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PROBLEMS WITH THE MEASUREMENT OF MONOAMINE OXIDASE A PROTEIN CONCENTRATION IN MITOCHONDRIAL PREPARATIONS

REVISED MOLECULAR ACTIVITIES AND IMPLICATIONS FOR ESTIMATING RATIOS OF MAO A:MAO B MOLECULES FROM RADIOCHEMICAL ASSAY DATA

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Abstract—There are significant discrepancies in the literature concerning the concentration of monoamine oxidase A (MAO A) from a number of tissue sources. Therefore, we compared the two principal techniques that have been used for quantitation of MAO A protein concentration: (1) titration of the enzyme with the MAO A-selective inhibitor clorgyline, and (2) saturation of the enzyme with [^{3}H]-pargyline followed by immunoprecipitation with an MAO A-specific monoclonal antibody. To determine which of the two techniques was likely to yield more reliable values for MAO A, MAO A protein concentrations in the same preparations were determined by quantitative immunoblotting. [^{3}H]Pargyline binding and quantitative immunoblotting yielded comparable values which were markedly lower than those obtained by titration of MAO A with unlabeled clorgyline. Therefore, clorgyline titration can seriously overestimate the concentration of MAO A protein in mitochondrial preparations. Since many literature values for the molecular activity of MAO A have relied upon enzyme concentrations determined by clorgyline binding, we reevaluated the molecular activities of MAO A and B for five important substrates. The ratio, MAO A molecular activity:MAO B molecular activity decreased in the order: serotonin (35:1) > tryptamine (12:1) > tyramine (3.3:1) > dopamine (2.4:1) > benzylamine (1:23). No comparable ratio was determined for β-phenylethylamine because of its previously described substrate inhibition of MAO B, although it is oxidized faster by MAO B over a wide range of concentrations. Comparison of molecular activities and K_m values for MAO A and B showed that with the exception of benzylamine and β-phenylethylamine, MAO A oxidizes the other tested substrates faster than MAO B over a wide range of concentrations. Therefore, measured ratios of MAO A:MAO B activity are generally greater than the ratios of MAO A:MAO B molecules in the preparations.

Monoamine oxidases (MAO; EC 1.4.3.4) oxidize a variety of exogenous and endogenous amines, including neurotransmitters such as serotonin, dopamine and norepinephrine (reviewed in Ref. 1) and the potent dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [2]. MAO A and B occur primarily in the outer mitochondrial membrane [3] and differ in substrate affinity [4, 5], inhibitor selectivity [6, 7] and immunological reactivity [8–10]. The two enzymes are approximately 70% identical in amino acid sequence and are coded by distinct but closely related genes [11].

While literature values for the concentration of MAO B molecules in a number of tissues are in reasonable agreement, comparable values for MAO A vary widely. For example, in homogenates of human cerebrum, the MAO B concentration has been reported to be approximately 3 pmol/mg protein [12, 13], while MAO A concentration has been variously reported to be 0.35 pmol/mg protein [13] and 9 pmol/mg [12]. The MAO B concentration in rat liver mitochondria is reportedly 12–13 pmol/mg protein [13, 14], while MAO A concentration has variously been reported to be 1.4 [13] and 11 [14] pmol/mg. Although it is conceivable that these discrepancies in MAO A concentration in the same tissue and species reflect biological variability, systematic problems in the quantitation technique may be at least in part responsible.

Most literature estimates of MAO A concentration in complex samples have relied upon the stoichiometric, covalent binding of irreversible inibitors to the FAD cofactor, which is bound to a single cysteine residue in each MAO subunit [15]. In one scheme, MAO activity is titered using known quantities of unlabeled, MAO A- or MAO B-selective, irreversible inhibitor, and the concentration of MAO A inferred from the quantity of inhibitor required to inhibit 100% of MAO activity [12, 14, 16, 17]. Alternatively, the substrate binding sites of MAO are saturated with radiolabeled, irreversible inhibitor, and the concentration of MAO inferred from the quantity of label bound [12, 13, 18–20]. [^{3}H]Pargyline, at sufficient concentrations, binds to both MAO A and MAO B, and is commercially available.

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For quantitation of MAO A and MAO B in preparations containing the two enzymes, [^{3}H]pargyline can be selectively bound to MAO A or B by inhibiting one of the enzymes with an appropriate selective inhibitor before labeling [12, 13, 18, 19], or [^{3}H]pargyline bound to each enzyme can be discriminated by immunoprecipitation of the [^{3}H]pargyline-labeled mixture with MAO A- or B-specific monoclonal antibodies [20].

In two cases where inhibitor titration and radiolabeled inhibitor binding were compared, the measured MAO A concentration was higher when determined by clorgyline titration than when determined by [^{3}H]pargyline binding, and it was suggested that values obtained using [^{3}H]pargyline binding were more likely to be correct [12, 18]. To test this conclusion directly, we have compared MAO A concentrations obtained by clorgyline titration, by [^{3}H]-pargyline binding, and by quantitative immunoblotting, which depends upon binding of an MAO A-specific monoclonal antibody. The results show that [^{3}H]pargyline binding and quantitative immunoblotting yield comparable and markedly lower values than does titration of MAO A with unlabeled clorgyline. Therefore, the [^{3}H]pargyline binding technque is probably the more reliable. Since many values in the literature for MAO A molecular activity and MAO A:MAO B ratio have relied upon clorgyline titration to quantitate MAO A concentration (for example, see Ref. 21), we have reevaluated the molecular activities of MAO A and B for several commonly used MAO substrates. The results show that the ratio of MAO A:MAO B activity determined by radiochemical assays is higher than the ratio of MAO A:MAO B molecules in the preparations.

MATERIALS AND METHODS

The following were obtained from New England Nuclear, Boston, MA: [^{14}C]serotonin binoxylate (60 Ci/mol, [^{14}C]β-phenylethylamine (PEA; 56 Ci/mol), [^{14}C]tyramine hydrochloride (44 Ci/mol), [^{3}H]tryptamine hydrochloride (37 Ci/mmol), [^{3}H]-dopamine hydrochloride (53 Ci/mmol), and [^{3}H]-pargyline hydrochloride (22.5 Ci/mmol). [^{14}C]Benzylamine hydrochloride (50 Ci/mol) was obtained from ICN Radiochemicals, Irvine, CA. Unlabeled dopamine, tyramine, tryptamine and serotonin were purchased from Research Biochemicals, Inc., Natick, MA. Benzylamine and PEA were purchased from the Sigma Chemical Co., St. Louis, MO.

Preparation of mitochondria and platelet membranes. Mitochondria from placenta and liver were isolated as previously described [20]. Term placentae were obtained from the Labor and Delivery Service of the Department of Obstetrics and Gynecology of the University of Texas Medical Branch, Galveston, TX. Human liver was obtained from Dr. Paul Bohr, Department of Pathology, University of Texas Medical Branch, Galveston, TX, washed in ice-cold isotonic buffer, cut into fragments and frozen at −80° until used.

Membranes from purified platelets obtained from Dr. Richard R. Fritz, Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, TX, were isolated as previously described [22].

Quantitation of MAO A by clorgyline titration. Mitochondria from liver (1.46 mg) or placenta (2.19 mg) were incubated for 3 hr at 30° in 0.05 M potassium phosphate buffer (pH 7.3) containing various concentrations of clorgyline (10–100 nM for liver, 0.1–1 μM for placenta). Residual MAO A activity was assayed with 100 μM [^{14}C]serotonin in 0.1 M potassium phosphate buffer (pH 7.3) as previously described [20]. Samples were diluted appropriately so that catalytic activities were linear with time over the 10-min assay interval. Samples were assayed in duplicate and each tissue was titered twice. Data are expressed as picomoles MAO A per milligram of protein after extrapolation to the picomoles of clorgyline necessary for 100% inhibition of MAO A. A stoichiometry of one molecule of clorgyline per FAD moiety, one FAD moiety per MAO A subunit, and two subunits per molecule were assumed[15].

Quantitation of MAO A and B active sites by [^{3}H]-*pargyline binding.* MAO A and B were quantitated using [^{3}H]pargyline as described previously [20]. Mitochondria (liver, 1.46 mg; placenta, 2.19 mg) or platelet membranes (1.6 mg) were incubated for 3 hr at 30° in 0.05 M potassium phosphate buffer (pH 7.3) containing 5 μM [^{3}H]pargyline HCl (22.5 Ci/mmol) from which the ethanol solvent had been evaporated under a stream of nitrogen gas. The preparations were washed by microcentrifugation to remove unbound label. Samples were assayed with 100 μM serotonin or 10 μM PEA to determine efficiency of [^{3}H]pargyline inhibition of MAO A and MAO B, respectively.

The quantity of pargyline bound to MAO was determined by extracting membranes with 1% Triton X-100 and immunoprecipitating MAO using an MAO A-specific (MAO A-4D3) or MAO-B-specific (MAO B-1C2) monoclonal antibody as previously described [20]. Pargyline was assumed to bind to MAO A with the same stoichiometry as clorgyline (one per subunit), and two subunits per molecule were assumed. Values were corrected for efficiency of inhibition by pargyline and efficiency of extraction of label from the membranes.

Quantitation of MAO A by immunoblotting. The immunoblotting procedure has been described previously [20]. Briefly, proteins were electrophoresed on sodium dodecyl sulfate (SDS)-polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. After blocking, the membranes were incubated in Tris-buffered saline (pH 7.3) containing 0.1% Tween-20 and primary antibody (1/200 dilution of MAO A-4D3 ascites fluid or a 1/1000 dilution of rabbit antiserum elicited to purified MAO A). Color was developed using an Elite ABC kit (Vector Laboratories, Burlingame, CA) or peroxidase-conjugated goat anti-rabbit IgG. The stained bands were visualized using a Hammamatsu C-1000 camera and True-Visicon MB frame grabber video board and analyzed by computerized image analysis using JAVA software with the "Analyzing" Utility (Jandell). Various quantities of liver (20–250 μg) and placental mitochondrial protein (12.5–100 μg) were compared to serially

diluted, known quantities of purified placental MAO A (0.125–1.0 μg; provided by Drs. Walter Weyler and James Salach, Department of Molecular Biology, Veterans Administration Medical Center, San Francisco, CA) run on the same gel. The subunit molecular weight calculated from a published human MAO A cDNA clone (59,400; [11]) was used to convert immunoblot values from nanograms to picomoles of MAO A, and two subunits per molecule of MAO A were assumed. The concentration of the MAO A in the purified sample was determined by flavin bleaching with dithionite, which detects the flavin moiety of MAO but does not require catalytic activity [23].

Determination of molecular activities of MAO A and B. MAO A molecular activity was determined in placental and liver mitochondrial preparations after preincubation with 1 μM deprenyl to inhibit MAO B. MAO B molecular activity was determined in liver after preincubation with 0.1 μM clorgyline to inhibit MAO A, and in platelet membranes (no preincubation). Minor cross-inhibition of MAO A by deprenyl and MAO B by clorgyline under these conditions was measured by assays with 100 μM serotonin and 100 μM benzylamine and used to correct the molecular activity values. These substrates are quite selective for MAO A and B in the preparations assayed.

The K_m values of MAO A and MAO B for each substrate were determined by assays over a range of concentrations: benzylamine (10–1300 μM), dopamine (10–1300 μM), PEA (MAO A: 10–1300 μM; MAO B, 2–25 μM), serotonin (10–1300 μM, MAO A only), tryptamine (1–150 μM) and tyramine (10–1300 μM). All assays were done in quadruplicate in 0.1 M potassium phosphate buffer (pH 7.3) for 10 min at 37°. Reaction products were extracted with water-saturated toluene, except water-saturated toluene/ethyl acetate (1:1) was used in the serotonin and dopamine assays. Specific activities are expressed as nanomoles per hour per milligram of protein. K_m values were derived from Lineweaver-Burk representations of the data.

Molecular activities of MAO A and B were calculated by dividing the number of molecules of substrate oxidized per minute by the number of molecules of MAO A or B determined by [^{3}H]pargyline binding. Enzyme activities for molecular activity measurements were performed at concentrations such that the measured velocities corresponded to at least 87% of the theoretical maximum molecular activities, as determined from the equation $V_{obs}[1 + (K_m/S)] = V_{max}$, and in all cases except PEA and MAO B, values were corrected to the theoretical molecular activities at substrate saturation. As noted by others, MAO B exhibits substrate inhibition for PEA [4, 24, 25], thereby making such a correction unreasonable. The molecular activity of MAO B for PEA reported in Table 3 was determined at 150 μM, and may reflect some substrate inhibition. To minimize variability, all tissues were assayed with all substrates at the same time. Results are expressed as means ± range of two experiments.

RESULTS

Quantitation of MAO A active sites. The concentrations of catalytically active MAO A molecules in liver and placental mitochondrial membranes were estimated by titrating serotonin-oxidizing activity with the MAO A-selective inhibitor clorgyline, and by saturation with [^{3}H]pargyline followed by immunoprecipitation with the MAO A-specific monoclonal antibody. As an independent measure of the concentration of MAO A protein in these preparations, samples were immunoblotted in parallel with serial dilutions of purified placental MAO A of known concentrations.

Table 1. Comparison of MAO A quantitation methods

Quantitation method	Concentration of MAO A (pmol/mg protein)	
	Liver	Placenta
Clorgyline titration	41.5 ± 0.5*	332 ± 24*
Immunoblot	< 17	108 ± 24
Pargyline labeling	3.2 ± 0.1	76 ± 2.9

Washed mitochondrial membranes from liver and placenta were assayed for MAO A concentration by three techniques described in Materials and Methods. Values are means ± SEM of three or more replicates, except as noted.
* Mean ± range of two independent titrations per tissue.

For quantitation by clorgyline titration, samples were incubated with increasing concentrations of the inhibitor as described in Materials and Methods. Control mitochondria incubated in buffer alone retained 90% (liver) to 100% (placenta) of initial MAO A activity, indicating that loss of activity was primarily or exclusively attributable to specific inhibition by clorgyline. In two independent experiments, extrapolation to 100% inhibition yielded values of 41.5 ± 0.5 pmol MAO A/mg protein for liver mitochondrial membranes, and 332 ± 24 pmol MAO A/mg protein for placental mitochondrial membranes (Table 1).

MAO A was also quantitated in the same liver and placental mitochondrial membrane preparations by [^{3}H]pargyline binding, as described in Materials and Methods. Assays with serotonin indicated that the pargyline labeling inhibited over 98% of serotonin oxidation, indicating that virtually all MAO A molecules had bound [^{3}H]pargyline. Triton X-100 extracted 90% of the [^{3}H]pargyline from the membrane. Measurement of the amount of [^{3}H]pargyline bound yielded MAO A concentrations far lower than the values obtained by clorgyline titration (3.2 ± 0.1 and 76 ± 2.9 pmol MAO A/mg protein for liver and placenta, respectively; Table 1). The discrepancy was most marked in the liver preparation (41.5 vs 3.2 pmol/mg protein by clorgyline titration and [^{3}H]-pargyline binding, respectively).

To determine which inhibitor-based method was more reliable, serial dilutions of placental mitochondria were electrophoresed on SDS-polyacrylamide gels in parallel with samples of serially diluted, purified MAO A, and transferred to nitrocellulose, and the MAO A was stained with monoclonal or polyclonal antibodies elicited to purified placental MAO A. Quantitation of staining intensity revealed similar MAO A concentrations with the

Table 2. Concentration of MAO A and B in mitochondrial preparations from three tissues determined by pargyline labeling

Tissue	Concentration (pmol/mg)	
	MAO A	MAO B
Liver	3.2 ± 0.10	10.7 ± 1.2*
Placenta	76 ± 2.9	3.5 ± 0.21
Platelets	ND†	5.4 ± 0.07

Mitochondrial preparations used for the molecular activity determinations were incubated in 0.05 M potassium phosphate buffer containing 5 μM [^{3}H]pargyline for 3 hr at 30° as described in Materials and Methods. Labeled mitochondria were extracted with 1% Triton X-100, immunoprecipitated with 1/100 diluted MAO A-4D3 or MAO B-1C2 ascites fluid, and the concentrations of MAO A and B determined. Values are means ± SEM of three or four replicates, except as noted.

* Mean ± range of duplicates.

† Not determined.

monoclonal and polyclonal antibodies (114 ± 35 and 100 ± 28 pmol/mg protein, respectively). The pooled average, 108 ± 24, shown in Table 1, is slightly higher than the concentration determined by [^{3}H]pargyline binding (76 ± 2.9), but much lower than the value obtained from clorgyline titration (332 ± 24; Table 1). The concentration of MAO A in liver mitochondrial preparations could not be determined by immunoblotting, because neither the polyclonal nor monoclonal antibody stained MAO A in immunoblots of up to 250 μg of liver mitochondrial membranes. The low specific activity of MAO A in liver compared to placental mitochondrial membranes (3–4% of that in placental mitochondrial membranes) suggests that lack of MAO A immunoblotting in liver mitochondrial preparations results from an MAO A concentration below the limit of detection under our conditions, which was 17 pmol/mg protein, or about 0.2% of applied protein by weight.

Determination of molecular activities of MAO A and B. Since many molecular activity values in the literature have been derived from MAO A concentrations determined by clorgyline titration, we reevaluated the molecular activities of MAO A and B for the oxidation of six important MAO substrates using two tissue sources for each MAO. Estimates of MAO A and B concentration were derived from [^{3}H]pargyline labeling data in Table 2. [^{3}H]Pargyline (5 μM) inhibited greater than 95% of the MAO A and B activity in all of the mitochodrial preparations, suggesting that the active sites of both enzymes were at least 95% saturated.

To further increase substrate selectivity, we used deprenyl to inhibit MAO B in placental and liver preparations when the molecular activity of MAO A was measured, and clorgyline to inhibit MAO A in liver when the molecular activity of MAO B was measured. Molecular activities were corrected for slight cross-inhibition of MAO A by deprenyl and MAO B by clorgyline, as described in Materials and Methods.

The molecular activities of liver and placental MAO A and liver and platelet MAO B for the six substrates tested are shown in Table 3 and the K_m values in Table 4. Note that the molecular activity of MAO B for PEA, determined at 150 μM substrate, was not corrected to infinite substrate concentration because PEA exhibits substrate inhibition of MAO B at high concentrations ([4, 24]; see Discussion). Serotonin had the highest molecular activity for MAO A (1802 and 1393 min^{-1} in liver and placenta, respectively) and benzylamine, the lowest (14.6 min^{-1} in placenta; Table 3). Benzylamine had the highest molecular activity for MAO B (446 min^{-1} for liver MAO B), only 25–33% of the molecular activity of MAO A for serotonin. Serotonin and tryptamine exhibited very low molecular activity for MAO B (44 and 64 min^{-1}, respectively, for liver MAO B).

The molecular activities for substrates which are commonly used to assay both MAO A and B (dopamine, tryptamine and tyramine) were 2.5-fold (dopamine, liver) to nearly 15-fold (tryptamine, liver) higher for MAO A than for MAO B (Table 3). Serotonin and benzylamine are the most selective substrate pair for assaying MAO A and B, respectively. The molecular activity of MAO B for benzylamine was only 25–33% that of MAO A for serotonin.

DISCUSSION

Quantitation of MAO A active sites. Data from quantitative immunoblotting confirms that [^{3}H]pargyline binding is more reliable than clorgyline titration for quantitating MAO A concentration, consistent with previous suggestions [12, 18]. Values for MAO concentration derived from immunoblotting are slightly higher than those determined by [^{3}H]pargyline labeling, perhaps because a small proportion of MAO A molecules in the preparations studied were catalytically inactive, and therefore unable to bind [^{3}H]pargyline. Binding of [^{3}H]pargyline to molecules other than MAO A and B in mitochondrial and platelet membranes was apparently minimal, since nearly 100% of the [^{3}H]pargyline which remained bound to the mitochondrial membranes after extensive washing was immunoprecipitable by a combination of MAO A and MAO B-selective antibodies. Our values for MAO A and B concentration, using MAO A- and B-specific antibodies to discriminate pargyline bound to each enzyme, agree well with values determined by [^{3}H]pargyline binding of selectively inhibited enzyme preparations [13, 26], suggesting that these approaches are probably interchangeable.

Revised molecular activities. Since many values reported in the literature for the molecular activity of MAO A for various substrates (and for MAO A:MAO B ratio) have relied upon MAO A concentrations determined by clorgyline titration, the molecular activities of MAO A and B were evaluated in placenta (primarily MAO A), platelets (MAO B only) and liver (MAO A and B).

The molecular activities of placental and liver MAO A differed by no more than 37% for all substrates tested (Table 3), suggesting that MAO A

Table 3. Molecular activities of MAO A and B from three tissues

Substrate	Molecular activity (mol substrate/mol enzyme/min)			
	MAO A		MAO B	
	Liver	Placenta	Liver	Platelets
Benzylamine	ND*	14.6 ± 1.4	446 ± 40.5	242 ± 20.5
Dopamine	628 ± 71.5	816 ± 41.5	246 ± 14.5	193 ± 56
PEA	398 ± 43.5	304 ± 24.5	233 ± 22.2†	133 ± 17†
Serotonin	1802 ± 105	1393 ± 250	43.5 ± 0.5	132 ± 16
Tryptamine	898 ± 130	572 ± 67	64.5 ± 3.5	77 ± 7
Tyramine	668 ± 77	700 ± 96	202 ± 28.5	174 ± 13.5

Molecular activities were determined by assaying the molecules of substrate oxidized per minute by samples of MAO A and MAO B whose concentrations were determined by [^{3}H]-pargyline binding (Table 2). Substrate concentrations used were chosen to yield at least 87% of the theoretical maximum activity at substrate saturation, as calculated from the K_m values in Table 4. In all cases except MAO B and PEA, values are corrected to the molecular activities predicted at infinite substrate concentration. Where necessary, values were also corrected for inhibitor cross-inhibition as described in Materials and Methods. Values are means ± range of two experiments in each of which all substrates and tissues were assayed.

* Not determined because of very low activity.

† Determined at 150 μM (ten times the measured K_m), and not corrected to infinite substrate concentration.

Table 4. K_m values for several MAO A and B substrates

Substrate	K_m (μM)	
	MAO A	MAO B
Benzylamine	138 ± 1	187 ± 93
Dopamine	408 ± 107*	649 ± 102
PEA	80 ± 13	15 ± 1†
Serotonin	145 ± 2	
Tryptamine	12 ± 0.5	20 ± 3
Tyramine	107 ± 8	154 ± 3

The K_m values for the oxidation of six commonly used substrates were determined by radiometric assay of each substrate over a wide range of concentrations as described in Materials and Methods. Values are means ± range of duplicate experiments, except as noted.

* Mean ± SEM of three experiments.

† PEA exhibited substrate inhibition for MAO B above 10 μM. The apparent K_m was determined by extrapolation of the Lineweaver–Burk plot through four points lying on a straight line representing 2–10 μM.

from these tissues is functionally similar. However, the molecular activity of platelet MAO B for serotonin appeared to be nearly 3-fold higher than that of the liver MAO B. Because of the high molecular activity of MAO A for serotonin, it would take very little MAO A in the platelet preparation (1/20 of the MAO B active site concentration) to account for the excess serotonin oxidation by the platelet enzyme. Human platelets are believed to contain only MAO B [27], although the presence of such a small quantity of MAO A might have gone undetected. Platelets from rabbit, the only species other than human and some primates known to have MAO in their platelets [28], contain both MAO A and MAO B [29]. Further work is necessary to determine whether platelet MAO B differs from liver MAO B in its ability to oxidize serotonin. Such a functional difference could stem from differences in the mitochondrial membrane environments of the platelet and liver enzymes, or conceivably to differences in the structures of the proteins themselves.

The catalytic activities of MAO A and MAO B are often compared in parallel assays. The K_m values and revised molecular activity data provide a way of estimating from catalytic activity data the absolute concentrations and relative ratios of MAO A and B molecules in the assayed preparations. Our data indicate that the ratios of MAO A:MAO B activity determined by most radiochemical assays are greater than the ratios of MAO A:MAO B molecules in assayed preparations. Ratios of MAO A:MAO B activity are typically determined by (a) selective inhibitor titration of MAO activity toward a substrate oxidized by both enzymes (such as tyramine or tryptamine), or (b) assay of each enzyme with a different, relatively selective substrate (often serotonin for MAO A and benzylamine or PEA for MAO B). Concerning titration studies (a, above), our data show that the molecular activities of tyramine, tryptamine and dopamine are 3- (dopamine), 3.3- (tyramine) and 10-fold (tryptamine) higher for MAO A compared to MAO B. Since the K_m values of each of these three substrates for MAO A and B are similar, a preparation containing equal concentrations of MAO A and B molecules would exhibit more MAO A than B activity at all concentrations of these substrates. Similarly, the molecular activity of MAO A for serotonin is more than 4-fold greater than the molecular activity of MAO B for benzylamine (Table 3), and K_m values of MAO A for serotonin and MAO B for benzylamine are similar (Table 4). Therefore, a preparation of equimolar MAO A and B would exibit approximately four times more serotonin- than benzylamine-oxidizing

activity over a wide range of substrate concentrations.

Since MAO A and B activities are also commonly compared using serotonin (at 100–1000 μM for MAO A) and PEA (at 10 μM for MAO B), it is also important to consider the relationship between the ratio of serotonin:PEA oxidizing activities and the concentration of MAO A:MAO B molecules. PEA concentrations are normally kept low because although PEA was originally proposed to be a specific substrate for MAO B [30], it is oxidized rapidly by MAO A at high concentrations [31]. Its specificity for MAO B is largely dependent on its lower K_m for B than MAO A (Table 4). Our measured K_m values for MAO B are somewhat higher than values reported in the literature for the human enzyme, although reported values vary widely (from 1.3 μM [32] to 7.2 and 12.2 [33] for human brain MAO B). The reasons for this variability in reported K_m values is unclear, but may be related to differing reaction conditions, since PEA oxidation by MAO B exhibits substrate inhibition, and low levels of residual MAO A activity in preparations containing both enzymes strongly affect the apparent K_m. The turnover values of MAO B for PEA reported in Table 3, measured at 150 μM, probably reflect significant substrate inhibition, since other experiments yielded 60% higher PEA-oxidizing activity by MAO B at 10 μM than at 150 μM (our unpublished data; conditions: 10-min assay, 37°, pH 7.5). The degree of substrate inhibition by PEA depends upon substrate concentration, pH, protein concentration and assay time [34]. Using the molecular activity and K_m for liver MAO A for serotonin (Tables 3 and 4) and the estimated turnover of PEA by liver MAO B at 10 μM (167% of the turnovers measured at 150 μM; Table 3), we estimate that a preparation having an equal concentration of MAO A and B molecules oxidizes serotonin (at 1 mM concentration) four times faster than PEA (at 10 μM). Therefore, for this substrate pair, as for the other assays discussed above, the ratio of MAO A:MAO B activity is considerably greater than the ratio of MAO A:MAO B molecules.

The K_m values and revised molecular activities for human MAO A and MAO B for a number of substrates will be useful in many studies of MAO. For example, they may reconcile an apparent discrepancy between data derived from catalytic assays and data from immunocytochemical studies, which depend on enzyme quantity rather than enzyme activity. Measurements of catalytic activity in the presence of various MAO-inhibiting drugs suggest that rat serotonergic neurons contain both MAO A and B activities [35, 36]. Furthermore, while serotonin is primarily oxidized by MAO A *in vivo*, MAO B can oxidize serotonin when MAO A has been selectively inhibited [37]. Consistent with this, it has been reported that 80% of the oxidation of 100 μM serotonin in rat serotonergic synaptosomes is by MAO A, and 20% by MAO B [36]. Despite strong evidence for MAO A in rodent serotonergic neurons, immunocytochemical studies readily reveal MAO B in serotonergic neurons of monkey and human, but no [38, 39] or marginal [40] staining for MAO A. Rat serotonergic neurons also stain intensely for MAO B but no MAO A antibody reagent is available to stain MAO A in rodent brain. While it is possible that a species difference might account for the failure to detect MAO A by immunocytochemistry in primate serotonergic neurons, it can be calculated from the molecular activities of liver MAO A and MAO B for serotonin, and the K_m values of serotonin for MAO A (Table 4) and MAO B (from Tipton *et al.* [4]), that a ratio of MAO A:MAO B molecules of only 1:76 in serotonergic synaptosomes would result in 80% oxidation of 1 mM serotonin by MAO A (1:186 at 100 μM serotonin). Such a low ratio of MAO A:B molecules is certainly consistent with intense immunocytochemical staining of MAO B and weak or negligible staining of MAO A in primate serotonergic neurons.

In conclusion, we have confirmed that under our conditions, [^{3}H]pargyline binding yields a more reliable estimate of MAO A concentration than does clorgyline titration, and that discrimination of MAO A and B by selective inhibition before labeling or by selective immunoprecipitation after labeling yields similar results. In addition, we have shown that unless substrates and conditions are carefully chosen with molecular activities and relative K_m values in mind, radiochemical assays of the ratio of amine oxidation by MAO A:MAO B may systematically overestimate the ratio of MAO A:MAO B molecules in preparations containing both human enzymes.

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REFERENCES

1. Waldmeier PC, Amine oxidases and their endogenous substrates, *J Neural Transm* [Suppl] **23**: 55–72, 1987.
2. Salach JI, Singer TP, Castagnoli N Jr and Trevor A, Oxidation of the neurotoxic amine 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) by monoamine oxidases A and B and suicide inactivation of the enzymes by MPTP. *Biochem Biophys Res Commun* **125**: 831–835, 1984.
3. Greenewalt JW and Schnaitman C, An appraisal of the use of monoamine oxidase as an enzyme marker for the outer mitochondrial membrane. *J Cell Biol* **46**: 173–179, 1970.
4. Tipton KF, O'Carroll A-M and McCrodden JM, The catalytic behaviour of monoamine oxidase. *J Neural Transm* [Suppl] **23**: 25–35, 1987.
5. White HL and Glassman AT, Multiple binding sites of human brain and liver monoamine oxidase: Substrate specificities, selective inhibitions, and attempts to separate enzyme forms. *J Neurochem* **29**: 987–997, 1977.
6. Johnston JP, Some observations upon a new inhibitor of monoamine oxidase in brain tissue. *Biochem Pharmacol* **17**: 1285–1297, 1968.
7. Knoll J and Magyar K, Some puzzling pharmacological effects of monoamine oxidase inhibitors. *Adv Biochem* **5**: 393–408, 1972.
8. Denney RM, Fritz RR, Patel NT and Abell CW,

Human liver MAO-A and MAO-B separated by immunoaffinity chromatography with MAO-B-specific monoclonal antibody. *Science* **215**: 1400–1404, 1982.
9. Denney RM, Patel NT, Fritz RR and Abell CW, A monoclonal antibody elicited to human platelet MAO. *Mol Pharmacol* **22**: 500–508, 1982.
10. Kochersperger LM, Waguespack A, Patterson JC, Hsieh C-CW, Weyler W, Salach JI and Denney RM, Immunological uniqueness of human monoamine oxidases A and B: New evidence from studies with monoclonal antibodies to human monoamine oxidase A. *J Neurosci* **5**: 2874–2881, 1985.
11. Bach AWJ, Lan NC, Johnson DL, Abell CW, Bembenek ME, Kwan S-W, Seeburg PH and Shih JC, cDNA cloning of human liver monoamine oxidase A and B: Molecular basis of differences in enzymatic properties. *Neurobiology* **85**: 4934–4938, 1988.
12. O'Carroll A-M, Anderson MC, Tobbia I, Phillips JP and Tipton KF, Determination of the absolute concentrations of monoamine oxidase A and B in human tissues. *Biochem Pharmacol* **38**: 901–905, 1989.
13. Kalaria RN and Harik S, Blood–brain barrier monoamine oxidase: Enzyme characterization in cerebral microvessels and other tissues from six mammalian species, including human. *J Neurochem* **49**: 856–864, 1987.
14. Fowler CJ and Oreland L, The nature of the substrate-selective interaction between rat liver mitochondrial monoamine oxidase and oxygen. *Biochem Pharmacol* **29**: 2225–2233, 1980.
15. Weyler W, Monoamine oxidase A from human placenta and monoamine oxidase B from bovine liver both have one FAD per subunit. *Biochem J* **260**: 725–729, 1989.
16. Arai Y and Kinemuchi H, Differences between monoamine oxidase concentrations in striatum and forebrain of aged and young rats. *J Neural Transm* **72**: 99–105, 1988.
17. Fowler CJ, Oreland L, Marcusson J and Winblad B, Titration of human brain monoamine oxidase -A and -B by clorgyline and L-deprenil. *Naunyn Schmiedebergs Arch Pharmacol* **311**: 263–272, 1980.
18. Parkinson D, Lyles GA, Browne BJ and Callingham BA, Some factors influencing the metabolism of benzylamine by type A and B monoamine oxidase in rat heart and liver. *J Pharm Pharmacol* **32**: 844–850, 1980.
19. Summers KM and Craig IW, The relationship between human monoamine oxidase A and B. *Biochem Soc Trans* **10**: 183–184, 1982.
20. Riley LA, Waguespack MA and Denney RM, Characterization and quantitation of monoamine oxidases A and B in mitochondria from human placenta. *Mol Pharmacol* **36**: 54–60, 1989.
21. Oreland L, Fowler CJ and Wiberg A, Titration of monoamine oxidase-A and -B and its clinical application. In: *Monoamine Oxidase Inhibitors: The State of the Art* (Eds. Youdim MBH and Paykel ES), pp. 77–85. John Wiley, New York, 1981.
22. Fritz RR, Abell CW, Denney RM, Denney CB, Bessman JD, Boeringa JA, Castellani S, Lankford DA, Malek-Ahmadi P and Rose RM, Platelet MAO concentration and molecular activity. I. New methods using an MAO B-specific monoclonal antibody in a radioimmunoassay. *Psychiatry Res* **17**: 129–140, 1986.
23. Weyler W and Salach J, Purification and properties of mitochondrial monoamine oxidase type A from human placenta. *J Biol Chem* **260**: 13199–13207, 1985.
24. Suzuki O, Katsumata Y and Oya M, Oxidation of β-phenylethylamine by both types of monoamine oxidases: Examination of enzymes in brain and liver mitochondria of eight species. *J Neurochem* **36**: 1298–1301, 1981.
25. Fowler CJ and Tipton KF, Concentration dependence of the oxidation of tyramine by the two forms of rat liver mitochondrial monoamine oxidase. *Biochem Pharmacol* **30**: 3329–3332, 1981.
26. Kalaria RNM, Mitchell MJ and Harik SI, Monoamine oxidases of human brain and liver. *Brain* **111**: 1441–1451, 1988.
27. Donnelly H and Murphy DL, Substrate- and inhibitor-related characteristics of human platelet monoamine oxidase. *Biochem Pharmacol* **26**: 853–858, 1977.
28. Garrick NA, Redmond DE Jr and Murphy DL, Primate-rodent monoamine oxidase differences. In: *Monoamine Oxidase: Structure Function and Altered Functions* (Eds. Singer TP, von Korff RW and Murphy DL), pp. 351–359. Academic Press, New York, 1979.
29. Edwards DJ and Chang SS, Multiple forms of monoamine oxidase in rabbit platelets. *Life Sci* **17**: 1127–1134, 1975.
30. Yang H-Y and Neff NB, β-Phenylethylamine: A specific substrate for type B monoamine oxidase of brain. *J Pharmacol Exp Ther* **187**: 365–371, 1973.
31. Kinemuchi H, Wakui Y, Toyoshima Y, Hayashi N. and Kamijo K, β-(PEA), a concentration-dependent preferential substrate for multiple forms of MAO. In: *Monoamine Oxidase: Structure Function and Altered Functions* (Eds. Singer TP, von Korff RW and Murphy DL), pp. 205–212. Academic Press, New York, 1979.
32. Schurr A, Ho BT and Schoolar JC, Human brain monoamine oxidase: One molecular entity—multiple binding sites? *J Pharm Pharmacol* **33**: 165–170, 1981.
33. Kinemuchi H, Wakui Y and Kamijo K, Substrate selectivity of type A and type B monoamine oxidase in rat brain. *J Neurochem* **35**: 109–115, 1980.
34. Kinemuchi H, Arai Y and Kamijo K, Inhibition of type B monoamine oxidase activity by β-phenylethylamine. In: *Monoamine Oxidase: Basic and Clinical Frontiers* (Eds. Kamijo K, Usdin E and Nagatsu T), pp. 59–170. Excerpta Medica, Princeton, 1982.
35. Azzaro AJ, Amedro JB, Brown LM, Smith DJ and Williams GM, The effect of selective type A or type B monoamine oxidase inhibition on the intrasynaptosomal deamination of (^{3}H)serotonin in rat spinal cord tissue. *Naunyn Schmiedebergs Arch Pharmacol* **338**: 9–13, 1988.
36. Fagervall I and Ross SB, A and B forms of monoamine oxidase within the monoaminergic neurons of the rat brain. *J Neurochem* **47**: 569–576, 1986.
37. Wolf WA, Youdim MBH and Kuhn DM, Does brain 5-HIAA indicate serotonin release or monoamine oxidase activity? *Eur J Pharmacol* **109**: 381–387, 1985.
38. Westlund KN, Denney RM, Kochersperger LM, Rose RM and Abell CW, Distinct monoamine oxidase A and B populations in primate brain. *Science* **230**: 181–183, 1985.
39. Westlund KN, Denney RM, Rose RM and Abell CW, Localization of distinct monoamine oxidase A and monoamine oxidase B cell populations in human brainstem. *Neuroscience* **25**: 439–456, 1988.
40. Konradi C, Svoma E, Jellinger K, Riederer P, Denney R and Thibault J, Topographic immunocytochemical mapping of monoamine oxidase-A, monoamine oxidase-B and tyrosine hydroxylase in human *post mortem* brain stem. *Neuroscience* **26**: 791–802, 1988.
41. Levitt P, Pintar JE and Breakefield XO, Immunocytochemical demonstration of monoamine oxidase B in brain astrocytes and serotonergic neurons. *Proc Natl Acad Sci USA* **79**: 181–183, 1985.