PURIFICATION OF MITOCHONDRIAL MONOAMINE OXIDASE AND ITS PROPERTIES

By

Moussa B.H. Youdim

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Allan Memorial Institute
McGill University
Montreal, Quebec

Department of Biochemistry
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PREFACE

Monoamine oxidase (oxidoreductase (deaminating 1.4.3.4,)) is defined as the enzyme which is responsible for the oxidative deamination of amines such as adrenaline, noradrenaline, serotonin, tryptamine, tyramine and alkyl monoamines. The enzyme was first described in 1928 by Hare-Bernheim.

The enzyme plays an important role in the metabolism of catecholamines. In spite of this importance our knowledge of the enzyme is limited to the recognition of certain types of compounds which act as either substrates or inhibitors. Some of these inhibitors have been useful in chemotherapy of mental disorders. Up to now, no adequate theory has been put forward as to how these inhibitors act on the enzyme. Recent advance in the chemistry of the enzyme provides strong evidence for the presence of more than one monoamine oxidase in the mitochondria.

The enzyme has until recently defied solubilization from the mitochondria. No adequate information as to the nature, cofactor requirements and composition of the enzyme can be obtained unless the enzyme is rendered soluble. Though certain investigators in this field have reported solubilization of the enzyme, no advance has been made in its chemistry.

Taking advantage of the stability of the enzyme, a new approach was taken to solubilize the enzyme. This involved

sonification of the mitochondria up to 100 minutes in the presence of a substrate to prevent heat-inactivation. The solubilized enzyme was obtained by centrifugation of sonicate at 160,000 x g for 90 minutes. The enzyme was precipitated with ammonium sulfate between 30 and 55 per cent saturation and easily dissolved in phosphate buffer.

This investigation includes studies of the solubilization and purification of liver monoamine oxidase from a number of animals. The properties of the enzyme are investigated, including cofactor requirement, i.e. whether the enzyme possesses a metal, the effect of copper-deficiency, riboflavin-deficiency on the activity of the enzyme.

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I. INTRODUCTION

A. Monoamine Oxidase

For a comprehensive review of monoamine oxidase (oxidoreductase (deaminating),1.4.3.4.) refer to the thesis by Moussa B.H. Youdim submitted to the Faculty of Graduate Studies, Department of Biochemistry, for the degree of Master of Science, August, 1964.

B. Purification of Monoamine Oxidase

In 1928 and later, Hare-Bernheim (1,2) described the presence of an enzyme called "tyramine oxidase" in mammalian tissues. At the same time Blaschko, Richter and Schlossmann (3) and Kohn (4) described an enzyme which was capable of oxidizing adrenaline. It was concluded independently by Blaschko's group (3), Kohn (4) and Pugh and Quastel (5) that "tyramine oxidase", "adrenaline oxidase" and "aliphatic amine oxidase" were identical. It was Zeller (6) who classified this enzyme as monoamine oxidase on the basis of what was then known about its substrate specificity.

Monoamine oxidase (EC. 1.4.3.4 , Monoamine O_2 oxidoreductase (deaminating)) catalyzes the following reaction:

$R\text{-}CH_2\cdot NH_2 + O_2 + H_2O \longrightarrow R - CHO + NH_3 + H_2O_2$ in which $R - CH_2\cdot NH_2$ represents mainly, but not exclusively, monoamines. It was Bernheim (2) who suggested the possibility

of an oxidastive deamination pathway and formulated the above reaction. However in crude tissue preparations catalase is usually present and the hydrogen peroxide is broken down as follows:

$$H_2O_2 \longrightarrow H_2O + \tfrac{1}{2}O_2$$

Therefore one atom of oxygen is used for each molecule of substrate oxidized.

The liver is one of the most active sources of the enzyme. The enzyme is characterized by its association with cellular organelles, the insoluble fraction of the tissue. Hawkins (7), in determining the monoamine oxidase activity of various fractions of rat-liver homogenate, found that about two-thirds of the enzymatic activity is present in the mitochondria and one-third in the microsomal fraction. Similar results have been obtained by Cotzias (8) and Blaschko et al. (9).

Ever since its discovery many attempts have been made to solubilize and purify the enzyme. It is only recently that some success has been made in this direction. A number of other amine oxidases, such as those of the pea seedling (10, 11), plasma (12, 13, 14, 15) and Aspergillus Niger (16) have been purified extensively. These enzymes differ from the mitochondrial monoamine oxidase by their great susceptibility to well-recognized carbonyl reagents and acylhydrazides such as hydrazine, semicorbazide and hydroxylamine. They are now known to have copper and pyridoxal phosphate as cofactors

(10-14, 16). The fact that mitochondrial monoamine oxidase is insensitive to most carbonyl reagents seems to eliminate pyridoxal phosphate as a co-factor.

Although mitochondrial monoamine oxidase has been studied intensively for more than 30 years, it has not been solubilized or purified nor has the nature of its prosthetic groups been established.

Hydrogen peroxide is a product of the reaction catalyzed by monoamine oxidase, so it has been suggested that this enzyme may be a flavoprotein (17). Hawkins (18), Sourkes (19), and Youdim (20) have demonstrated that there is a decrease in the monoamine oxidase activity of livers of riboflavin-deficient rats. Other indirect evidence for the role of a flavin in the action of monoamine oxidase comes from the fact that atebrin, an antagonist of flavin enzymes, inhibits the oxidation of adrenaline (21) as well as of isoamylamine, tyramine (19) and kynuramine (20).

The finding that liver monoamine oxidase is inhibited by chelating agents suggested that the enzyme is metalloprotein. Gorkin (22) has been able to inhibit reversibly monoamine oxidase of rat liver and brain mitochondria with various chelating agents such as 8-hydroxyquinoline, cyclohexanediamine tetraacetate and diethyldithiocarbamate. Barbato and Abood (23) found that the enzyme is inhibited by O-phenathroline. Results similar to those of Gorkin (22, 24) have been found by Youdim and Sourkes (20), who have

also shown that the enzyme is also inhibited by 2,2' dipyridyl and thenoyltrifluoroacetone. On the basis of the inhibition by metal-chelating agents one can suggest the presence of iron, molybdenum or copper in the prosthetic group of the enzyme. Green (25), studying the effect of hydrazine derivatives of monoamine oxidase inhibitors on its activity, has postulated that the monoamine oxidase is a copper-containing enzyme and that the inhibition by hydrazine derivatives results from a copper-catalyzed liberation of free radicals in the vicinity of the enzyme's active centre. The essential role of copper in other amine oxidases (11, 12, 16, 26) suggests that this metal may be important in the function of the present enzyme. In fact the presence of copper has been reported by Yasunobu (27), who purified the enzyme partially. The copper content of his enzyme preparation is of the same order as that of other amine oxidases (10-12, 15, 16, 26).

A number of investigators have reported purification of mitochondrial monoamine oxidase. Ganrot and Rosengren (28) reported 600-fold purification of pig liver monoamine oxidase. They were able to extract monoamine oxidase activity from the mitochondria with the use of bile salts. The enzyme was further purified on column of calcium phosphate, the eluant being increasing gradient of potassium phosphate containing sodium cholate. A 20-fold purification was reported by Barbato and Abood (29) using Triton X-100 to solubilize the

enzyme. However when the enzyme was precipitated with ammonium sulfate it could not be redissolved. Similar results were obtained by Gorkin (30) using a Triton-x type of non-ionic detergent (OP-10) at a final concentration of 1.25% to solubilize the mitochondria. Beef-liver monoamine oxidase was purified 17-fold by Sakamoto *et al.* (31), using a combination of ultrasonic oscillation and cholic acid.

Guha and Krishna Murti (32) in a recent communication reported 350-fold purification of rat liver monoamine oxidase using ultrasonic oscillation. Ganrot and Rosengren (28) have already reported that ultrasonic oscillation brings monoamine oxidase into such a state that it remains in the supernatant after centrifugation at 140,000 x *g* for 2 hours. The preparation however could not be used as a starting material for purification of the enzyme. It should be noted that attempts at purification of the enzyme by the method of Guha and Krishna Murti have failed (32). Gabay (33) has purified rabbit-liver monoamine oxidase 250-fold using a method of ultrasonic oscillation and extraction with Triton x-100. Further purification has been achieved on a DEAE-cellulose and calcium phosphate column. The enzyme is eluted from these columns using phosphate buffer that contained 0.4% Triton X-100. Gabay reported (33) that he could not repeat the purification procedure reported by Guha and Krishna Murti.

Recently Nara et al. (34) and Yasunobu (27) reported purification of beef-liver monoamine oxidase, stating that the enzyme is a copper-flavoprotein. The flavin content was estimated to be one mole of flavin per 200,000 gm of protein.

C. Nature and Scope of This Investigation

As stated earlier in the preface, we lack much basic information such as the nature, co-factor requirements and the composition of the enzyme, whether the enzyme has a metal associated with it. In order to clarify these questions, it has become necessary to study these properties with a solubilized preparation of the enzyme.

Taking advantage of the stability of the enzyme, ultrasonic oscillation was used to bring monoamine oxidase into a soluble form. Though ultrasonic oscillation by itself did not solubilize mitochondrial monoamine oxidase, it was the starting point. Bile salts have been very useful in solubilization of mitochondrial protein. Hence it was thought that a combination of ultrasonic oscillation and bile salts might render the enzyme soluble and that this soluble preparation could be used for further purification.

This work consists of studies with inhibitors, chelating agents, -SH compounds, riboflavin-deficiency, pyridoxine-deficiency, copper-deficiency on the activity of liver - monoamine oxidase.

Using these techniques, it is hoped that subtle

differences will provide an interpretation as to the nature and co-factor requirements of the liver-mitochondrial monoamine oxidase.

II. EXPERIMENTAL

A. Chemicals

1. Substrates of Monoamine Oxidase

Kynuramine dihydrobromide was purchased from Regis Chemical Company, Chicago, Ill. Benzylamine and furfurylamine were obtained from Metheson Coleman and Bell Company, Inc., Norwood, Ohio.

2. Inhibitors of Monoamine Oxidase

Thenoyltrifluoroacetone, o-phenanthroline, 2,2'-dipyridyl, 8-hydroxyquinoline and sodium diethyldithiocarbamate were obtained from Fisher Scientific Company, Montreal. D-Penicillamine hydrochloride was obtained from California Corporation for Biochemical Research, Los Angeles, California. Sodium pyrophosphate and potassium citrate were obtained from Fisher Scientific Company, Montreal. Versene Fe^{+3} specific was purchased from The Dow Chemical Company, Framingham, Massachusetts.

Phenylcyclopropylamine sulfate (Parnate, tranylcypromine), S.K.F. 385 A (Trans) was obtained from Research Laboratories, Smith, Kline and French Labs., Philadelphia. Iproniazid (Marsilid) was obtained from Hoffmann-La Roche Limited, Montreal. Pargyline (M0911) was obtained from Abbott Laboratories, North Chicago, Ill. Catron, 2-phenylisopropylhydrazine was a gift from Schering Corporation, Montreal.

3. Diets

Riboflavin-deficient and pyridoxine-deficient diets were purchased from General Biochemicals, Chagrin Falls, Ohio. Skim milk powder (Crino) was obtained from local stores.

4. Materials for Column Chromatography

All brands of Sephadex were obtained from Pharmacia, Uppsala, Sweden. DEAE-cellulose and hydroxylapatite were purchased from Bio-Rad Laboratories, Richmond, California.

B. Methods

1. Preparation of Liver Monoamine Oxidase

Livers were obtained from adult male rats of the Sprague-Dawley strain. The livers were chilled over cracked ice immediately upon removal and forced through a Latapie mincer to remove connective tissue. The liver pulp was weighed and homogenized in a small volume of ice cold 0.3M sucrose solution in a glass homogenizer with Teflon pestle for 5 minutes at one-minute intervals to allow cooling. The final volume of the homogenate was adjusted so that 1 gm or 2.5 gm of tissue was present in 10 ml of homogenate. This was called a 10% or 25% homogenate preparation.

(a) Centrifugation procedure

The method is essentially that of Hawkins (7). The homogenate in o.3M sucrose was centrifuged at 2000 RPM (580 x g) for 20 minutes to sediment unbroken liver cells,

large cell fragments, nuclei and red blood cells. The sediment was washed once by resuspending it in a 0.3M sucrose solution up to the original volume and by recentrifuging it at the same speed for 10 minutes. The sediment was discarded.

The supernatant obtained from this fraction was combined with the original supernatant and subjected to high speed centrifugation at 11,000 RPM (14,000 x g) for one hour. The sediment was suspended in the original volume to give a 10% or 25% preparation and labelled the mitochondrial fraction.

The above procedure was used to prepare rabbit and monkey liver mitochondria and rat brain mitochondria.

2. Preparation of Riboflavin-Deficient and Pyridoxine-Deficient Animals

Two groups of adult male (Sprague-Dawley) rats were fed commercial riboflavin-deficient and pyridoxine-deficient diets, respectively. At the end of one week the rats of each group were divided into two subgroups. The control groups received 30 mg per kg of diet of riboflavin-5-phosphate mixed into the deficient diet or similarly prepared diet containing pyridoxine hydrochloride. The diet of the experimental group, in case of riboflavin-deficient, contained 2 gm per kg galactoflavin. At intervals rats from each group were killed; their livers and brains removed, and mitochondria prepared. Also collected was blood from the control and pyridoxine-deficient rats. The heparinized blood was centrifuged, the

plasma was collected and retained for further studies.

3. Preparation of Copper-Deficient Rats

The method used is modelled after that of Gray and Daniels (35).

Young male albino rats, weighing about 40 gm, were fed copper-low diet. After 14 days they were divided into two groups. One of these, the deficient group, continued to receive the diet, consisting of the following: skim milk powder (0.1 µg/gm copper by analysis), 1 Kg; ferric sulfate, 0.1 gm; manganese sulfate, 0.03 gm; ammonium molybdate, o.10 gm; DL - methionine, 10 gm; 2500 I.U. vitamin A, 160 I.U. vitamin D, 400 µg thiamine, 240 µg riboflavin, 160 µg niacinamide and 240 ug pyridoxine. The animals of the other group ("control") were given the above diet to which had been added 0.039 gm of copper sulfate per kg of milk powder. At intervals rats were killed and monoamine oxidase activity of their liver and plasma was measured.

4. Preparation of Sephadex Gel-Filtration Column

The experiments described below were performed with Sephadex gel-filtration medium G-200 (lot No. T03314, particle size 40-120 (U.S. Standard)). The powder was suspended in water and allowed to swell overnight. Small particles were removed carefully by decanting. The gel was then washed several times with 0.05M phosphate buffer, pH 7.4 and allowed

to stand for one week at 2-3°. The column was prepared by pouring the gel particles into the chromatographic tube, which was already partly filled with the same buffer, and then allowing the particles to settle to a height of about 100 cm. For the estimation of molecular weight 3 mg samples of three proteins with different molecular weights were dissolved in 1 ml of the buffer for application to the top of the column. Fractions of 5 ml of eluate were collected using the LKB 3400 Radi-Rad Fraction collector. All column runs were carried out at 3-5°. The void volume of the column, i.e. the volume of material excluded from the particle pores, was determined according to the manufacturer's direction using India ink. Cytochrome C, one of the standard proteins used, was measured by its absorbance at 412 mμ.

5. Preparation of DEAE-Sephadex Column

DEAE-Sephadex A-50 (lot No. T03632, particle size 100-270 mesh (dry basis, U.S. Standard sieves)). DEAE-Sephadex was allowed to swell in distilled water and was then washed repeatedly with o.5N sodium hydroxide, on a Buchner funnel until free of chloride. The excess of sodium hydroxide was removed by rinsing well with glass distilled water. Then the ion exchanger was suspended in a large excess of initial eluant buffer (0.05M phosphate buffer, pH 7.4), for 24 hours. Almost complete equilibrium was reached after this period. The gel was then poured into chromatographic column (2.2x40 cm).

6. Preparation of Hydroxylapatite Column

Pure dehydrated hydroxylapatite (calcium phosphate) was suspended in 0.005M phosphate buffer, pH 6.8, for 24 hours. The column was prepared by pouring the material as a slurry and equilibrating it with the initial buffer by passing buffer through the bed over a period of at least 12 hours. Phosphate buffers were used with a stepwise increase in concentration while the pH (6.8) was held constant.

7. Preparation of Reagents for Iron Determination

(a) Acetate buffer, 0.5M, pH 4.0. Prepared 0.5M sodium acetate and o.5M acetic acid. Mixed 8 volumes of the acid and 2 volumes of the acetate.

(b) 0.1M sodium sulphite in water, prepared freshly every few days.

(c) 2,2'-dipyridyl, 0.5 percent in pH4.0 acetate buffer.

(d) Standard solution containing 3 ug iron per ml.

Iron Determination

The method is that of Ramsay (36).

In all analytical work to be described, glass distilled water was used. All glassware were washed with hot hydrochloric acid and rinsed several times with tap water and four times with glass distilled water. To the sample of protein was added 1 ml of 2 '2 dipyridyl followed by 1 ml of the sodium sulphite solution and made up to 7.5 ml with distilled water.

The blank was prepared the same way except that protein was replaced with distilled water. The tubes were then placed in boiling water bath for 20 minutes. After removal the tubes were cooled, centrifuged and made up to the 7.5 ml mark with distilled water. The colour, which was quite stable, was read against the blank at 520 mμ.

8. Preparation of Reagents for Copper Determination

(a) Concentrated sulphuric acid and 60% perchloric acid.

(b) Saturated sodium pyrophosphate and ammonium hydroxide.

(c) 0.5% sodium diethyldithiocarbamate.

(d) Normal amyl alcohol.

(e) Working standard of copper sulfate.

Copper Determination

An adapted method of Eden and Green (37) was used to determine copper. A known quantity of the sample was placed in a 30 ml Kjeldahl flask and 1 ml concentrated sulfuric acid and 2-4 ml of 60% perchloric acid. The digestion was carried out on an electric burner, until the sample became and remained colourless. A blank containing all reagents was treated simultaneously throughout the whole procedure. The digest was removed to 50 ml tubes with 10 ml distilled water. Ten ml saturated sodium pyrophosphate and 5 ml ammonium hydroxide were added to neutralize the acid. The colour was developed

by the addition of 2 ml 0.5% sodium diethyldithiocarbamate. The copper-diethyldithiocarbamate complex was extracted with 5 ml normal amyl alcohol. The amyl alcohol layer was dried with sodium chloride and its optical density was read against the blank in a Coleman junior spectrophotometer at 440 mμ. The values for copper were determined from a curve made by digestion of samples containing known amounts of copper sulphate.

9. Preparation of Reagents for Protein Estimation

Reagent A, 2% sodium carbonate in 0.10N sodium hydroxide. Reagent B, 0.5 per cent copper sulfate (hydrated) in 1 per cent sodium or potassium tartate. Reagent C, alkaline copper solution was freshly prepared, by mixing 50 ml of reagent with 1 ml Reagent B. Discard after one day. Reagent D , diluted Folin reagent. Folin-Ciocalteau phenol reagent diluted 1:1 with water to make it 1N in acid. Working standard prepared from human serum albumin, 400 γ/ml.

Lowry's Protein Measurement

The procedure was a modification of that of Lowry (38). 0.2 ml of a solution containing 5-100 ug of protein is added to 1.0 ml of Reagent C in a 10 ml test tube, mixed well and allowed to stand for 10 minutes. 0.1 ml of Reagent D is added very quickly and mixed as soon as possible. The tubes are left to stand for 30 minutes and then read in a Coleman junior spectrophotometer at 690 mμ. A blank containing everything

except protein is also prepared. With each estimation a standard curve of human serum albumin, 400 µg per ml, is prepared from which the amount of protein can be calculated. Protein was also measured spectrophotometrically at 280 mµ.

10. Estimation of Flavin

The method is essentially that of King et al.(39). All the procedures were carried out at 0-4°. 10 ml of the enzyme preparation or 10 percent mitochondrial preparation in phosphate buffer 0.05M, pH 7.4 were mixed with 10 ml of 20% trichloroacetic acid. The mixture was homogenized with Teflon pestle and allowed to stand for 30 minutes. It was then centrifuged for 20 minutes at 14,000 x g. The volume of the clear, yellow supernatant solution was measured and recorded. Thus, a factor was used in subsequent calculations; in this case $\frac{20}{\text{volume of preparation}}$*. The solution was extracted three times with 20 ml portions of peroxide-free ether to remove the trichloroacetic acid. The aqueous phase was adjusted to pH 7.0 with N NaOH.

Spectrophotometric Method

The absorbances at 450 mµ for the oxidized and the reduced forms of the flavins were recorded before and after addition of sodium dithionite to the sample. Absorbance index of 6.7×10^{-6} $cm^2 \times mole^{-1}$ was used for the acid-extractable flavins. The values for estimation of flavin were obtained from a standard curve of FAD.

* Taking into account the decrease in the volume due to precipitation of protein with trichloroacetic acid.

11. Measurement of Mitochondrial Monoamine Oxidase Activity

(a) Kynuramine Assay

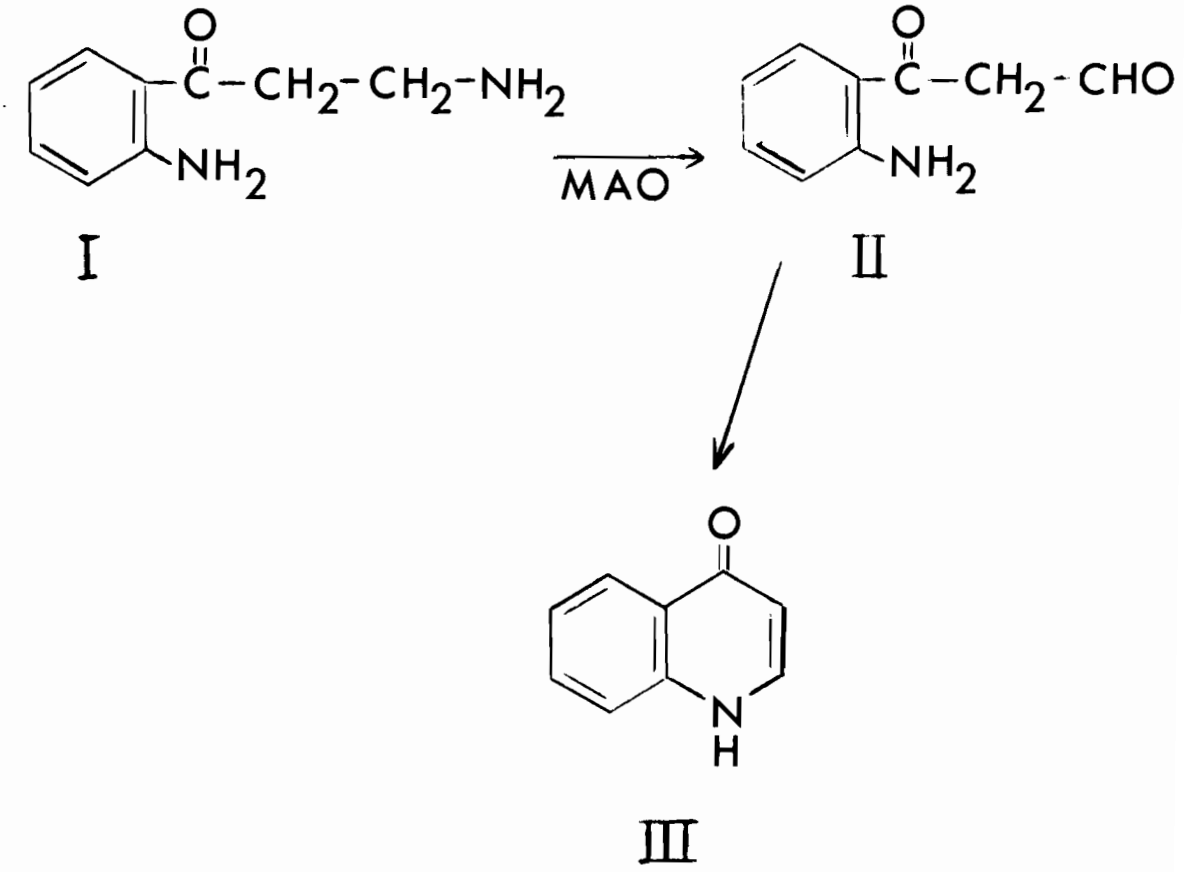
The method of Weissbach et al.(40) was used.

The substrate, kynuramine (I) is oxidatively deaminated by the enzyme to the aldehyde (II) which undergoes intramolecular (non-enzymic) condensation to 4-hydroxyquinoline (4 (1H)-quinoline, III). The rate of activity can be measured by the disappearance of Kynuramine at 360 mμ or appearance of 4-hydroxyquinoline at 315 - 329 mμ in a Beckman spectrophotometer.

All incubations were carried out in 20 ml beakers. The reaction mixture contained 0.2 ml of enzyme preparation, kynuramine 6.7×10^{-5}M final concentration, 1.0 ml of phosphate buffer or 1 ml of borate buffer 0.033 final concentration and water to a total volume of 3.0 ml. A blank was prepared in the same manner, except that kynuramine was omitted. All beakers were preincubated for 5 minutes and a further 20 minutes after the addition of the substrate at 37° C. An initial reading was made at 315 mμ . All reactions were stopped at the end of 20 minutes with 1 ml of 5% zinc sulfate.

(b) Benzylamine Assay

The method is that of Tabor (41) with modification to suit our purpose. The assay system is similar to the above kynuramine method. Benzylamine 1.67×10^{-3} M final concentra-



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tion was used. The enzyme mixture was preincubated 5 minutes and a further 30 minutes with the substrate. An initial reading was taken at 250 mμ. The enzymic activity was measured by the increase in absorbance at 250 mμ in a Beckman spectrophotometer.

12. Measurement of Plasma Monoamine Oxidase

The method is the same as the "benzylamine assay" except that 1 ml plasma is incubated with 1.67×10^{-3} M benzylamine for 2 hours.

III. RESULTS

1. Survey of Possible Methods for Preparing Soluble Monoamine Oxidase

(a) Enzymic Methods

Treatment of mitochondrial enzyme with pronase (42), a highly active hydrolytic enzyme, failed to render the enzyme soluble. At the same time most of the monoamine oxidase activity was lost.

(b) Ultrasonic Disintegration of Mitochondria

Hogeboom and Schneider (43) reported the use of the ultrasonic oscillator in a study of the distribution of enzymes in the mitochondria. The following experiments were designed to test the feasibility of this method for preparing soluble monoamine oxidase. Mitochondria were prepared as described under "Methods". They were then treated with a Branson Sonifier S75 ultrasonic oscillator for 20 minutes at 1 - 4 and 20 kilocycles. The preparation was centrifuged at 176,000 x g for 90 minutes. This yielded a supernatant fluid that was red in colour and the surface contained a milky layer. The results of several experiments of this type indicated that, while ultrasonic oscillation inactivates monoamine oxidase, about 45% of the activity was recovered in the supernatant.

(c) The Effect of Ultrasonic Oscillation on Monoamine Oxidase Activity

The effect of heating on the activity of monoamine oxidase has already been studied (20). Preliminary experiments have shown that the enzyme loses its activity with ultrasonic oscillation. The effect of time of ultrasonic oscillation on the activity of monoamine oxidase is shown in Table I. It can be seen that the enzyme loses a great amount of its activity in the first 10 minutes of sonification. The loss in activity may be due to local heating produced at the tip of the sonifier.

Previous studies (20) have shown that the enzyme can be protected from heat-inactivation if a substrate is present. The effect of ultrasonic oscillation on the activity of the enzyme in the presence of 0.003M benzylamine is shown in Table II. It can be seen that there is little loss in activity in the presence of benzylamine, even after 100 minutes of sonification. Therefore, for the preparation of soluble monoamine oxidase by the ultrasonic oscillation, benzylamine was always included at a concentration of 0.003M in the ensuing experiments.

(d) The Effect of Ultrasonic Oscillation on the Release of Monoamine Oxidase in Soluble Form

It is important to obtain the maximal release of monoamine oxidase in soluble form from the mitochondria.

Table I

The Effect of Ultrasonic Oscillation on Rat Liver Monoamine Oxidase* Activity

Experiment No.	Time of ultrasonic oscillation† (mins) 0	10	20	30	40	50	60	70
1	1.30**	0.55	0.53	0.52	0.47	0.45	0.43	0.39
2	0.60	0.41	0.39	0.33	-	-	-	-

* A 10 per cent (W/V) mitochondrial preparation was used.

** Monoamine oxidase activity is given in terms of increase in optical density at 315 mμ using kynuramine at 6.7×10^{-5} M final concentration.

† Ultrasonic oscillation was carried out using a Branson Sonifier S75 at 20 KC (8 amperes).

Table II

The Effect of Ultrasonic Oscillation on the Activity of Monoamine Oxidase in the Presence Of Benzylamine.

Experiment No.	Control	Sonified Enzyme	Time of Sonification (mins.)
1	0.56*	0.54**	60
2	1.35	1.20	80
3	1.35	1.30	100

The procedure was the same as in Table I.

* Activity of rat liver mitochondrial monoamine oxidase before sonification.

** Enzymic activity after sonification at 20 KC in the presence of 0.003M benzylamine.

Experiments were therefore designed to obtain the optimal time of sonification. Mitochondria were prepared as described earlier. They were suspended in 0.3M sucrose solution containing phosphate buffer 0.0125M, pH 7.4 and benzylamine 0.003M. The mixture was then subjected to sonification, for varying times. After this the enzyme preparations were centrifuged at 160,00 x g for 90 minutes. The milky layer above the supernatant was removed and discarded. The supernatant was collected and activity measured. Table III gives the result of 3 experiments. Up to 86% of activity was obtained in the supernatant with 100 minutes of sonification. A longer period of sonification did not increase the amount of enzyme in soluble form. We have therefore used this time, 100 minutes, in subsequent sonification.

(e) Extraction with Organic Solvents

It is true that ultrasonic oscillation brought monoamine oxidase into such a form that it remained in the supernatant after centrifugation at 160,000 x g for 90 minutes. The preparation however could not be used as a starting material for purification of the enzyme. The supernatant from 160,000 x g could be passed through a Sephadex G-200 column, followed by elution with 0.05 M phosphate buffer, pH 7.4 and all the activity was precipitated with ammonium sulfate 35% saturation. The precipitate was soluble in 0.05M phosphate buffer, pH 7.4, however it could not be eluted from a column

Table III

The Effect of Ultrasonic Oscillation on the Solubility of Rat Liver Monoamine Oxidase

Experiment No.	Duration of Sonification (mins.)	Activity of		Per cent of enzyme activity solubilized.
		Control	Sonicate*	
1	60	0.58	0.32	55
2	80	0.31	0.23	70
3	100	1.03	0.90	86

* Enzymic activity was measured in the supernatant solution obtained after sonification of crude rat liver mitochondria for the indicated time, followed by centrifugation at 160,000 x g for 90 minutes. Activity is given in terms of increase in optical density at 315 mμ during the first 20 minutes of incubation with 6.7×10^{-5} M kynuramine.

of DEAE-cellulose or DEAE-Sephadex A-50. Many attempts to elute the enzyme from these columns by varying the pH and molarity of effluent failed. It therefore became necessary to render the enzyme truly soluble. It seemed possible that though the enzyme is soluble it may be bound by such mitochondrial constituents as lipids, nucleic acid and proteins. If this were the case, truly soluble monoamine oxidase might be obtained by treatment of supernatant obtained from 160,000 x g, with acetone or n-butanol, or ethanol.

Cold (-12°) acetone was added to 40 ml of 160,000 x g supernatant obtained after sonification of mitochondrial monoamine oxidase preparation. The mixture was stirred for 10 minutes. It was filtered in a Buchner funnel and the cake was washed with more cold acetone. The cake was dried by suction in a Buchner funnel. The extraction from acetone powder was carried out by treatment with Tris buffer. The acetone powder was dispersed in 50 ml of 0.2M Tris buffer, pH 8.9 and stirred frequently for 15 minutes. The mixture was then homogenized and allowed to stand for 30 minutes. It was centrifuged at 144,000 x g in Spinco L model ultracentrifuge for 60 minutes. About a third of the original activity was obtained in the supernatant. This extracted monoamine oxidase could not be eluted from a DEAE-cellulose column.

Ethanol extractions were carried out at -4°. 95% ethanol was cooled to -12°. With the addition of ethanol to

the enzyme, the mixture was stirred mechanically. After extraction the mixture was centrifuged at 14,000 x g for 20 minutes and supernatant was discarded. The precipitate, though active, failed to dissolve in 0.05M phosphate buffer, pH 7.4.

The procedure for extraction with n-butanol was the same as ethanol, except that the sonified mitochondria is used. After extraction, the precipitate was centrifuged at 106,000 x g for 30 minutes. Both the supernatant and precipitate were inactive.

(f) The Effect of Detergents

Non-ionic detergents such as Triton X-100 and 'O-P-10' have been used by other investigators (29, 30, 31, 33) in this field and others to solubilize mitochondrial enzymes. The use of these detergents does not render the enzyme soluble, because when the enzyme is precipitated with ammonium sulfate it can not be redissolved (29) and, secondly, these detergents inactivate the enzyme (29). Therefore these detergents have not been used.

Ganrot and Rogengren (28) reported the use of bile salts for the purification of monoamine oxidase. Though they did not solubilize the enzyme, they obtained a highly active monoamine oxidase from mitochondria. They used sodium cholate in their phosphate buffer to elute monoamine oxidase activity from a column of DEAE-cellulose.

Preliminary experiments using cholate (sodium cholate, pure ox-bile) gave satisfactory results, i.e. the enzyme was not inactivated.

To the sonified (100 minutes) mitochondrial preparations, cholic acid was added to give a concentration ranging from 0.1% to 1.5%. The mixture was then allowed to stand for 30 minutes and finally centrifuged at 160,000 x g for 90 minutes. The supernatants from these preparations were then subjected to ammonium sulfate salt fractionation. The optimal condition was found to be with 1.0% cholic acid final concentration. More than 60% of the activity was found in salt fraction between 30 and 55% saturation. This fraction was then subjected to column chromatography on DEAE-Sephadex and hydroxylapatite. No difficulty was encountered in eluting the enzyme from these columns with phosphate buffer.

2. Purification of Mitochondrial Monoamine Oxidase

All operations were carried out at 2 - 4°.

Step 1. Sonification of liver mitochondria - Fresh rat liver freed of connective tissue by squeezing it through a tissue press (Harvard Apparatus Co., Dover, Mass.), were homogenized in 0.3M sucrose solution, using a Teflon pestle. Phosphate buffer, pH 7.4 was added to a final concentration of 0.0125M, and benzylame to a final concentration of 0.003M. A volume that would give 25% (W/V) preparation was used. The homogenate was then centrifuged for 20 minutes,

at 600 x g ; the activity of monoamine oxidase in the supernatant was measured and was considered the "original activity" in the purification studies. This supernatant fraction was subjected to ultrasonic oscillation using the Branson Sonifier S75 at 20KC (8 amperes) for 100 minutes. During sonification the enzyme preparation was kept at 4° with the use of an ice bath. At the end of this period cholic acid was added to give a final concentration of 1% and the mixture was allowed to stand for 30 minutes. The enzyme mixture was then centrifuged at 160,000 x g for 90 minutes. The milky layer above the supernatant was removed gently and discarded. The supernatant was collected and subjected to salt fractionation.

Step 2. First Ammonium Sulfate Fractionation. Solid ammonium sulfate was added to achieve 30 per cent saturation. After standing overnight, the precipitate was removed by centrifugation at 14,000 x g for 20 minutes and discarded. More ammonium sulfate was then added to give 55 per cent saturation. After standing overnight, the precipitate was collected by centrifugation at 14,000 x g for 20 minutes and dissolved in 0.05M phosphate buffer, pH 7.4. The solution was dialyzed for 24 hours against 4 litres of 0.005M phosphate buffer, pH 7.4. Any inactive precipitate which was formed was removed by centrifugation.

Step 3. Sephadex G-200 gel-filtration. The dialyzed enzyme solution was placed on a Sephadex G-200 column (2.2 x 100 cm) and eluted with 0.05M phosphate buffer, pH 7.4.

Fractions (5 ml volume) were collected at the rate of 15 ml per hour. The elution pattern for monoamine oxidase is shown in Fig. 1. The activity was eluted between fractions 21 to 34. Cytochrome C was eluted between fraction 48 and 62. More than 90 per cent of the amine oxidase activity was recovered at this stage.

The fractions containing monoamine oxidase activity were pooled and subjected to ammonium sulfate salt fractionation as follows: solid ammonium sulfate was added to give 55 per cent saturation. After standing for 30 - 60 minutes at 2 - 4° the precipitate was removed by centrifugation and dissolved in a small volume of 0.05M phosphate buffer, pH 7.4. Insoluble precipitate was removed by further centrifugation at 14,000 x g for 10 minutes.

Step. 4. DEAE-Sephadex Column Chromatography. The enzyme solution was subjected to DEAE-Sephadex A-50 column chromatography. The adsorbent was prepared as described under the section of "Methods" and packed into a column (2.2 x 15 cm). The column was equilibrated with 0.05M phosphate buffer, pH 7.4. The enzyme solution was passed through the column, effluent being 0.05M phosphate buffer, pH 7.4 containing increasing concentration of sodium chloride ranging from 0.05M to 0.2M. The elution of the protein was followed by the measurement of protein at 280 mμ as well as by the determination of enzymic activity (Fig. 2). All the

Figure 1

Elution pattern of solubilized rat monoamine oxidase from a Sephadex G-200 column. The supernatant fraction obtained by centrifugation of sonicate at 160,000 x g was placed on a Sephadex column (2.2 x 100 cm) and eluted with 0.05M phosphate buffer, pH 7.4. Activity was measured using kynuramine (6.7×10^{-5} M) as substrate. Fractions of 5 ml were collected at a flow rate of 15 ml per hour. (-.-.-.-.-) represents protein and (————) represents monoamine oxidase activity.

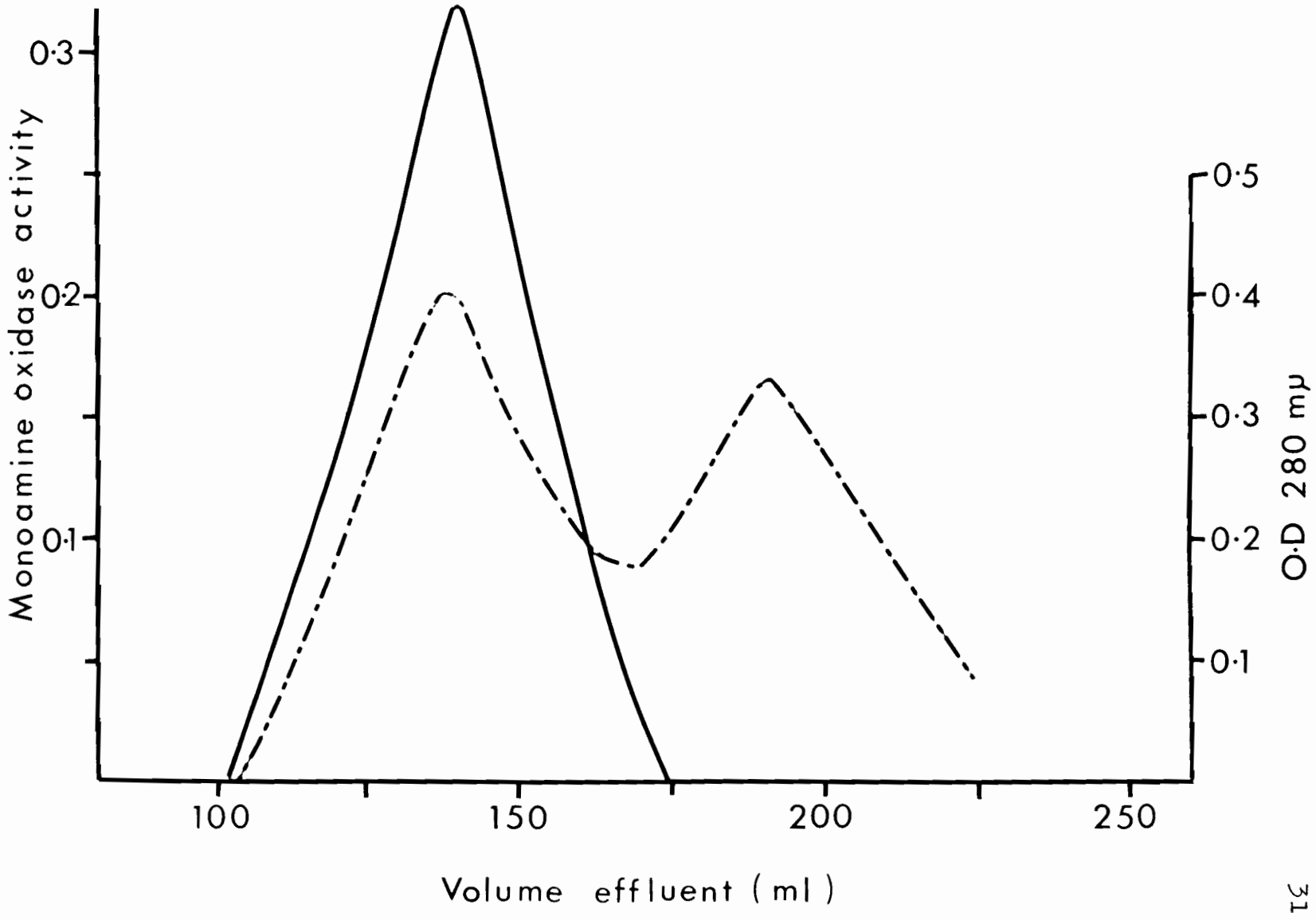
Monoamine oxidase activity
0·3
0·2
0·1
0·5
0·4
0·3
0·2
0·1
O·D 280 mμ
100
150
200
250
Volume effluent (ml)

Figure 1

Figure 2

The column chromatography of partially purified rat liver monoamine oxidase on a DEAE-Sephadex A-50. The enzyme solution was applied to a column (2.2 x 15 cm) and eluted with phosphate buffer 0.05M, pH 7.4, containing increasing concentration of sodium chloride. 5 ml fractions were collected at a flow rate of 5 ml per hour. (_____) represents protein and (-------) represents monoamine oxidase activity.

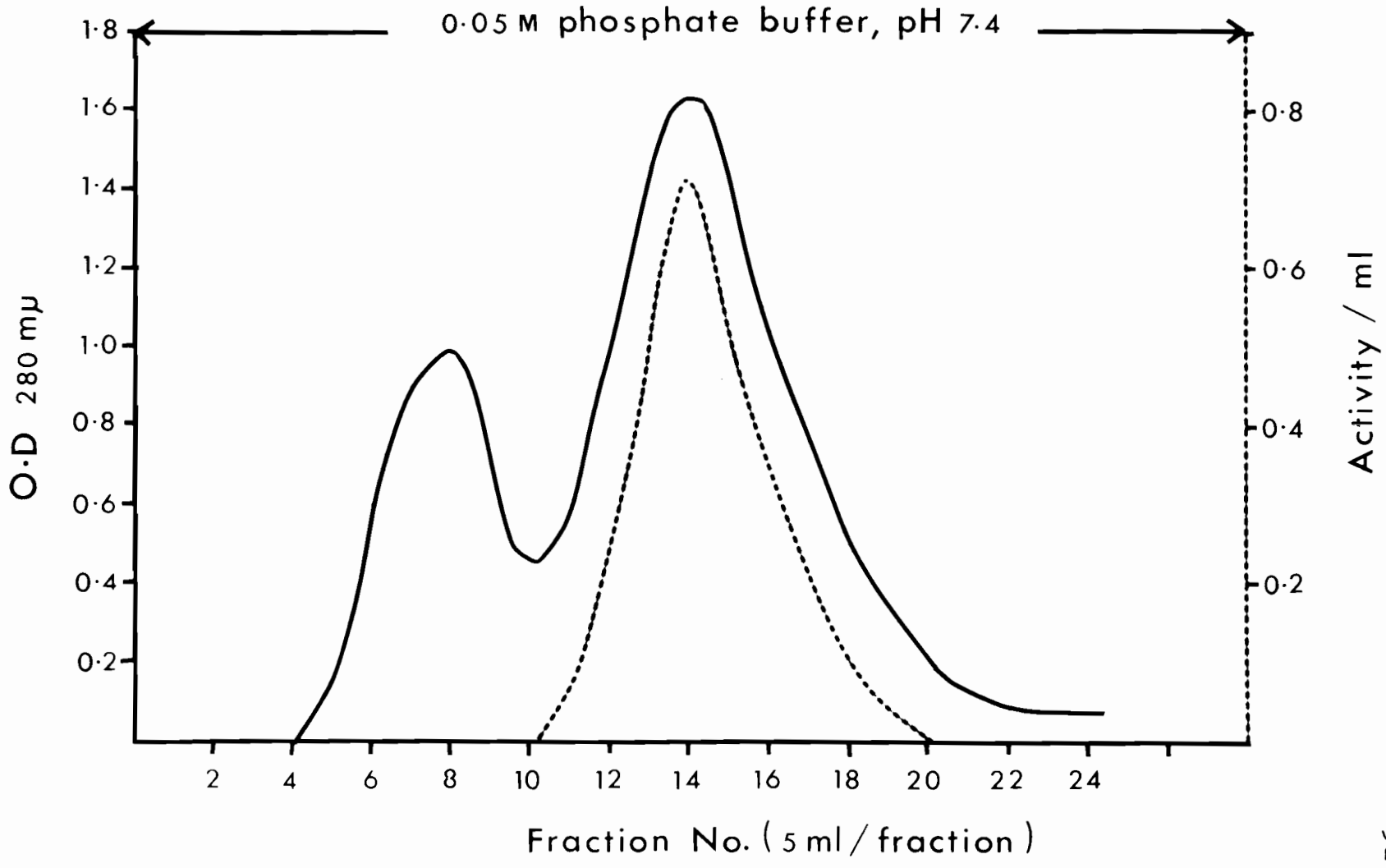
0·05 M phosphate buffer, pH 7·4
O·D 280 mμ
Activity / ml
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0·8
0·6
0·4
0·2
2
4
6
8
10
12
14
16
18
20
22
24
Fraction No. (5 ml / fraction)

Figure 2

enzymic activity that was going to be eluted was eluted with 0.05M phosphate buffer, pH 7.4, between fractions 10 and 20. 5 ml fractions were collected at a flow rate of 5 ml per hour. The fractions containing monoamine oxidase activity were pooled. The active material was precipitated with ammonium sulfate (55 per cent saturation). The precipitate was redissolved in 0.05M phosphate buffer, pH 7.4 and dialyzed overnight against 2 litres of 0.005M phosphate buffer, pH 7.4.

Step 5. Hydroxylapatite Chromatography. The dialyzed enzyme solution was subjected to hydroxylapatite column chromatography. The hydroxylapatite was packed into a column (2.2 x 15 cm) and equilibrated for at least 12 hours with 0.005M phosphate buffer, pH 6.8. The enzyme solution was placed on the column and eluted stepwise with increasing concentration of phosphate buffer, pH 6.8 from 0.005M to 0.5M. 5ml fractions were collected. Enzyme activity was obtained in a single peak with 0.5M phosphate. The elution pattern is shown in Fig. 3. The fractions containing monoamine oxidase activity were pooled and subjected to ammonium sulfate salt fractionation. Ammonium sulfate was added to give a 35 per cent saturation; if any precipitate was formed, it was removed by centrifugation at 14,000 x g for 20 minutes. More solid ammonium sulfate was added to give 55 per cent saturation. The precipitate was collected by centrifugation and dissolved in small amount of

Figure 3

Column chromatography of partially purified mitochondrial monoamine oxidase on hydrosylapatite. Enzymic protein was applied to a column (2.2 x 15 cm) which was equilibrated with 0.005M phosphate buffer, pH 6.8. Fractions of 5 ml were collected. (_____) represents protein and (--------) represents monoamine oxidase activity.

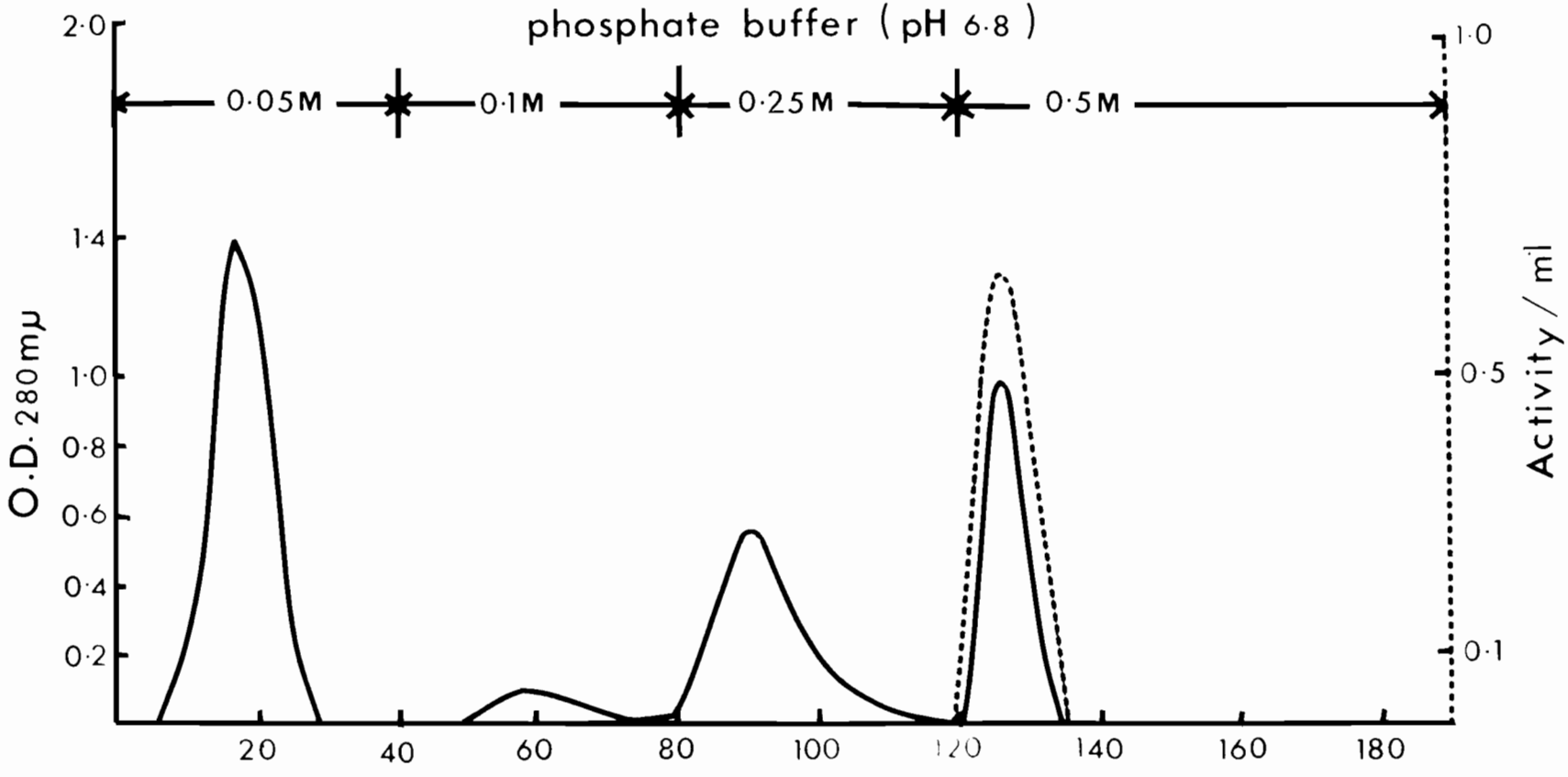
phosphate buffer (pH 6·8)
0·05M
0·1M
0·25M
0·5M
O.D. 280mμ
Activity / ml
2·0
1·4
1·0
0·8
0·6
0·4
0·2
1·0
0·5
0·1
20
40
60
80
100
120
140
160
180
FRACTION NUMBER (5ml / fraction)

Figure 3

phosphate buffer 0.05M, pH 7.4. This was the purified monoamine oxidase. Up to 25 per cent of the original activity was recovered.

The results of the purification of liver monoamine oxidase from rat, rabbit and monkey are given in Tables IV, V and VI. The greatest specific activity was obtained with the rat-liver monoamine oxidase. Table IV shows a 208-fold purification over that of the starting material, microsomal-mitochondrial preparation. Rabbit-liver monoamine oxidase was purified 140-fold and monkey-liver enzyme was purified up to 54-fold by gel-filtration using Sephadex G-200 column. No further purification of this enzyme was carried out.

Rabbit-liver monoamine oxidase was purified using the same method. The elution pattern for the rabbit enzyme from a hydroxylapatite column is shown in Fig. 4. Enzymic activity was eluted with 0.5M phosphate buffer, pH 6.8, in a single peak.

3. Properties of Monoamine Oxidase

(a) Stability

Purified preparations of the enzyme from rat and rabbit can be stored at 2 - 4° in 0.3M sucrose solution or 0.05M phosphate buffer, pH 7.4 with little loss in activity for 3 - 4 weeks. Rat liver monoamine oxidase is stable in the pH range 6.0 - 9.0. Outside this range, however, the rate of inactivation is increased. It should be remembered that the mitochondrial preparation (not solubilized enzyme) is

Figure 4

Column chromatography of partially purified rabbit liver monoamine oxidase on hydroxylapatite. Enzymic protein was applied to a column (2.2 x 15 cm) which was equilibrated with 0.005M phosphate buffer, pH 6.8. Fractions of 5 ml were collected. (————) represents protein and (--------) represents monoamine oxidase activity.

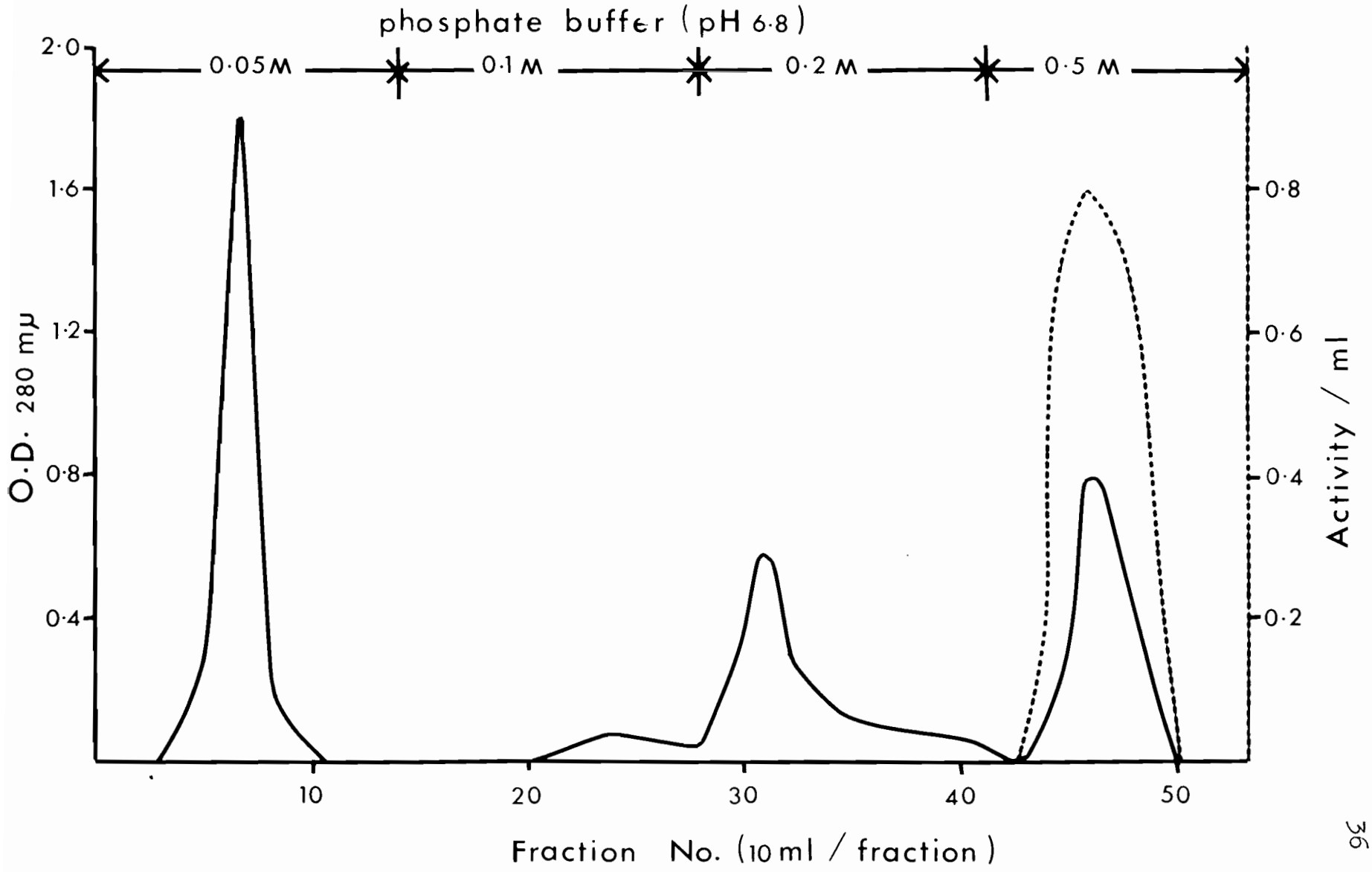
phosphate buffer (pH 6·8)
0·05 M
0·1 M
0·2 M
0·5 M
2·0
1·6
1·2
0·8
0·4
O.D. 280 mμ
0·8
0·6
0·4
0·2
Activity / ml
10
20
30
40
50
Fraction No. (10 ml / fraction)

Figure 4

more stable. Rat liver monoamine oxidase loses 10 per cent of its activity in two weeks.

(b) Homogeneity

Ultracentrifugation of purified rat liver monoamine oxidase showed a single symmetrical peak. Ultracentrifugation was carried out in 0.05M phosphate buffer, pH 7,4. Figures 5 and 6 show the Schlieren patterns obtained for two different preparations of enzyme, during a sedimentation velocity run, using a synthetic boundary cell. From these experiments a sedimentation coefficient of 6.5×10^{-13} Svedberg units was calculated.

(c) Determination of Molecular Weight

Andrews (44) has shown that the gel filtration method of molecular weight determination does not require that a protein be purified; hence the size of a particular enzyme can be estimated even when that enzyme is present in a mixture. Sephadex G-200 gel-filtration column was prepared as described earlier. In this work a standard curve relating elution volume to the logarithm of molecular weight was prepared, using five proteins of known molecular weight. This curve is illustrated in Fig. 7. When partially purified rat liver monoamine oxidase was placed on the Sephadex G-200 column, it was eluted by 145 ml of buffer. By interpolation on the standard curve, this would correspond to a molecular weight of 290,000. The purified enzyme gave the same estimated

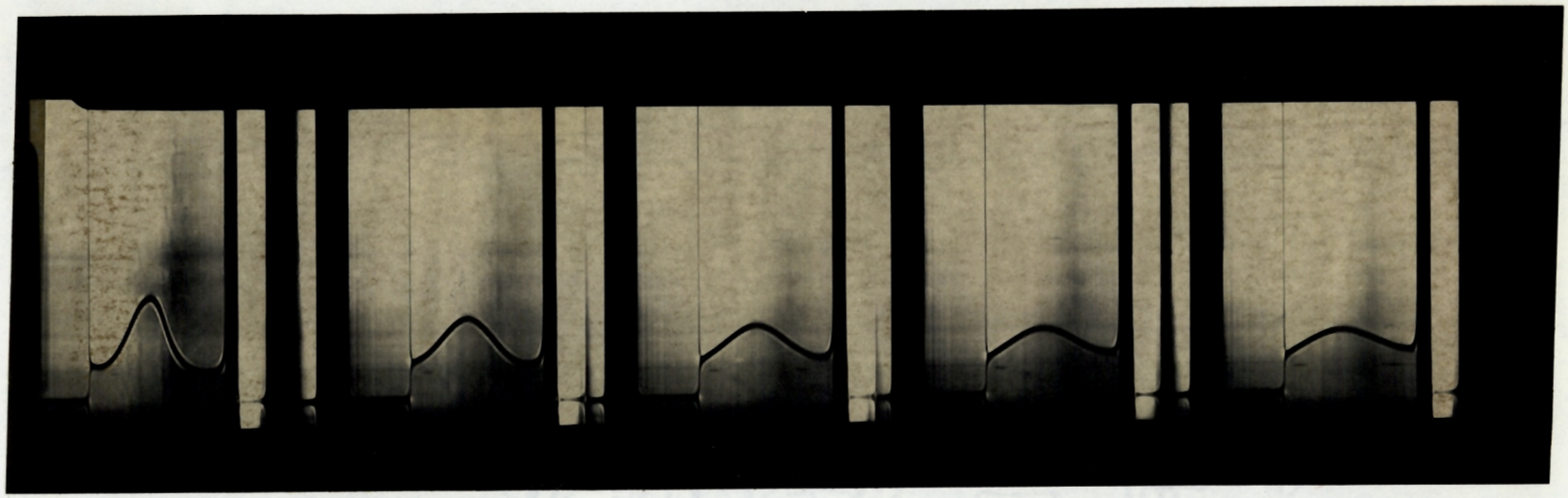

Fig. 5. Ultracentrifugation pattern of rat liver monoamine oxidase at a concentration of 5 mg per ml in 0.05M phosphate buffer, pH. 7.4. The pictures were recorded at 3, 12, 21, 30 and 39 minutes at 59,780 r.p.m. in a synthetic boundary cell at a temperature of 20 .

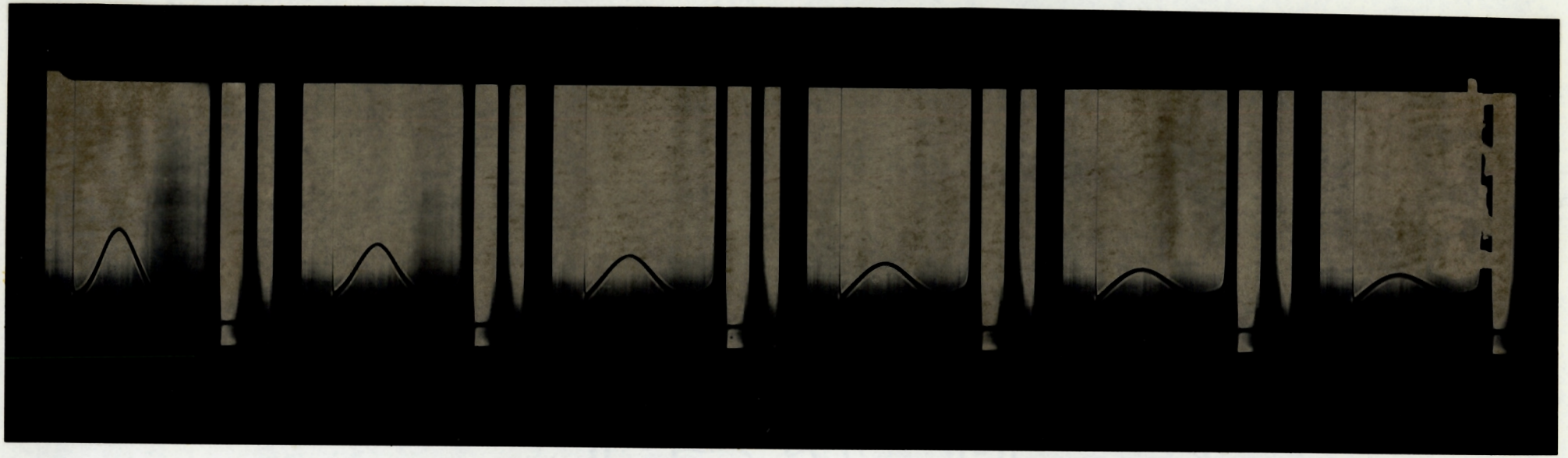

Fig. 6. Ultracentrifugation pattern of rat liver monoamine oxidase at a concentration of 5 mg per ml in 0.05M phosphate buffer, pH 7.4. The pictures were recorded at 9, 18, 27, 36 and 45 minutes at 59,780 r.p.m. in a synthetic boundary cell at a temperature of 20 .

Figure 7

Molecular weight determination by Sephadex G-200 gel-filtration method. The following proteins (source, supplier and molecular weight given) were used in preparing the standard curve: A, cytochrome c from horse heart, Sigma Chemical Co., St. Louis, Miss., 12,400 (68); B, bovine serum albumin, Fraction V, Sigma Chemical Co., 67,000 (69); C, glyceraldehyde 3-phosphate dehydrogenase (from rabbit muscle), Calbiochem, 120,000 (70); D, bovine serum globulin, Fraction II, Nutritional Biochemicals Corp., Cleveland, Ohio, 160,000 (69); E, horse spleen apoferritin, twice recrystallized, Nutritional Biochemicals Corp., 480,000 (71).

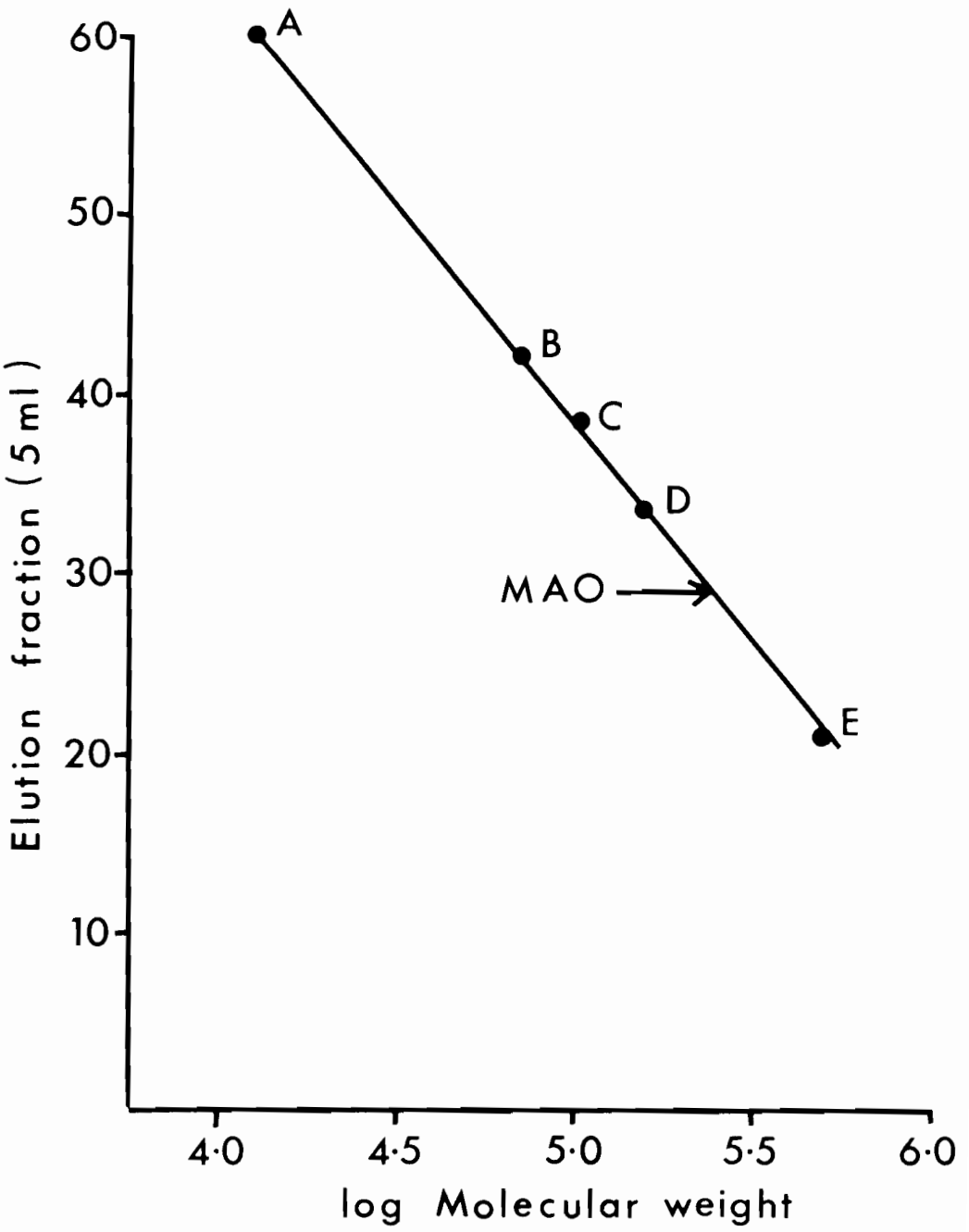
A
B
C
D
MAO
E
60
50
40
30
20
10
Elution fraction (5 ml)
4·0
4·5
5·0
5·5
6·0
log Molecular weight

Figure 7

Table IV

Purification of rat liver monoamine oxidase

Step	Units*	Protein	Specific Activity**	Yield
		mg		%
1. Crude mitochondrial fraction	595,000	42,220	14.1	100
2. Ultracentrifugation	475,000	18,270	26	80
3. 30-55% Ammonium sulfate	271,250	2,768	98	46
4. Sephadex gel-filtration	270,000	385	702	45
5. DEAE-Sephadex chromatography	200,000	160	1255	34
6. 30-55% Ammonium sulfate	195,000	142	1370	33
7. Hydroxylapatite chromatography	168,000	59	2850	28

* A unit of activity is defined as a change in O.D of 0.001, in 20 minutes at 37°C. The substrate was kynuramine, 6.7×10^{-5} M, final concentration.

** Activity per mg protein.

Table V

Purification of rabbit liver monoamine oxidase

Step	Units*	Protein	Specific Activity**	Yield
		mg		%
1. Homogenate	300,000	30,000	10	100
2. Ultracentrifugation	230,000	9,200	25	77
3. 30-55% Ammonium sulfate	112,500	2,250	50	38
4. Sephadex gel-filtration	110,000	268	400	37
5. DEAE-Sephadex chromatography	98,850	116	850	33
6. 30-55% Ammonium sulfate	85,500	88	970	28.5
7. Hydroxylapatite chromatography	72,000	51	1405	24

* The same as Table IV

** The same as Table IV

Table VI

Purification of monkey liver monoamine oxidase

Step	Units*	Protein	Specific Activity**	Yield
		mg		%
1. Crude mitochondrial fraction	420,000	10.500	40	100
2. Ultracentrifugation	390,000	4,600	85	
3. 30-55% Ammonium sulfate	182,500	445	410	
4. Sephadex gel-filtration	177,500	82	2150	

*, ** The same as Table IV

Figure 8

Absorption spectra of purified rat-liver monoamine oxidase in 0.05M phosphate buffer, pH 7.4. Enzyme purified 208-fold was used at a concentration of 1 mg per ml (x———x) represents the oxidized form of the enzyme and (------) represents the reduced form of the enzyme, using sodium dithionite for reduction. (-x-x-x-x) represents spectra difference (oxidized minus reduced).

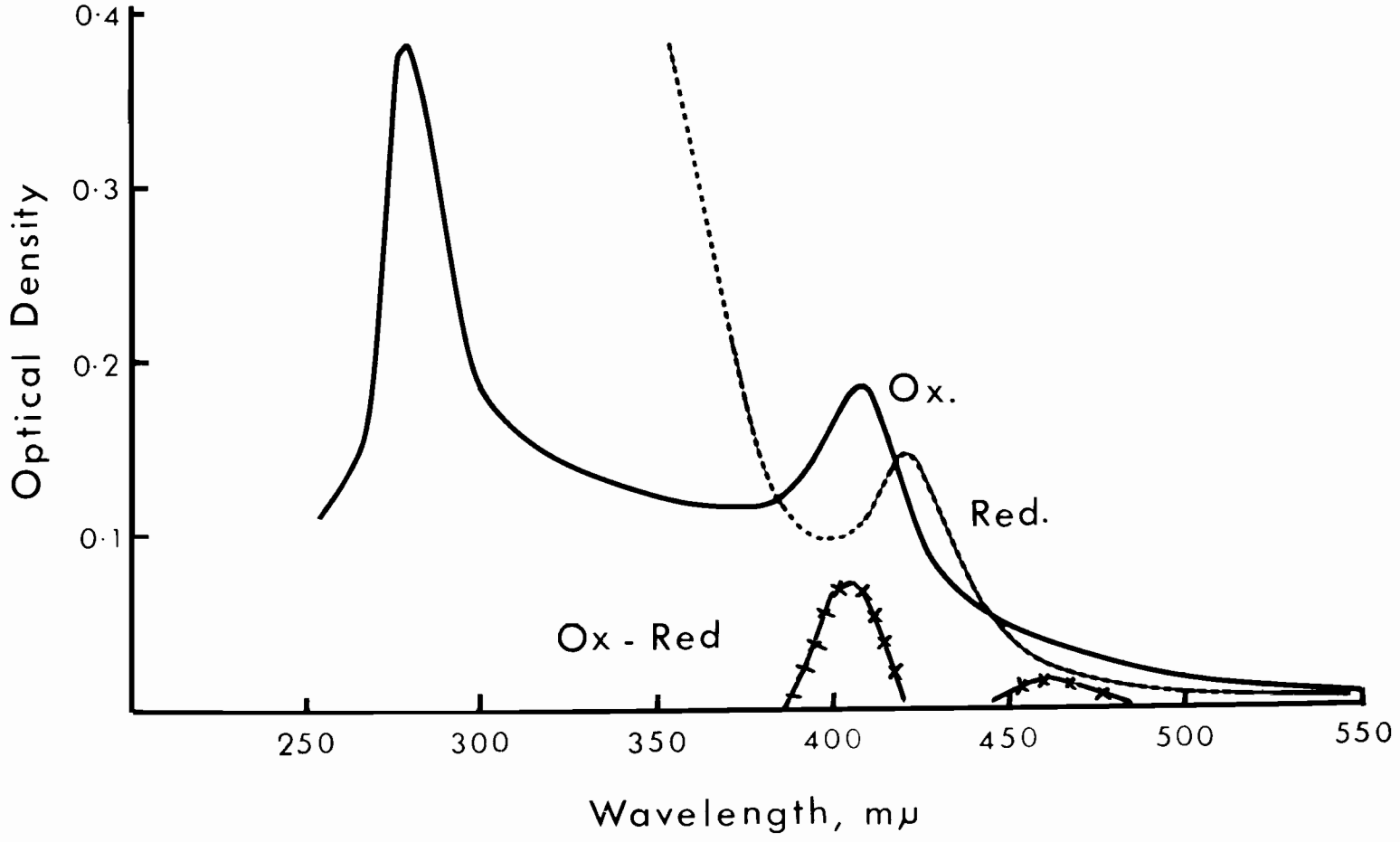
0·4
0·3
0·2
0·1
Optical Density
Ox.
Red.
Ox - Red
250
300
350
400
450
500
550
Wavelength, mμ

Figure 8

molecular weight. In the case of rabbit liver monoamine oxidase, the molecular weight of the purified enzyme was estimated to be 260,000.

(d) Absorption Spectrum

The absorption spectrum of rat and rabbit liver monoamine oxidase are shown in Figures 8, 9. The native (oxidized) enzyme exhibits two maxima at about 280 mμ and 410 mμ. The yellow colour of the enzymes probably corresponds to the latter peak. The latter absorption band is partially bleached by addition of a reducing agent such as sodium dithionite or sodium borohydride and the maximum at 410 mμ is shifted to 420 mμ. In the case of rat, difference spectrum of the native (oxidized) enzyme and the reduced showed two maxima, at 405 mμ and 460 mμ. With the rabbit monoamine oxidase similar results were obtained. It should be remembered, though not shown the reduced enzyme is autoxidizable with oxygen.

4. Action of Chelating Agents

Metal-binding agents such as o-phenanthroline, α,α'-dipyridyl and 8-hydroxyquinoline are known to inhibit the enzyme (20, 22, 23), although the extent of inhibition is somewhat variable, depending on the preparation, degree of purification and the time of preincubation with the enzyme, as shown in Table VII. A greater inhibition was

Figure 9

Absorption spectra of purified rabbit-liver monoamine oxidase in 0.05M phosphate buffer, pH 7.4. Enzyme purified 208-fold was used at a concentration of 1 mg per ml (x———x) represents the oxidized form of the enzyme and (------) represents the reduced form of the enzyme, using sodium dithionite for reduction. (-x-x-x-x) represents spectra difference (oxidized minus reduced).

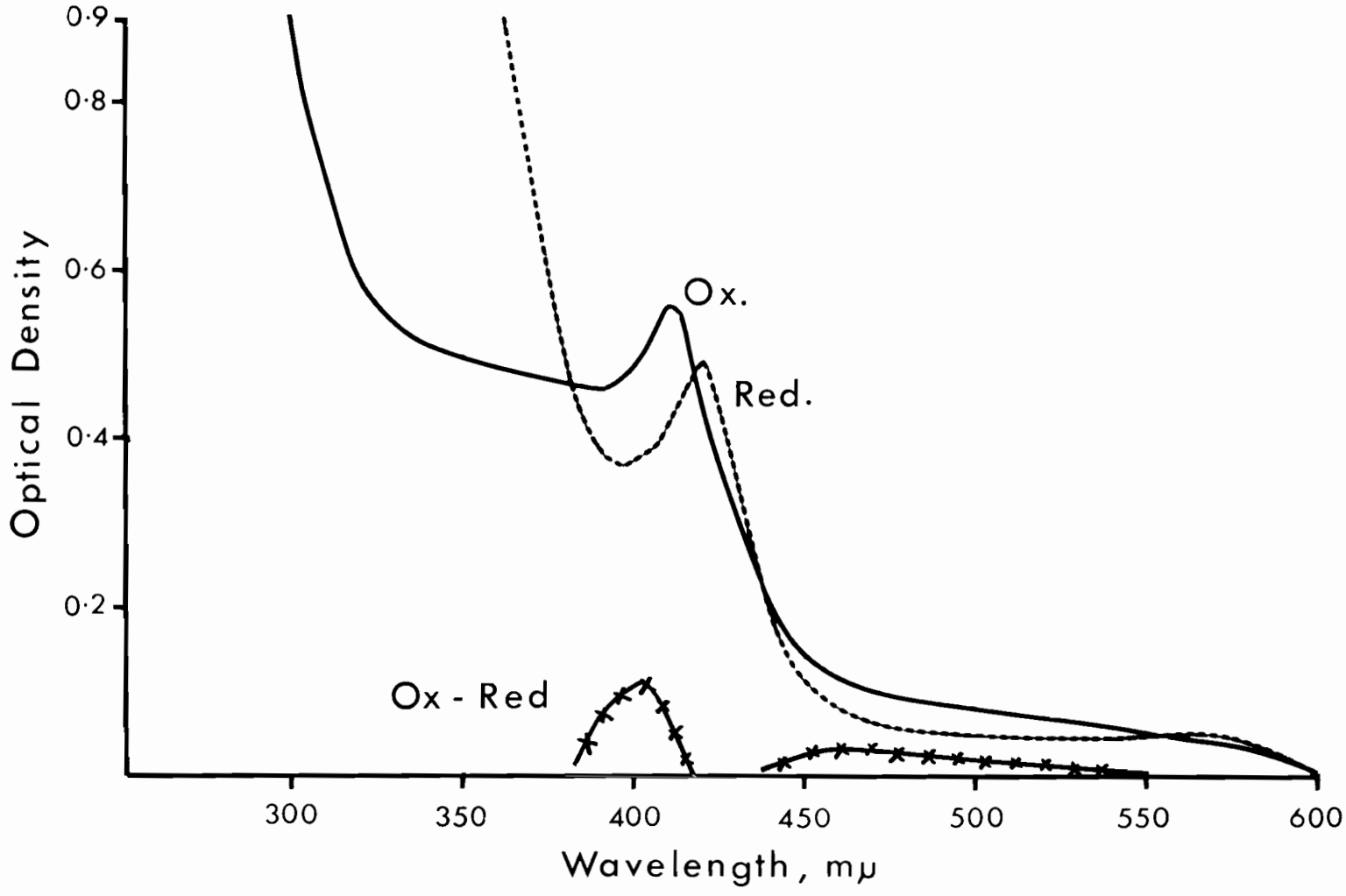
0·9
0·8
0·6
0·4
0·2
Optical Density
Ox.
Red.
Ox - Red
300
350
400
450
500
550
600
Wavelength, mμ

Figure 9

Table VII

Inhibition of rat liver monoamine oxidase
by chelating agents

Enzyme with a specific activity of 500 (62-fold purified) was used.

Chelating agents*	10 mins. preincubation	20 mins. preincubation
Control	0.23**	0.25
Versene Fe^{+3}	0.175 (24)†	0.16 (36)
Diethyldithiocarbamate	0.20 (13)	0.185 (26)
Sodium pyrophosphate	0.15 (35)	0.14 (44)
Potassium citrate	0.125 (46)	0.135 (46)
Thenoyltrifluoroacetone	0.125 (46)	0.150 (40)
O-Phenanthroline	0.185 (20)	0.115 (54)
2, 2′ Dipyridyl	0.10 (57)	0.07 (75)
8-Hydroxyquinoline	0.08 (67)	0.05 (80)

* All inhibitors at 1.67×10^{-4}M, except versene Fe^{+3} specific (0.2 ml of 1.0% in 3.0 ml), concentration added to the enzyme in 0.1M phosphate buffer, pH 7.4. The mixture was then preincubated for indicated time at 37 and a further 20 minutes with the substrate, kynuramine 6.7×10^{-5} final concentration.

** Enzymic activity, increase in optical density in the first 20 minutes incubation with the substrate.

† Figures in parenthesis represent per cent inhibition.

Table VIII

Iron content during purification of liver monoamine oxidase

Step	Rat		Rabbit	
	Specific Activity*	Iron** Content	Specific Activity	Iron Content
1. Crude mitochondrial fraction	14.1	0.04	10	0.06
2. Ultracentrifugation	26	0.02	25	0.045
3. 30-55% Ammonium sulfate	98	0.07	50	0.09
4. Sephadex gel-filtration	702	0.29	400	0.29
5. DEAE-Sephadex chromatography	1255	0.65	850	0.68
6. Hydroxylapatite chromatography	2850	1.2	1405	1.1

* units/mg protein

** µg iron/mg protein

obtained with the chelators which are known to chelate iron. These include o-phenanthroline, a,a'-dipyridyl, 8-hydroxyquinoline and thenoyltrifluoroacetone. Diethyldithiocarbamate had very little inhibitory effect; this agent is considered to be a chelator of copper, primarily. This study shows that the accessibility of the metal on the enzyme to chelating agents is low and so is the extent of dissociation of the metal from the enzyme (this will be discussed later).

5. The Relationship of Iron Content to Enzyme Activity

Iron content of the enzyme preparation was measured at each step of purification; it was observed that there was an increase in iron content that was proportional to the increase in specific activity. Table VIII shows the typical result obtained with one preparation. Considerable contamination was obtained at the initial steps of purification, since many other mitochondrial enzymes are known to contain iron. The contaminant was separated by Sephadex gel-filtration and DEAE-Sephadex. The direct relationship observed between monoamine oxidase activity and iron content is shown in Figures 10, 11 for rat and rabbit respectively.

6. Iron and Other Metal Content

Rat liver monoamine oxidase purified 208-fold, which showed a single symmetrical peak in the ultracentrifuge, was found to contain 0.12 per cent iron (Figure 10 and Table VIII) and 0.034 per cent copper, because the molecular weight was found to be 290,000 by the method of gel-filtration. Rabbit liver monoamine oxidase purified 140-fold contained 0.11 per cent of iron.

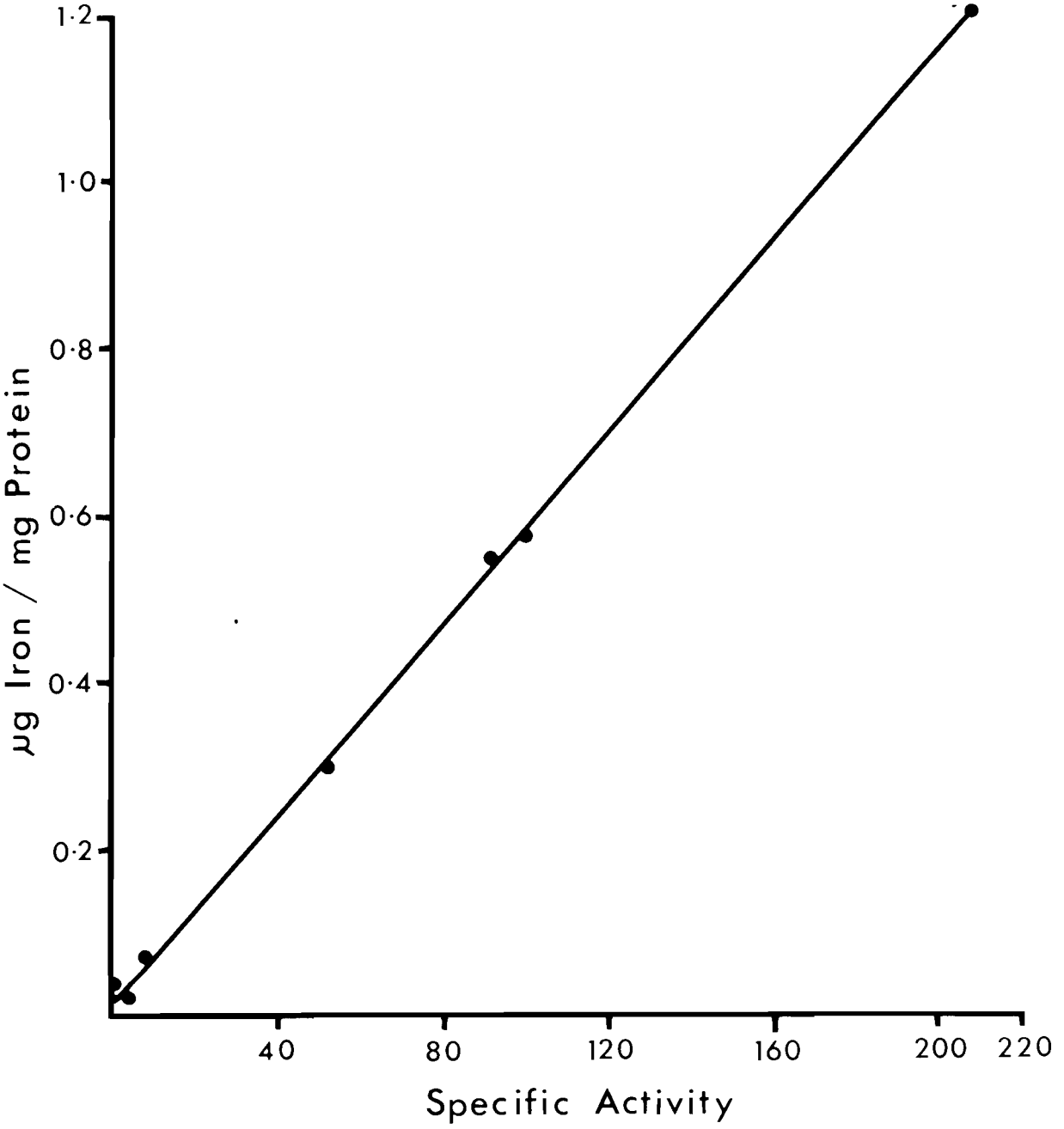

Fig. 10. Proportionality between iron content and specific activity during the purification of rat liver monoamine oxidase. Specific activity here is defined as the number of times the activity is increased over that of the initial.

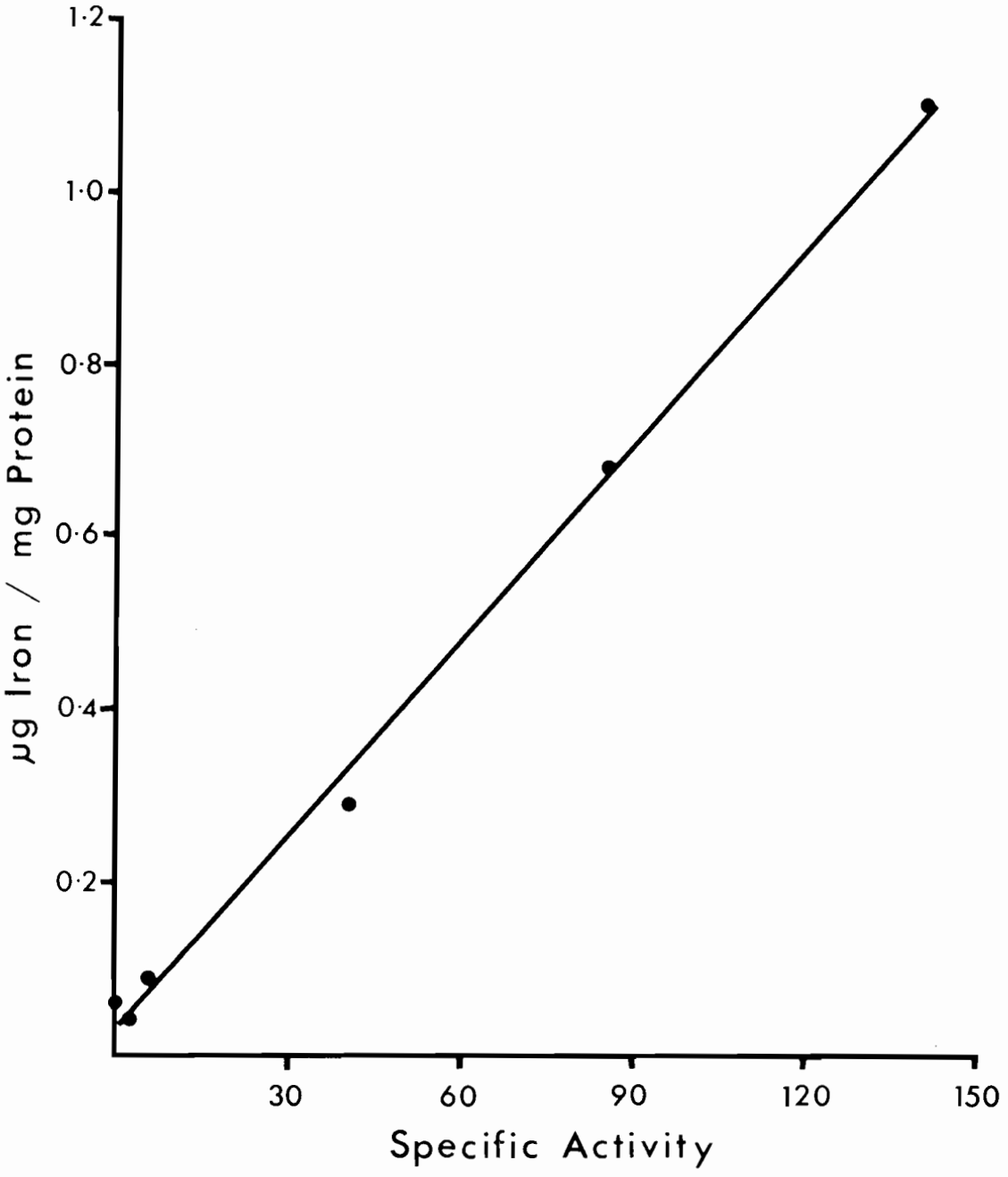

Fig. 11. Proportionality between iron content and specific activity during the purification of rabbit liver monoamine oxidase. Specific activity here is defined as the number of times the activity is increased over that of the initial.

7. Dialysis Against Chelating Agents

Dialysis against 8-hydroxyquinoline did not result in loss of activity, though this chelating agent was very effective in inhibiting the enzyme. But dialysis against diethyldithiocarbamate resulted in concomitant loss of activity, Table IX. Enzyme with a specific activity of 2740 was used. Of the enzyme solution, 5 ml was dialyzed for 20 hours against 200 ml of 0.05M phosphate buffer, pH 7.4, containing 0.01 or 0.1 M sodium diethylcithiocarbamate and then dialyzed for 20 hours against 2 litres of the buffer alone to remove the excess chelating agent. The fine yellow precipitate of the metal-diethyldithiocarbamate complex was removed by centrifugation at 14,000 x g for 30 minutes. Up to 60% of the activity was lost during dialysis against diethyldithiocarbamate. Attempts at restoration of the activity to the original with 1.67×10^{-5} M copper sulfate failed (Table IX), in fact copper sulfate inactivated the enzyme.

8. Effect of Copper-Deficiency on Amine Oxidases of Rat Plasma and Liver.

Young male albino rats, weighing about 40 gms. initially, were used in these experiments. In two experimental series they were fed a solid diet that was low in copper and animals were killed after 15, 28, 35 and 50 days (series 1), and after 43 and 47 days (series 2) for determination of the plasma and liver enzymes. In the third series, a liquid diet was used, animals being killed at 49 and 91 days. The solid

Table IX

Dialysis of rat liver monoamine oxidase

against chelating agent

The enzyme with specific activity 2740 was used. The enzyme solution 5 ml containing 6 mg of protein were dialyzed against 200 ml of 0.05M phosphate buffer, pH 7.4 containing 0.1M of diethyldithiocarbamate for 20 hours. Then redialyzed against 2 litres of 0.005M phosphate buffer, pH 7.4 for another 20 hours.

Concentration of chelating agent	Specific activity	Effect of copper sulfate*
	units/mg protein	activity/mg protein
None	2740	0
0.10	1069	0

* Incubation of dialyzed and non-dialyzed enzyme with 1.67×10^{-5} M copper sulfate.

diet, modelled after one of those used by Gray and Daniels (35) to obtain very low concentrations of hepatic copper, has been described under section "Methods".

The liquid milk diet consisted of commercial evaporated canned milk (Farmer's Wife brand, Number 2, partly skimmed milk, concentrated with vitamins C and D added, manufactured by Cow and Gate (Canada) Limited, Brockville, Ontario), diluted 1:1 (v/v) with glass-distilled water ("dietary milk"). The brand of milk used was selected from several that were analyzed in this laboratory for copper content. It contained less than 10 µg of copper per 100 ml of evaporated milk. This low content relative to other brands may be attributable to the fact that the liner of this variety was lacquered. All rats received a supplement of iron and manganese in the following amounts, added to the volume of milk they could be expected to drink in one day: 400 µg of iron (Fe^{++}) and 50 µg of manganese (Mn^{++}), as the sulfates. After three days, the animals were divided into deficient and control groups. The latter received the same diet as the deficient rats, but with the addition of 50 µg of copper, as the sulfate, to the above supplement.

The rats were killed by decapitation; blood was collected in ice-cooled test tubes containing heparin. The heparinized blood was then centrifuged at 14,000 x g for 15 minutes. The plasma was collected and used for the enzymic assays.

Table X

Copper content and amine oxidase activity of plasma and liver, and ceruloplasmin of copper-deficient rats

Series	Days on copper def.diet	Copper content				Amine oxidase activity				Ceruloplasmin[a]	
		Plasma[b]		Liver[c]		Plasma[d]		Liver			
		Control	Deficient	Control	Deficient	Control	Deficient	Control	Deficient	Control	Deficient
1	15									0.08	0.00,0.10
										0.26	0.15,0.13
	28			6.3	-	0.46	0.16				
	35	1.5	0.3	8.5	1.2	0.55	0.12	0.92[e]	0.76		
								0.95[d]	0.90		
	50			5.3	1.4	0.52	0.00	1.30[e]	1.20		
								1.45[d]	1.20		
2	43	1.5	1.3			0.58	0.42			0.08±0.02	0.02±0.01
	47	1.7	0.1			0.35	0.14				
3	49	1.5	0.4			0.51	0.18				
	91	1.5	0.4	5.3	1.6	0.45	0.09	1.40[e]	1.50		
	91			5.3	1.0	0.55	0.40	1.40[e]	1.30		

Table X (Footnotes)

[a] Activity given in absorbance units (Ravin, 1956). Individual values at 15 days; mean ± S.E. at 43 days for 10 control and 6 deficient rats.

[b] µg of copper per gm of fresh liver

[c] µg of copper per ml of plasma

[d] Substrate was benzylamine, 1.67×10^{-4}M (final concentration).

[e] Substrate was kynuramine, 6.7×10^{-5} M (final concentration).

The inorganic salts used in the dietary supplements were of the highest purity that was commercially available.

A summary of the results obtained with animals fed copper deficient diets is presented in Table X. In all three series the deficiency of dietary copper led to a distinct lowering of the concentration of copper in plasma and liver, as well as of the benzylamine oxidase activity of the plasma. In contrast to these findings the monoamine oxidase activity of the liver mitochondria ranged from 83-107% of the control activity in various stages of deficiency.

The ceruloplasmin (45) activity of rat plasma is variable and there was overlapping of the values for controls and deficient animals. However, at 43 days in series 2 the mean difference was statistically significant (P 0.05), thus confirming the establishment of the deficiency state by this time.

A single experiment in which copper sulfate (6.7×10^{-6}M) was added to "deficient" plasma (series 2, 43 days) indicated that no restoration of enzymic activity using benzylamine as substrate could be achieved in this way.

9. Effect of Pyridoxine-deficiency on Amine Oxidases of Rat Plasma and Liver

Rats were made pyridoxine-deficient as described under "Methods". Blood plasma was collected in similar fashion as in copper-deficiency and a similar study carried out with

Table XI

The effect of pyridoxine-deficiency on amine oxidases of plasma and liver

Days on pyridoxine-deficient diet	Plasma amine oxidase		Liver amine oxidase	
	Pyridoxine-supplemented	Deficient	Pyridoxine-supplemented	Deficient
28	0.36[a]	0.13 (36)[b]	0.24	0.25
42	0.42	0.10 (24)	-	-
60	0.42	0.08 (19)	-	-

[a] Substrate was benzylamine, 1.67×10^{-4}M (final concentration).

[b] Figures in parentheses represent per cent of control activity.

pyridoxine-deficient animals revealed, as shown in Table XI, a sharp decrease in amine oxidase activity of rat plasma, but no change in the monoamine oxidase of the liver. Although the latter activity has been determined only at 4 weeks of the deficiency, the results agree with those obtained earlier in this laboratory with rats fed pyridoxine-deficient diet for up to 7 weeks, and using tyramine and isoamylamine as substrates (46).

10. The Effect of Galactoflavin and Riboflavin-deficiency on Rat Mitochondrial Monoamine Oxidase.

If monoamine is in some way dependent upon adequate riboflavin nutrition of the animal, it would be expected that the enzyme prepared from the liver and brain of riboflavin-deficient rats would oxidise a smaller proportion of the substrate than would rats supplemented with the vitamin. The results of one set of experiments are shown in Table XII.

The enzyme prepared from the livers of galactoflavin treated rats showed significant reduction in activity after 14 days. There was no effect on the activity of brain monoamine oxidase. After 42 days of treatment with galactoflavin the liver enzyme had lost up to 81% of its activity in contrast to only a loss of 30% in brain. Though galactoflavin hastens the onset of riboflavin-deficiency, the results obtained are not significantly different from simple riboflavin deficiency (20).

Table XII

The effect of galactoflavin and riboflavin-deficiency on rat mitochondrial monoamine oxidase

Days on deficient diet	Liver		Brain	
	Supplemented	Deficient	Supplemented	Deficient
14	1.04*	0.70 (33)**	0.48	0.48 (0)
28	0.90	0.31 (66)	0.46	0.33 (28)
42	1.00	0.19 (81)	0.48	0.33 (31)

The deficient animals received a riboflavin-deficient diet which contained 2 gm/kg galactoflavin. The control animals received the same diet with a supplement of riboflavin, 30mg/kg diet. Animals were killed at intervals; livers and brains were removed and activities were determined.

* Activity is given in terms of increase in optical density at 315 mμ during the first 20 minutes of incubation with 6.7×10^{-5} kynuramine.

** Figures in the brackets represent per cent loss in activity.

The results obtained with riboflavin-deficiency had a number of features; (a) a limiting lower level below which enzyme activity will not fall on a riboflavin-free diet, (b) the same enzyme (in this case monoamine oxidase) may be more severely affected in one tissue than in another, and (c) depressed enzyme activities are not restored to normal by addition of the coenzymes in vitro, suggesting that the apoenzyme has been lost, Table XIII.

11. Isolation of Chromophore From Rat Liver Monoamine Oxidase

Treatment of the purified enzyme with 20% trichloroacetic acid resulted in the liberation of the chromophore. The method for the isolation of the chromophore is described under "Methods".

The absorption spectrum of the isolated chromophore was taken in 0.05M phosphate buffer, pH 7.4. The spectrum was very similar, in fact identical with that of authentic flavinadenine dinucleotide under the same experimental conditions (Figure 12). In 0.05M phosphate buffer, the isolated chromophore showed peaks at 265, 370 and 460 mμ similar to that of FAD.

12. Fluorescence Properties of Isolated Chromophore

The fluorescence spectrum of the isolated chromophore was measured in 0.05M phosphate buffer, pH 7.4 and the fluorescence spectra of isolated chromophore and flavinadenine dinucleo-

Table XIII

The effect of flavins on the activity of riboflavin-deficient rat liver monoamine oxidase

In all the experiments the beakers containing the enzyme and flavin (1.7×10^{-5} M) preparations were preincubated for 30 minutes at 37° C in a Dubnoff shaker before the addition of kynuramine (6.7×10^{-5} M). Blanks were prepared in the similar manner except that kynuramine was omitted.

	No Cofactor	FAD	FMN	Riboflavin
Control	0.98*	0.95	0.99	0.98
Deficient	0.30	0.30	0.32	0.30

* Optical density for the first 20 minutes incubation with the substrate.

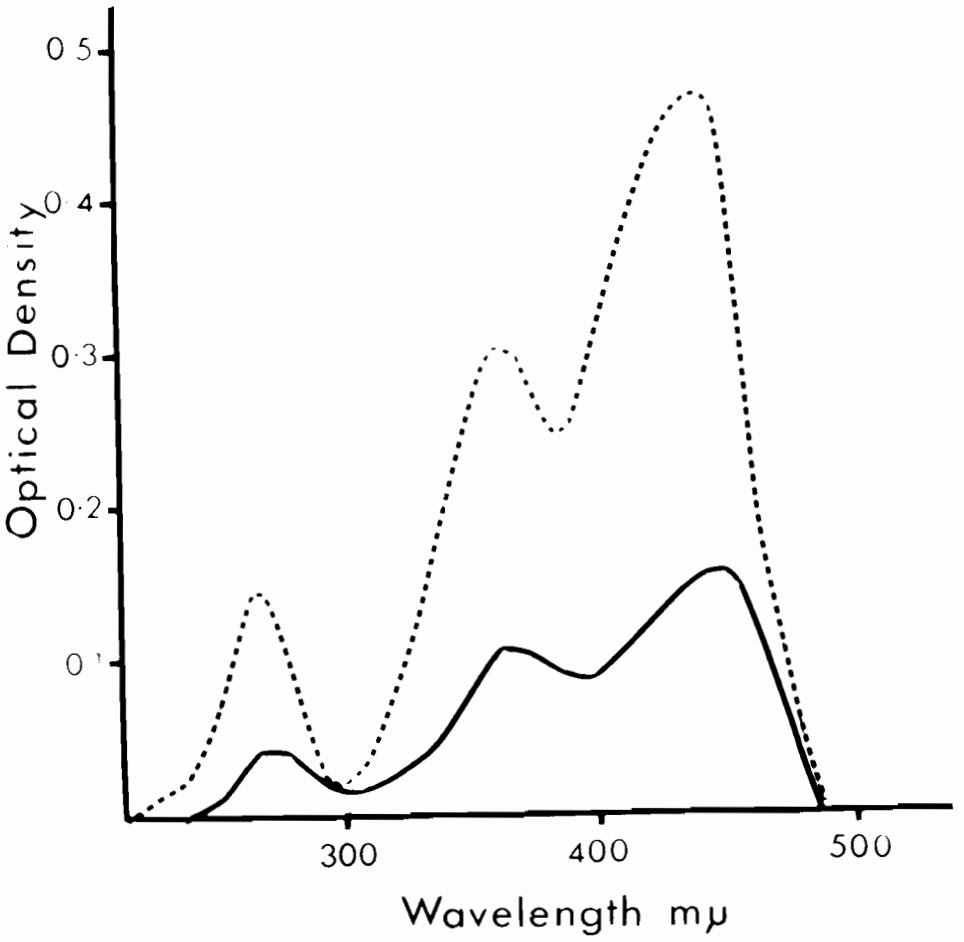

Fig. 12. Absorption spectra of the isolated chromophore (solid lines) and flavinadenine dinucleotide (broken lines) in 0.05 M phosphate buffer, pH 7.4.

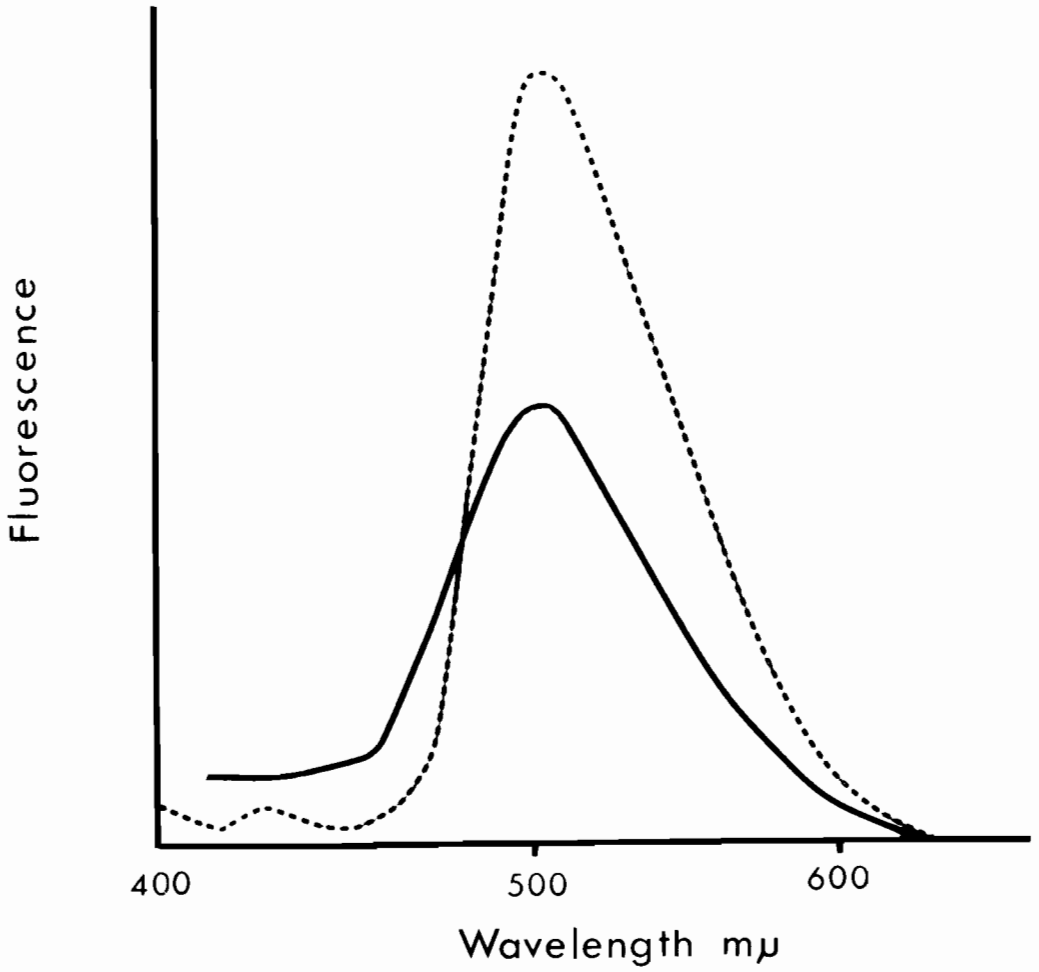

Fig. 13. Fluorescence emission spectra of the isolated chromophore (solid lines) and flavinadenine dinucleotide (broken lines). The spectra were taken at pH 7.4 in 0.05 M phosphate buffer. The excitation wavelengths were 455 mμ for the chromophore and 460 mμ for flavinadenine dinucleotide.

tide are shown in Figure 13. The spectrum is very similar to that of pure flavinadeninedinucleotide under the same experimental conditions. The fluorescence spectrum of the isolated chromophore can be described as showing maximal excitation at 455 mμ and maximal emission at 515 mμ ; similar values for flavinadenine dinucleotide are maximal excitation at 460 mμ and maximal emission at 520 mμ .

13. Effect of Thiol Compounds on Rat Liver Monoamine Oxidase

-SH compounds have already been shown to inhibit monoamine oxidase (47, 48, 49). The present study not only confirms those results but also shows that the purified enzyme is even more sensitive to these compounds. The results of these experiments are shown in Table XIV. Of the metals studied, copper sulfate is the most interesting since this metal compound inhibits the enzyme by 100 per cent at 6.7×10^{-6} M. BAL, which in usual reaction mixture undergoes autoxidation, inhibited amine oxidase activity.

All thiol compounds exerted inhibitory effect on rat-liver monoamine oxidase.

14. The Effect of Chelating Agents and Inhibitors on the Rat and Rabbit Monoamine Oxidase at Various pH's

The purpose of the experiments described in this section is to compare the properties of rat and rabbit monoamine oxidase. Previous studies (20, 23) with rat liver monoamine oxidase have

Table XIV

The effect of -SH compounds and metals on the activity of purified rat liver monoamine oxidase

Enzyme with a specific activity of 1255 (96-fold purified) was used.

-SH compounds or salt*	Activity	% Inhibition
Control	553	-
Mercuric acetate	85	85
Silver nitrate	190	65
Cadmium chloride	267	52
Copper sulfate (a) 1.67×10^{-4}M	0	100
(b) 6.7×10^{-6}M	0	100
2,3-dimercaptopropanol,(BAL)	155	72
Thioglycollic acid	205	63
p-Chloromercurobenzoic acid	59	89

* All flasks were preincubated for 20 minutes with 1.67×10^{-4}M -SH compound before the addition of kynuramine, 6.7×10^{-5} M final concentration.

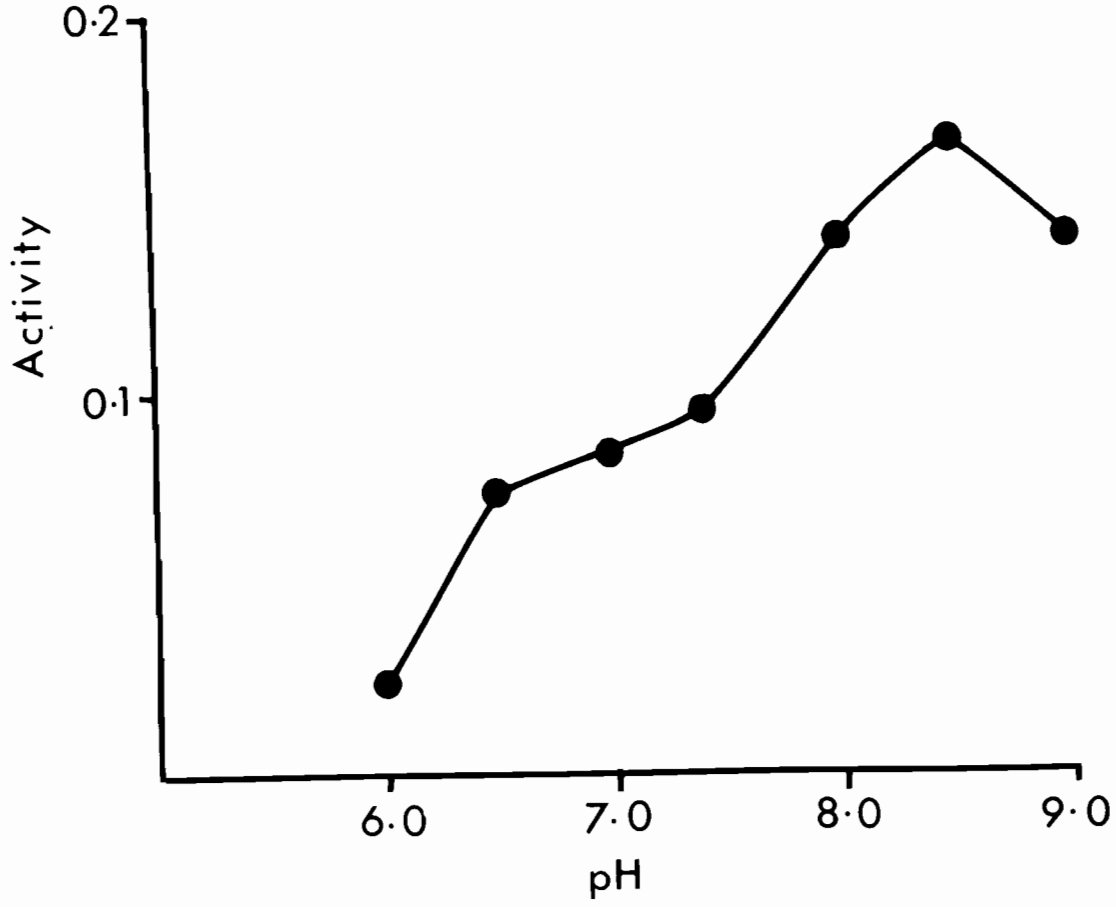

Fig. 14. The activity-pH curve of rat liver monoamine oxidase using kynuramine 6.7 x 10^{-5} M as substrate.

shown that the activity-pH curve has a shoulder at pH 6.5-7.0 and a peak at about pH 8.5, but the partially heat inactivated enzyme shows two optima, at pH's 6.5 and 7.4. Studies with chelating agents and inhibitors of monoamine oxidase on the two optima have led to that conclusion that there exists more than one monoamine oxidase in the mitochondrial membrane.

Rat and rabbit liver monoamine oxidase were solubilized and partially purified as described earlier. Their activity-pH is shown in Figures 14 and 15, using kynuramine 6.7×10^{-5} M as substrate. The rat monoamine oxidase showed a shoulder at pH 6.5-7.4 and a peak at pH 8.5 while the enzyme from rabbit showed a single optimum at pH 8.0. Using benzylamine 1.6×10^{-3} M as substrate, the rabbit enzyme showed similar activity-pH curve with the optimum between pH's 7.4 and 8.0 (Fig. 15 B).

The effect of chelating agents on the activity of rat and rabbit monoamine oxidase at different pH's are shown in Tables XV and XVI. A number of chelating agents such as thenoyltrifluoroacetone, 2, 2-dipyridyl, 8-hydroxyquinoline and o-phenanthroline were used. With the rat liver monoamine oxidase a greater inhibition was obtained at pH's 6.5 and 7.0 than at pH 8.5, Table XV. At a concentration of 1.7×10^{-4} M 8-hydroxyquinoline had an inhibitory effect of 88%, 82% and 28% at pH's 6.5, 7.0 and 8.1. o-Phenanthroline had a similar effect at the same concentration, Figure 16.

In contrast to rat monoamine oxidase, the inhibition produced by chelating agents on the rabbit enzyme were similar

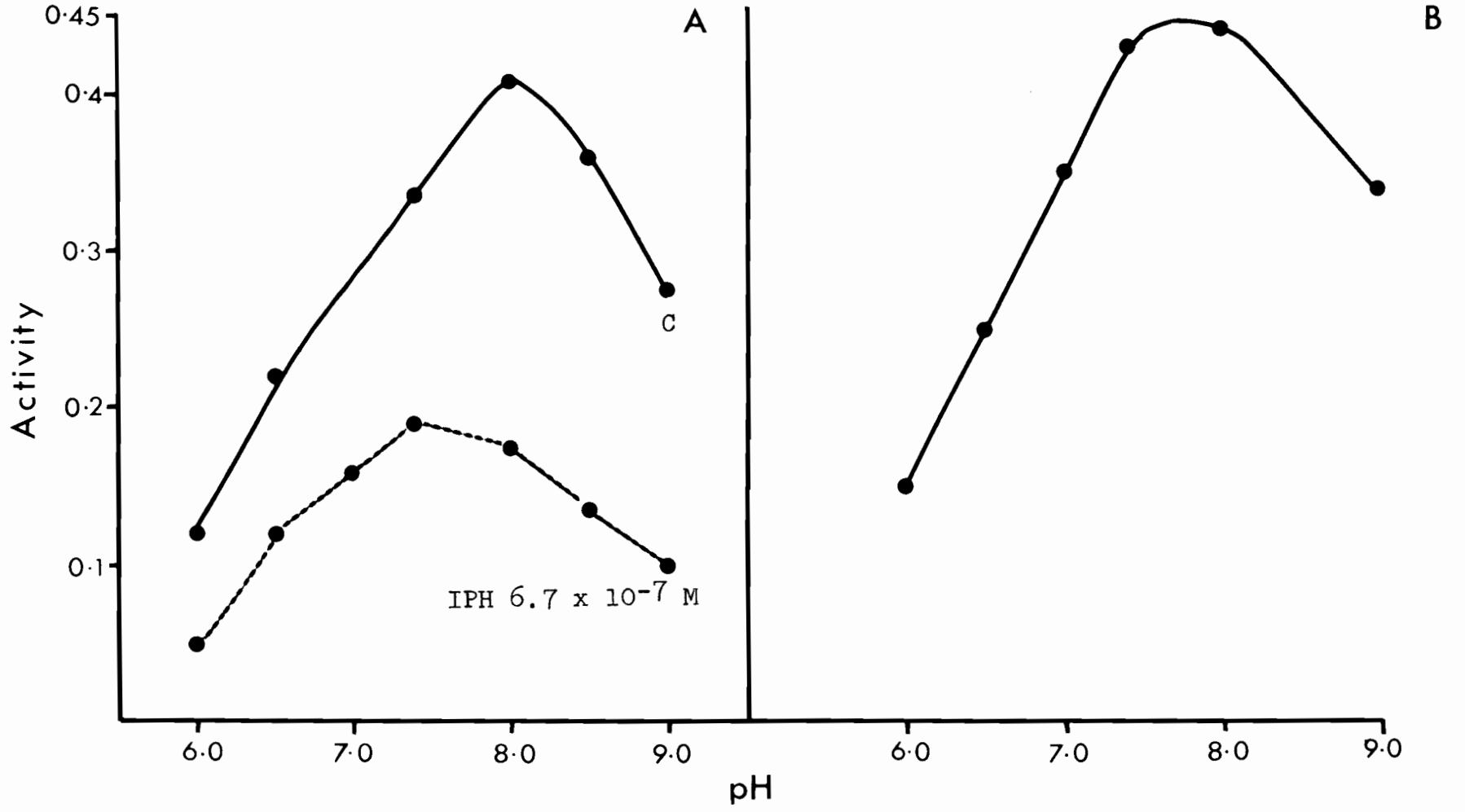

Fig. 15. A. Activity-pH curve of rabbit monoamine oxidase with (IPH) and without (C) 2-phenylisopropylhydrazine, using kynuramine, 6.7 x 10^{-5} M as substrate.

B. Activity-pH curve using 1.6 x 10^{-3} M benzylamine as substrate.

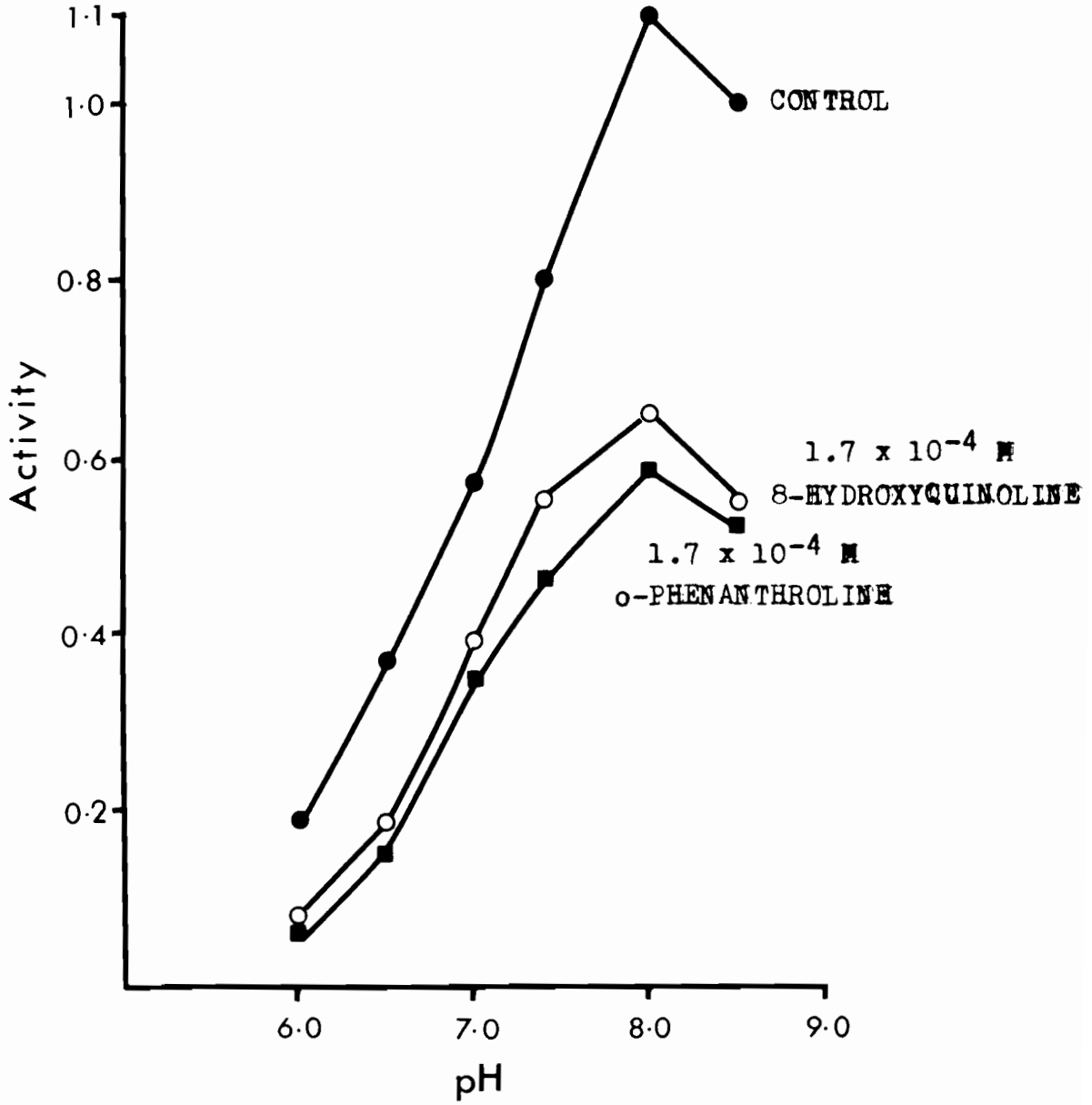

Fig. 16. Activity-pH curve of purified rabbit liver monoamine oxidase with and without chelating agents.

at pH's 6.5, 7.0 and 8.0. 8-Hydroxyquinoline at a concentration of 1.7×10^{-4} M was the most effective with inhibitions of 59%, 42% and 45% at pH's 6.5, 7.0 and 8.0 respectively, Table XVI. Thenoyltrifluoroacetone was the least effective. The effect of 8-hydroxyquinoline and o-phenanthroline on the activity-pH curve of rabbit liver monoamine oxidase is shown in Figure 17.

15. The Effect of Inhibitors of Monoamine Oxidase

Phenylcyclopropylamine (tranylcypromine, Parnate), a non-hydrazine inhibitor of monoamine oxidase inhibited the rat liver enzyme at all pH's, but to different extents. At a concentration of 1.7×10^{-7} M there was a rapid fall in activity at the higher pH's, Table XVII. This resulted in the appearance of a second peak at pH 6.5 and a broad peak at pH's 8.0-8.5. Inhibition of monoamine oxidase by a hydrazine derivative, 2 -phenylisopropylhydrazine, was similar to that of phenylcyclopropylamine. There was a much greater inhibition on the alkaline side of the pH, Table XVII and Figure 18.

The inhibition produced by phenylcyclopropylamine and 2 -phenylisopropylhydrazine on the rabbit monoamine oxidase was different. These inhibitors inhibited the enzyme at all pH's to the same extent, Table XVII and Figure 18. Unlike that of rat monoamine oxidase, the activity-pH curve of inhibited enzyme resembled that of the control, Figure 15 A.

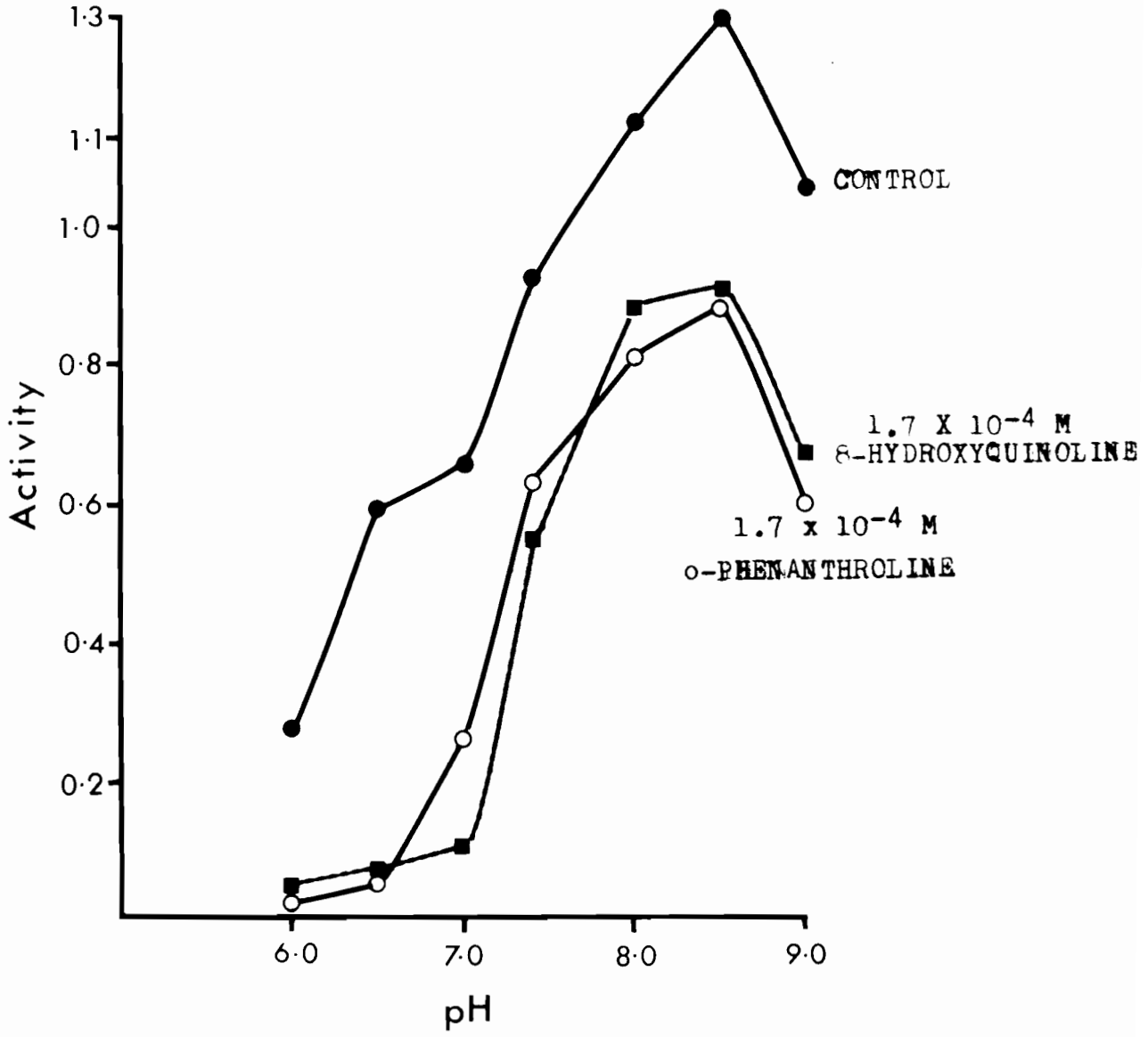

Fig. 17. Activity-pH curve of purified rat liver monoamine oxidase with and without chelating agents.

Table XV

The effect of chelating agents on the activity-pH curve of rat liver monoamine oxidase

In all the experiments the beakers containing the enzyme with specific activity 1255, and chelating agent (1.7×10^{-4}M) preparation were preincubated for 20 minutes at 37 C in a Dubnoff shaker before the addition of kynuramine (6.7×10^{-5}M). Blanks were prepared in the similar manner except that kynuramine was omitted.

Chelating Agents	Control pH 6.5	7.0	8.5	Experimental 6.5	7.0	8.5
Thenoyltrifluoro-acetone	0.59[a]	0.65	1.30	0.09(85)[b]	0.18(72)	1.05(19)
2'2,dipyridyl	0.59	0.65	1.30	0.05(92)	0.15(77)	0.72(45)
8-hydroxy-quinoline	0.57	0.62	1.25	0.07(88)	0.11(82)	0.90(28)
O-phenanthroline	0.57	0.62	1.25	0.05(91)	0.12(81)	0.89(29)

[a]Optical density for the first 20 minutes incubation with the substrate.

[b]Figures in parentheses represent per cent inhibition.

Table XVI

The effect of chelating agents on the activity-pH curve of rabbit liver monoamine oxidase

The experimental procedure was the same as in Table XV

Chelating Agents	Control pH 6.5	7.0	8.0	Experimental 6.5	7.0	8.0
Thenoyltrifluoro-acetone	0.33[a]	0.56	1.05	0.31(6)[b]	0.50(11)	1.0 (5)
2'2,dipyridyl	0.33	0.56	1.05	0.28(15)	0.45(20)	0.85(19)
8-hydroxy-quinoline	0.37	0.58	1.10	0.15(59)	0.34(42)	0.60(45)
o-phenanthroline	0.37	0.58	1.10	0.19(49)	0.39(33)	0.65(40)

[a]Optical density for the first 20 minutes incubation with the substrate.

[b]Figures in parentheses represent per cent inhibition.

Table XVII

The effect of tranylcypromine and 2-phenylisopropylhydrazine on the activity-pH curve of rat and rabbit liver monoamine oxidase

The experimental procedure was the same as in Table XV

pH	Rat			Rabbit	
	Control	TCP	IPH	Control	TCP
5.8	0.30[a]	0.25 (17)[b]	0.27 (10)	0.19	0.12 (37)
6.5	0.60	0.45 (25)	0.49 (18)	0.33	0.20 (39)
7.0	0.67	0.28 (58)	0.31 (54)	0.56	0.35 (37)
7.4	0.88	0.32 (64)	0.25 (72)	0.77	0.50 (35)
8.0	1.10	0.35 (68)	0.20 (82)	1.05	0.65 (38)
8.5	1.15	0.35 (70)	0.17 (85)	1.00	0.43 (57)
9.0	1.00	0.19 (19)	0.10 (90)		

TCP, tranylcypromine, was used at a final concentration of 1.7×10^{-7} M and IPH, 2-phenylisopropylhydrazine, at 6.7×10^{-7} M.

[a]Optical density for the first 20 minutes incubation with the substrate.

[b]Figures in parentheses represent per cent inhibition.

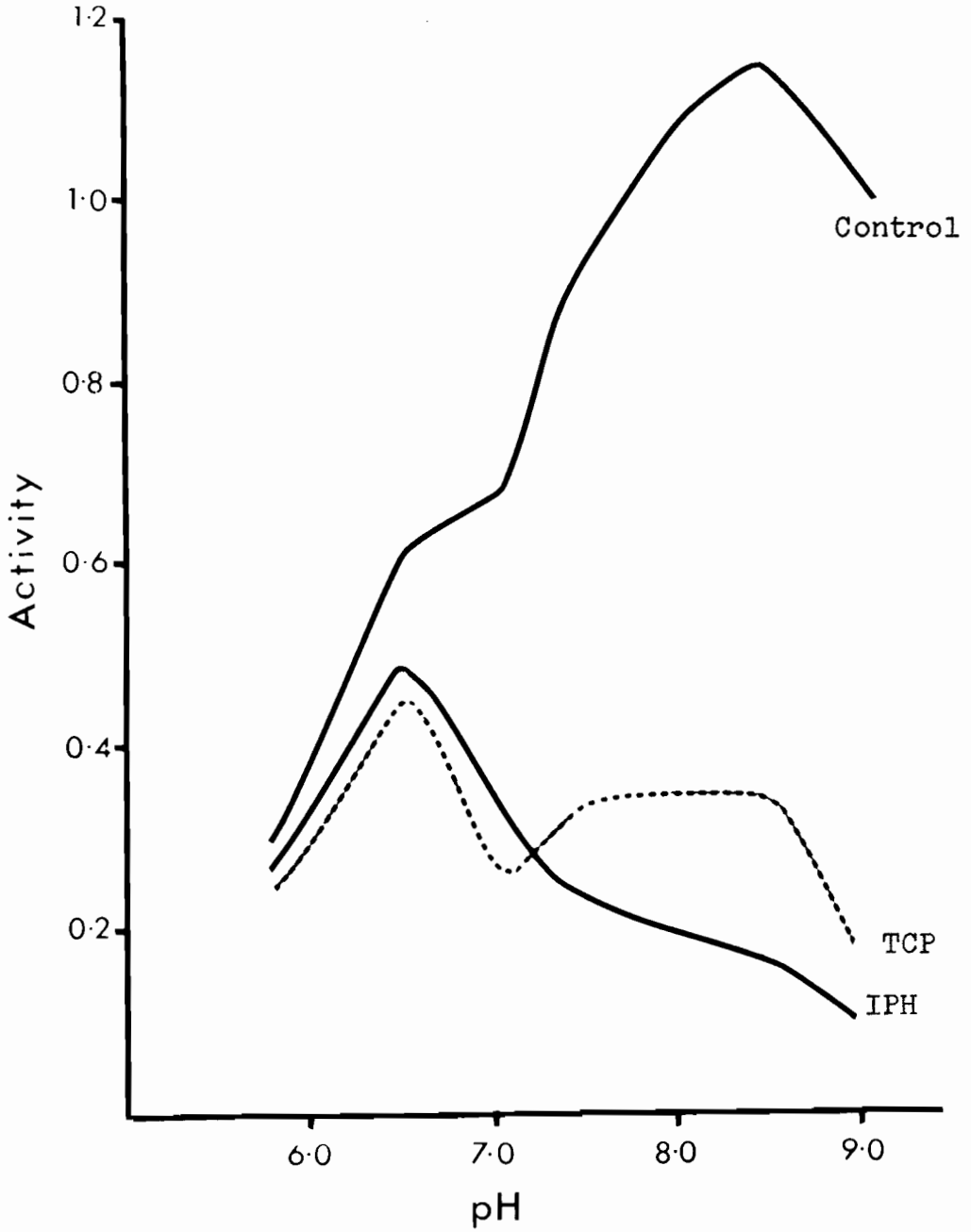

Fig. 18. The effect of monoamine oxidase inhibitors, tranylcypromine (TCP) 1.7 x 10^{-7} M and 2-phenylisopropylhydrazine (IPH) 6.7 x 10^{-7} M final concentration on activity-pH curve of purified rat liver monoamine oxidase.

16. The Effect of pH and Thermal Inactivation on Rabbit Liver Monoamine Oxidase

The mitochondrial preparation, contained in thin uniform walled test-tubes stoppered with rubber bungs, was immersed in a constant temperature bath, using a "Bronwill" Constant Temperature Circulator. To study the effect of thermal inactivation of at different pH's, the enzyme and buffer (phosphate buffer, 0.066M final concentration, pH's 5.8-7.4 and borate buffer the same concentration, pH 7.4-9.0) in a ratio of 1:2 were heated at 50 C for 30 minutes.

The activity-pH curve of the native enzyme preparation using kynuramine as substrate, 1.6×10^{-4} M, showed a bell-shaped curve with a peak at pH 8.0, while that of heated preparation showed reduced activity at all pH's with a peak at pH 7.4, Figure 19.

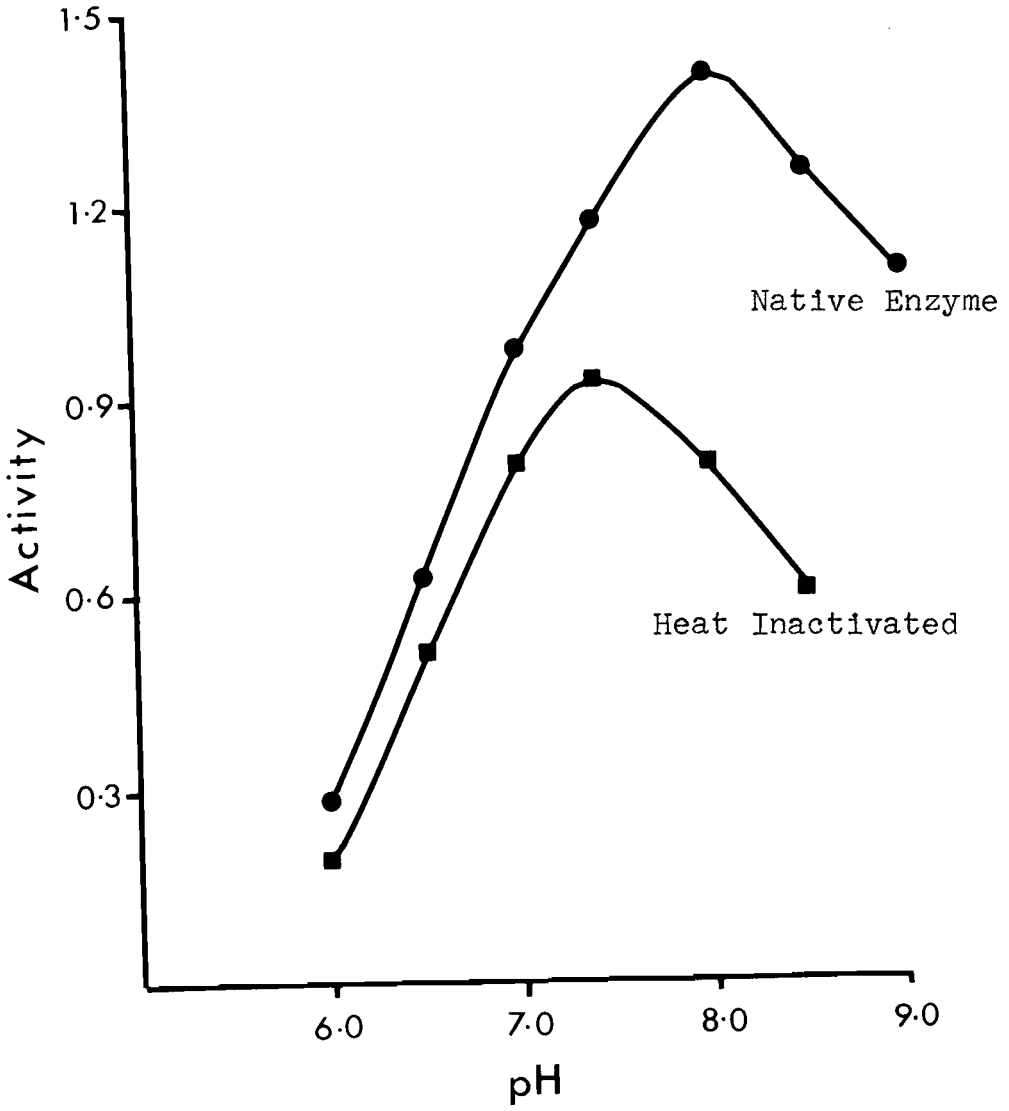

Fig. 19. Activity-pH curve of native and partially heat inactivated (50°, 30 mins.) using kynuramine 1.6×10^{-4} M as substrate.

IV. DISCUSSION

Most of the investigators (29, 30, 32, 33) who have reported on the solubilization of the enzyme have used a non-ionic detergent to solubilize the enzyme; however, when the enzyme was precipitated with ammonium sulfate, it could not be dissolved. In this work, I have aimed at obtaining preparations of monoamine oxidase that would not deposit the enzyme from solution after centrifugation at 160,000 x g for at least 90 minutes and which are readily soluble in aqueous media without the use of non-ionic dispersing agents, after precipitation with substances (e.g. ammonium sulfate) which do not inactivate the enzyme.

The purest preparations of liver monoamine oxidase hitherto reported were made by Guha and Krishna Murti (32) by sonification and fractionation on DEAE-cellulose columns. The method reported gave a 350-fold purification of the enzyme. Attempts at purification by the above method have failed in this laboratory. The method now described, in this thesis, involving sonification and chromatographic fractionation on Sephadex G-200 gel-filtration, DEAE-Sephadex and hydroxylapatite, yields pure and stable preparations of monoamine oxidase. Rat liver monoamine oxidase was purified 208-fold, over that of the crude mitochondrial preparation, while with rabbit and monkey liver enzymes 140- and 56-fold respectively were obtained.

Exact comparison of activities with other preparations (31-34) is rarely possible because different methods are used to express specific activities, substrates used are different and the source of the enzymes are different. Nara et al.(34) have reported that their enzyme had a specific activity of 3400 using benzylamine as substrate and beef liver enzyme. In my preparation, rat liver monoamine oxidase had a specific activity of 2850 using kynuramine as substrate. This may suggest that beef enzyme is more active than rat.

The results of chromatographic experiments with Sephadex G-200 gel-filtration, DEAE-Sephadex, hydroxylapatite and ultracentrifugation revealed that the partially purified and pure preparations of liver monoamine oxidase can not be resolved into more than one discrete, enzymatically active fraction. Though Gorkin (30) using "solubilized" rat liver monoamine oxidase on a "Brushite" column has been able to obtain two amine oxidases capable of oxidizing two different amines, further experiments are required to clarify this property.

Spectrophotometric studies of the final enzyme preparations suggest that the enzyme is yellow; the intensity of the band with a maximum at 410 mμ is related to the concentration of enzyme in these preparations. The yellow compound is partially bleached and the activity of the enzyme is easily reduced by sodium dithionite or sodium borohydride and the

reduced form is auto-oxidizable; it may function as a hydrogen carrier in the enzyme-catalyzed reactions and may be involved in the catalytic activity of the enzyme.

The difference spectrum (oxidized minus reduced spectrum) shows two maxima, one at 405 mμ and 460 mμ, which correspond to the maximum of flavin nucleotide. Extraction of yellow compound for flavin by the method of King et al. (39) revealed the presence of a flavin whose absorption spectra and fluorescence emission spectra ressembled that of flavinadenine dinucleotide. The native enzyme does not fluoresce. Nara et al. (34) have reported very similar results. To obtain the chromophore they have extracted their enzyme preparation with trichloroacetic acid and then digested it with trypsin. The flavin may be bound by a covalent bond to the protein. This may be why it is so extremely difficult to liberate it. Nara's preparations contain 1 mole of flavinadenine dinucleotide per 200,000 gm of protein. We have obtained small amounts of flavinadenine dinucleotide in the purest preparations. Guha (50) has suggested that the enzyme contains pyridoxal phosphate on the basis that the enzyme absorbs maximally at 410 mμ and reacts with hydrazine. This can hardly be acceptable since carbonyl reagents such as semicarbazide hydrazine and hydroxylamine do not inhibit the enzyme (31) and enzyme obtained from livers of rats put on pyridoxine deficient diet do not show a drop in activity (46). But rat plasma amine oxidase provides data that parallel those

of Yamada and Yasunobu (13, 26), Buffoni and Blaschko (12) and Blaschko et al.(51) showing that amine oxidase of beef and pig plasma are pyridoxal phosphate dependent enzyme. The absorption spectra of my rat and rabbit enzyme preparations are not typical of an enzyme containing flavinadenine dinucleotide in its prosthetic group. Enzymes which contain flavinadenine dinucleotide in their prosthetic groups show the characteristic absorption bands of this compound, whose maxima at 375 and 450 mμ are usually shifted some 5-20 mμ, generally towards longer wavelengths, by combination with the apoenzymes. Thus sarcosine oxidase (52) absorbs at 410 mμ and 440-480 mμ, 377 and 455 mμ with glucose oxidase (notatin) (53) and at 389 and 465 mμ with L-amino acid oxidase of mocassin venom (54). For my enzyme preparations the maxima for flavin becomes apparent when one studies the difference spectrum (oxidised minus reduced). The maximum at 410 mμ is shifted towards longer wave length in the presence of hydrosulfite.

Further evidence for the role of flavin in monoamine oxidase is given by the effect of galactoflavin, a known anti-metabolite of riboflavin, on the activity of the enzyme. It is already known that enzyme obtained from the livers of rats put on riboflavin-deficient diet showed a substantial decrease in activity (20). In the experiments described under "Results" rats were given galactoflavin in their daily diet. Though galactoflavin caused a decrease in enzymic activity it could

not completely inactivate the enzyme. One explanation may be that galactoflavin could not deplete all the stores of flavins. It is interesting that galactoflavin had a greater inhibitory effect on the activity of liver monoamine oxidase than the brain. Depressed enzyme activities are not restored to normal by the addition of coenzymes, flavinadenine dinucleotide, or riboflavin 5-phosphate or riboflavin *in vitro*, suggesting that the apoenzyme has been lost. Hawkins (18) in her studies with riboflavin-deficient rats, has suggested that the vitamin may be contained in enzyme protein. With inositol present in the diet, riboflavin was more effective in restoring amine oxidase activity. There was no rapid restoration of the enzymic activity after the rats received riboflavin. Hawkins (18) has further suggested that inositol is required either for the synthesis of a prosthetic group, or of a new enzyme protein.

The classification by Nara (34) of rat liver monoamine oxidase as a flavoprotein has been on the colour of purified preparations and on the difference absorption spectrum and the fluorescence spectra of the isolated chromophore. The enzyme however has not been resolved into apoenzyme and flavin. The precise nature of the flavin component is unknown. The work of Nara *et al.* (34) has shown that most of the flavin of beef-liver monoamine oxidase is liberated only after tryptic digestion, suggesting that the flavin may be covalently bound to the enzyme and is not released from the enzyme even by trichloroacetic acid.

Inhibition by chelating agents suggests the presence of a metal in the enzyme. Gorkin (22), studying the effect of chelating agents on the enzyme, came to the conclusion that the enzyme had a metal as a prosthetic group. He was able to antagonise the effect of inhibition produced by chelating agents with metal ions. He was unable to reach a definite conclusion as to which metal is involved in monoamine oxidase of rat liver. Lagnado and Sourkes (55) have studied the effect of various metal ions on the activity of monoamine oxidase. With some metals, at high concentration, the enzyme was inhibited and while with other metals at lower concentration, monoamine oxidase activity was manifested. Green (25) with his studies on the mechanism of inhibition of monoamine oxidase by hydrazine inhibitors, that monoamine oxidase is a copper containing enzyme. His theory is based upon the assumption that inhibition by hydrazine derivatives results from a copper-catalyzed liberation of free radicals in the vicinity of the enzyme's active centre. The theory that hydrazine inhibitors react with a metal is further strengthened by the result of Gorkin (56) that when tyramine is used as the substrate, the irreversible inhibition of monoamine oxidase activity by iproniazid can be prevented by pretreatment of the enzyme with the chelating agent apteamine.

The present results with copper-deficiency show that the plasma amine oxidase of the rat is , like that of pig (51),

dependent upon a dietary source of copper for its activity. On the other hand, the stability of rat liver monoamine oxidase activity during the time that the plasma activity had fallen to clearly subnormal values indicate that this enzyme in the mitochondria of rat liver either does not depend upon copper nutrition or, if it is a cupro-protein, holds its copper very tenaciously.

The data shown in Tables X, XI and XII demonstrate the ability of experiments using nutritionally deficient diets, as well as purely kinetic studies (Sakamato et al. 31), to provide a clear differentiation between two enzymes performing ostensibly the same function, although with somewhat different spectra of substrates. The evidence does not exclude roles for either copper or pyridoxal phosphate in the activity of mitochondrial monoamine oxidase but makes such roles less likely. The decisive data will come with the purification of the enzyme so that its chemical and functional properties can be studied in association with one another.

The essential role of copper in other amine oxidases (10, 11, 12, 13, 16) suggests that this metal may be important in the function of the present enzyme. In fact small amounts of copper, as well as iron, have been found in the purest preparation. This is offset by another observation that under conditions of copper-deficiency in the rat the activities of plasma amine oxidase and of ceruloplasmin (p-phenylenediamine

oxidation) drop rapidly to low levels whereas mitochondrial monoamine oxidase activity remains at 83-107% of controls. According to Nara et al.(34), this enzyme, prepared from beef liver, is a copper-flavoprotein. One must consider the fact that the present method of preparation not only yields a considerably more purified enzyme than hitherto reported but contains only 0.034% of copper. This is less than the content reported by Yasunobu (27, 57).

Because of the presumed sulphydryl "active group" in the enzyme, Lagnado and Sourkes (55) have investigated the actions of some -SH compounds on mitochondrial preparations of monoamine oxidase. They reported inhibition of monoamine oxidase by a number of sulfhydryl compounds such as dimercaprol, thioglycollic acid, cysteine and cystine. They confirm the suggestion of Friedenwald and Herrmann (58) that the enzyme possesses an -SH group which functions in the oxidation of the substrate and that an excess of some -SH compound or of a disulfide could inhibit monoamine oxidase at one stage of the catalytic process. One of the aims of the studies with the purified enzyme was to survey the effect of various metal and -SH compounds on the enzymic activity. The results obtained, as summarized in Table XIV, indicated that the enzyme is inhibited by a number of heavy metals such as mercury, silver, cadmium and copper sulfate and by -SH compounds. Inhibition produced by these compounds are greater than reported previously (55). Mercury, cadmium and silver are all known to be capable

of inhibiting a number of enzymes having functional -SH groups (59). The results with copper sulfate are interesting. Firstly, they confirm the findings of Bernheim (60) and Perry(61) and Lagnado (55) who suggested that this metal is an -SH inhibitor. Secondly, a much lower concentration of copper sulfate than that reported by Lagnado (55) is needed to produce inhibition. Thirdly, Nara et al. (34) and Yasunobu (27) have reported that the enzyme is a copper-flavoprotein.

The inhibitory action of the -SH compounds 2,3-dimercaptopropanol, thioglycollic acid, p-chloromercurobenzoic acid tested on rat liver monoamine oxidase is consistent with the previous concept that this enzyme possesses a sulfhydryl group capable of functioning reversibly in an oxidation-reduction process.

Horita (62), studying the influence of pH on serotonin metabolism in various tissue homogenates, has indicated the presence of at least two enzyme systems in the heart tissue of rat which are capable of metabolizing serotonin. In his studies with inhibitors, he found that KCN inhibited the heart enzyme at pH 9.5 while phenylisopropylhydrazine inhibited the enzyme at the physiological, pH 7.4. In my own studies (M.Sc. thesis, 20), I found similar results with the rat liver mitochondrial monoamine oxidase using kynuramine as substrate. Phenylcyclopropylamine, phenylisopropylhydrazine and isproniazid inhibited the enzyme at pH 7.4 and 8.1 but there was

little inhibition at pH 6.5. The opposite effect was obtained with chelating agent and pargyline (a non-hydrazine inhibitor of monoamine oxidase), i.e. there was a greater degree of inhibition at pH 6.5 than at pH's 7.4 or 8.1. Furthermore the activity-pH curve of partially heat-inactivated enzyme showed two optima, one at pH 6.5 and the other at pH 7.4. Studies with inhibitors and chelating agents on the activity-pH curve of partially heat-inactivated enzyme showed similar results as described for the native enzyme. Whatever the explanation, it is clear from these studies that at pH levels of 6.5 and 8.1, rat liver mitochondrial contains two systems which can metabolize kynuramine.

With the solubilization and purification of monoamine oxidase from rat and rabbit liver, it was of interest to see first, whether the rat enzyme would behave the same as to the effect of inhibitors and chelating agents on its activity-pH curve and second, whether the rabbit enzyme shows the same properties. In my studies with the purified rat monoamine oxidase I have obtained results which confirm the earlier work on the activity-pH curve. Purified rat liver monoamine oxidase still showed a shoulder at pH 6.5-7.0 and a peak at about pH 8.5 (Figure 14). Unlike the rat enzyme, the rabbit monoamine oxidase did not show a shoulder at pH 6.5-7.0 and had a peak at pH 8.0 **using** kynuramine as substrate (Figure 15). Partially heat-inactivated enzyme from the rabbit showed

only one optimum at pH 7.4. This is unlike the rat enzyme (20) which showed two optima at pH 6.5 and pH 7.4. Further evidence that the enzyme from rat and rabbit livers show species differences can be judged from the differences in the shape of the activity-pH curves and the effects of phenylcyclopropylamine and phenylisopropyl hydrazine and of chelating agents for the two types of preparation. This difference may be more apparent than real, because the rat liver "enzyme" may consist of more than one species. Evidence of which is slowly accumulating (20, 63-68). Nagatsu (68) has shown in the course of studies on heat stability of rat liver monoamine oxidase, using serotonin as substrate, that the activity of disappearance of serotonin by rat liver homogenate remained after 10 minutes heating at 60 C. However, properties of this activity differed from those of fresh homogenate, especially in the fact that it could not be inhibited by excess monoamine oxidase inhibitors. Further purification and studies of the enzyme will be important for the resolution of multiplicity of mitochondrial monoamine oxidase.

V SUMMARY

In order to investigate the properties of rat liver monoamine oxidase, a method was devised which would result in solubilization and purification of the enzyme from mitochondria. This method has also been applied for the purification of rabbit and monkey liver mitochondrial monoamine oxidase.

Liver mitochondrial monoamine oxidase was purified up to 208-fold in the case of rat, and 140 and 56-fold in the case of rabbit and monkey, respectively, by a procedure involving distruption of mitochondrial by sonic oscillation and cholic acid treatment, ammonium sulfate fractionation, Sephadex gel-filtration, chromatography on DEAE-Sephadex and hydroxylapatite. The procedure yielded preparations of enzyme which were soluble; which could not be sedimented by centrifugation at 160,000 x g for 90 minutes, and which are readily soluble in aqueous media without the use of non-ionic dispersing agents, after precipitation with substances (e.g. ammonium sulfate) which do not inactivate the enzyme.

Iron and copper-chelating agents inhibited the enzyme. The metal analysis of the purified preparations of the enzyme indicated that the rat liver monoamine oxidase contained 0.12% iron and 0.034% copper. Rats made copper-deficient did not show a decrease in liver monoamine oxidase activity, while the activity of plasma monoamine oxidase decreased sharply.

Studies with riboflavin-deficiency in rat and the

feeding of galactoflavin, on the enzyme activity indicated that riboflavin is necessary for the activity of the enzyme, though the enzyme has not been separated into apoenzyme and cofactor. Small amounts of flavin have been found in the purest preparation of rat liver enzyme.

The molecular weights of rat and rabbit liver monoamine oxidase as determined by Sephadex gel-filtration are put at 290,000 and 260,000 respectively. Ultracentrifugation of purified rat liver monoamine oxidase gave a sedimentation coefficient of 6.51×10^{-3}S.

The enzyme is inhibited by -SH compounds indicating the essential role of -SH group for its activity.

Studies on the effect of inhibitors and heat on the activity-pH curves of rat and rabbit monoamine oxidase indicate that, while in the rat enzyme, there are two systems involved in the oxidation of substrate, the rabbit enzyme does not show this property.

VI. CLAIMS TO ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

1. Rat and rabbit liver monoamine oxidases have been solubilized and purified using a combination of sonic oscillation and cholic acid, chromatography on Sephadex gel-filtration, DEAE-Sephadex and hydroxylapatite. Up to 25 per cent of the original activity is recovered by this method. Monkey liver monoamine oxidase has been partially purified.

2. Small amounts of copper (0.034%) as well as iron (0.12%) have been found in the purest preparation of rat liver monoamine oxidase.

3. Extraction of the purified rat liver monoamine oxidase by trichloracetic acid revealed the presence of a chromophore identical to flavinadenine dinucleotide in respect to absorption and fluorescence spectra. The native enzyme does not fluoresce. The difference in absorption spectra (oxidized minus reduced) of native enzyme revealed two peaks at 405 mμ and 460 mμ.

4. The molecular weights of the enzyme prepared from rat and rabbit liver by gel-filtration are put at 290,000 and 260,000. The sedimentation coefficient of rat liver monoamine oxidase by ultracentrifugation is put at 6.51×10^{-13} S.

5. Copper-deficiency in rats substantially reduced plasma amine oxidase activity, whereas no change was observed on the activity of liver monoamine oxidase.

6. Rat and rabbit liver monoamine oxidases were found to be different in respect to activity-pH curve and the effect of heat and inhibitors on their activity-pH curve.

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