Mitochondrial Monoamine Oxidase

I. PURIFICATION AND CHARACTERIZATION OF THE BOVINE KIDNEY ENZYME*

(Received for publication, March 27, 1967)

V. GENE ERWIN‡ AND LESLIE HELLERMAN

From the Department of Physiological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

SUMMARY

Monoamine oxidase of bovine kidney mitochondria has been purified to high specific activity by solubilization with digitonin, ammonium sulfate fractionation, and calcium phosphate gel-cellulose column chromatography.

Absorption and fluorescence spectra indicated strongly that mitochondrial monoamine oxidase is a flavoenzyme. Under anaerobic conditions, benzylamine reduced the flavin at 460 mp apparently to 75% of the total reduction observed with sodium dithionite. The enzyme preparation contained approximately 1 mole of flavin per 100,000 g of protein. The flavin prosthetic group was not characterized precisely, due to the tight association of this group with the protein.

Analysis of monoamine oxidase exhibited 7 to 8 sulfhydryl equivalents per 10^5 g of protein. When varying amounts of silver nitrate or p-chloromercuribenzoate were incubated with the enzyme, the activity was observed to decrease as a linear function of the amount of inhibitor added; complete inhibition was obtained at a ratio of 7.3 moles of thiol-characterizing reagent per 10^5 g of protein. Similarly, the extent of reduction of the flavoenzyme by benzylamine, at 460 mp, was proportional to the metallic reagent-enzyme ratio, indicative of a selective inhibitory process.

The copper content of the purified enzyme was found by a procedure of atomic absorption spectrophotometry to be 0.14 to 0.15 µg per mg of protein. When the copper content and the flavin content there would be 4 or 5 eq of catalytically active flavin per atom of copper, indicating the nonessentiality of copper for activity of this oxidase. These observations are in agreement with conclusions offered recently by other authors. In addition, there was no observable correlation between the inhibition of enzyme activity by appropriate chelating agents and the ability of these agents to chelate copper. Other interpretations for the inhibition produced by certain of these agents are proposed.

* This work was supported by Research Grants CA 03186 and CA 00392 from the National Cancer Institute, National Institutes of Health, United States Public Health Service.‡ United States Public Health Service Fellow (F2-GM-21). Present address, University of Colorado School of Pharmacy, Boulder, Colorado 80302.

Early reports (1, 2) of the enzymatic oxidation of certain amines in various tissues, including brain, liver, and kidney, have stimulated numerous investigations (3-5) of the role of monoamine oxidase in the metabolism of catecholamines and serotonin. In addition, it has been shown that a number of pharmacologically active compounds, including certain antihypertensive and antidepressant drugs, inhibit monoamine oxidase in vivo and in vitro (6, 7). However, few studies have been concerned with the purification and characterization of mitochondrial monoamine oxidase and little is known about the mechanisms of inhibition of this enzyme or the detailed mechanism of catalysis.

Barbato and Abood (8) have purified monoamine oxidase 20-fold from bovine liver mitochondria and Guha and Krishna Murty (9) have achieved a 180-fold purification from rat liver mitochondria; in these studies the nature of the enzyme was not determined. Nara, Gomes, and Yasunobu (10) reported a 58-fold purification of bovine liver mitochondrial monoamine oxidase and suggested that the enzyme is a copper-containing protein. These investigators also presented preliminary data from which they concluded that a flavin is associated with the enzyme (11). In this paper we present our recent observations bearing on the copper and flavin aspects of mitochondrial monoamine oxidase as well as on other properties of the enzyme. In addition, we have outlined a simple preparative procedure which gives a highly concentrated and active enzyme. Availability of this enzyme preparation is facilitating studies of inhibitory processes and of the catalytic mechanism of amine oxidation (cf. References 12-14).

EXPERIMENTAL PROCEDURE

Materials and Methods—Digitonin A (Calbiochem) was recrystallized once from absolute ethanol, dried, and ground to a fine powder. Digitonin micellar suspensions ("solutions") were prepared immediately before use by addition of warm 0.25 M sucrose to the powdered material, brief mixing, and treatment for 1 to 2 min in an ultrasonic bath. The resulting water-clear solutions remained clear for several hours at 0°C. Benzylamine hydrochloride was recrystallized twice from isopropyl alcohol. Bio-Gel HT mixture (hydroxyapatite for chromatography, Bio-Rad Laboratories, Richmond, California), 125 g, and Schleicher
and Schuell cellulose, 100 g, were diluted to 1.5 liters with water, in a suction flask, mixed well, and placed under a vacuum for 10 min; alternatively, calcium phosphate gel (Sigma) was used.

Monoamine oxidase activity was assayed by the spectrophotometric method of Tabor, Tabor, and Rosenthal (15), usually with benzylamine (hydrochloride) as substrate or by the method of Weissbach et al. (16) conducted at 37° with kynurnine dihydrobromide (Sigma) as substrate. A unit of enzyme activity is defined as the amount of enzyme catalyzing change in absorbance at 250 nm of 0.001 optical density unit per min in a 2.2-ml reaction mixture containing 3 mM benzylamine hydrochloride and 0.05 M phosphate buffer, pH 7.6 (prepared by the addition of NaOH to a solution of KH2PO4 to give the desired pH value). Protein was determined by the Folin-Biuret method with crystalline bovine serum albumin as standard. In the concentrations used digitonin did not interfere with the protein determinations.

Spectrophotometric determinations were performed with a Bausch and Lomb Spectronic 600 spectrophotometer equipped with a Sargent SRL recorder and AT-20 digital readout attachment.

Phospholipids were extracted from aqueous suspensions of the various fractions by the method of Folch et al. (17). The washed chloroform-methanol extracts were dried and the residues were heated to 60-70° for 5 to 10 min to denature any remaining protein. The phospholipid dissolves in chloroform-methanol (2:1 by volume) and aliquots were digested with H2SO4 in the presence of H2O2. Total phospholipid was determined by the method of Gomori (18).

Copper analyses of the enzyme samples were performed with a Technicon atomic absorption spectrophotometer by standard techniques. Fluorescence studies of the enzyme were performed with the Amino Bowman spectrophotofluorometer.

Ultracentrifugal analysis of the enzyme was conducted in the Spincoc model E ultracentrifuge. A sample of enzyme that contained 3 mg of protein per ml in 0.1 M phosphate buffer, pH 7.6, was analyzed in a standard 1-ml, 4-degree sector cell at 20° with 59,780 rpm.

Determination of protein sulphydryl residues was performed spectrophotometrically (19) with p-chloromercuribenzoate or by a suitable procedure with silver nitrate.¹

Purification of Monoamine Oxidase—Examination of various tissues for monoamine oxidase activity revealed that the specific activity of this enzyme in mitochondria from bovine kidney cortex was 4-fold greater than in rat liver mitochondria. Accordingly, bovine kidney cortex was used as a starting material for purification of large amounts of the enzyme.

Bovine kidneys were obtained from a local slaughter house and were cut into small pieces, weighed, and iced promptly in polyethylene bags. The cortical tissue was removed in a cold room at 2°, cut into small pieces, weighed, and iced promptly in polyethylene bags. The mitochondria were isolated by the method of Schneider and Hogeboom (20). All purification procedures were conducted at 0-4°.

Mitochondria were suspended in sufficient 0.25 M sucrose to give approximately 100 mg of protein per ml. An equal volume of cold 1.5% digitonin in 0.25 M sucrose was added with stirring to the suspension, a digitonin to protein ratio of 0.15:1 being optimal for this step. After a 20-min incubation, the suspension was diluted with 0.25 M sucrose (1:3), centrifuged at 40,000 × g for 90 min, and the supernatant fluid was discarded. The resulting pellet, which contained all of the monoamine oxidase activity, was resuspended in sufficient 0.25 M sucrose, containing 0.02 M phosphate buffer, pH 7.6, to give the volume of the original mitochondrial suspension. An equal volume of 3.0% digitonin in 0.05 M phosphate buffer, pH 7.6, was added, with stirring, to the suspension. Centrifugation of this suspension at 144,000 × g for 1 hour yielded a clear, yellow-brown supernatant fluid which contained approximately 80% of the total monoamine oxidase activity. This fluid was treated with solid ammonium sulfate and the precipitate at 0.65 to 0.9 saturation was sedimented by centrifugation at 144,000 × g for 2 hours. The precipitate was dissolved in approximately 100 ml of 0.5 M phosphate buffer, pH 7.6, and dialyzed against 1,000-fold excesses of 5 M phosphate buffer, pH 7.6, for 4 and 12 hours, successively. Of the dialyzed fraction, approximately 300 mg of protein were placed on a calcium phosphate gel-cellulose column (4 × 10 cm) (21) (see also "Materials and Methods" above). The column was then washed with 100 ml of 0.05 M phosphate buffer, pH 7.6, containing 0.5% digitonin, followed by a similar wash with 0.15 M phosphate buffer, pH 7.6. The enzyme activity was eluted with 0.5 M phosphate buffer containing 0.5% digitonin. Fractions with the highest specific activity were combined, dialyzed as previously described, placed on a second calcium phosphate gel-cellulose column, and eluted as before. The enzyme was placed on a third calcium phosphate gel-cellulose column and washed with 100-ml quantities of 0.05 M and 0.15 M phosphate buffer, pH 7.6, without added digitonin, and then eluted with 0.5 M phosphate buffer, pH 7.6. This procedure left a green band at the top of the column which was not observed in the previous steps. The enzyme preparation obtained in this step was a clear, yellow liquid and for all practical purposes could be treated as a solution. However, dilute solutions of the enzyme treated with phosphotungstic acid were observed by electron microscopy as small, oval-shaped particles with a diameter of approximately 75 Å, this value being in agreement with the apparent Stokes radius (see below). In addition, digitonin was detected in the enzyme sample by the anthrone method for polysaccharides (22), which suggested further that the enzyme preparation was not a "true" solution. Results of a typical purification are shown in Table I. In these studies, samples of the enzyme were kept in the cold room at 2° for 4 to 6 weeks without loss of activity. The specific activity of the purified enzyme, determined under conditions used by other investigators, was considerably higher than previously reported. The rate of the enzyme-catalyzed reaction was linear with protein concentration over a wide range with benzylamine or kynurnamine as substrate, and the change in absorbance was linear to 15 min. Addition of Sigma crystalline catalase to test mixtures did not alter the reaction rate. The enzyme was completely inactivated when held at 60° for 5 min.

TABLE I

Purification of monoamine oxidase from bovine kidney mitochondria

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Purification</th>
<th>Yield</th>
<th>Phospholipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digitonin-treated mitochondria</td>
<td>6,520 mg</td>
<td>2,400,000</td>
<td>368</td>
<td>1.0</td>
<td>100</td>
<td>0.15</td>
</tr>
<tr>
<td>Digitonin extract, (NH₄)₂SO₄ (0.65 to 0.9 saturation)</td>
<td>2,712 mg</td>
<td>1,960,000</td>
<td>725</td>
<td>2.0</td>
<td>81</td>
<td>0.28</td>
</tr>
<tr>
<td>First calcium phosphate gel-cellulose chromatogram</td>
<td>616 mg</td>
<td>1,020,000</td>
<td>1,659</td>
<td>4.6</td>
<td>43</td>
<td>0.48</td>
</tr>
<tr>
<td>Second calcium phosphate gel-cellulose chromatogram</td>
<td>70 mg</td>
<td>385,000</td>
<td>5,500</td>
<td>15.0</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Third calcium phosphate gel-cellulose chromatogram</td>
<td>40 mg</td>
<td>320,000</td>
<td>8,000</td>
<td>21.7</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20 mg</td>
<td>252,000</td>
<td>12,600</td>
<td>34.2</td>
<td>10</td>
<td>0.06</td>
</tr>
</tbody>
</table>

* Enzyme activity is expressed as a change in absorbance of 0.001 per min at 250 μm with 3 mM benzylamine hydrochloride as substrate in a 2.2 ml reaction mixture containing 50 mM phosphate buffer, pH 7.6, at 37°C.

Possibly attributable to the presence of digitonin in the preparation. Monoamine oxidase from rat liver mitochondria was purified by a method similar to the method described above. The most highly purified fraction had a specific activity of 5700 with a 7% yield. The properties of the enzyme from rat liver appeared to correspond to those from bovine kidney cortex.

Substrates—Our highly purified kidney enzyme was found to oxidize secondary and tertiary amines as well as primary amines (Fig. 2). However, histamine was not oxidized, which indicated the absence of histaminase or diamine oxidase in the preparation. The relative rates of oxidation of various substrates (Fig. 2) differed slightly from published results with a bovine liver mitochondrial monoamine oxidase (10), attributable to the use here of initial rates rather than a 30-min value. As shown in Fig. 2, benzylamine oxidation had practically stopped after 20 min, while oxidation of other substrates continued almost linearly. The abrupt change here in oxidation rate, when approximately 50% of the substrate had been oxidized, may have resulted in part by removal of substrate through condensation with the product, benzaldehyde.

The stoichiometry of the enzyme-catalyzed reaction was determined for several substrates (Table II). Based on these results, the process in the absence of catalase may be summarized as follows:

\[ R-\text{CH}_2-\text{NH}_2 + \text{O}_2 + \text{H}_2\text{O} \rightarrow R-\text{C}(_2\text{H}_4\text{O}) + \text{NH}_2 + \text{H}_2\text{O}_2 \]

This reaction sequence was adduced previously from data obtained with crude preparations (1, 2).

Phospholipid Content of Enzyme—It has been shown that monoamine oxidase is tightly bound to the mitochondrial membrane fraction (23) and that the enzyme is inactivated by various methods of organic solvent extraction (8). In addition, the work of Schnaitman, Erwin, and Greenawalt (24) has indicated strongly that the enzyme is associated with the outer mitochondrial membrane which is rich in phospholipid (0.5 μmole per mg of protein). Accordingly, phospholipid determinations were performed on various fractions during purification of the enzyme to determine whether or not the enzyme is a phospholipoprotein. As shown in Table I, the concentration of phospholipid in the initial purification steps was 0.15 to 0.48 μmole per mg of protein. However, the most highly purified fraction contained only 0.06 μmole of phospholipid per mg of protein. Addition of mitochondrial phospholipid, extracted by the chloroform-methanol
procedure of Folch et al. (17) had no effect on the enzyme activity. Furthermore, full activity was recovered after incubation of the purified enzyme with phospholipase A. These results indicate that monoamine oxidase has no specific phospholipid requirement.

Copper Content of Purified Monoamine Oxidase—In view of the report that bovine liver monoamine oxidase is a copper protein (10), it was considered desirable to determine the copper content of this purified enzyme. This was accomplished by use of atomic absorption spectrophotometry. Dilute enzyme solutions (approximately 0.5 mg of protein per ml used in these studies) were concentrated to 2 mg of protein per ml by pressure dialysis. As shown in Table III, there was no correlation between the copper content and the specific activity of the enzyme. The amount of copper in the enzyme fractions initially increased with purification; however, in the final calcium phosphate gel-cellulose step the copper content had dropped to 0.017% with a concomitant increase in specific activity of the enzyme. Three separate preparations of the enzyme with specific activities of approximately 12,000 were found to contain 0.15 to 0.19 pg of copper per mg of protein, which is equivalent to 1 mole of copper for approximately 450,000 g of protein.

In order to determine if the small amount of copper in the purified enzyme could be removed without a loss in activity, the enzyme was treated with 5 mM 8-hydroxyquinoline, a reversible substrate-competitive inhibitor (cf. Reference 25) (Table IV). The mixture was passed through a Sephadex G-25 column and concentrated by pressure dialysis, after which it was analyzed for copper. This treatment lowered the copper content only slightly, to a value of 0.14 µg per mg of protein and without loss in activity.

Effects of Various Chelating Agents—Because of the low copper content of our purified enzyme, and in view of reports that chelating agents inhibit mitochondrial monoamine oxidase (8, 10, 25), it was necessary to examine here the effects of various chelating agents. As shown in Table V, only 8-hydroxyquinoline, of those agents that are known to form stable copper chelates, proved to be an effective inhibitor. Sodium diethyldithiocarbamate and 1,10-phenanthroline at concentrations of 10 mM and 1 mM, respectively, produced only weak inhibition when incubated with the enzyme for 10 min at 37°. Moreover, under these conditions, cuprizone, when freshly prepared, displayed little inhibitory action. However, if cuprizone solutions were allowed to stand overnight, their inhibitory potency increased. However, this increase in inhibitory action on the enzyme was accompanied by a decrease in the absorbance of the cuprizone solution at 250 µM, possibly attributable to hydrolysis or decomposition of the reagent.

As stated, 8-hydroxyquinoline was found to be the only chelator of the group that effectively inhibited the purified enzyme; this prompted a more detailed study of the effects of a series of hydroxyquinoline analogues and structurally related compounds. As shown in Table IV, there was no correlation between any copper-chelating capacity of hydroxyquinolines and their clear-cut inhibitory effects on the enzyme. 2-Hydroxyquinoline, which is a poor chelating agent (26), is a more effective inhibitor (K_i, 2.5 x 10^{-4} M) than 8-hydroxyquinoline (K_i, 6.5 x 10^{-4} M). In addition, the structural analogues, α-naphthol and β-naphthol, produced 50% inhibition at 2 x 10^{-4} M and 1 x 10^{-3} M, respectively. Inhibition by these compounds was found to be reversible by dialysis. Furthermore, inhibition of monoamine oxidase by 8-hydroxyquinoline can be completely reversed by the addition of Zn^{2+}, Ni^{2+}, or Cu^{2+} ions; inhibition by 2-hydroxyquinoline, α-naphthol or β-naphthol cannot be reversed by these ions. When Zn^{2+}, Ni^{2+}, or Cu^{2+} ions were added in equimolar amounts with 8-hydroxyquinoline, the resulting complexes precipitated, effectively removing the inhibitor.

Double reciprocal plots (Figs. 3 and 4) reveal that inhibition of monoamine oxidase by the hydroxyquinolines, α-naphthol, or β-naphthol is competitive with substrate. Values of K_i were quite comparable when the data were examined by either v/(S) versus (1/v) or (S)/v versus v plots.

Spectral Properties of Mitochondrial Monoamine Oxidase—The absorption spectrum of monoamine oxidase possessed a maximum

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Amount</th>
<th>Catalase</th>
<th>Oxygen uptake</th>
<th>Aldehyde formed</th>
<th>Ammonia liberated</th>
<th>H_{2}O_{2} produced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmoles</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tryptamine</td>
<td>2.0</td>
<td>Absent</td>
<td>1.90</td>
<td>1.88</td>
<td>1.90</td>
<td>1.93</td>
</tr>
<tr>
<td>Tryptamine</td>
<td>2.0</td>
<td>Present</td>
<td>0.99</td>
<td>1.92</td>
<td>1.91</td>
<td>0.98</td>
</tr>
<tr>
<td>Tyramine</td>
<td>2.0</td>
<td>Absent</td>
<td>1.87</td>
<td>1.85</td>
<td>1.93</td>
<td>1.90</td>
</tr>
<tr>
<td>Tyramine</td>
<td>2.0</td>
<td>Present</td>
<td>1.00</td>
<td>1.90</td>
<td>1.90</td>
<td>1.92</td>
</tr>
<tr>
<td>Kynuramine</td>
<td>2.0</td>
<td>Absent</td>
<td>1.96</td>
<td>1.94</td>
<td>1.87</td>
<td>1.84</td>
</tr>
<tr>
<td>Kynuramine</td>
<td>2.0</td>
<td>Present</td>
<td>1.96</td>
<td>0.97</td>
<td>1.92</td>
<td>0.00</td>
</tr>
</tbody>
</table>

* Copper analysis was performed by atomic absorption at 3247 A with protein concentrations of approximately 2 mg per ml, as described in the text.

Table II

Stoichiometry of monoamine oxidation

Oxygen uptake was measured by Warburg manometry. Monoamine oxidase (specific activity, 12,000), 200 units, was added to a 2.8-ml reaction mixture containing 0.05 M phosphate buffer, pH 7.6. Substrate was tipped from the side arm after a 10-min equilibration period. At the completion of the reaction, H_{2}O_{2} was determined by tipping catalase from a second side arm and estimating the O_{2} evolved. Aliquots were removed (after completion of the run) and N_{2}, substrate, or aldehyde was determined. Ammonia was determined by Conway diffusion methods. Kynuramine was assayed spectrophotometrically at 360 m_{µ}. Aldehyde was determined by adding an aliquot to a 3-ml reaction mixture which contained excess aldehyde dehydrogenase and NAD in 0.01 M sodium pyrophosphate buffer, pH 8.6. Formation of NADH was followed spectrophotometrically.
TABLE IV

Inhibition of monoamine oxidase by hydroxyquinolines and naphthols

Table IV provides the inhibition of monoamine oxidase by hydroxyquinolines and naphthols. Enzyme was used with benzylamine as substrate, and inhibition was measured with kynuramine as substrate. The processes were followed spectrophotometrically and their values were determined as described in the text and in Figs. 3 and 4. Ni++, Zn++, or Co++ ions, when added, were in a concentration equivalent to the inhibitor concentration. (Note: Cu++ ions added in the presence of chelating agent caused further inhibition and not reversal.) Temperature, 37°.

| Substrate and inhibitor | Kia | Reversible by Ni++, Zn++, Co++ | %
|-------------------------|-----|-------------------------------|---
| 8-Hydroxyquinolines     | 6.5 | 100                           |   |
| 2-Hydroxyquinolines     | 2.5 | 0                             |   |
| 4-Hydroxyquinolines     | 2   | 0                             |   |
| α-Naphthol              | 1   | 0                             |   |
| β-Naphthol              |     |                               |   |

Kynuramine

| Substrate and inhibitor | Ki | Reversible by Ni++, Zn++, Co++ | %
|-------------------------|----|-------------------------------|---
| 8-Hydroxyquinolines     | 3.5 | 100                          |   |
| 2 Hydroxyquinolines     | 2.3 | 0                            |   |
| 4-Hydroxyquinolines     | 3.6 | 0                            |   |
| α-Naphthol              | 1.6 | 0                            |   |
| β-Naphthol              |     |                               |   |

a Inhibitor constants, Ki, were determined from double reciprocal plots with inhibitor concentrations of 2 x 10^-4 M and 4 x 10^-4 M. Inhibition was competitive with substrate and was 100% reversible by dialysis.

b Ki = 1.2 x 10^-4 M.

c Cf. Reference 25.

d 4-Hydroxyquinoline was not inhibitory.

TABLE V

Effects of various chelating agents on monoamine oxidase

Table V lists the effects of various chelating agents on monoamine oxidase. Kynuramine and benzylamine concentrations were 5 x 10^-3 M. Chelating agents were dissolved in 0.05 M phosphate buffer; the pH was adjusted when necessary to 7.6. Of the inhibitor solution, 0.3 ml was added to a cuvette containing 0.05 M phosphate buffer and 3 to 6 mg of enzyme protein (specific activity, 12,600) in a total volume of 2.2 ml at 37°. After mixing of the contents, substrate was added to initiate the reaction. The reaction rate was determined as described in Table I and in the text.

<table>
<thead>
<tr>
<th>Substrate and chelator</th>
<th>Final concentration</th>
<th>Inhibitiona</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kynuramine</td>
<td>1.0</td>
<td>5</td>
</tr>
<tr>
<td>Cuprizone</td>
<td>1.0</td>
<td>13</td>
</tr>
<tr>
<td>Sodium diethyldithiocarbamate</td>
<td>10.0</td>
<td>17</td>
</tr>
<tr>
<td>8-Hydroxyquinoline</td>
<td>1.0</td>
<td>55</td>
</tr>
<tr>
<td>EDTA</td>
<td>1.0</td>
<td>0</td>
</tr>
</tbody>
</table>

| Benzylamine            | 1.0                 | 13          |
| Cuprizone              | 1.0                 | 15          |
| Sodium diethyldithiocarbamate | 10.0          | 20          |
| 8-Hydroxyquinolines    | 1.0                 | 30          |
| EDTA                   | 1.0                 | 0           |

a Incubation at pH 7.6 and 37° for 10 min before addition of the substrate produced the same degree of inhibition.

Fig. 3. Kinetics of inhibition of benzylamine oxidation by α-naphthol and β-naphthol. The reaction mixture contained 3.0 mg of enzyme protein (specific activity, 12,600). Assay conditions are described in the text, and in Table I. The abscissa gives the reciprocal of the molarity of benzylamine and the ordinate gives the reciprocal of the change in absorbance at 250 μM per min at 37°.
FIG. 4. Kinetics of inhibition of kynuramine oxidation by hydroxyquinolines. The reaction mixture contained 6 μg of enzyme protein (specific activity, 12,600). Assay conditions are described in the text and in Table I. The ordinate gives the reciprocal of the change in absorbance at 360 μM per min at 37° and the abscissa gives the reciprocal of the molarity of kynuramine.

After reduction with dithionite reflected the shift in the 412 μM maximum to 425 μM. After reduction of the enzyme with benzylamine, there was no evidence of semiquinone formation, as indicated by the absence of an increase in absorption at 530 μM (29).

Fluorescence Properties of Mitochondrial Monoamine Oxidase—As shown in Fig. 6, the highly purified enzyme gave an emission peak at 320 μM when activated at 450 μM, characteristic of the properties of flavins and flavoenzymes (30). The fluorescence at 530 μM was markedly depressed by the addition of sodium dithionite or by reduction of the enzyme with benzylamine hydrochloride under anaerobic conditions. It has been well established that reduced flavins (FAD or FMN) do not fluoresce at 520 μM when activated at 450 μM (30). This assumption has been found valid for certain other flavoenzymes (27). The results point to the flavoprotein character of the enzyme, the 520 μM fluorescence of the enzyme-bound flavin being quenched approximately 50-fold as compared to free FAD.

The purified enzyme contained approximately 1 eq of flavin per 100,000 g of protein. This value was calculated from the absorbance at 455 μM and from the change in absorbance at 455 μM produced by sodium dithionite and benzylamine. It will be recalled that an "equivalent weight" of the enzyme, based on copper content, was approximately 450,000. These results...
precipitation with 20% trichloracetic acid. Removal of flavin form-methanol (2:1) removed any of the flavin. After the flavoprotein. Neither acid ammonium sulfate precipitation, of attempts were made to remove the flavin group from the enzyme. Flavoprotein strongly support the conclusion that the enzyme is not a copper-requiring flavoenzyme.

50 mM phosphate buffer, pH 7.6, at 25° was obtained with an Aminco Bowman spectrophotofluorometer. The FAD (2.6 x 10^{-6} M) spectrum was obtained at a 50-fold lower sensitivity adjustment under similar conditions.

In order to characterize further the nature of the flavin moiety, a number of attempts were made to remove the flavin group from the flavoprotein. Neither acid ammonium sulfate precipitation, boiling of buffered enzyme for 10 min, nor extraction with chloroform-methanol (2:1) removed any of the flavin. After the 10-min boiling, the denatured protein was sedimented and redissolved in 1% sodium dodecylsulfate, and this was followed by precipitation with 20% trichloracetic acid. Removal of flavin was resisted also in this procedure. Another sample of denatured flavoprotein (2 mg) obtained after the boiling procedure was digested for 48 hours at 37° with 5 mg of Pronase (Calbiochem). Control samples containing 2 mg of bovine serum albumin plus FAD or FMN were treated similarly. After digestion, the samples were centrifuged and the supernatants concentrated under reduced pressure. The dried samples were dissolved in a pyridine-glacial acetic acid-water (1:10:1890) buffer, pH 3.5, spotted on paper, and subjected to electrophoresis for 4 hours. The FAD and FMN in the control samples moved about 10 cm toward the anode, while the flavin associated with the enzyme moved slightly toward the cathode. The flavin from the enzyme was still associated with amino acids, peptides, or possibly unhydrolyzed protein, as shown by the presence of ninhydrin-positive material, whereas the free FAD and FMN spots were not so associated.

Nature of Binding of Flavin Prosthetic Group—In order to characterize further the nature of the flavin moiety, a number of attempts were made to remove the flavin group from the flavoprotein. Neither acid ammonium sulfate precipitation, boiling of buffered enzyme for 10 min, nor extraction with chloroform-methanol (2:1) removed any of the flavin. After the 10-min boiling, the denatured protein was sedimented and redissolved in 1% sodium dodecylsulfate, and this was followed by precipitation with 20% trichloracetic acid. Removal of flavin was resisted also in this procedure. Another sample of denatured flavoprotein (2 mg) obtained after the boiling procedure was digested for 48 hours at 37° with 5 mg of Pronase (Calbiochem). Control samples containing 2 mg of bovine serum albumin plus FAD or FMN were treated similarly. After digestion, the samples were centrifuged and the supernatants concentrated under reduced pressure. The dried samples were dissolved in a pyridine-glacial acetic acid-water (1:10:1890) buffer, pH 3.5, spotted on paper, and subjected to electrophoresis for 4 hours. The FAD and FMN in the control samples moved about 10 cm toward the anode, while the flavin associated with the enzyme moved slightly toward the cathode. The flavin from the enzyme was still associated with amino acids, peptides, or possibly unhydrolyzed protein, as shown by the presence of ninhydrin-positive material, whereas the free FAD and FMN spots were not so associated.

Physical Properties of Mitochondrial Monoamine Oxidase—The enzyme of specific activity 12,000 sedimented as if it were a "homogeneous species" in the analytical ultracentrifuge, displaying a sedimentation constant of 10.6. The eluant from the enzyme, passed through a Sephadex G-200 column (1 x 50 cm), was recovered as a single sharp peak; an apparent diffusion constant (Dapp) of 3.5 x 10^{-7} cm^2 sec^{-1} was determined by the method of Ackers (31) after use of crystalline bovine serum albumin and crystalline catalase for calibration of the column. On assumption of a partial specific volume of 0.75, an apparent molecular weight of 290,000 was calculated for the enzyme, a value consistent with observations for rat liver monoamine oxidase reported recently (32). However, the assumptions underlying these methods with the particular enzyme preparations under consideration here obviously introduce uncertainty concerning particle weight values.

Inhibition of Monoamine Oxidase by Thiol Reagents—As shown in Fig. 8, the native enzyme possessed approximately 8 titratable sulfhydryl residues per 10^4 g of protein. Similarly, 8 sulfhydryl residues were titratable in the presence of 4 m guanidine hydrochloride or 0.3% sodium dodecyl sulfate. Various preparations of monoamine oxidase are inhibited by thiol reagents, the nature of the inhibition of the highly active enzyme was explored in a somewhat preliminary fashion. When monoamine oxidase and varying amounts of silver nitrate or p-chloromercuribenzoate were admixed in phosphate buffer and incubated as described in Fig. 9, enzymatic activity was observed to decrease as a linear function of the amounts of inhibitor added to the enzyme; complete inhibition was attained at a ratio of 7.3 moles of thiol reagent per 10^4 g of protein. Similar results have been reported for other highly purified polysulfhydryl enzyme systems (33-35). In order to determine whether the linear relationship was associated with "selective inhibition" as described by Hollerman, Coffey, and Neims (33), an all-or-none assay involving reduction of the flavoenzyme by substrate at varying molar ratios of thiol reagent to protein was performed. As shown in Table VI, the amount of flavoenzyme reduced at 460 mp by benzylamine, under anaerobic conditions (N2), was proportional to the inhibitor-enzyme ratio. Inasmuch as the extent of, and not the ratio of, flavoenzyme reduction was determined, these results suggest that...
samples were then made anaerobic and the extent of reduction of monoamine oxidase in 0.05 M phosphate buffer, pH 7.6, and the mercuribenzoate (X) were added seriatim to 3-fig portions of mixtures were incubated at 25° for 1 min and 30 min, respectively. Enzyme activity was assayed spectrophotometrically, as described in the text and in Table I.

Varying amounts of silver nitrate were added to four 1.0-ml samples of monoamine oxidase (180 mg per ml; specific activity 12,000). After 1 min a 0.02-ml aliquot was removed for assay of enzyme activity, as described in the text and in Table I. The samples were then made anaerobic and the extent of reduction by addition of benzylamine was determined as described in Fig. 5.

Effect of Ag⁺ ions on reduction of enzyme-bound flavin in presence of benzylamine

Varying amounts of silver nitrate were added to four 1.0-ml samples of monoamine oxidase (180 mg per ml; specific activity 12,000). After 1 min a 0.02-ml aliquot was removed for assay of enzyme activity, as described in the text and Table I. The samples were then made anaerobic and the extent of reduction by addition of benzylamine was determined as described in Fig. 5.

<table>
<thead>
<tr>
<th>Ag⁺ per 10⁶ g of protein</th>
<th>Control activity</th>
<th>%</th>
<th>%</th>
<th>Extent of reduction of flavoenzyme at 460 µm&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>100</td>
<td>0.100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>69</td>
<td>0.672</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>92</td>
<td>0.050</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>25</td>
<td>0.020</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

* Flavin reduction for the inhibited enzyme is defined as 100%.

### DISCUSSION

Although some differences have been noted (see below), the data presented in this paper concerning substrate specificity, pH optimum, and stoichiometry of reaction indicate that such general properties of monoamine oxidase isolated from bovine kidney cortex mitochondria were comparable to those displayed by the enzyme obtained from rat or bovine liver mitochondria, and suggest also that the enzyme is distinct from diamine oxidase and histaminase.

It was shown by Gorkin (23) that monoamine oxidase is tightly bound to mitochondrial membranes; and Schnaitman, Erwin, and Greenawalt (24) found that the enzyme most probably is associated with the outer mitochondrial membrane which can be dispersed preferentially by digitonin. Digitonin was used in this study to "solubilize" monoamine oxidase in the initial stages of purification. Although monoamine oxidase is a membroous protein and is inhibited by various organic solvent extraction procedures (8), the purified enzyme is not inhibited by phospholipase A and apparently does not require phospholipid for activity. However, the possibility of a nonspecific lipid requirement that might be fulfilled by digitonin is not ruled out.

As shown in "Results," there was no correlation between the specific activity of monoamine oxidase and the copper content of the enzyme (Table II). The final purification step here (cf. Reference 36) resulted in a large decrease in the copper content with a concomitant increase in specific activity. The copper content of 0.15 µg per mg of protein differs markedly from the value of 0.7 µg of copper per mg of protein previously reported for bovine liver monoamine oxidase (10), and is far lower than the copper content reported for other copper proteins (37). If based on the copper analysis, the protein combining weight of the most highly purified fractions would be approximately 450,000. As described in "Results," the minimum molecular weight based on substrate and dithionite-reducible flavin is approximately 100,000. These findings indicate that there would be at least 4 eq of catalytically active flavin per g atom of copper and suggest strongly that mitochondrial monoamine oxidase does not require copper for activity. The apparent molecular particle weight (290,000) determined by physical methods might be taken to support this conclusion. A comparable particle weight value for rat liver mitochondrial monoamine oxidase was reported recently by Youdin and Sourses (32). In addition, these investigators found that highly purified rat liver monoamine oxidase contained only 0.034% copper and that mitochondrial monoamine oxidase activity was not decreased by feeding animals a copper-free diet, whereas serum monoamine oxidase and ceruloplasmin activities were markedly lowered by this treatment.

Additional evidence that monoamine oxidase does not require copper was obtained by a study of the effects of various chelating agents with respect to enzyme activity. Of those chelating agents used, only 8-hydroxyquinoline produced marked inhibition of the enzyme activity (Table V) and inhibition produced by a series of hydroxyquinolines did not correlate with the capability of these agents to chelate copper (Table IV). Moreover, α-naphthol and β-naphthol were potent inhibitors and produced the same type of inhibition (competitive with substrate) as the hydroxyquinolines. Inhibition of monoamine oxidase activity by the above compounds may be related to the phenolic nature of these agents. Frisell, Lowe, and Hellerman (38) have shown that phenols inhibit another flavoenzyme, 3-amino acid oxidase, and Fleischman and Tollin (39) have shown that phenols, including naphthols, form charge transfer complexes with FAD.

Absorption and fluorescence data shown in Fig. 5, 6, and 7 establish that mitochondrial monoamine oxidase is a flavoenzyme (cf. Reference 27). The precise nature of the flavin prosthetic group was not determined owing to the tight and possibly covalent linkage between the flavin and protein similar to earlier
observations with succinate dehydrogenase. Succinate dehydrogenase activity was not detectable in our purified preparations.

The material absorbing at 412 μm may not actually be associated with the catalytic activity of our enzyme. Certainly, benzylamine did not appreciably alter this peak, whereas dithionite produced a shift from 412 μm to 425 μm. In addition, a small peak at 565 μm appeared after reduction of the enzyme with dithionite. These results suggest that the material absorbing at 412 μm might be a heme, possibly a cytochrome b. Spectral changes that have been observed for cytochrome b purified from mitochondria and reduced with dithionite (40) are suggestive.

As shown in this study, purified monoamine oxidase is markedly sensitive to thiol-characterizing reagents; inhibition is complete when all of the detectable sulfhydryl groups have been titrated (Figs. 7 and 8). In addition, the data suggest that the enzyme is selectively inhibited by these reagents. Denaturation of the enzyme with 4 M guanidine hydrochloride or 0.3% sodium dodecyl sulfate does not increase the number of titratable sulfhydryl groups (cf. Reference 33). Consequently, either all of the sulfhydryl groups associated with the native protein are accessible, or, after initial attack by the thiol-characterizing reagents, there is sufficient conformational change in the protein to make the total sulfhydryl group complement available. The latter proposal is in keeping with the concept of selective inhibition (see “Results,” “Inhibition of Monoamine Oxidase by Thiol Reagents”), as described by Hellerman, Coffey, and Neims (33); the initial attack of the thiol-characterizing reagent would be rate-limiting in the process.

REFERENCES