

The Nature of the Electrophoretically Separable Multiple Forms of Rat Liver Monoamine Oxidase

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1. Treatment of a partly purified preparation of rat liver monoamine oxidase with the chaotropic agent sodium perchlorate caused the enzyme to migrate as a single band of activity on polyacrylamide-gel electrophoresis, whereas the untreated enzyme separated into a number of bands. 2. Treatment with the chaotropic agent caused no loss of enzyme activity towards benzylamine, dopamine or tyramine. 3. The activities of the untreated preparation towards different substrates were inhibited to different extents by heat treatment and by some inhibitors. No such differences could be detected after the enzyme preparation had been treated with sodium perchlorate. 4. Lipid material, which could be separated by gel filtration, was liberated from the enzyme preparation by sodium perchlorate treatment. 5. The molecular weight of the treated enzyme was found to be 380000 ± 38000 . 6. Perchlorate treatment altered the solubility of the enzyme. 7. A continuous assay method for monoamine oxidase is described.

There has been a large number of reports to indicate that there may be more than one form of the enzyme monoamine oxidase [monoamine- O_2 oxidoreductase (deaminating) EC 1.4.3.4] in rat liver and in a number of other sources (see Gorkin, 1966, and Youdim, 1972, for reviews). The activities of monoamine oxidase towards different substrates are inhibited to different extents by heat treatment (Oswald & Strittmatter, 1963), the reversible inhibitor harmine (Gorkin & Tatyankenko, 1967) and the irreversible inhibitors clorgyline [*N*-methyl-*N*-propargyl-3-(2,4-dichlorophenoxypropylamine)] (Hall *et al.*, 1969; Johnston, 1968; Squires, 1972) and phenethylhydrazine (Tipton, 1972). Recently a number of bands of enzyme activity have been resolved by polyacrylamide-gel electrophoresis and these have been reported to differ in their substrate specificities and in their sensitivities to heat treatment and to certain inhibitors (see e.g. Youdim *et al.*, 1970; Youdim, 1972). It has been claimed that these multiple forms are not an artifact of the preparation method employed, because they can be separated reproducibly from different preparations of the enzyme (Youdim *et al.*, 1969). An alternative suggestion is that the multiple forms may represent a single enzyme species to which different amounts of membrane material are attached (Veryovkina *et al.*, 1964) and this view received support from the finding that the electrophoretically separable forms of rat liver monoamine oxidase have widely different phospholipid contents (Tipton, 1972).

A number of compounds, including the perchlorate and thiocyanate anions, are capable of disrupting protein-membrane complexes. These compounds, which have been termed chaotropic agents, cause a

weakening of hydrophobic bonds between proteins and lipid material, and this has been discussed in detail by Hanstein *et al.* (1971). Chaotropic agents have been used to separate proteins such as succinate dehydrogenase (Davis & Hatefi, 1971) and the enzymic assembly of complex I of the ox liver mitochondrial electron transport system (Davis & Hatefi, 1969) from complexes with membrane material. In the present paper the effects of the chaotropic agent sodium perchlorate on the properties of a partly purified preparation of rat liver mitochondrial monoamine oxidase are reported.

Experimental

Methods

Enzyme preparations. Rat liver mitochondrial monoamine oxidase was partly purified by a modification of the procedure of Youdim & Sandler (1968). Mitochondria were prepared from 160 g of rat liver by the method of Hawkins (1952) and were suspended in 10 mM-sodium phosphate buffer, pH 7.2, to give a total volume of 230 ml. The suspension was stored frozen for at least 3 days. After thawing benzylamine hydrochloride was added to give a final concentration of 3 mM. The mixture was then exposed to sonic oscillations for 90 min by using a Dawe Soniprobe (Dawe Instruments Ltd., London) fitted with a 13 mm (0.5 in) diam. flat-profile probe at an output amperage of between 6 and 7. The suspension was cooled in ice during the sonication. After this treatment 20 ml of the buffer, which contained a sufficient quantity of the detergent Triton X-100 to make the final detergent concentration 2.0% (w/v) was added. The sonication was continued for a further 10 min and the suspension

was then left at room temperature for 30 min before being centrifuged at 160000g for 90 min at 4°C. The supernatant was cooled in ice and solid (NH₄)₂SO₄ was added to 30% saturation. The mixture was stirred for 20 min before centrifugation at 35000g for 20 min. The clear supernatant was made to 65% saturation with solid (NH₄)₂SO₄ and, after stirring for 20 min, the mixture was centrifuged as before. The precipitate was taken up in about 20 ml of 50 mM-Tris-HCl buffer, pH 8.2, and the solution was passed through a column of Sephadex G-25 (bed volume 500 ml) that had been equilibrated in the same buffer.

The final protein concentration of this preparation was in the range 25–35 mg/ml. The specific activity of the preparation assayed with 1.0 mM-benzylamine as the substrate at 30°C and in 0.05 M-Tris-HCl buffer, pH 8.2, was 2.0 nmol of substrate oxidized/min per mg of protein, which represented a sevenfold purification over the original mitochondrial suspension, and the yield of monoamine oxidase activity was about 45%.

This preparation appears to be similar to that used by Collins *et al.* (1972) and Youdim *et al.* (1969, 1970) for their studies of the electrophoretically separable multiple forms of rat liver and brain monoamine oxidase except that a wider (NH₄)₂SO₄ fraction was taken and Sephadex G-25 was used instead of Sephadex G-200.

Ox liver aldehyde dehydrogenase was partly purified by a modification of the method of Deitrich *et al.* (1962). The acetone-dried powder of ox liver was stored *in vacuo* over dried silica gel and Carbowax at -12°C. The ethanol fractionation step was performed with 96% ethanol as recommended by Deitrich *et al.* (1962) except that the rates of addition of ethanol were halved and the mixture was kept at -20°C overnight after the addition of the second aliquot of ethanol and before centrifugation at -10°C. The precipitate at this stage was quickly rinsed with ice-cold 3.6 mM-EDTA-NaOH buffer, pH 7.0, before being dissolved in the same buffer (80 ml per '50 ml' starting volume of acetone-dried powder). This solution was dialysed at 4°C against 10 litres of the same buffer for at least 6 h. The pH of the solution was then adjusted to 6.0 with 0.1 M-acetic acid and a 5 g 'cake' of CM-cellulose that had been equilibrated in 2 mM-sodium phosphate buffer, pH 6.0, was added. After stirring this mixture in ice for 20 min the mixture was centrifuged. The supernatant was stored frozen in small portions. When a portion was thawed for use its pH was adjusted to 8.2 with 0.1 M-NaOH.

Assay methods. Unless otherwise stated monoamine oxidase activity was assayed by a coupled assay system in which the aldehyde produced is used to reduce NAD⁺ in the presence of aldehyde dehydrogenase. The assay mixture contained, in a final volume of 1 ml, 0.12 international units of aldehyde dehydrogenase, 1 mg of NAD⁺, 10 mM-Tris hydrochloride,

pH 8.2, 30 mM-glycine-NaOH buffer, pH 8.2, 0.7 mM-EDTA and 0.002% sodium deoxycholate. The reaction was started by the addition of monoamine oxidase and the change in E₃₄₀ was followed with time.

The activity of monoamine oxidase towards benzylamine was also assayed by the spectrophotometric method of Tabor *et al.* (1954). Pyruvate kinase was assayed in a mixture containing 0.1 mg of NADH/ml, 0.05 mg of phosphoenolpyruvic acid/ml, 0.1 mg of ADP/ml, 1 mM-MgSO₄, 0.02 M-KCl, 2 units of lactate dehydrogenase/ml in 0.05 M-Tris-HCl buffer, pH 7.2. The decrease in E₃₄₀ was followed with time after the addition of enzyme. Lactate dehydrogenase was assayed by following the decrease in E₃₄₀ when this enzyme was added to a mixture containing 0.15 mg of pyruvic acid/ml, 0.1 mg of NADH/ml in 20 mM-sodium phosphate buffer, pH 7.2. β-Galactosidase was assayed by following the decrease in E₄₁₂ when the enzyme was added to a 3 mM solution of *o*-nitrophenylgalactoside in 20 mM-sodium phosphate buffer, pH 7.2.

Assays were done at 30°C in 1 cm light-path cuvettes in a Gilford 240 recording spectrophotometer.

Protein concentration was determined by the microbiuret method (Goa, 1953) with bovine serum albumin as a standard. The elution of catalase, aldolase and apoferritin from Sepharose columns was followed by measuring the E₂₈₀ of the effluent.

Polyacrylamide-gel electrophoresis. This was carried out with 5% (w/v) polyacrylamide gels in a continuous buffer system consisting of 0.05 M-Tris-HCl buffer, pH 9.1, as described by Youdim *et al.* (1969). Protein samples were layered directly on the surface of the gels immediately before electrophoresis was commenced. In some experiments the sample was made 1.0% (w/v) with sucrose to facilitate layering. The presence of sucrose had no effect on the electrophoretic behaviour of the enzyme preparations. Electrophoresis was carried out for from 1.25–1.75 h at about 60 V/cm. The gels were stained for monoamine oxidase activity by the method of Glenner *et al.* (1957). Enzyme activity could be eluted from sections of the gel by standing the sections overnight at 4°C in either 0.05 M-Tris-HCl buffer, pH 8.2, containing 0.01% sodium deoxycholate or in 0.05 M-glycine-NaOH buffer, pH 8.2. The activity in the eluates was determined with [¹⁴C]tyramine by the method of Squires (1972) except that 2-mercaptoethanol and NaCN were omitted from the assay mixture. Gels were stained for protein with Amido Black [1% (w/v) in 7% (v/v) acetic acid] and the excess of stain was removed by soaking the gels in 7% (v/v) acetic acid.

Measurement of phospholipid content. Samples containing between 1 and 3 mg of protein were extracted by the method of Folch *et al.* (1951). The solvent

extracts were then evaporated to dryness and 500 μ l of perchloric acid and 30 μ l of H₂O₂ (100 vol.) were added. Each mixture was then heated for 15 min at 200°C on an oil bath. The samples were then removed and allowed to cool before their P_i contents were determined by the method of Michelsen (1957). A standard calibration curve was prepared from digestion mixtures with added NaH₂PO₄ in the range 0–3.0 μ g of P.

Lipid analysis. Samples at pH 8.2 were extracted three times with 8 vol. of chloroform–methanol (2:1, v/v). The mixture was then adjusted to pH 2.0 with HCl and the extraction procedure was repeated. Extracts were evaporated to dryness, dissolved in the chloroform–methanol mixture and stored in sealed tubes under an atmosphere of N₂ at –12°C. T.l.c. was carried out on glass plates coated with 2.5 mm thick layers of Kieselguhr G nach Stahl. The plates were developed at 20°C in an atmosphere saturated with the mobile phase. The following mixtures were used for development: chloroform–methanol–water (65:25:4, by vol.) (system I, to separate phospholipids) and light petroleum (b.p. 60–80°C)–diethyl ether–acetic acid (80:20:1, by vol.) (system II, to separate other lipids). The chromatograms were stained by spraying with perchloric acid–molybdate to detect phosphate (Wagner *et al.*, 1961) or with 2',7'-dichlorofluorescein to detect lipids (Mangold & Kammerick, 1962).

Gel-filtration experiments. Gel filtration was carried out on either a 40 cm \times 2.2 cm column of Sepharose 4B with a flow rate of approximately 20 ml/h or a 50 cm \times 2.6 cm column of Sepharose 6B with a flow rate of approximately 15 ml/h. Columns were equilibrated with 50 mM-Tris–HCl buffer, pH 8.2, which contained, in some experiments, 0.01% sodium deoxycholate. Molecular weights were estimated by using the linear calibration curves of elution volume versus log mol.wt. which were obtained with the following standard proteins: pyruvate kinase, lactate dehydrogenase, β -galactosidase, catalase, aldolase and apoferritin. The molecular weights assumed for these standards were taken from Darnall & Klotz (1972). The elution of monoamine oxidase activity from the columns was assayed by the method of Tabor *et al.* (1954).

Treatment of monoamine oxidase with sodium perchlorate. Samples (0.8 ml) of partly purified monoamine oxidase (25–35 mg/ml) in 50 mM-Tris–HCl buffer, pH 8.2, were incubated in test-tubes at 30°C for 8.5 min with 0.1 ml of 7.0 M-sodium perchlorate, 0.1 ml of 10 mM-benzylamine hydrochloride and 4 μ l of mercaptoethanol. The tubes were then placed in an ice-bath until the temperature of the contents reached 4°C. The mixture was immediately applied to a 12 cm \times 2 cm column of Sephadex G-25 that had been equilibrated with 50 mM-Tris–HCl buffer, pH 8.2, containing 0.01% sodium deoxycholate and was

eluted at room temperature with this buffer–detergent mixture.

Preparation of a mitochondrial outer membrane fraction. This was prepared by the swelling and sonication method of Sottocasa *et al.* (1967) as modified by Craven *et al.* (1969). The 'light' and 'soluble' subfractions separated by the discontinuous-gradient centrifugation were combined to minimize loss of monoamine oxidase activity. This combined fraction, which represents the outer membranes of the mitochondria, the contents of the intermembrane space and some soluble matrix constituents (Sottocasa *et al.*, 1967), contained some 90 \pm 5% of the monoamine oxidase activity that was in the original rat liver mitochondrial preparation as determined with benzylamine as the substrate.

Chemicals

Catalase, lactate dehydrogenase, pyruvate kinase, aldolase, β -galactosidase, alkaline phosphatase and NAD⁺ were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. Phenethylhydrazine sulphate (Phenelzine, Nardil) was obtained from Fluka A.G. Chemische Fabrik, Buchs, Switzerland. 2',7'-Dichlorofluorescein and 2-mercaptoethanol were obtained from Eastman–Kodak Co. Ltd., Kirkby, Liverpool, U.K. Clorgyline [*N*-methyl-*N*-propargyl-3-(2,4-dichlorophenoxypropyl)amine] was a kind gift from May and Baker Ltd., Dagenham, Essex, U.K. Tyramine hydrochloride, dopamine hydrochloride and tryptamine hydrochloride were obtained from Ralph N. Emmanuel Ltd., Wembley, Middx., U.K., and [1-¹⁴C]tyramine hydrochloride was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Sephadex and Sepharose gels were obtained from Pharmacia Fine Chemicals Ltd., Uppsala, Sweden and Kieselguhr G nach Stahl was obtained from Camlab (Glass) Ltd., Cambridge, U.K. All other chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., and were of the highest purity available from this source.

Benzylamine was converted into its hydrochloride and was recrystallized twice from acetone–water or ethanol before use. All substrate and inhibitor solutions were freshly prepared on the day that they were to be used.

Results

Assay of monoamine oxidase with aldehyde dehydrogenase

The preparation of aldehyde dehydrogenase contained no detectable quantities of monoamine oxidase or alcohol dehydrogenase. Aldehyde dehydrogenase has been previously used to determine the aldehyde formed by the action of monoamine oxidase (Weissbach *et al.*, 1957; McEwen *et al.*, 1970). The K_m values

of this enzyme for aldehydes are low; Deitrich *et al.* (1962) found the K_m value for benzaldehyde, for example, to be $2.0\mu\text{M}$ at pH9.6 and we estimated a value of $1.0\mu\text{M}$ for this substrate under the assay conditions used in the monoamine oxidase assay. Thus this enzyme is well suited for use in a continuous coupled assay system. In the assay system described the velocity of the reaction was linear for at least 5 min and was proportional to the monoamine oxidase concentration. Comparison of the results of this assay with the spectrophotometric assay for benzylamine oxidation (Tabor *et al.*, 1954) showed that one molecule of NADH was produced by the action of aldehyde dehydrogenase for each molecule of benzaldehyde formed in the absence of this enzyme. The monoamine oxidase inhibitors harmine, clorgyline and phenethylhydrazine had no effect on the activity of the aldehyde dehydrogenase at the final concentrations used in these studies, although considerably higher concentrations of phenethylhydrazine are known to inhibit pig brain aldehyde dehydrogenase both directly and by reaction with the aldehyde substrate (Duncan & Tipton, 1971).

Effect of sodium perchlorate on monoamine oxidase activity

Treatment of the monoamine oxidase preparation with sodium perchlorate resulted in a loss of activity unless mercaptoethanol and a substrate were also present. A number of substrates, including tyramine, dopamine and tryptamine at a final concentration of 1.0mM , were as efficient at protecting the activity as 1.0mM -benzylamine. Incubation of the enzyme with perchlorate for periods of up to 10 min under the conditions described resulted in no loss of enzyme activity but at longer incubation times the activity steadily declined.

After treatment with perchlorate the solubility of the enzyme is altered and precipitation occurs at pH

values of less than 7.8. This process occurs at protein concentrations as low as 0.25mg/ml and results in complete loss of activity from the supernatant. The precipitation process could be conveniently followed by observing the increase in E_{250} as turbidity developed when a sample of the preparation was added to the appropriate buffer. At pH values above 6.0 suspensions of the precipitated material appear to retain the full activity and the precipitate may be redissolved with complete recovery of activity in solution by raising the pH to 8.2. At pH values below 6.0 the activities of both the treated and untreated preparations were irreversibly lost. At pH7.2 precipitation from 10mM -sodium phosphate buffer was more rapid than precipitation from 0.05M -Tris-HCl buffer or 0.05M -glycine-NaOH buffer. The addition of bivalent cations such as Ca^{2+} and Fe^{2+} (added as the chloride salts) to final concentrations of 1mM caused immediate precipitation of a treated enzyme preparation in 0.05M -Tris-HCl buffer, pH8.2, although these salts had no effect on the untreated enzyme under similar conditions.

The presence of the ionic detergent sodium deoxycholate at a final concentration of 0.01% stabilized the preparation but the non-ionic detergent Triton X-100 was ineffective at concentrations of up to 2% (w/v). The presence of 0.01% sodium deoxycholate had no effect on the K_m and V_{max} values of the treated enzyme determined with tyramine, dopamine and benzylamine in 0.05M -Tris-HCl buffer, pH8.2.

Treatment of monoamine oxidase with sodium perchlorate as described caused little alteration in the Michaelis parameters of the enzyme towards tyramine, dopamine and benzylamine as shown in Table 1.

'Mixed substrate' experiments

The use of mixtures of two substrates is a useful method for the detection of the presence of two

Table 1. *Substrate specificity of perchlorate-treated and untreated monoamine oxidase*

K_m and V_{max} values for the oxidation of the three substrates were determined at 30°C and at pH8.2 by using the coupled assay with aldehyde dehydrogenase. The information was obtained from reciprocal plots covering a substrate concentration range from 2mM to $50\mu\text{M}$ for dopamine and tyramine and from 2mM to $10\mu\text{M}$ for benzylamine. The V_{max} values are expressed as a percentage of that determined for benzylamine, which was taken as 100% . The absolute V_{max} value for both preparations oxidizing benzylamine was 2nmol of product formed $\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$.

Substrate	Perchlorate-treated enzyme		Untreated enzyme	
	K_m (μM)	V_{max}	K_m (μM)	V_{max}
Benzylamine	60	100	80	100
Tyramine	120	77	145	73
Dopamine	200	58	210	41

enzymes with differing specificities (see e.g., Dixon & Webb, 1964). The initial velocities of oxidation of the substrates benzylamine, tyramine and dopamine were determined at concentrations of these substrates near the K_m value and compared with the initial velocities obtained when pairs of these substrates, each present at their K_m concentrations, were used. The results are shown in Table 2. The specific assay for benzylamine oxidation (Tabor *et al.*, 1954) was used to show that

the substrates tyramine, dopamine and tryptamine each acted as an apparent competitive inhibitor of the oxidation of benzylamine. Fig. 1 shows reciprocal plots for the apparent inhibition of the perchlorate-treated enzyme; the plots given with the untreated enzyme were similar. K_i values were determined from Dixon plots (Dixon, 1953) and the values obtained with the treated enzyme compared with those obtained with the untreated enzyme, given in parentheses,

Table 2. Oxidation of substrate mixtures by monoamine oxidase

The initial velocities of oxidation of the three substrates when present at their K_m concentrations (see Table 1) were determined by using the coupled assay with aldehyde dehydrogenase. The initial velocities were then determined for mixtures of pairs of these substrates both present at their K_m concentrations. The ratio of the initial rate obtained with the mixture to the sum of the initial rates obtained when each of the substrates was assayed alone is shown as a percentage. In all cases the expected value for this ratio calculated on the basis of a single enzyme being involved would be 66.7% (Dixon & Webb, 1964).

Substrate mixture	Ratio of combined to separate activities (%)	
	Perchlorate-treated enzyme	Untreated enzyme
Benzylamine+dopamine	67.1	70.0
Tyramine+dopamine	67.6	68.0
Tyramine+benzylamine	65.0	68.0

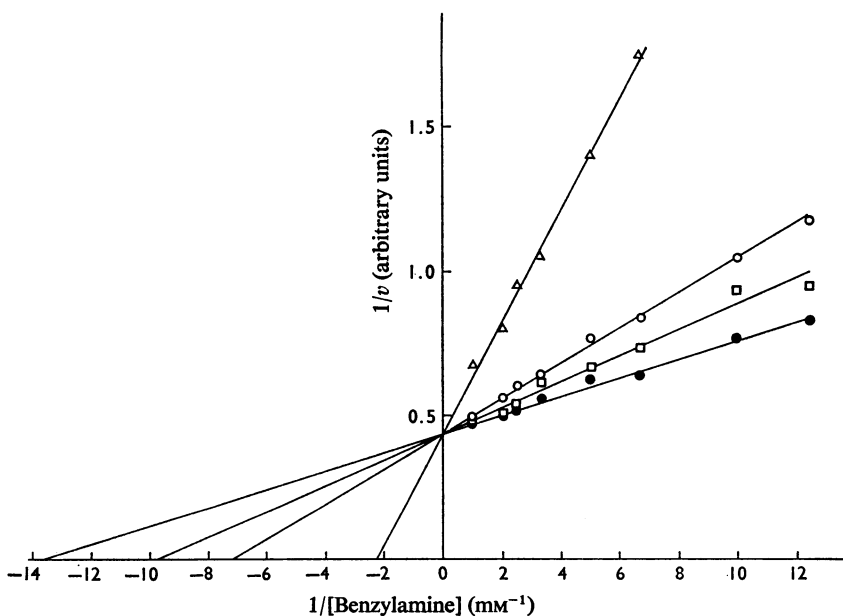


Fig. 1. Inhibition of the monoamine oxidase activity towards benzylamine by other substrates

The oxidation of benzylamine by a preparation of the perchlorate-treated enzyme was followed by the spectrophotometric assay of Tabor *et al.* (1954) in 50mM-Tris-HCl buffer, pH 8.2. The reciprocal plots were in the presence of: 100 μM-tryptamine (Δ), 100 μM-tyramine (○), 100 μM-dopamine (□) and no other substrate (●).

were $114\mu\text{M}$ ($120\mu\text{M}$) for tyramine, $32\mu\text{M}$ ($30\mu\text{M}$) for tryptamine and $200\mu\text{M}$ ($210\mu\text{M}$) for dopamine. The K_i values are close to the respective K_m values for tyramine and dopamine acting as substrates shown in Table 1 and that for tryptamine compares well with the K_m value of $25\mu\text{M}$ determined with both the treated and untreated enzyme preparations.

Heat treatment

The time-courses of loss in enzyme activity towards benzylamine, tyramine and dopamine during preincubation of the enzyme at 40°C were determined for both the perchlorate-treated and untreated preparations (Fig. 2).

Effect of pH on enzyme activity

The effects of pH on the activities of both the treated and the untreated enzyme preparations are shown in Fig. 3. Neither enzyme preparation showed a definite pH optimum in the range studied although the activity increased as the pH increased. However, at pH values above pH9.0 the stability of both enzyme preparations decreased markedly and this could result in the observation of an apparent pH optimum unless great care is taken to determine the true initial rate of the reaction. Variations of the ionic strength of the medium with NaCl indicated that the variation of ionic strength encountered

because of the buffer systems employed in this study would have no detectable effect on enzyme activity.

Inhibitors of monoamine oxidase

Inhibition of both treated and untreated monoamine oxidase by harmine was competitive towards benzylamine, tyramine and dopamine. The inhibitor constants (K_i) calculated from Dixon plots (Dixon, 1953) are shown in Table 3. Time-courses of the inhibition of the activities of both preparations by phenethylhydrazine are shown in Fig. 4 and the effects of clorgyline concentration on the activities of the two preparations with the same three substrates are shown in Fig. 5.

Polyacrylamide-gel electrophoresis

Electrophoresis of the partly purified preparation of rat liver monoamine oxidase by the method described by Youdim *et al.* (1970) produced results similar to those reported by the above workers and by others (Sierens & D'Iorio, 1970; Shih & Eiduson, 1971): three bands of activity migrated into the gel towards the anode and a single band of activity migrated into the gel towards the cathode in experiments in which the polarities were reversed. However, if the enzyme was loaded directly on the surface of the gel no detectable activity remained at the origin of the gel, whereas if the enzyme was loaded as a paste with

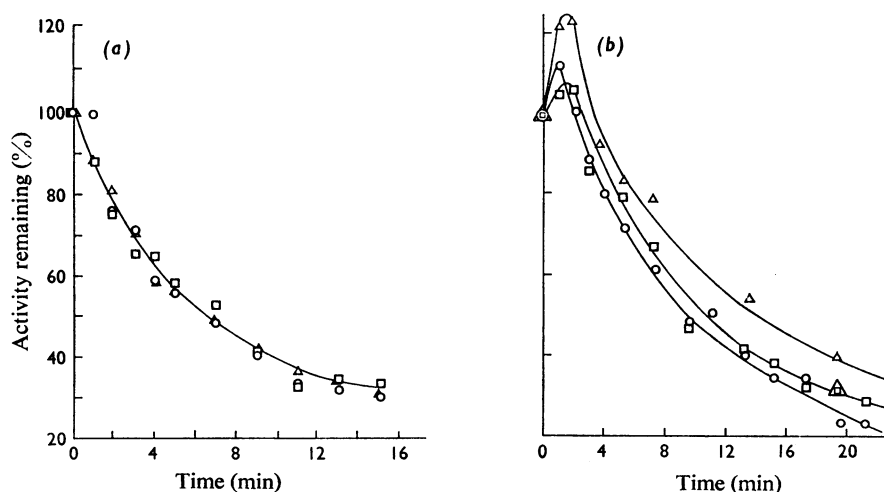


Fig. 2. Effect of heat treatment on the activity of the monoamine oxidase preparations

Samples of (a) perchlorate-treated and (b) untreated monoamine oxidase were incubated at 40°C in 50mM-Tris-HCl, pH8.2, at a protein concentration of 10mg/ml in a shaking water bath. At the indicated times samples were removed and assayed at 30°C by using the coupled assay with aldehyde dehydrogenase. The substrates used were: benzylamine (O), tyramine (□) and dopamine (Δ).

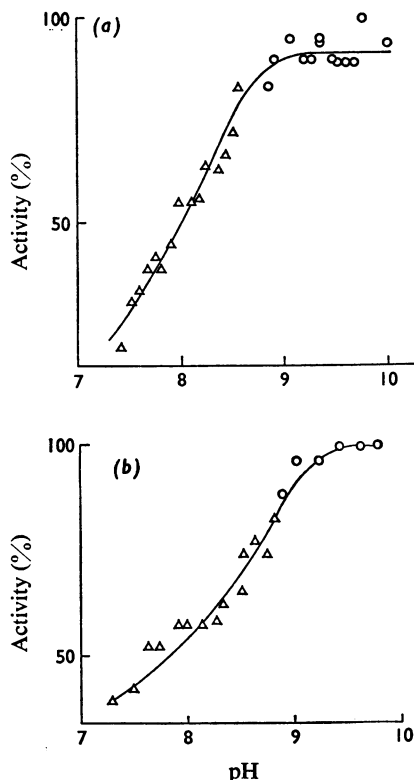


Fig. 3. Effect of pH on the activity of the monoamine oxidase preparations

The activities of (a) perchlorate-treated enzyme and (b) untreated enzyme were assayed at various pH values by using the spectrophotometric method for benzylamine oxidation (Tabor *et al.*, 1954) and the initial velocities expressed as a percentage of the maximum velocity obtained are plotted as a function of pH. Two buffer systems were used: (Δ) 50mM-Tris-HCl buffer and (○) 50mM-glycine-NaOH buffer.

Sephadex G-200 as recommended by Youdim *et al.* (1969) a band of enzyme activity remained at the origin when the electrophoresis was performed towards the anode. Some preparations of the rat liver monoamine oxidase separated into four bands migrating to the anode as shown in Fig. 6(a). In all cases sectioning of the gel followed by elution of the enzyme and assay of its activity towards tyramine revealed that some 90% of the activity that could be eluted corresponded in position to the slowest moving of the bands which migrated towards the anode (band 1 in Fig. 6a). Fig. 6(b) shows gels that had been stained for protein, which show that, by careful sectioning of a gel, it is possible to isolate the electrophoretic bands containing monoamine oxidase activity without gross contamination from other major protein bands. The accurate sectioning of gels was facilitated by the presence of yellowish-brown bands in the unstained gels that bore a fixed relationship to the positions of the bands of activity (Collins *et al.*, 1968).

When the crude preparation of mitochondrial outer membranes was subjected to the electrophoretic procedure only a single band of activity could be detected either after staining the gels or in the eluates from a sectioned gel. This band migrated towards the anode with a mobility similar to that of band 1 from the partly purified preparation (Fig. 6). Dialysis of the crude outer-membrane preparation against 50mM-Tris-HCl buffer, pH8.2, before electrophoresis had no effect on this electrophoretic behaviour. A large amount of protein material remained at the origin when electrophoresis was carried out towards the anode but the monoamine oxidase activity at the origin was negligible.

After sodium perchlorate treatment of the partly purified preparation of monoamine oxidase only a single band of activity could be detected either by direct staining or after elution of a sectioned gel. This band migrated towards the anode and corresponded in position to band 1 of the untreated preparation (Fig. 6). As with the outer-membrane

Table 3. Inhibition of the monoamine oxidase preparations by harmine

Inhibition by harmine was studied by using the coupled assay with aldehyde dehydrogenase. The type of inhibition was shown from reciprocal plots to be competitive in all cases and the K_i values shown were calculated from Dixon plots (Dixon, 1953).

Substrate	Inhibitor constant, K_i (μM)	
	Perchlorate-treated enzyme	Untreated enzyme
Benzylamine	32	125
Tyramine	31	100
Dopamine	31	95

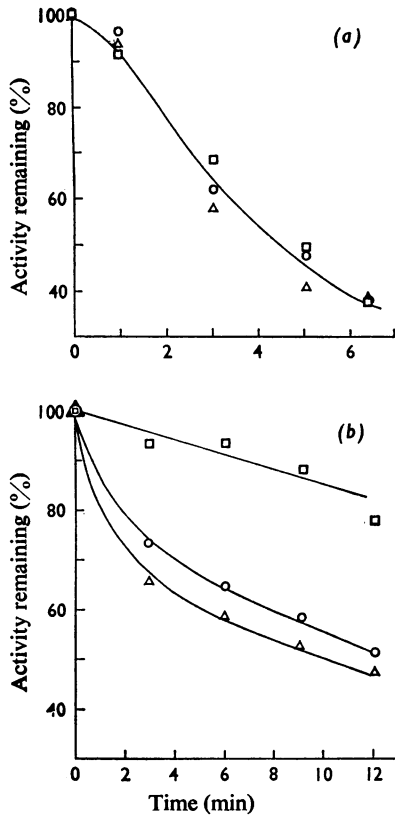


Fig. 4. Effect of phenethylhydrazine on the activity of the monoamine oxidase preparations

Enzyme preparation (500 μl, 10 mg/ml) in 50 mM-Tris-HCl buffer, pH 8.2, containing 0.01% sodium deoxycholate was incubated at 30°C in the presence of 150 μM-phenethylhydrazine. At the time-intervals indicated samples were removed and assayed by using the coupled assay with aldehyde dehydrogenase with equal relative concentrations ($K_m \times 8$) of tyramine (○), benzylamine (Δ) or dopamine (□). No inactivation of either of the enzyme preparations occurred over the incubation periods used when phenethylhydrazine was absent. (a) Perchlorate-treated enzyme; (b) untreated enzyme.

preparation no band of activity that migrated towards the cathode could be detected. Samples of the partly purified enzyme preparation were incubated with the perchlorate-benzylamine-mercaptoethanol-buffer medium for periods of 2, 6, 8, 10 and 20 min before gel filtration into 50 mM-Tris-HCl buffer, pH 8.2, containing 0.01% sodium deoxycholate. Polyacrylamide-gel electrophoresis showed that the multiple bands of activity characteristic of

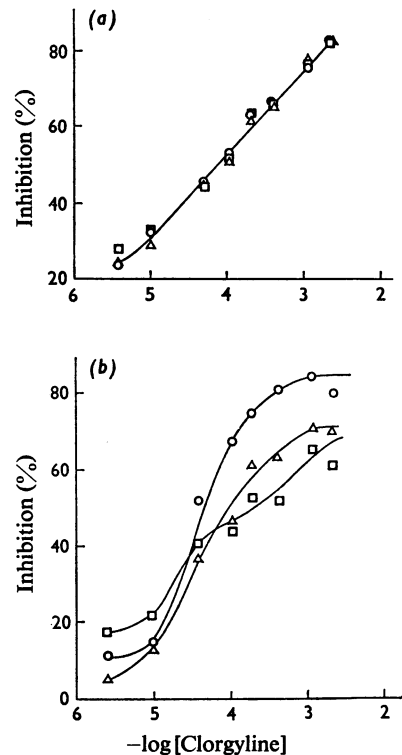


Fig. 5. Effect of clorgyline on the activity of the monoamine oxidase preparations

Portions (450 μl) of (a) the perchlorate-treated or (b) the untreated monoamine oxidase preparations (10 and 24 mg/ml respectively) were incubated at 20°C for 30 min with 50 μl of a clorgyline solution in water to give the final concentration indicated. After this incubation time no further time-dependent loss of activity was observed with any of the substrates used. The activities of the substrates used [benzylamine (○), tyramine (□) and dopamine (Δ)] were determined by using the coupled assay with aldehyde dehydrogenase. Control experiments indicated that no loss of activity toward any of the substrates occurred if either enzyme preparation was incubated under these conditions in the absence of clorgyline.

the untreated preparation were lost after 2 min of incubation with the chaotropic agent.

Gel-filtration experiments

When sodium perchlorate-treated monoamine oxidase was subjected to gel filtration on a column of either Sepharose 6B or Sepharose 4B that had been equilibrated with 0.05 M-Tris-HCl buffer, pH 8.2,

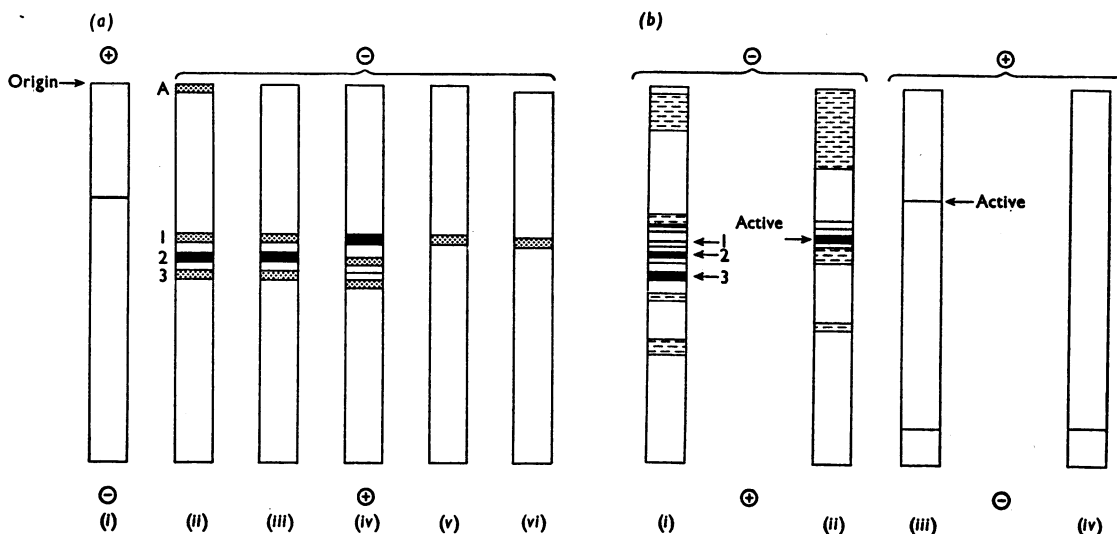


Fig. 6. Polyacrylamide-gel electrophoresis of monoamine oxidase preparations

(a) Electrophoresis was carried out as indicated in the text and the gels were stained for monoamine oxidase by the method of Glenner *et al.* (1957). Similar results were obtained if either tyramine or tryptamine was the substrate used in the assay mixture. (i) Partly purified monoamine oxidase was used with electrophoresis towards the cathode. A band of activity migrating towards the cathode was not seen with any of the other preparations. In the experiments represented by the other diagrams electrophoresis was carried out towards the anode. (ii) Partly purified monoamine oxidase was applied to the gel, dry Sephadex G-200 was added before buffer was added, and electrophoresis was then commenced (as recommended by Youdim *et al.*, 1969). Band A is the artifact produced by this method of loading. (iii) Partly purified monoamine oxidase was loaded directly on the surface of the gel before electrophoresis was commenced. (iv) These four anodic bands were seen with some preparations of partly purified monoamine oxidase. (v) Perchlorate-treated monoamine oxidase. (vi) The mitochondrial outer-membrane preparation. (b) Electrophoresis was carried out as indicated in the text and the gels were stained for protein with Amido Black. The numbering of the bands of activity is the same as that in Fig. 6(a). (i) Partly purified monoamine oxidase with electrophoresis towards the anode. (ii) Perchlorate-treated monoamine oxidase with electrophoresis towards the cathode. (iii) Partly purified monoamine oxidase with electrophoresis towards the cathode. (iv) Perchlorate-treated monoamine oxidase with electrophoresis towards the cathode.

about 50% of the activity applied was eluted in a position corresponding to the void volume of the column, whereas the remainder was retarded and appeared in a volume corresponding to a molecular weight of about 400 000. If 0.01% sodium deoxycholate was included in the equilibration buffer more than 90% of the monoamine oxidase was retarded by the gel (Fig. 7). In the case of untreated monoamine oxidase all the activity emerged as a single retarded peak and the presence of 0.01% sodium deoxycholate had no effect on this pattern. The molecular weight values calculated from these experiments for treated monoamine oxidase were 370 000 with Sepharose 6B and 390 000 with Sepharose 4B. Gel filtration of the untreated enzyme preparation on Sepharose 4B gave an elution volume corresponding to a molecular weight of 400 000.

A strikingly opalescent fraction was eluted in the

void volume of the Sepharose column when the sodium perchlorate-treated enzyme preparation was used but this fraction only contained substantial monoamine oxidase activity in the absence of sodium deoxycholate. This fraction was never observed when untreated monoamine oxidase was subjected to gel filtration. This opalescent fraction was collected from the eluate of a Sepharose 4B column through which treated enzyme was passed in the presence of 0.01% sodium deoxycholate. The void-volume fraction was extracted with chloroform-methanol as described under 'Methods' and the combined organic-solvent extracts were investigated chromatographically. T.l.c. with system II revealed four fluorescent spots after staining with 2',7'-dichlorofluorescein. One of these spots, which remained at the origin, was stained by the perchloric acid-molybdate spray indicating that it probably contained phospholipid

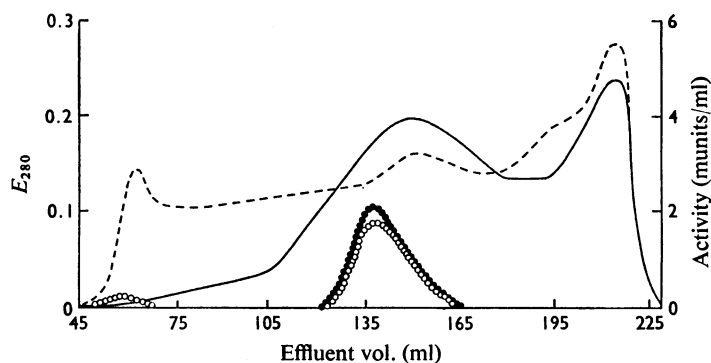


Fig. 7. Gel filtration of monoamine oxidase on Sepharose 4B

A 40cm \times 2.2cm column was equilibrated with 50mM-Tris-HCl buffer, pH 8.2, containing 0.01% sodium deoxycholate. Enzyme samples were applied to the column and the protein content of the effluent was estimated by measuring the E_{280} . Samples (1.4 ml) were collected and the enzyme activity in each was measured by the method of Tabor *et al.* (1954). The presence of 1mM-dithiothreitol had no effect on the elution patterns obtained. ---, E_{280} of the perchlorate-treated preparation; —, E_{280} of the untreated preparation; ○, activity of the perchlorate-treated preparation; ●, activity of the untreated preparation.

Table 4. Phospholipid contents of monoamine oxidase preparations

The extraction and determination of phospholipid P was done as described in the text. The numbering of the electrophoretically separated bands of monoamine oxidase is as in Fig. 6 (a, ii). The material that migrated towards the cathode on electrophoresis was insufficient for accurate determination of its phospholipid P content. The fractions containing monoamine oxidase activity eluted from Sepharose 4B used were fractions of maximum and constant specific activity in each case. Values are the means \pm s.d. of four determinations.

Sample	Phospholipid P content (μ g of P/mg of protein)
Untreated preparation	0.61 ± 0.02
Electrophoretically separated bands from untreated preparation:	
band 1	1.5 ± 0.10
band 2	0.6 ± 0.05
band 3	0.5 ± 0.05
Electrophoretically separated band from perchlorate-treated preparation	0.5 ± 0.05
Sepharose gel eluate from untreated preparation	0.7 ± 0.05
Sepharose gel eluate from perchlorate-treated preparation	0.5 ± 0.05

material. The other spots migrated with R_F values of 2.8, 17.0 and 95.0 (as compared with the solvent front position, which was taken as 100) and did not stain with the perchlorate-molybdate mixture.

The phospholipids were examined by t.l.c. with solvent system I. Spraying with the perchloric acid-molybdate reagent revealed spots with R_F values of 25.0, 42.0, 65.0, 75.0 and a more diffuse spot with an

R_F value of 93.5. These were tentatively identified by comparison with standard lipid solutions and by their reaction with ninhydrin or Dragendorf's reagent (Wagner *et al.*, 1961) as phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, an unknown compound and either phosphatidic acid or cardiolipin or a mixture of both. These components represent the major phospholipids in this fraction

Table 5. *Relative amounts of phospholipid P in electrophoretically separable bands from partly purified monoamine oxidase preparations*

The electrophoresis and phospholipid P determination procedures used were as described in the text. The procedures used to prepare the monoamine oxidase were identical except that Tipton (1972) used 1.5% Triton X-100 rather than the 2.0% used here. The phospholipid P content of each band is expressed relative to that of band 1, which is taken as 100. The absolute values for the phospholipid P contents of band 1 were 1.5 $\mu\text{g}/\text{mg}$ of protein for the preparation used in this work and 0.23 $\mu\text{g}/\text{mg}$ of protein for the preparation used by Tipton (1972). The phospholipid P content of band A was not determined in this work because it has been shown to be an artifact of the gel loading procedure.

Electrophoretic band	Relative amount of phospholipid P	
	Present work	Tipton (1972)
A	—	196
1	100	100
2	40	48
3	33	31
Perchlorate-treated	33	—

but compounds occurring at concentrations of less than 10% of the total would probably not be detected by the staining techniques employed.

Phospholipid analysis

Table 4 shows the amounts of phospholipid P associated with the bands of monoamine oxidase activity that could be separated by polyacrylamide-gel electrophoresis. It was not possible to obtain enough of the material that moved towards the cathode for an accurate determination. The total amount of phospholipid P associated with the partly purified preparation of monoamine oxidase was lower if a lower concentration of Triton X-100 was used in the solubilization procedure. With 1.5% Triton X-100, rather than the 2.0% used here, Tipton (1972) obtained a preparation that contained 0.24 μg of phospholipid P/mg but which gave a series of multiple forms on polyacrylamide-gel electrophoresis similar to those shown in Fig. 6(a). Although the total phospholipid content can apparently vary quite considerably the relative amounts of phospholipid P associated with the electrophoretically separable bands of monoamine oxidase activity appears to remain relatively constant as shown in Table 5, in which the contents of the separated bands are expressed as a percentage of that of the slowest moving band that migrated towards the anode (band 1 in Fig. 6a). This relative constancy is remarkable in view of the marked difference between the total phospholipid P associated with band 1 found in this work (1.5 $\mu\text{g}/\text{mg}$ of protein, Table 4) and that obtained when the lower Triton X-100 concentration was used in the enzyme extraction (0.24 $\mu\text{g}/\text{mg}$ of protein; Tipton, 1972).

Table 4 also shows values for the phospholipid contents of samples of monoamine oxidase that had been subjected to gel filtration on Sepharose 4B. Fractions of maximum and constant specific monoamine oxidase activity (about 10 units/mg in both cases) were collected from the Sepharose columns and treatment with sodium perchlorate produced a decrease in the phospholipid P content associated with fractions containing monoamine oxidase activity. The opalescent material eluted in the void volume when perchlorate-treated monoamine oxidase was subjected to gel filtration on Sepharose 4B equilibrated with 5 mM-Tris-HCl buffer, pH 8.2, containing 0.01% sodium deoxycholate contained some 30% of the total phospholipid P that was applied in the monoamine oxidase preparation. In contrast, no phospholipid phosphorus could be detected in the void volume when the untreated monoamine oxidase preparation was subjected to gel filtration under identical conditions.

Discussion

Polyacrylamide-gel electrophoresis of a partly purified preparation of rat liver monoamine oxidase separated three bands of activity that moved towards the anode and one that moved toward the cathode (Fig. 6a). In contrast with the results of Youdim *et al.* (1969, 1970) and Youdim (1972) no band of activity remained at the origin when the enzyme preparation was applied directly to the surface of the gel. Such a band was, however, seen when the monoamine oxidase preparation was applied to the gel as a mixture with Sephadex G-200 as recommended by Youdim *et al.* (1969). These results indicate that the band of

activity that remains at the origin is probably an artifact of the loading method and is perhaps due to aggregation or precipitation of the enzyme when it is mixed with the dry Sephadex.

Treatment of the enzyme preparation with sodium perchlorate for short periods under the conditions described had little effect on the activity of the enzyme or on its Michaelis parameters measured with three different substrates (Table 1). The enzyme treated in this way only revealed one band of activity on polyacrylamide-gel electrophoresis and this band migrated towards the anode. Polyacrylamide-gel electrophoresis of the crude outer-membrane preparation from rat liver mitochondria revealed that only a single band of monoamine oxidase activity could be detected. This band corresponded in mobility to that of the band given by perchlorate-treated monoamine oxidase and to that of the major band of activity that could be separated from the partly purified monoamine oxidase preparation. The apparent correspondence between the mobilities of the monoamine oxidase activity bands may be coincidental, although it is possible that the electrophoretic procedure causes the enzyme to dissociate from the outer membranes.

The absence of any electrophoretically separable multiple forms of monoamine oxidase activity from the outer-membrane preparation suggests that their presence in the partly purified preparation of the enzyme may be an artifact of the purification procedure used. This procedure involves exposure of a mitochondrial preparation to sonic oscillations over a prolonged period followed by treatment with the non-ionic detergent Triton X-100 (Youdim & Sandler, 1968). The vigorous sonication procedure may release the enzyme with different proportions of membrane material attached to individual molecules. Such a process could account for the different phospholipid contents of the electrophoretically separable forms of partly purified monoamine oxidase (Tipton, 1972). Alternatively, the solubilization procedure could result in the formation of complexes between the enzymes, Triton X-100 and lipid material.

The bands of monoamine oxidase activity that could be separated from the partly purified preparation by electrophoresis contained different proportions of phospholipid P, whereas the single band of activity detected after treatment with sodium perchlorate contained an amount of phospholipid P similar to that present in the fastest-moving anodic component from the untreated material. It has already been pointed out that the different electrophoretic mobilities of the multiple forms of monoamine oxidase cannot simply be charge effects due to phospholipid binding (Tipton, 1972) and a detailed analysis of the lipid and detergent contents of the individual bands of activity would be necessary for

a fuller understanding of the role of lipids in the generation of these multiple forms. Since, however, the individual bands of activity that can be extracted from sectioned gels are unlikely to be homogeneous little useful information could be gained from detailed analyses of them.

Treatment with sodium perchlorate does not completely remove the phospholipid material associated with the peak of maximal monoamine oxidase activity that can be eluted from Sepharose 4B although it does decrease it. A considerable amount of lipid material can be separated from perchlorate-treated monoamine oxidase by gel filtration on Sepharose 4B but it is not possible to say which of these lipids have been released from monoamine oxidase rather than from other components of the preparation; because of this no detailed analysis of its composition was undertaken. The release of phospholipid material is, however, of interest since Olivecrona & Oreland (1971) have shown that such compounds may be involved in the binding of monoamine oxidase to the outer membrane of the mitochondrion.

The perchlorate-treated enzyme was similar in molecular weight to the untreated enzyme and the values obtained are close to those reported for monoamine oxidase from ox brain and human brain (Nagatsu *et al.*, 1972) and to the disaggregated form of the enzyme from ox liver (Gomes *et al.*, 1969). The treated enzyme aggregated readily in solution and was stabilized by low concentrations of the ionic detergent deoxycholate but not by the non-ionic detergent Triton X-100. Since enzyme activity was not lost on aggregation at pH values above 6.0 deoxycholate appears to prevent aggregation. As the partly purified preparation of monoamine oxidase is relatively stable in a solution containing Triton X-100 it would be interesting to see if the perchlorate-treated enzyme could be stabilized by a combination of anionic lipids plus a non-ionic detergent.

From studies with a crude preparation of rat liver monoamine oxidase Severina & Sheremet'evskaya (1967, 1969) concluded that the relative hydrophobicity of the substrate used was the principal factor that governed the apparent differential sensitivity of the enzyme to heat treatment and to some inhibitors. The results reported here are in accord with this view in that heat treatment and incubation with phenethylhydrazine or with low concentrations of clorgyline cause increasing inhibition of the enzyme activity in the order benzylamine > tyramine > dopamine. Such effects, which disappear after perchlorate treatment, may be attributable to effects of bound lipid on both the stability and specificity of the enzyme.

A number of treatments have been reported to provide evidence for the existence of multiple forms of rat liver monoamine oxidase. The experiments reported here do not confirm the observations of

Gorkin & Tatyanko (1967) that the reversible inhibitor harmine inhibits the oxidation of different substrates to different extents. Heat treatment and treatment with the irreversible inhibitors clorgyline and phenethylhydrazine did result in differential inhibition of the activities towards different substrates when the partly purified enzyme preparation was used. These differential effects, however, could not be detected in the perchlorate-treated preparation. The failure of the mixed-substrate experiments to detect the presence of more than one enzyme in either the perchlorate-treated or in the untreated preparation is in agreement with the results obtained by Oswald & Strittmatter (1963) with a crude preparation of rat liver mitochondria. The failure of this method to indicate heterogeneity when dopamine was used as one of the substrates was of particular interest because the band of monoamine oxidase activity that migrates towards the cathode on electrophoresis has been reported to be specific for this substrate (see, e.g., Youdim, 1972).

The fact that both the mitochondrial outer-membrane preparation and the perchlorate-treated preparation show no evidence of inhomogeneity on polyacrylamide-gel electrophoresis suggests that the multiple forms that can be separated by electrophoresis of the partly purified monoamine oxidase preparation may represent artifacts of the preparative procedure used. This conclusion would be consistent with the failure of Collins *et al.* (1972) to observe any correlation between the differential effects of inhibitors on the activities of the electrophoretically separable forms of rat liver and human brain monoamine oxidase and the effects of these inhibitors *in vivo*. It is not possible to conclude from these studies that multiple forms of rat liver monoamine oxidase activity do not exist *in vivo* since the yield of enzyme activity in the purification procedure was less than 50%, although the electrophoretic homogeneity of the mitochondrial outer-membrane preparation (which accounted for some 90% of the total mitochondrial activity) would argue against this. It is also possible that the lipid environment of the enzyme may not be homogeneous throughout any given organ and this could result in modifications of the properties of the enzyme *in vivo*. It would be very difficult to demonstrate conclusively the occurrence of multiple forms of monoamine oxidase *in vivo* because of the difficulties in accounting for permeability barriers, but the present work indicates that attempts to understand the behaviour of monoamine oxidase *in vivo* from studies on the electrophoretically separable forms of this enzyme are unhelpful.

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