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A discontinuous luminometric assay for monoamine oxidase

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Abstract—A simple, sensitive and convenient discontinuous luminometric assay for monoamine oxidase (MAO) is described. It is based on measurement of the light production from the peroxidase-catalysed chemiluminescent oxidation of 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) by the hydrogen peroxide produced in the MAO reaction. The procedure is suitable for use with a wide range of MAO substrates, although 5-hydroxytryptamine, adrenaline and noradrenaline are too readily oxidized by hydrogen peroxide to be used. A particular advantage of this procedure is that it is applicable to the oxidation of substrates which do not yield products, such as an aldehyde or free ammonia, which form the basis of several alternative substrate-independent assay procedures. The application of the procedure to assay the oxidation of benzylamine, tyramine and 2-*n*-pentylaminoacetamide (milacemide) by a crude mitochondrial preparation from rat liver and purified ox liver MAO-B is demonstrated.

Monoamine oxidase [amine: oxygen oxidoreductase (deaminating) flavin-containing, MAO,* EC 1.4.3.4] catalyses the deamination of various aromatic and long chain aliphatic amines and numerous methods are available for determining its activity [see 1 for review]. However, many of these impose limitations on the choice of substrate. Assays based on monitoring the disappearance of specific substrates or the appearance of specific products, such as the direct spectrophotometric assays with kynuramine [2], benzylamine [3] or its derivatives [see 1], suffer such limitations. Furthermore, assays using benzylamine are only applicable to MAO-B [for review on MAO specificities see 4]. Radiochemical assays which measure the production of the labelled aldehyde product [5, 6] have been the most widely used because of their relative ease of use and high sensitivity. However, they are restricted to substrates that are available in appropriately labelled form and pose hazards due to the use of radioisotopes and organic solvents. Aldehyde production may also be monitored by a coupled spectrophotometric assay in which the aldehyde formed is used to reduce NAD^+ in the presence of aldehyde dehydrogenase (ALDH; EC 1.2.1.3) [7] or by detection of the aldehyde formed using 2,4-dinitrophenylhydrazine [8]. However, the latter procedure is somewhat cumbersome and insensitive whereas the former can only be used with amines that form products that are effective substrates for ALDH. Ammonia production may be monitored by microestimation of ammonia using a Warburg manometric apparatus [9], an indophenol colour reaction after diffusion [10], an ammonia- or ammonium ion-selective electrode [see 1, 11] or by coupling its formation to NAD(P)H oxidation through the glutamate dehydrogenase reaction [see 1]. The sensitivities of such assays are often limited by the relatively high levels of ammonia present in many tissue samples and the enzymic procedure is not applicable for use with N-substituted amines such as adrenaline.

In addition to the limitations of the above-mentioned assays for aldehyde and ammonia formation, it is now recognised that the broad substrate specificities of the MAO include amine derivatives that yield neither ammonia nor an aldehyde as products. Thus, the oxidation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and its derivatives to the corresponding dihydropyridinium and pyridinium derivatives [see e.g. 12] yield neither of these products and the oxidation of milacemide (2-*n*-pentylaminoacetamide) forms pentylamine and glycinamide [13].

Assay procedures involving the determination of the disappearance of oxygen or the formation of hydrogen peroxide should be universally applicable since these are common to all MAO-catalysed reactions. However, direct manometric or polarographic determination of oxygen consumption [14] is relatively insensitive. H_2O_2 production may be monitored by a coupled fluorimetric or spectrophotometric assay in which the H_2O_2 formed is used to oxidize a dye in the presence of peroxidase [see 15–18]. Problems can arise with crude tissue preparations in such assays and the more sensitive fluorimetric assay can suffer interference from endogenous fluorescent or quenching materials.

Because of the disadvantages inherent in these currently used assay methods we have developed a luminometric technique for monitoring MAO activity. It was hoped that the new assay would approach the sensitivity of the commonly used radiochemical assays, would pose fewer health or environmental hazards and that it would be applicable to a wide range of MAO substrates and MAO preparations, ranging from mitochondrial to the purified enzyme. The procedure is a simple discontinuous assay in which the peroxidase-catalysed chemiluminescent oxidation of 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) is used to determine the production of hydrogen peroxide. A paper briefly referring to the use of this assay has recently been published [19], and details of the procedure, and its limitations, are presented here.

Materials and Methods

Materials. Horse radish peroxidase (HRP, EC 1.11.1.7), luminol, sodium azide, benzylamine hydrochloride, tyramine hydrochloride and 5-hydroxytryptamine creatinine sulphate (5-HT) were obtained from the Sigma Chemical Co. (Poole, U.K.). Hydrogen peroxide (30%) was obtained from BDH Chemicals Ltd (Poole, U.K.). 5-Hydroxytryptamine [side-chain-2-^{14}C]creatinine sulphate and *p*-hydroxyphenyl [2-^{14}C]ethylamine hydrochloride were obtained from Amersham International plc. (U.K.). Milacemide was a kind gift from Dr P. Dostert, Farmitalia Carlo Erba (Milan, Italy).

Preparation of enzymes. Rat liver mitochondria were prepared by the method of Kearney *et al.* [20]. Purified MAO-B from ox liver was prepared by the method of Salach [21]. ALDH was partly purified from ox liver by a modification [22] of the method of Deitrich *et al.* [23].

Spectrophotometric assays. Absorbances and rates of change of absorbance were measured using a Pye Unicam PU 8800 UV/VIS spectrophotometer or a Pye Unicam

* Abbreviations: MAO, monoamine oxidase; ALDH, aldehyde dehydroxygenase; luminol, 5-amino-2,3-dihydro-1,4-phthalazinedione; HRP, horse radish peroxidase; 5-HT, 5-hydroxytryptamine creatinine sulphate.

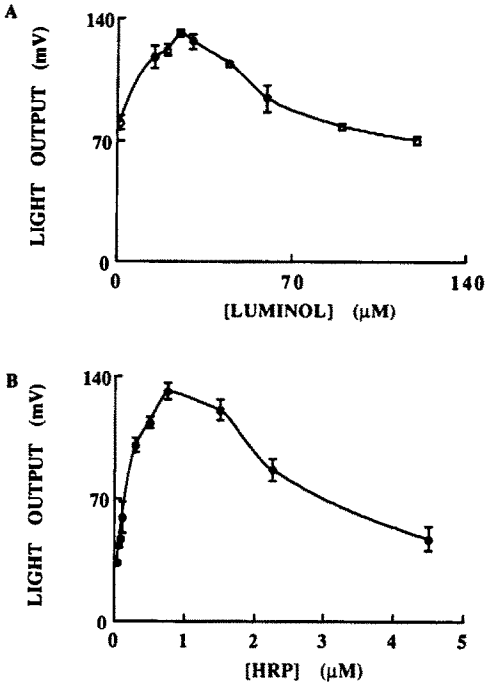

Fig. 1. The effect of varying the concentration of luminol and HRP on the intensity of light production in the luminescence assay. (A) The luminescent signal (○) resulting from the injection of H_2O_2 into mixtures containing Tris–HCl, pH 8.0, HRP and various concentrations of luminol, as described in the text. (B) The luminescent signal (●) resulting from the injection of H_2O_2 into a mixture containing Tris–HCl, pH 8.0, luminol and various concentrations of HRP as described in the text. Each point is the mean ± range of duplicate determinations from single experiments.

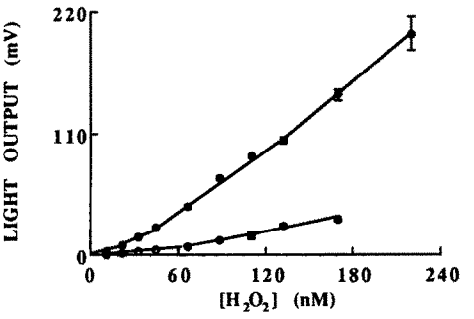

Fig. 2. Standard curve for hydrogen peroxide determination by luminometry. The luminescent signal resulting from the injection of a known amount of hydrogen peroxide, giving the indicated final concentration, into a solution containing luminol, HRP and Tris–HCl buffer, was determined at pH 8.0 (●) and at pH 7.2 (○) as described in the text. Each point is the mean ± range of duplicate determinations from single experiments.

SP6-450 UV/VIS spectrophotometer connected to a TECAM TE-7 Tempette circulating water-bath and a Philips PM 8252 recorder. In all cases the final reaction volume was 3 mL and assays were performed at pH 7.2 and at 37°. MAO activity was assayed either directly [3] or by the coupled spectrophotometric assay [7].

Radiochemical assays. MAO activity was assayed at 37° and pH 7.2 by the radiochemical procedure described previously [6]. The radioactivity of the product formed was determined by liquid scintillation spectroscopy in either a Packard Tri-Carb 300C or a Packard Tri-Carb 1500C liquid scintillation counter.

Luminometric assays. Equipment: luminometric assays were carried out in glass reaction vessels using an LKB Wallac 1250 luminometer connected to an LKB Wallac 1250 display unit and a Philips 8252 recorder. The temperature was maintained at 37° using a Tecam TE-7 waterbath.

Reagents: stock solutions of 10 mM luminol in 50% (v/v) dimethyl sulphoxide and 25 μM HRP were prepared daily. A M_r of 40,000 for HRP was used to calculate the molarity of solutions [see 24]. Stock solutions of H_2O_2 30% (w/v) were diluted to 0.5 μM for estimation of the standard curve. The absolute concentration of a 1 mM solution was determined daily on the basis of a molar extinction coefficient of 43.6 $M^{-1} \cdot cm^{-1}$ at 240 nm [see 25].

Optimum concentration of luminol and HRP: the reaction mixtures, in final volumes of 1 mL, contained 67 mM Tris–HCl, pH 8.,0, 750 nM HRP and various concentrations of luminol (1–120 μM) or 25 μM luminol and various concentrations of HRP (50 nM–4.5 μM). Reactions were initiated by the injection of 150 nM H_2O_2 via an air-tight Hamilton microsyringe. The instantaneous luminescent signals were recorded.

Standard curve for hydrogen peroxide determination: the reaction mixtures contained 27–67 mM Tris–HCl, pH 8.0, 25 μM luminol and 750 nM HRP. Reactions were initiated by the injection of H_2O_2 (11–225 nM) into the reaction mixtures as described above.

The effect of azide on H_2O_2 determination: the effect of azide on H_2O_2 determinations in the presence of rat liver mitochondria and purified MAO-B was examined. Reaction mixtures in final volumes of 3 mL containing either 83.3 mM potassium phosphate buffer, pH 7.2, 540 nM H_2O_2 and 3.1 mM azide or 90 mM potassium phosphate buffer, pH 7.2 and 540 nM H_2O_2 were incubated at 37°. Samples were withdrawn and assayed for H_2O_2 by the luminometric assay. Mitochondria (0.15 mg) or MAO-B (15.5 μg) were added and 100 μL aliquots were withdrawn at the indicated time points and assayed for H_2O_2.

Discontinuous luminometric assay for MAO: the MAO reaction mixture contained in a final volume of 3 mL, 93.3 mM potassium phosphate buffer, pH 7.2, 3.1 mM sodium azide and an appropriate concentration of mitochondria or MAO-B. The assay mixture was incubated in a cuvette for 5–7 min at 37°. The reaction was initiated with an appropriate MAO substrate, i.e. 333 μM benzylamine or 833 μM milacemide. At specific time intervals 50–100 μL aliquots were withdrawn and injected into vials containing 67 mM Tris–HCl buffer, pH 8.0, 750 nM HRP and 25 μM luminol. The instantaneous luminescent signal was recorded and the absolute concentration of hydrogen peroxide present in the MAO reaction at that time of the assay was calculated by reference to the standard curve.

Results

The intensity of light production was found to be dependent on both the luminol (Fig. 1A) and the HRP (Fig. 1B) concentration. The optimum concentration of luminol was found to be 25 μM. The inhibition of luminescence at higher concentrations of luminol could be due to the inhibition of HRP or to precipitation of luminol at these high concentrations. The optimum concentrations

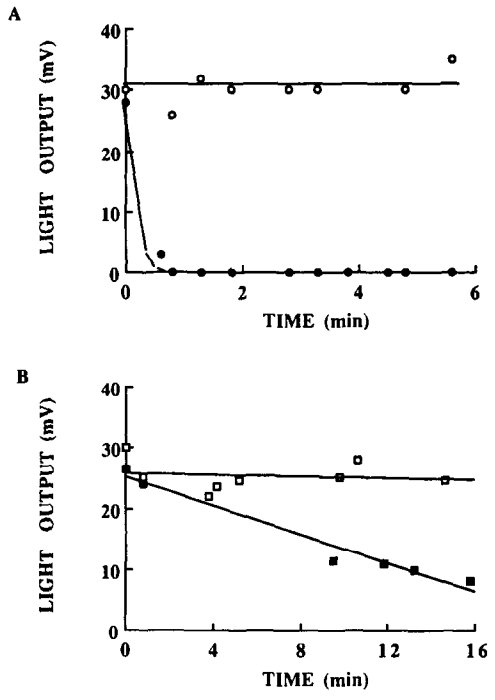

Fig. 3. Effect of azide and tissue preparations on H_2O_2 determinations. Reaction mixtures, containing either potassium phosphate buffer, pH 7.2, H_2O_2 and azide or potassium phosphate buffer, pH 7.2, and H_2O_2, were incubated at 37°. Samples were withdrawn and assayed for H_2O_2 by the luminometric assay (zero time point). Mitochondria (0.15 mg) or MAO-B (15.5 μg) were added and aliquots withdrawn at the indicated time points and assayed for H_2O_2. Results are presented as the luminometric signal obtained in the presence (open symbols) or absence (closed symbols) of azide using (A) mitochondria (●, ○) or (B) purified MAO-B (■, □).

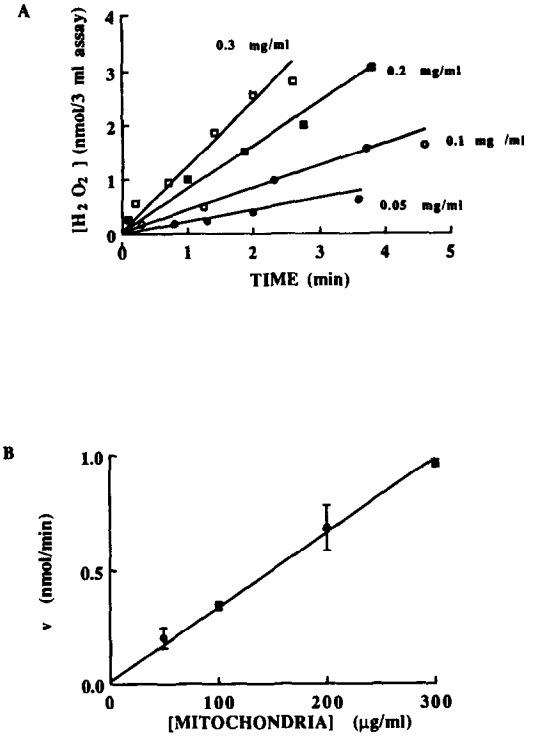

Fig. 4. The effect of rat liver mitochondrial concentration on the initial velocity of milacemide oxidation, as assayed by the discontinuous luminometric assay. (A) Time-courses were determined at 37° in a reaction mixture containing potassium phosphate buffer, pH 7.2, azide, 833 μM milacemide and the final concentrations of mitochondria as indicated. Samples were withdrawn at the indicated times and injected into reaction mixtures containing Tris–HCl, pH 8.0, luminol and HRP. The luminescent signal was converted to H_2O_2 concentration (nmol) by means of the standard curve. (B) The effects of enzyme concentration on the initial velocity of milacemide oxidation was determined from the slopes of the lines shown in Fig. 4A. Each point is the mean ± range of two separate experiments.

of HRP was found to be 750 nM. The inhibition of luminescence at higher concentrations of HRP might be due to its catalase activity or the reaction of HRP with one or more of the intermediate products in the pathway of light production.

The peak light output (mV) plotted against the [H_2O_2] (nM) yielded a non-linear standard curve (Fig. 2). It is notable that the HRP-catalysed peroxidation of luminol exhibit a marked pH dependence; when the reaction was performed at pH 7.2 (the standard pH of the MAO assay) much lower light signals were received than at a pH of 8.0. Thus, the pH of the MAO assay was maintained at 7.2 but samples were transferred to a medium at pH 8.0 for luminescence determinations. The standard calibration curves were used to relate all light output signals to absolute hydrogen peroxide concentration either graphically or using a computer program.

An important aspect of the development of this assay was its applicability to all enzyme preparations. However, some crude preparations, i.e. rat liver mitochondria, contain catalase (EC 1.11.1.6) which catalyses the rapid removal of hydrogen peroxide (Fig. 3A). Hence, in rat liver mitochondrial preparations sodium azide was used at a final concentration of 3.1 mM [see 18]. This concentration of azide did not affect MAO activity towards tyramine, as assessed by the radiochemical assay (data not shown), and prevented the rapid removal of hydrogen peroxide as assessed by the luminometric assay (Fig. 3A). Azide (3.1 mM) was also included in assays with purified preparations of MAO-B as experiments incubating hydrogen peroxide and the MAO-B preparation indicated that something in the MAO-B preparation was removing the hydrogen peroxide (Fig. 3B). This was possibly some catalase contamination or alternatively the presence of iron in the MAO-B preparation [see 21]. This concentration of azide did not affect the MAO-B activity as assessed by the radiochemical assay (data not shown) and prevented the removal of hydrogen peroxide as assayed by the luminometric assay (Fig. 3B). In general, it was found that the hydrogen peroxide levels in the presence of azide and rat liver mitochondria were stable for up to 6 min and levels in the presence of azide and purified MAO-B were stable for 10 min. After these time periods there was a decrease in the light output implying a decrease in the level of hydrogen peroxide. Hence, the MAO reaction was only assayed within the time periods where no decrease in the known hydrogen peroxide levels was observed.

Figures 4 and 5 show the application of the discontinuous luminescent assay to the "unusual" MAO substrate, milacemide, using rat liver mitochondria and to the

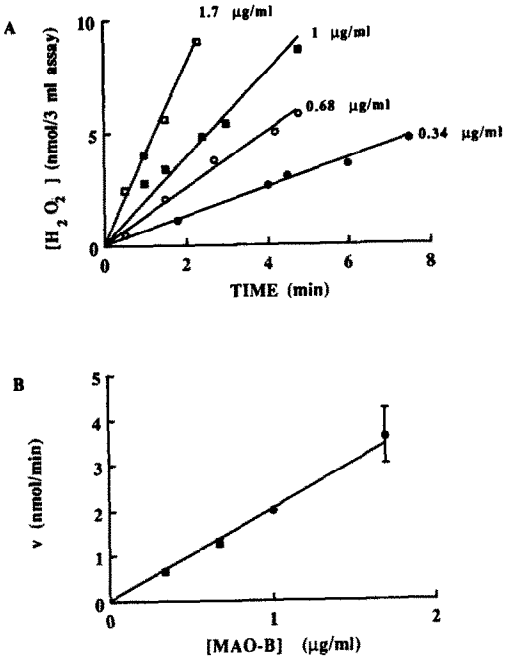

Fig. 5. The effect of purified ox liver MAO-B concentration on the initial velocity of benzylamine oxidation, as assayed by the discontinuous luminometric assay. (A) Time-courses were determined at 37° with 333 μM benzylamine as described in the legend to Fig. 4. (B) The effects of enzyme concentration on the velocity of benzylamine oxidation were determined from the slopes of the lines shown in Fig. 5A. Each point is the mean ± range of two separate experiments.

"classical" MAO substrate, benzylamine, using a purified MAO-B preparation. In both cases, the initial rates of oxidation were shown to be linear functions of enzyme concentration. Table 1 compares the specific activities of rat liver mitochondria preparations and purified MAO-B preparations as determined by different assay methods using different MAO substrates.

Discussion

The peroxidase-catalysed chemiluminescent oxidation of luminol has been subject to numerous mechanistic studies [26]. A generally accepted reaction scheme involves the oxidation of luminol by a complex between oxidant and peroxidase to produce a luminol radical. Luminol radicals then undergo further reactions resulting in the formation of an endoperoxide. This decomposes to yield an electronically excited 3-aminophthalate dianion emitting light on return to the ground state. In 1985 Tenne *et al.* [27] developed a rapid and sensitive bioluminescence assay for MAO activity in which the aldehyde formed was a substrate for bacterial luciferase. However, that procedure was greatly restricted in applicability, since it was restricted to use with long-chain aliphatic amine substances. The luminometric assay described here is more generally applicable. However, in common with other peroxidase-coupled assays [see 15], it does impose some restrictions on the choice of MAO substrates. It is a requirement that the substrate chosen is not oxidized more readily by H_2O_2 in the presence of HRP than luminol. In the present work the assay was shown to be unsuitable for use with 5-HT and thus one can conclude that it would also be inapplicable with adrenaline and noradrenaline since these are more readily oxidized than 5-HT.

Several factors can interfere with the luminometric assay. However, knowledge and diligent control of these effects allows its successful use. Sensitivity can be compromised by the presence of luminogenic contaminants, i.e. contaminating H_2O_2 in the reagents, water and the reaction vessels. To avoid such spurious effects, the light emission for the reaction mixture should first be measured as an appropriate blank before initiating the reaction with substrate. The use of glass reaction vessels is desirable. Some samples of disposable plastic cuvettes were found to be unsuitable, due to spurious light signals caused by static electricity. To obtain good reproducibility from day to day, the pH must be carefully controlled. It is recommended that a standard curve is included in each day's work with the relevant solutions to be used for that day's study.

There are many advantages associated with this novel assay. With the exception of luminol the reagents used in this assay are stable. Furthermore, they do not provide health or environmental hazards. The assay is precise with duplicate determinations differing on average by less than 5%.

The specificity and accuracy of the method are illustrated in Table 1. The specific activities of rat liver mitochondria or purified MAO-B from ox liver measured by the discontinuous luminometric assay paralleled those determined by the coupled spectrophotometric assay and the radiochemical assay, when tyramine and milacemide were used as substrates. With benzylamine as substrate, however, the specific activities obtained with the luminometric assay were less than those obtained with the direct and coupled spectrophotometric assays. Azide is known to inhibit MAO as an analogue of the product ammonia [28] and, using the direct spectrophotometric assay, Barwell and Ebrahimi [29] have recently reported the MAO activity towards this substrate to be inhibited by azide. Since azide was not present in the spectrophotometric assays initially used in the present work, these determinations were repeated in the presence of 3.1 mM sodium azide. A mitochondrial preparation with a specific activity towards 333 μM benzylamine of 2.11 ± 0.08 nmol/min/mg protein (mean ± SEM, N = 3) gave a specific activity of 1.04 ± 0.12 in the presence of 3.1 mM sodium azide. Thus, the apparent inhibition of MAO-B activity towards benzylamine by azide adequately accounts for the apparent discrepancy between the values obtained by the two different assay methods. The reasons why such inhibition affects the results obtained with benzylamine but not with milacemide or tyramine remains to be resolved, although it may be related to differences in the kinetic mechanism followed with these substrates [see 30]. Replacement of azide by the catalase inhibitor 3-amino-1,2,4-triazole, which is without effect on the activity of MAO-B [29], may circumvent this problem.

The detection limit of the assay, defined as the lowest concentration of H_2O_2 which can be reliably measured and distinguished from a suitable blank (the product concentration at which the analysis becomes feasible) and corresponding to the minimum value more than three standard deviations from the appropriate blank value, was found to be 0.7 nmol H_2O_2 present/3 mL MAO assay. This value was calculated on the basis of there being a 3.5 ± 0.9 mV blank value associated with mitochondrial preparations. Hence, the lowest possible signal that is valid is 9.45 mV. This corresponds to a H_2O_2 concentration of 23.6 nM in the luminometer tube and thus 0.7 nmol H_2O_2 must be accumulated in the MAO assay for reliable detection by the discontinuous luminometric assay.

Thus, this assay is reliable and sensitive, although in practice it was found to be more time consuming than the spectrophotometric and radiochemical assays. However, its simplicity and sensitivity make it a useful adjunct to the existing methods of MAO determinations, particularly in

Table 1. Comparison of MAO activities of two different preparations as determined by different assay methods

Enzyme preparation	Substrate	Specific activity (nmol/min/mg) Radiochemical assay	Direct spectrophotometric assay	Coupled spectrophotometric assay	Luminometric assay
Rat liver mitochondria	Tyramine (150 μM)	4.9 ± 0.27 (6)	—	4.4 ± 0.48 (4)	4.4 ± 0.19 (3)
Rat liver mitochondria	Milacemide (833 μM)	—	—	0.9 ± 0.1 (2)	1.2 ± 0.10 (4)
Rat liver mitochondria	5-HT (100 μM)	2.9 ± 0.1 (3)	—	3.3 ± 0.35 (2)	No activity detected
Rat liver mitochondria	Benzylamine (333 μM)	ND	ND	2.36 ± 0.77 (3)	1.31 ± 0.1 (9)
Purified MAO-B	Tyramine (150 μM)	488 ± 11 (3)	—	430 ± 25 (3)	499 ± 36 (3)
Purified MAO-B	Milacemide (833 μM)	—	—	522 ± 20 (2)	536 ± 21 (4)
Purified MAO-B	Benzylamine 333 μM)	ND	1593 ± 36 (6)	1202 ± 58 (5)	705 ± 36 (7)

Specific activities quoted are the means ± SEM or range from a number of different experiments, as indicated in parenthesis.

ND, not determined.

cases where no aldehyde product is formed in the MAO-catalysed reaction, or when any possible aldehyde product is not, or is not known to be, a substrate for ALDH.

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Department of Biochemistry
Trinity College
Dublin 2
Ireland

EIMEAR M. O'BRIEN*
KATRINA A. KIELY
KEITH F. TIPTON†

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* Present address: Xenova Limited, 240 Bath Road, Slough SL1 4EF, U.K.
† Corresponding author.

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