems that Compound I was inactivating the enzyme during the reaction, and that ferrous ion or GSH could prevent the inactivating effect of Compound I, possibly by forming a complex with Compound I. The four functional groups in Compound I could easily complex ferrous ion, and GSH might react with oxidized substrate.

# CHAPTER IV

## SUMMARY

The enzyme assay method has been re-investigated, and it has been concluded that the routine enzyme assay method should consist of incubation of the enzyme with ferrous ion at pH 3.5 and measurement of the enzyme reaction at pH 7.0.

The chromatography of the enzyme on DEAE-cellulose column has achieved 166-fold purification over the homogenate step with 11 per cent recovery of activity when applied after partial purification. The highest specific activity observed was 264,000 units per mg protein.

Although this enzyme has been found to be a relatively stable one, it could be readily changed to an inactive form or active form under various conditions. It has been proposed that the active form is a protein-ferrous ion complex and the inactive form is either a protein-ferric ion complex or protein without the metal. Reduced glutathione could preserve the enzyme activity probably by preventing the oxidation of the bound ferrous ion.

Although the maximum velocity of the enzyme reaction was a function of the pH, at which it was incubated with ferrous ion, the apparent Michaelis constant for ferrous ion was not dependent on the pH of incubation. It has been proposed that the acid activation (pH 3.5 incubation) and probably the heat and salt activation as well were the result of an increase in the number of active sites on the enzyme.

The protective effect of ferrous ion and 3-hydroxyanthranilic acid on the PCMB inhibition suggested that this enzyme is a "sulfhydryl enzyme." It has also been proposed that the ferrous ion was bound to the protein through thiol-metal linkage.

The studies on the progress curve of the enzyme reaction showed that free ferrous ion or reduced glutathione was required in the reaction solution to prevent the rapid reduction of the rate after the initial stage. One cause of this falling off in the rate seemed to be the inhibition of the enzyme by the reaction product.

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