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INHIBITION OF MITOCHONDRIAL MONOAMINE OXIDASE OF THE RAT UTERUS AND LIVER BY CLORGYLINE, PARGYLINE AND HARMINE

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Abstract—The inhibition of mitochondrial monoamine oxidase (MAO) activity in rat uterus and liver by clorgyline, harmine and pargyline is reported. MAO activity is shown to be present in mitochondria of the rat uterus by rate-zonal centrifugation on a sucrose gradient. Each inhibitor was tested for its ability to inhibit the oxidation of tyramine (TYN), 5-hydroxytryptamine (5HT) and β-phenylethylamine (PEA). TYN deamination by uterine organelles was inhibited in two distinct steps by clorgyline and harmine, whereas in liver mitochondria only clorgyline manifested the two-step inhibition pattern. Elimination of TYN oxidation by pargyline occurred as a single sigmoid curve. Single sigmoid inhibition curves with all three inhibitors were also observed for 5HT and PEA in both tissues. For uterine and liver mitochondria the relative effectiveness of each inhibitor toward the oxidation of the three substrates was as follows: (a) clorgyline and harmine, 5HT > TYN > PEA; (b) pargyline, PEA > TYN > 5HT. It was concluded that, as has been previously demonstrated in liver, two forms of MAO exist in mitochondria isolated from the rat uterus. This conclusion is based upon (1) the biphasic inhibition of TYN deamination by clorgyline and harmine and (2) the reversal of the relative inhibitory effectiveness of the two classes of MAO inhibitors, (a) clorgyline and harmine and (b) pargyline, toward the three substrates. Semicarbazide did not inhibit the oxidation of any of the substrates. This indicates that the mitochondrial enzyme activity from the uterus, as in the liver, is a true monoamine oxidase.

Mitochondrial monoamine oxidase (MAO; E.C. 1.4.3.4.) is believed to exist in multiple forms in a variety of tissues from various species. This interpretation has been drawn from reports of (1) biphasic inhibition patterns of the enzyme activity by drugs which specifically inhibit MAO [1–4], and (2) the separation of multiple bands of activity on polyacrylamide gels [5]. Johnston [1] concluded that two forms of MAO must exist in rat brain mitochondria on the basis of the biphasic inhibition of tyramine oxidation by clorgyline (M & B 9302). That portion of the activity which was inhibited by low concentrations of clorgyline was labelled as A and the remaining activity as B. MAO-A preferentially oxidizes 5-hydroxytryptamine (5HT) [7] and norepinephrine [6] and is also inhibited by harmine [2]. MAO-B is inhibited by pargyline [2] and deprenyl[7] and demonstrates a high affinity for β-phenylethylamine (PEA) [7].

Recent reports have correlated alterations in MAO activity in the rat uterus with variations in the endocrine state of the animal [8, 9]. The changes were found to be substrate dependent [9] suggesting that more than one form of MAO may be present in the rat uterus. Multiple forms of MAO have been demonstrated in liver mitochondria [3, 5].

We present evidence for the existence of multiple forms of MAO in mitochondria isolated from the rat uterus on the basis of the response of the enzyme activity to MAO inhibitors.

MATERIALS AND METHODS

Female Sprague–Dawley rats originally derived from Charles River Breeding Laboratories, Wilmington, Massachusetts, U.S.A., were bred in our own colony for these studies. Animals were housed on a 14/10 light cycle (lights on from 05:00 to 19:00 hr). Purina Laboratory Chow and water were available *ad lib.* Tissues were taken from 90–110-day-old animals killed by decapitation. Animals from all stages of the estrous cycle were used.

Preparation of mitochondria. Minced livers were homogenized in 3 vol of 0.32 M sucrose medium containing 0.1 mM EDTA at pH 7 by a motor-driven Potter–Elvehjem homogenizer with Teflon pestle and then diluted to 10 vol and centrifuged in a Sorvall Model RC2-B refrigerated centrifuge at 3000 *g* for 3 min 20 sec timed from the point at which the rotor attained speed to the time of shutdown in an SS-34 rotor. Supernatant was then centrifuged at 35,000 *g* for 15 min and the pellet resuspended using a Dounce homogenizer and washed twice in 0.32 M sucrose.

Minced uteri were subjected to enzymatic digestion according to the method of Williams and Gorski [10] prior to homogenization. Incubations were done in air at 37° in Krebs–Ringer phosphate buffer medium, pH 7.4, containing 10 mg/ml Ficoll. Approximately 200 mg/ml of uterine tissue, wet weight, was incubated for 30 min in the presence of 20 mg/ml trypsin and 11.5 mM $CaCl_2$. The tissue was centrifuged and the sediment washed twice with buffer. The washed tissue fragments were then incubated 30 min with a volume of buffer equal to the original volume containing 4 mg/ml trypsin and 7 mg/ml collagenase. The tissue fragments were sedimented and washed twice with buffer containing soybean-trypsin inhibitor, 0.2 mg/ml and a third time with 0.1 mg/ml. The digested tissue was suspended in sucrose medium, 3 vol, calculated on the basis of the original tissue weight, homogenized and treated as for the preparation of liver

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mitochondria. The relative specific activities of undigested and digested tissue for the three substrates were the same. Total activities in digested tissue were somewhat higher, presumably due to the reduced effort required to disrupt the tissue fragments. In the absence of enzymatic digestion of the tissue only a small per centage of the MAO activity co-sediments with cytochrome *c* oxidase on a sucrose gradient. The majority of the activity remains at the top of the gradient.

Rate-zonal sucrose gradient centrifugation. Mitochondria suspended in 0.32 M sucrose were layered atop a linear sucrose gradient, 1.05, 1.25 g/cm^3. The gradient was centrifuged in a Beckman Model L2-65B ultracentrifuge for 30 min at 25,000 rpm in an SW 27 rotor (88,900 *g*). Each fraction, approx 2 ml, was assayed for protein content, cytochrome oxidase activity and monoamine oxidase activity.

Monoamine oxidase assay. A modification of the radiochemical assay of Otsuka and Kobayashi [11] was employed to measure MAO activity. Assays were carried out in 0.1 M borate buffer, pH 7.8, in a final volume of 0.5 ml for 30 min in a shaking water bath at 37°. Each assay tube contained 5 μg of amine (free base), approx. 50,000 dis/min of [^{14}C]amine, 1 mM EDTA and an aliquot of mitochondrial preparation equivalent to 125 μg of protein for liver mitochondria and 225 μg of protein for uterine mitochondria. The reaction was stopped by adding 0.2 ml of 2 M citric acid to each tube and radioactive product was extracted into 5 ml of anisole containing 0.4% Omnifluor (98% 2,5-diphenyloxazole and 2% *p*-bis-(*O*-methylstyryl)-benzene), cooled at 4° and centrifuged in a table-top centrifuge. An aliquot of extract was removed and counted in a liquid scintillation counter, Beckman Instruments, Model LS 100-C. The extraction efficiency of anisole for the oxidation products of all substrates was approximately 90%.

Cytochrome oxidase assay. Enzyme activity was determined by measuring the rate of oxidation of cytochrome *c* at 550 nm [12] with the aid of a Beckman DU-2 spectrophotometer and Honeywell Electronic 194 recorder. Assays were conducted in 0.1 M potassium phosphate buffer, pH 7, containing 0.1% Tween 80 at 23°.

Protein assay. Protein was estimated by the method of Lowry *et al.* [13], using bovine serum albumin as a standard.

Inhibition of enzyme activity. An aliquot of frozen and thawed mitochondria was placed in the assay tube along with inhibitor and incubated for 15 min at 23°. Substrate solution, 0.1 ml in buffer, was added and the tubes incubated 30 min at 37°. Each assay was done in duplicate.

Chemicals. Tyramine-1-[^{14}C]hydrochloride, 9.2 mCi/m-mole, 5-hydroxytryptamine-2-[^{14}C]binoxalate, 51.7 mCi/m-mole, and β-phenylethylamine-1-[^{14}C]hydrochloride, 9.9 mCi/m-mole, were obtained from New England Nuclear, Boston, Mass., U.S.A. Tyramine hydrochloride, 5-hydroxytryptamine creatinine sulfate, β-phenylethylamine, trypsin, Type III, collagenase, Type I, soybean trypsin inhibitor, Type I-S, cytochrome *c*, Type III, bovine serum albumin and Ficoll were purchased from Sigma Chemical Company, St. Louis, Missouri, U.S.A. Anisole and disodium EDTA were from Fisher Scientific Co., Boston, Mass., U.S.A. Tween 80 was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Semicarbazide hydrochloride was from Eastman Chemical Co., Rochester, N.Y., U.S.A. Pargyline (*N*-methyl-*N*-propargylbenzylamine) and harmine hydrochloride hydrate were purchased from Aldrich Chemical Co., Milwaukee, Wis., U.S.A. Clorgyline (M & B 9302; *N*-methyl-*N*-propargyl-3-(2,4-dichlorophenoxy) propylamine hydrochloride) was a gift from D. R. Maxwell of May & Baker Ltd., Dagenham, Essex, RM10 7XS, England.

RESULTS

Mitochondrial enzyme activities. The specific activities for TYN, 5HT and PEA of the mitochondrial preparations from the liver and uterus are presented in Table 1. Under the conditions used in these studies the specific activities for TYN and PEA were greater than for 5HT in organelles from both tissues. Specific activities were greater for the three amines in liver mitochondria than in uterine mitochondria.

Rate-zonal sucrose gradient centrifugation. Rat uterine monoamine oxidase co-sediments with the inner membrane marker enzyme cytochrome oxidase at a sucrose density of 1.185 g/cm^3, Fig. 1, demonstrating that the MAO activity measured in these studies was of mitochondrial origin. The peaks of enzyme activity are broad indicating that some disruption of the mitochondrial membranes has occurred during isolation. The muscular nature of the uterus makes the isolation of intact mitochondria impossible using standard techniques. By employing an enzymatic pre-digestion of the minced uteri the yield of mitochondrial enzyme activity was tripled and a preparation with greatly reduced membrane disruption was obtained. Inhibitor studies utilized frozen and thawed mitochondria. We do not believe that the biphasic inhibition curves shown in Fig. 3 are simply the result of the presence of two populations of mitochondria in the preparations with different degrees of disruption of their outer membranes.

Inhibition of liver mitochondrial MAO. Inhibition of liver mitochondrial monoamine oxidase by pargyline, clorgyline and harmine is depicted in Fig. 2. Each compound was tested for its ability to inhibit the oxidation of TYN, 5HT and PEA. Each curve represents the mean for two independent experiments utilizing different mitochondrial preparations.

Pargyline was most efficient in inhibiting PEA deamination and least effective against 5HT, Fig. 2a.

Table 1. Monoamine oxidase activity of rat liver and uterine mitochondria.

Tissue	Substrate (nmoles/30 min/mg protein) TYN	5HT	PEA
Liver	52.3	25.7	67.5
Uterus	49.5	15.8	40.5

Enzyme activity measured with tyramine (TYN), 5-hydroxytryptamine (5HT) and β-phenylethyl-amine (PEA). Each value represents the mean from two mitochondrial preparations.

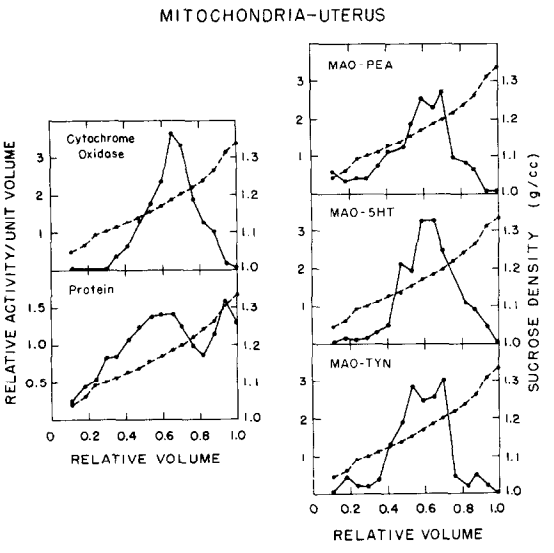

Fig. 1. Rate-zonal centrifugal analysis of rat uterine mitochondria on a linear sucrose gradient. Mitochondria suspended in 0.32 M sucrose, 12 mg protein/5 ml, was layered atop a linear sucrose gradient, 27 ml of 1.05 1.25 g/cm^3 sucrose, and centrifuged for 30 min at 25,000 rpm (88,900 g) in the SW 27 rotor in the L2-65B ultracentrifuge. Each fraction, approximately 2 ml, was assayed for protein, cytochrome oxidase activity and monoamine oxidase activity with tyramine (TYN), 5-hydroxytryptamine (5HT) or β-phenylethylamine (PEA) as substrates. Relative vol = accumulated fraction vol/total vol. Relative activity/unit vol = per cent activity in fraction/per cent vol in fraction.

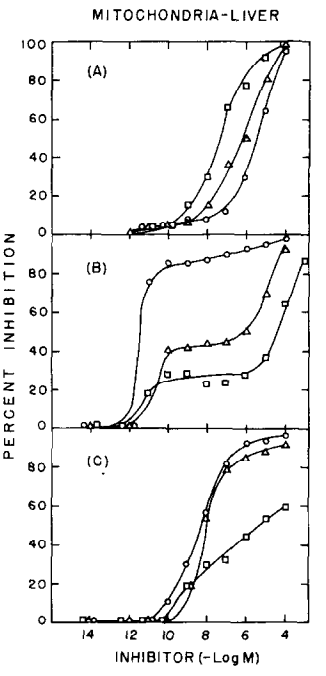

Fig. 2. The inhibition of rat liver mitochondrial monoamine oxidase by (A) pargyline, (B) clorgyline and (C) harmine. Each curve represents the inhibition of MAO using either [^{14}C]5-hydroxytryptamine (○), [^{14}C]tyramine (△) or [^{14}C]β-phenylethylamine (□) as substrate. An aliquot of mitochondrial enzyme preparation was pre-incubated 15 min at 26° in the presence of inhibitor after which substrate was added and the assay carried out at 37° for 30 min. Each point is the mean for two separate experiments. Enzyme activities for the three substrates are as shown in Table 1. Activity in the presence of inhibitor was compared to these values to determine the per cent inhibition.

Concentrations of pargyline giving 50% inhibition of enzyme activity, i.e. pI_{50}, were as follows, PEA = 7.5, TYN = 6.0, 5HT = 5.5.

Clorgyline inhibition, Fig. 2b, differed in two important aspects from that observed for pargyline. First the inhibition of TYN and PEA oxidation occurred in two distinct steps. Approximately 30–50% of the enzyme activity toward PEA and TYN was inhibited below 10^{-10} M clorgyline. The remaining activity was not eliminated until a concentration of 10^{-4} M was reached. For PEA pI_{50} (A) = 11 and (B) = 4 and for TYN(A) = 10.5 and (B) = 5. Over 80% of 5HT oxidizing capacity was inhibited at 10^{-10} M clorgyline, $pI_{50} \simeq 11.5$. The remaining 20% of the activity was not eliminated until a concentration of 10^{-4} M was reached. Secondly, the relative

Table 2. Inhibition of MAO activity of rat liver and uterine mitochondria.

Tissue	Inhibitor	[M]	Per cent Inhibition Substrate TYN	5HT	PEA
Liver	Semicarbazide	10^{-5}	0	0	0
		10^{-3}	0	13	0
	Clorgyline	10^{-5}	77	98	40
		10^{-3}	99	99	70
	Pargyline	10^{-6}	98	97	97
		10^{-4}	100	100	100
Uterus	Semicarbazide	10^{-5}	0	8	3
		10^{-3}	23	18	20
	Clorgyline	10^{-5}	65	77	28
		10^{-3}	96	100	58
	Pargyline	10^{-6}	94	89	33
		10^{-4}	97	95	90

Semicarbazide, clorgyline or pargyline was incubated with an aliquot of mitochondria for 15 min at 23°. Tyramine (TYN), 5-hydroxytryptamine (5HT) or β-phenylethylamine (PEA) was then added and the assay run for 15 min at 37°. Values represent the loss of enzyme activity compared to mitochondria incubated in the absence of inhibitor. M = moles/l.

order of inhibitory capacity toward the three substrates was inverse to that of pargyline.

Our data demonstrate similar effects of clorgyline on the oxidation of 5HT and TYN by rat liver mitochondrial MAO to those reported earlier by Hall *et al.* [3]. The pI_{50} values for 5HT and TYN inhibition by clorgyline are lower than previously reported for the liver. Differences in assay conditions such as substrate and protein concentrations are likely to be the reason for any apparent discrepancies. Furthermore, many studies in the past have been done on tissue homogenates [1–3] rather than purified organelles.

The relative inhibitory activity of harmine toward the three amines was the same as observed for clorgyline. PEA metabolism was the least effectively inhibited, never reaching a value greater than 60% at 10^{-4} M. TYN and 5HT had nearly the same $pI_{50} \simeq 8$. The inhibition curve for 5HT was shifted to slightly lower concentrations than that for TYN, however.

Semicarbazide did not inhibit amine oxidation even at a concentration of 10^{-3} M, Table 2.

Inhibition of uterine mitochondrial MAO. Pargyline, clorgyline and harmine inhibition of MAO activity in uterine mitochondria is shown in Fig. 3. Each curve represents the mean for two independent experiments utilizing different mitochondrial preparations.

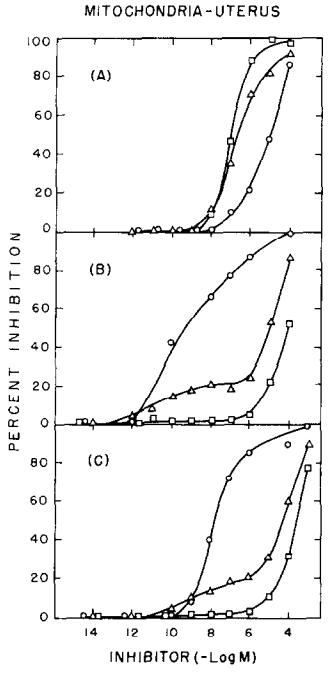

Fig. 3. The inhibition of rat uterine mitochondrial monoamine oxidase by (A) pargyline, (B) clorgyline and (C) harmine. Each curve represents the inhibition of MAO using either [^{14}C]5-hydroxytryptamine (○), [^{14}C]tyramine (△) or [^{14}C]β-phenylethylamine (□) as substrate. An aliquot of mitochondrial enzyme preparation was incubated 15 min at 26° with inhibitor after which substrate was added and the assay carried out at 37° for 30 min. Each point is the mean for two separate experiments. Enzyme activities for the three substrates are as shown in Table 1. The activity in the presence of inhibitor is compared to these values to determine per cent inhibition.

The patterns of inhibition of amine oxidation in uterine mitochondria by pargyline, clorgyline and harmine were analogous to those observed with liver organelles. Pargyline, Fig. 3a, exhibited a $pI_{50} = 7$ with PEA, 6.5 with TYN and 5 with 5HT. These are very close to the respective values of 7.5, 6.0 and 5.5 for liver preparations.

As was the case for the liver, the order of inhibition of metabolism of 5HT, TYN and PEA by clorgyline and harmine was reversed with respect to that of pargyline. 5HT oxidation was inhibited at much lower concentrations than was PEA oxidation. The pI_{50} values for clorgyline were 5HT = 9.5 and PEA = 4. For harmine these same figures were 5HT = 8 and PEA = 3.5. TYN deamination was inhibited in two steps by both clorgyline, pI_{50} (A) = 10.5 and (B) = 5, and harmine, pI_{50} (A) = 9 and (B) = 4.

Semicarbazide is ineffective as an inhibitor of TYN, 5HT or PEA oxidation by uterine mitochondria, Table 2. Enzyme activity is nearly completely inhibited at 10^{-3} M clorgyline or pargyline whereas semicarbazide at 10^{-3} M abolishes only 20% of the enzyme activity.

Differences in the inhibition patterns for liver and uterine mitochondrial MAO are apparent in comparisons of Figs 2 and 3. Clorgyline inhibition of TYN and PEA oxidation was characterized by biphasic inhibition curves in liver mitochondria, whereas only TYN deamination was inhibited in two stages in uterine organelles. Oxidation of 5HT was inhibited at lower concentrations of clorgyline in liver, $pI_{50} = 11$, than in uterus, $pI_{50} = 9$. Another distinguishing feature of the uterine enzyme was the biphasic inhibition of TYN oxidation by harmine which was not observed in the liver.

The relative specific activity for 5HT, in comparison to TYN or PEA, Table 1, was lower in the uterus than in the liver. The ratio MAO-A:MAO-B was lower in the uterus, 20:80, Fig. 3, than in the liver, 40:60, Fig. 2. It is possible that the difference in the ratios of MAO-A:MAO-B in the two tissues was the reason for the difference in the relative specific activities for 5HT in the two tissues.

DISCUSSION

On the basis of our studies it is apparent that monoamine oxidase activity of rat uterine mitochondria exists in at least two different forms possessing distinct substrate and inhibitor specificities. In the terminology of Johnston [1], one form, MAO-A, is inhibited at low concentrations of clorgyline and exhibits a high affinity for 5HT. The other form, MAO-B, is most active toward PEA and is less sensitive to clorgyline than is MAO-A but it is more susceptible to pargyline inhibition. Both forms metabolize tryramine.

These results compare favorably with reports of the occurrence of multiple forms of MAO in the brain [1, 4], intestine [2], kidney [2] and liver [3] as well as tissue specific MAO activities in sympathetic ganglion and pineal gland [4, 6].

Semicarbazide, an inhibitor of the pyridoxal-linked enzyme 'benzylamine oxidase' [14], did not inhibit

the oxidation of TYN, 5HT or PEA by uterine mitochondria to any appreciable extent. This also supports the conclusion that the amine oxidase activity of the uterine mitochondria is a true monoamine oxidase.

The uterus is a heterogeneous tissue consisting of a muscular outer layer and a glandular inner layer. It is richly innervated [15–17] and the nerve supply undergoes alterations as the hormonal state of the animal changes [16–17]. The uterus and other organs of the reproductive tract are atypical in that they possess a dual adrenergic nerve supply composed of the usual 'long' neurons and 'short' neurons which arise from ganglia within or near the organ in question.

Neuron-specific MAO activities have been reported [4]. MAO in the uterus varies with changes in the endocrine state of the rat [8, 9] and is a substrate-dependent phenomenon [9]. It is believed that only the 'short' neurons of the guinea pig uterus respond to changes in hormone levels [18]. The two types of uterine mitochondrial MAO observed in these studies may be located in different types of adrenergic neurons with distinct roles in the mechanisms responsible for regulating the physiological activities of the uterus.

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