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PREPARATION AND PROPERTIES OF AN ELECTROPHORETICALLY  
HOMOGENEOUS MONOAMINE OXIDASE FROM MONKEY INTESTINE

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## SUMMARY

The monoamine oxidase (monoamine:O<sub>2</sub> oxidoreductase (deaminating), EC 1.4.3.4) from monkey small intestinal mitochondria has been purified by using (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and Sepharose 4B fractionation, and the purified preparation was homogeneous by electrophoresis on polyacrylamide gels. The enzyme was optimally active at pH 7.6 and exhibited maximal rates with kynuramine as substrate. The enzyme was moderately inhibited by metal chelating agents and severely inhibited by *p*-hydroxymercuribenzoate. Several monoamine oxidase inhibitors such as Parnate, Iproniazid and Niamide showed complete inhibition at 0.1 mM, while 1-phenyl-2-isopropylhydrazine (0.1 mM) showed only 50% inhibition. There was no evidence of any multiplicity of the enzyme during fractionation.

## INTRODUCTION

It is now well established that the main pathway in the metabolism of serotonin and other monoamines in the body is oxidative deamination catalyzed by monoamine oxidase, (monoamine:O<sub>2</sub> oxidoreductase (deaminating), EC 1.4.3.4). Serotonin is synthesized in several tissues through the decarboxylation of 5-hydroxytryptophan which is formed from tryptophan. A large proportion of the body serotonin is present in the gastrointestinal tract and a study of the metabolism of serotonin in the small intestine is, therefore, of great significance. A preliminary report<sup>1</sup> on the enzymatic decarboxylation of 5-hydroxytryptophan, the immediate precursor of serotonin, was made and the enzyme has been partially purified and its properties studied (D. K. MURALI AND A. N. RADHAKRISHNAN, unpublished results). In the present report the preparation and properties of monoamine oxidase from monkey small intestine are given. The purified preparation of monoamine oxidase was homogeneous in disc electrophoresis on polyacrylamide gels.

## MATERIALS AND METHODS

*Animals*

Normal adult male monkeys (*Macaca mullata* or *Macaca radiata*) were used.

There was no significant difference in the enzyme activities of these two monkey species.

### *Chemicals*

The following chemicals were obtained commercially as indicated. Kynuramine · 2HBr, 5-hydroxytryptamine creatinine sulphate, tryptamine, tyramine, FAD,  $\beta$ -mercaptoethanol, *p*-hydroxymercuribenzoate, DL-penicillamine, nitro-blue tetrazolium and Coomassie brilliant blue, crystalline bovine serum albumin (Sigma Chemical Co., U.S.A.); Cleland's reagent (dithiothreitol) from Calbiochem, U.S.A.; enzyme grade sucrose and  $(\text{NH}_4)_2\text{SO}_4$  (Mann Research Labs., U.S.A.); [ $1\text{-}^{14}\text{C}$ ]tryptamine · HCl, 43.7 mC/mmol, 5-hydroxy[3- $^{14}\text{C}$ ]tryptamine creatinine sulphate, 40 mC/mmol (Radiochemical Centre, Amersham, England); [2- $^{14}\text{C}$ ]tryptamine bisuccinate, 10.9 mC/mmol (New England Nuclear Corp., U.S.A.).

The following were obtained as gifts as mentioned: Marsilid (Iproniazid) from Hoffmann-LaRoche Ltd., Basle; Parnate (tranylcypromine from Smith Kline and French, India Ltd.); 1-phenyl-2-isopropylhydrazine, (Lakeside Labs., U.S.A.); Niamide (Pfizer-Dumex Ltd., India); Triton X-100 (Rohm and Haas, U.S.A.).

Other chemicals were commercially available reagent-grade products.

### *Tissue preparation*

After anaesthetizing by injecting Nembutal, the monkey was sacrificed and the small intestine removed and washed with isotonic KCl (1.15%). The mucosa was scraped off with a blunt knife and used for the preparation of homogenate.

### *Enzyme assay*

Monoamine oxidase activity in suitable aliquots of the enzyme preparations was determined at pH 7.6 spectrophotometrically by following the rate of disappearance of kynuramine at 360 m $\mu$  by the method of WEISSBACH *et al.*<sup>2</sup> as adopted by NAGATSU<sup>3</sup>. One unit of enzyme is defined as the amount causing disappearance of 1  $\mu$ mol of kynuramine in 1 h (or formation of 1  $\mu$ mol of product in the case of  $^{14}\text{C}$ -labelled substrates).

Protein was determined by the procedure of LOWRY *et al.*<sup>4</sup> using crystalline bovine serum albumin as standard.

### *Disc electrophoresis*

The electrophoresis was carried out using polyacrylamide gel essentially according to DAVIS<sup>5</sup> using a Buchler apparatus, but using 3.5% gel and effecting polymerization of both the spacer gel and separating gel by riboflavin instead of ammonium persulphate.

After the electrophoretic run, the gel columns were separately stained for protein and for the enzyme. The monoamine oxidase enzyme was stained by the histochemical procedure of GLENNER *et al.*<sup>6</sup> using nitro-blue tetrazolium, and protein was stained with 0.05% solution of Coomassie brilliant blue in 12.5% trichloroacetic acid<sup>7</sup>.

## RESULTS

*Subcellular fractionation*

Monkey intestinal homogenate (14%) was prepared in 0.3 M sucrose adjusted to pH 7.6 with  $\text{KHCO}_3$ . A teflon homogenizer was employed and the homogenate was filtered through a nylon cloth and the subcellular fractions prepared essentially according to the procedure of HUBSCHER *et al.*<sup>8</sup> except for the microsomal sediment which was obtained at a lower speed ( $105\,000 \times g$ , 60 min).

Monoamine oxidase activity was determined in the subcellular fractions obtained above, and it was found that the bulk of the enzyme activity (65%) was present in the mitochondrial fraction with 29% in the microsomal, 12% in the nuclear and 6% in the supernatant fractions. The mitochondrial fraction was, therefore, used for further purification.

*Solubilization*

In most of the purification procedures reported earlier, solubilization of the mitochondrial enzyme has been achieved either by sonication<sup>9-11</sup> or by treatment with detergents<sup>3,12-16</sup>. In the present study various attempts such as papain treatment, treatment with elastase, freezing and thawing (using liquid  $\text{N}_2$  or by deep-freezing), sonication, and sonication in presence of detergent were made for solubilizing the enzyme. It was finally found that repeated sonication and freezing, a method used for the solubilization of pig brain mitochondrial enzyme<sup>11</sup>, was the most suitable procedure for the solubilization of mitochondrial monoamine oxidase from monkey intestine.

*Purification of monkey intestinal monoamine oxidase*

*Preparation of sonicate.* The intestinal homogenate in 0.3 M sucrose (340 ml, Fraction 1) was subjected to subcellular fractionation, and the mitochondrial fraction obtained was washed with ice-cold distilled water and centrifuged at  $22\,000 \times g$  for 45 min. The pellet thus obtained was suspended in an equal volume of 0.01 M potassium phosphate buffer (pH 7.6) containing Cleland's reagent (0.1 mM) and frozen for a period of about a week. The frozen material was allowed to thaw out and was diluted with the same buffer used earlier to get a final protein concentration of 10-15 mg/ml. The suspension was then sonicated at  $0-5^\circ$  for 45 min using a sonicator (Mullard; 20 kc/sec). The sonicate was first centrifuged at  $105\,000 \times g$  for 60 min and then the carefully decanted supernatant was again centrifuged at  $105\,000 \times g$  for 60 min. The cream-coloured opalescent supernatant was separated and left in the deep-freeze ( $-20^\circ$ ). The combined residue fractions from the above step were suspended in the buffer used above to give a protein concentration of 10-15 mg/ml and again subjected to sonication as before. The sonicated suspension was stored frozen overnight and thawed out and centrifuged as before. The procedure of sonication, freezing and thawing was continued 5 times to get the maximum recovery of the enzyme in the supernatant fraction (294 ml, Fraction 2). The recovery of enzyme activity during each sonication step was not always reproducible, but the total recovery in the 5 steps averaged about 35% of the activity in the mitochondrial fraction.

With this solubilized preparation, a considerable effort was expended for the purification of monoamine oxidase, on the basis of earlier procedures using alumina

Cy gel<sup>13</sup>, calcium phosphate gel<sup>10,12,14,16</sup>, bentonite and alcohol fractionation<sup>11</sup>, chromatography on DEAE-cellulose<sup>3,9,11,13,16</sup>, DEAE-Sephadex<sup>10</sup> or CM-Sephadex. All these efforts were unsuccessful. Finally  $(\text{NH}_4)_2\text{SO}_4$  fractionation followed by gel filtration on Sepharose 4B column was adopted.

*$(\text{NH}_4)_2\text{SO}_4$  fractionation.* To the supernatant that was obtained above (150 ml of Fraction 2) Triton X-100 (10%) was added to give a final concentration of 0.05% and solid  $(\text{NH}_4)_2\text{SO}_4$  was then added in small amounts with stirring to give a final 30% saturation. The mixture was kept stirring for another 30 min and then centrifuged at  $22\,000 \times g$  for 15 min. The precipitate was dissolved in 15 ml of a 'fortified buffer' which contained 0.01 M potassium phosphate buffer (pH 7.6), Triton X-100 (0.05% final), 1  $\mu\text{M}$  FAD, and 0.1 mM Cleland's reagent. The  $(\text{NH}_4)_2\text{SO}_4$  fraction (Fraction 3) was then dialyzed against fortified buffer without Triton X-100 but with Cleland's reagent (10  $\mu\text{M}$ ) and FAD (0.1  $\mu\text{M}$ ).

The fortified buffer has been used throughout as a precaution against possible precipitation of the enzyme during column fractionation and poor recoveries<sup>3,16</sup>.

*Gel filtration on Sepharose 4B.* Gel filtration of the dialyzed Fraction 3 on Sephadex G-200 columns showed a single peak of monoamine oxidase activity appearing within the void volume, with no significant improvement in specific activity. However, it suggested that the enzyme preparation had a high molecular weight, and therefore, fractionation on Sepharose 4B was attempted.

The  $(\text{NH}_4)_2\text{SO}_4$  fraction (Fraction 3, 5 ml) was applied on a column of Sepharose 4B (2.3 cm  $\times$  25.2 cm) equilibrated with fortified buffer, and the elution carried out with the same buffer. Fractions (3 ml) were collected at a flow rate of approx. 13 ml/h. The monoamine oxidase activity appeared as a single peak (Fig. 1) just a few fractions after the void volume (Fraction 4).

Blue dextran 2000 was used to determine void volume in both Sephadex G-200 and Sepharose 4B columns.

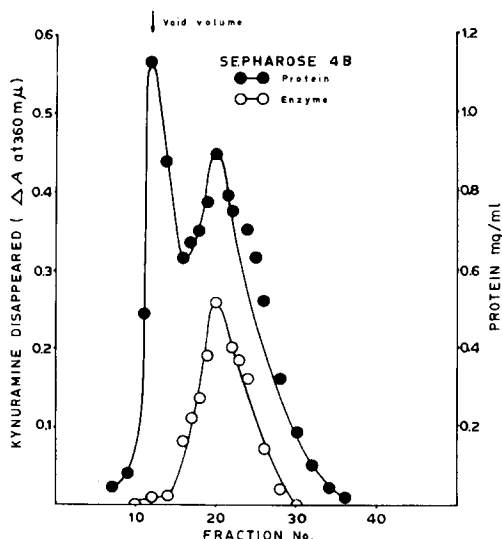


Fig. 1. Gel filtration of Fraction 3 on a Sepharose 4B column; for details see text.

TABLE I

PURIFICATION OF MONOAMINE OXIDASE FROM MONKEY SMALL INTESTINE  
Standard assay conditions using kynuramine as substrate.

Fraction	Total protein (mg)	Enzyme activity		Recovery (%)
		Total units (in thousands)	Units/mg protein	
1. Homogenate	7500	800	107	100
2. Sonic extract	810	194	240	25
3. (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (30% satn.)	234*	172	735	22
4. Sepharose 4B	100	178	1780	22

\* Corrected volumes.

The summary of the purification steps is given in Table I. The enzyme was purified about 17-fold with an overall recovery of 22% and was electrophoretically homogeneous as shown below.

#### Disc electrophoresis

The purified enzyme (Fraction 4) was subjected to gel electrophoresis on polyacrylamide gel as described in MATERIALS AND METHODS. Initially when a 7.5% gel (polymerization by ammonium persulphate) was used, there was a diffuse zone staining for monoamine oxidase very near the point of application. Also, in view of the high molecular weight of the enzyme preparation, it was considered desirable to increase the pore size by decreasing the gel concentration to 3.5% and using riboflavin for polymerization to avoid possible loss of enzyme activity due to the action of ammonium persulphate. The riboflavin-polymerized 3.5% gels were fragile and required careful manipulation. When electrophoresis was carried out using 3.5% gels and stained for the enzyme, monoamine oxidase appeared as a single pink band moving anodically, 13.5 cm away from the junction of the spacer and separation gels. When the column was stained for protein, only one band corresponding to the enzyme band was obtained (Fig. 2), showing that the monoamine oxidase preparation was electrophoretically homogeneous.

#### Properties of the purified enzyme

*Time-course and effect of enzyme concentration.* When an appropriate amount of the enzyme was used, the rate of disappearance of kynuramine was proportional to the time of incubation at 37° up to 1 h. The rate was linear over an 8-fold range of protein (28–220 µg).

*pH optimum.* The enzyme was optimally active at pH 7.6 (Fig. 3).

*Substrate specificity and K<sub>m</sub> value for kynuramine.* The specificity of the enzyme has been tested using the following substrates (all at 0.1 mM): kynuramine, [2-<sup>14</sup>C]-tryptamine, [1-<sup>14</sup>C]tyramine and 5-hydroxy[3-<sup>14</sup>C]tryptamine. The enzyme activity using the three labelled substrates was determined by measurement of radioactivity in the deamination products of the reaction after separating them from the amine on CG-50 (H<sup>+</sup>) columns as described by ROBINSON *et al.*<sup>17</sup>. The results are summarized

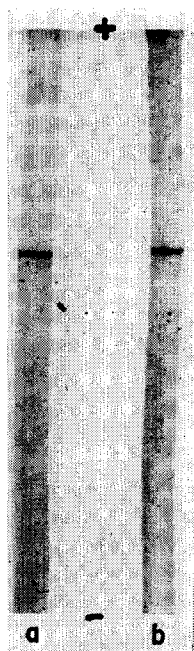


Fig. 2. Polyacrylamide disc electrophoresis of Fraction 4. Sepharose 4B fraction (about 500  $\mu$ g protein in 20% sucrose) was applied on gel columns (1 cm  $\times$  21 cm) packed with polyacrylamide gel (3.5%) polymerized with riboflavin, and the electrophoresis was carried out in 0.05 M borate buffer (pH 8.6) at 4–5 mA per tube, for 12 h in the cold (0–5°). The gel columns were stained for monoamine oxidase (a) and for protein (b).

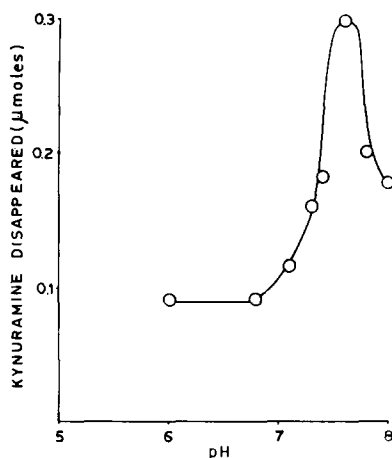


Fig. 3. pH optimum for monoamine oxidase from monkey intestine. Standard assay conditions with phosphate buffer of varying pH.

in Table II. It is found that kynuramine was the most active substrate followed by tyramine, tryptamine and serotonin in that order. A similar order in the activity of the enzyme has been noted in the case of bovine kidney<sup>14</sup>. However, the relative order and the magnitude of the enzyme activity with various substrates varies widely depending on the tissue and the animal species employed<sup>3,13,14</sup>.

TABLE II

SUBSTRATE SPECIFICITY OF MONOAMINE OXIDASE FROM MONKEY INTESTINE

Standard assay conditions for kynuramine and conditions for the <sup>14</sup>C-labelled substrates as given in the text. Sepharose 4B fraction (Table I), which had lost some activity on storage, was used.

	Enzyme activity (units/mg protein)
Kynuramine	850
[1- <sup>14</sup> C]Tyramine	122
[2- <sup>14</sup> C]Tryptamine	23.2
5-Hydroxy[3- <sup>14</sup> C]tryptamine	9.9

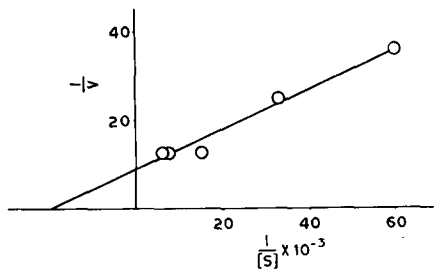


Fig. 4.  $K_m$  for kynuramine. Standard assay conditions.

The  $K_m$  value for kynuramine as calculated from a Lineweaver-Burk plot was found to be  $50 \mu\text{M}$  (Fig. 4) and the optimal concentration of this substrate was  $0.1 \text{ mM}$ .

*Effect of various inhibitors.* Several inhibitors of monoamine oxidase especially of clinical and pharmacological interest were examined to see the direct action of these compounds on the purified monkey intestinal enzyme (Table III). It was found that hydrazine derivatives such as iproniazid (1-isonicotinyl-2-isopropylhydrazine) severely inhibited the activity even at low concentrations. It is interesting to note that 1-phenyl-2-isopropylhydrazine (JB-516), another hydrazine derivative, was not as potent an inhibitor as iproniazid. In the case of monoamine oxidase from beef brain<sup>3</sup>, however, both 1-phenyl-2-isopropylhydrazine and iproniazid were reported to be powerful inhibitors. Niamide and Parnate were also found to be powerful inhibitors of the monkey intestinal monoamine oxidase.

Chelating agents such as *o*-phenanthroline, sodium diethyldithiocarbamate, 8-hydroxyquinoline and *aa*-dipyridyl inhibited the enzyme activity (Table III).

TABLE III

EFFECT OF VARIOUS INHIBITORS ON MONKEY INTESTINAL MONOAMINE OXIDASE

Inhibitors were preincubated with enzyme at  $37^\circ$  for 15 min before addition of the substrate. Standard assay conditions using kynuramine as substrate.

<i>Inhibitor</i>	<i>Concn.</i> ( $\mu\text{M}$ )	<i>Relative</i> <i>activity</i>
None	—	100
1-Phenyl-2-isopropylhydrazine	100	47
	10	84
Iproniazid	100	0
	10	10
Niamide	100	0
	10	8
Parnate	100	0
Sodium diethyldithiocarbamate	1000	47
<i>o</i> -Phenanthroline	1000	52
<i>aa</i> -Dipyridyl	1000	26
8-Hydroxyquinoline	1000	37
DL-Penicillamine	1000	84
<i>p</i> -Hydroxymercuribenzoate	10	0
<i>p</i> -Hydroxymercuribenzoate + $\beta$ -mercaptoethanol	1000	75
<i>p</i> -Hydroxymercuribenzoate + Cleland's reagent	100	68

Penicillamine, even at 1 mM, had very slight inhibitory effect. A similar observation, that penicillamine does not significantly inhibit the monoamine oxidase activity (from rat or human skin), has recently been made by LOVENBERG *et al.*<sup>18</sup>. *p*-Hydroxymercuribenzoate (10  $\mu$ M) severely inhibited the enzyme activity and the inhibition could be partly reversed by sulphydryl compounds such as  $\beta$ -mercaptoethanol and Cleland's reagent.

#### DISCUSSION

An active monoamine oxidase is present in monkey small intestine. The enzyme is found predominantly in the mitochondrial fraction. Out of several procedures employed for the solubilization of the enzyme, the method of repeated freezing and sonication was found to be the most suitable, a procedure similar to that employed for pig brain mitochondria<sup>11</sup>.  $(\text{NH}_4)_2\text{SO}_4$  fractionation followed by gel filtration on Sepharose 4B gave a preparation which was homogeneous in polyacrylamide-gel electrophoresis.

The enzyme was optimally active at pH 7.6, similar to mitochondrial monoamine oxidase from various other sources. The  $K_m$  for kynuramine, 50  $\mu$ M, was of the same order as reported for the bovine kidney enzyme<sup>14</sup>. However, OSWALD AND STRITTMATTER<sup>19</sup> have shown that  $K_m$  values for tyramine and serotonin vary not only between species but also between organs from the same species.

Several monoamine oxidase inhibitors severely affected the enzyme activity. 1-Phenyl-2-isopropylhydrazine was not so effective. In the case of the beef brain enzyme it has been shown<sup>3</sup> that isopropylhydrazine was a potent inhibitor. On the basis of this, the inhibition by isopropylhydrazine derivatives like 1-phenyl-2-isopropylhydrazine and iproniazid has been attributed to the isopropylhydrazine moiety of these compounds (see also ref. 20). In the case of monkey intestinal enzyme the inhibition using 1-phenyl-2-isopropylhydrazine (10  $\mu$ M) was only 16% compared to about 90% with iproniazid, thus suggesting that other factors may be involved.

The inhibition of the monkey intestinal enzyme by *p*-hydroxymercuribenzoate indicates that a sulphydryl group may be essential for the activity of monoamine oxidase. A number of chelating agents moderately inhibited the monoamine oxidase preparation of monkey intestine suggesting the involvement of a metal ion for enzyme activity, but this needs further evaluation. Earlier workers have considered the possibility of a metal involvement (for review see ref. 21).

The gel filtration data using Sepharose 4B columns<sup>22</sup> can be used to make a rough evaluation of the molecular weight of the monoamine oxidase preparation. This gave a value of about  $1 \cdot 10^6$  which is higher than the reported values for monoamine oxidase from rat liver<sup>10</sup> and bovine kidney<sup>14</sup> ( $2.4 \cdot 10^5$ ) and from pig brain<sup>11</sup> ( $1.02 \cdot 10^5$ ). Further studies are needed for a more definitive conclusion on the molecular weight of the monkey intestinal monoamine oxidase preparation, especially to see if the pure enzyme might still be a larger complex held in solution by Triton X-100.

In addition to this difference in molecular weight, the purified monkey intestinal preparation moved as a single band in disc electrophoresis and did not reveal the existence of heterogeneity. Recently COLLINS *et al.*<sup>23</sup> using disc electrophoresis on polyacrylamide gel have provided evidence for the existence of four isoenzymes of monoamine oxidase in the case of both rat liver and human liver. Earlier YODIM



AND SANDLER<sup>24</sup>, utilizing the same technique, were able to show two isoenzymes with a human placental preparation. But McEWEN *et al.*<sup>15</sup>, based on their kinetic data on the partially purified monoamine oxidase from human liver, have ruled out the presence of multiple monoamine oxidase activities, although they do not exclude the possibility that the intact mitochondria from human liver may contain multiple monoamine oxidase activities. In the present work, there was no evidence for heterogeneity of monoamine oxidase by gel filtration studies using Sephadex G-200 or Sepharose 4B or by disc electrophoresis on polyacrylamide gel. TIPTON AND SPIRES<sup>25</sup> have interpreted their kinetic findings as indicating that the majority of the monoamine oxidase activity in pig brain mitochondria is due to a single enzyme and suggest that the multiplicity of monoamine oxidase may not be a general phenomenon.

The monoamine oxidase from monkey intestine can thus be obtained in an electrophoretically homogeneous state by relatively simple fractionation techniques. The tedious solubilization step may not be a handicap, since the enzyme at this stage is quite stable in the deepfreeze and enables a batchwise operation to obtain a large initial sample. It is hoped that this procedure can be employed to isolate the homogeneous enzyme in sufficient quantity for a detailed study of its physicochemical properties.

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