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THE EFFECT OF LIPOPHILIC COMPOUNDS UPON THE ACTIVITY OF RAT LIVER MITOCHONDRIAL MONOAMINE OXIDASE-A AND -B

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Abstract—The effect of various lipophilic compounds on the activity of monoamine oxidase (MAO) was determined. The local anaesthetics procaine, procainamide, tetracaine and lignocaine were all MAO-A selective inhibitors, whereas benzyl alcohol, butan-1-ol, hexan-1-ol and octan-1-ol inhibited MAO-B selectively. Procaine was found to be a competitive inhibitor of the deamination of 5-hydroxytryptamine (5-HT), tyramine, β-phenethylamine and benzylamine. Benzyl alcohol was competitive towards β-phenethylamine and benzylamine, but a mixed-type inhibitor towards 5-HT and tyramine. The same patterns of inhibition for both drugs were found when the activity was assayed under atmospheres of either oxygen or air. The inhibition produced by both compounds was fully reversible. Triton X-100 appeared to inhibit the activity of MAO-A selectively when preincubated with the enzyme for 30 min at 30°. This selectivity was lost when the preincubation temperature was raised to 37°. The inhibition of MAO activity by Triton X-100 after preincubation at 37° was found to be irreversible. Sodium deoxycholate and SDS were also found to inhibit the activity of MAO after preincubation with the enzyme at 37°. The significance of these results is discussed.

The use of the irreversible inhibitor, clorgyline [*N*-methyl-*N*-propargyl-3-(2,4-dichlorophenoxy) - propylamine], enabled Johnston [1] to define two forms of monoamine oxidase (Monoamine: O_2 oxidoreductase, MAO, EC 1.4.3.4) that differed in their substrate specificities and inhibitor sensitivities. In the rat liver, the form that was more sensitive to inhibition, termed MAO-A, was found to be active towards 5-hydroxytryptamine (5-HT) and tyramine, whereas the less sensitive form, MAO-B, was found to be active towards tyramine, β-phenethylamine and benzylamine [2, 3]. Other inhibitors, such as PCO [5-phenyl-3-(*N*-cyclopropyl)-ethylamine-1,2,4-oxadiazole] [4] and Lilly 51641 {*N*-[2-(*o*-chlorophenoxy)-ethyl]-cyclopropylamine} [5] act in a way similar to clorgyline, whereas deprenyl (phenyl-isopropyl-methyl-propinylamine) and pargyline (*N*-methyl-*N*-benzyl-propinylamine) appear to inhibit the activity of MAO-B preferentially [6, 7]. However, this binary classification of MAO may be an oversimplification, and the substrate specificities of the two forms appear to vary considerably from tissue to tissue (see ref 8).

The nature of the different forms of the enzyme are still a matter of controversy (see refs. 9 and 10 for reviews), but there is evidence that the nature of the lipid environment influences its specificity and inhibitor sensitivity. The inhibitor sensitivity and kinetic mechanism obeyed by the enzyme has been shown to be altered by the use of the detergent Triton X-100 [10–12] which can act by breaking protein–membrane lipid interactions (see refs. 13–15). Local anaesthetics and alcohols, which are also thought to interact with membrane lipids (see refs. 16 and 17) have been reported to inhibit the activity of MAO [18–28]. In this paper, the substrate-selective inhibition of MAO by lipophilic compounds has been investigated.

MATERIALS AND METHODS

The mitochondrial membrane vesicles used in this study were prepared as described previously [29]. Briefly, livers from rats were homogenized (1:8 w/v) in sucrose buffer (0.25 M sucrose, buffered with 10 mM potassium phosphate, pH 7.8) in an MSE Atomix blender for 10 sec. The homogenates were then centrifuged at 600 *g* for 15 min to remove nuclei and cell debris, and then centrifuged at 6500 *g* for 20 min to sediment the mitochondrial membranes. These membranes were resuspended in sucrose buffer and recentrifuged at 6500 *g* for 20 min. The resultant pellet was layered on to a linear sucrose gradient (40–70 % w/v, buffered with 10 mM potassium phosphate, pH 7.8, made with an MSE gradient former) and centrifuged at 50,000 *g* for 135 min in a swing-out rotor. A tight band of protein formed two-thirds of the way down the gradient which was collected, resuspended in 10 mM potassium phosphate, pH 7.8 (in order to reduce the sucrose concentration without any change in the ionic phosphate concentration), and centrifuged at 18,000 *g* for 30 min. The pellet was resuspended in sucrose buffer to a protein content of 1 mg ml^{-1} to yield a preparation of mitochondrial membranes with only moderate contamination from matrical, microsomal and

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lysosomal marker enzymes [29]. Care was taken to ensure that the MAO in the vesicle fractions and the respective crude homogenates had the same substrate specificities, ratios of activity when assayed under an atmosphere of oxygen/activity in air ('oxygen ratio'), sensitivities to inhibition by Tris–HCl, and K_m values towards the amines used in this study. Furthermore, the biphasic curves of inhibition by clorgyline of the oxidation of tyramine in the two fractions were similar [29]. The vesicle fractions were stored frozen until used for assay.

Reversibility experiments. Reversibility was investigated by the method described previously [30]. After incubation of a mixture of the enzyme preparation with the drug, the mixture was diluted so that both the enzyme and drug concentrations were reduced. Dissociation of a freely reversible inhibitor would be expected to cause the specific activity of the enzymes to rise to the value that would have been obtained if the preparation had originally been incubated in the presence of drug at this dilution.

Effects of temperature upon the absorbance of vesicles. Three millilitres of each vesicle preparation were placed in a glass cuvette. These were then placed in a heated cuvette holder in a Pye-Unicam SP500 Spectrophotometer, and the absorbance at 450 nm (A_{450}) measured at different temperatures.

Assay methods. MAO activity was assayed at 37° by the radiochemical method of McCaman *et al.* [31] as modified by Callingham and Laverty [32] with 5-HT, tyramine, β-phenethylamine and benzylamine as substrates. In some cases (shown in the text), the vesicles were preincubated with the drug for 30 min prior to the addition of the substrate. In these cases, the appropriate concentration of drug was also added with the substrate, so that the preincubation and incubation concentrations were the same. In all cases, incubation times were chosen so that the initial velocity was measured. Protein content was determined by the method of Lowry *et al.* [33], with bovine serum albumin as standard.

Materials. The radioactive substrates for MAO, 5-hydroxytryptamine-[G-^{3}H]-creatinine sulphate and tyramine-[side chain-1,2-^{3}H]-hydrochloride were obtained from the Radiochemical Centre, Amersham, U.K. β-Phenethylamine-[ethyl-1-^{14}C]-hydrochloride was obtained from New England Nuclear GMBH, Dreieichenchain, Germany, and benzylamine-[methylene-^{14}C]-hydrochloride from ICN Pharmaceuticals, Hersham. U.K. Benzylamine-[ring-3-^{3}H]-hydrochloride was a custom synthesis by the Radiochemical Centre, Amersham, U.K. The two benzylamine isotopes behaved identically towards MAO, and, unless otherwise shown, the ^{14}C-isotope was used. Aldehyde dehydrogenase (K^+ activated, from Baker's yeast), sodium deoxycholate, procaine hydrochloride and tetracaine hydrochloride were obtained from Sigma London, Kingston-upon-Thames, U.K. Benzyl alcohol, octan-1-ol and Triton X-100 were obtained from Fisons Scientific Apparatus, Loughborough, U.K. Benzaldehyde (distilled before use), butan-1-ol, hexan-1-ol and sodium dodecyl sulphate (SDS) were obtained from British Drug Houses, Poole, U.K. Nicotine adenine dinucleotide (NAD) was obtained from Boehringer Mannheim, Mannheim, Germany; procainamide hydrochloride from Astra Chemicals Ltd., Watford, U.K; and lignocaine hydrochloride from the Pharmaceutical Manufacturing Company, Bolton, U.K. All other reagents were of analytical grade wherever possible. Male Wistar rats were obtained from A. J. Tuck and Son, Rayleigh, U.K.

RESULTS

Dose-response curves with each of the compounds tested showed that in general the local anaesthetics (procaine, procainamide, tetracaine and lignocaine)

Table 1. IC_{50} values for the inhibition of rat liver MAO by local anaesthetics, alcohols and detergents*

	IC_{50} (mM)				
	5-HT (0.25 mM)	Tyramine (0.25 mM)	β-Phenethylamine (0.05 mM)	Benzylamine (0.25 mM)	Local anaesthetic concentration (mM)
Procaine	0.03	0.5	12.5	12.5	2–12.5
Procainamide	0.035	8.0	50	50	~0.18
Tetracaine	0.0015	0.005	0.05	0.05	0.1
Lignocaine	1.5	7.3	20	20	1–3.8
Benzyl alcohol	2.2	0.5	0.2	0.2	35
Butan-1-ol	50	7.9	7.9	7.9	68–110
Hexan-1-ol	5.0	0.3	0.3	0.3	
Octan-1-ol	1.6	0.25	0.4	0.4	~0.45
Triton X-100 (30°)	1.0	1.4	~10	~10	
Triton X-100 (37°)	0.4	0.5	1.0	1.0	
Sodium deoxycholate	1.9	2.2	3.0	3.0	
SDS	~0.6	~0.6	~0.6	~0.6	~0.1

* Eight to ten point curves were drawn through the means (± S.E.R.) of duplicate determinations of the per cent MAO activity remaining in the presence of the drug at varying concentrations, and the IC_{50} values derived from these curves. In all cases, the curves for benzylamine and β-phenethylamine were similar, and so were analysed together. The vesicles were preincubated for 30 min at 37° (unless otherwise shown) with the drug before the addition of substrate and appropriate concentration of drug. Substrate concentrations were chosen so as to be near their respective K_m values in oxygen [29]. Three groups of vesicles, each derived from the livers of two rats (body weight 365 ± 4 g) were used. Local anaesthetic concentrations given are the ranges for a variety of nerve preparations [data from ref. 16]. The ^{3}H-isotope of benzylamine was used in these experiments.

were more potent inhibitors of the deamination of 5-HT than of the deamination of β-phenethylamine and benzylamine, whereas the converse was true for the alcohols (benzyl alcohol, butan-l-ol, hexan-l-ol and octan-l-ol). The results of these studies are summarised in Table 1, and, as an example, the curves obtained with procaine are shown in Fig. 1. In each case, the samples were preincubated with the drugs for 30 min at 37° before addition of the substrate solution (containing the same concentration of the drug). The responses found for procaine and benzyl alcohol were the same in the absence of preincubation. Substrate concentrations were chosen as to be near their respective K_m values in oxygen [29]. Dilution experiments showed that the inhibition of MAO activity by procaine and benzyl alcohol was reversible with all four substrates used.

The inhibition of MAO activity by procaine and benzyl alcohol was determined at different substrate concentrations. Procaine was found to be a competitive inhibitor in all cases, with a markedly lower K_i towards 5-HT oxidation than towards the oxidation of the other substrates. Benzyl alcohol was a competitive inhibitor when β-phenethylamine and benzylamine were used as substrates, and a mixed inhibitor towards 5-HT and tyramine oxidation. The mode of inhibition and the K_i values were not significantly altered when the experiments were carried out in an atmosphere of air rather than oxygen. The results are summarised in Table 2, and, as an example, the data for benzyl alcohol inhibition, plotted as $1/v$ against $1/S$, is shown in Figs. 2 a–d.

Preliminary experiments with benzaldehyde on these vesicle fractions gave IC_{50} values of 0.4 mM towards the oxidation of 5-HT, 0.05 mM for tyramine, and 0.04 mM towards the oxidation of β-phenethylamine and benzylamine, respectively. Substrate concentrations were the same as in Table 1. Preincubation with NAD^+ and aldehyde dehydrogenase (1 mM and 5 U . ml^{-1}, respectively) did not alter the inhibtion of MAO activity by benzyl alcohol with any of the four substrates.

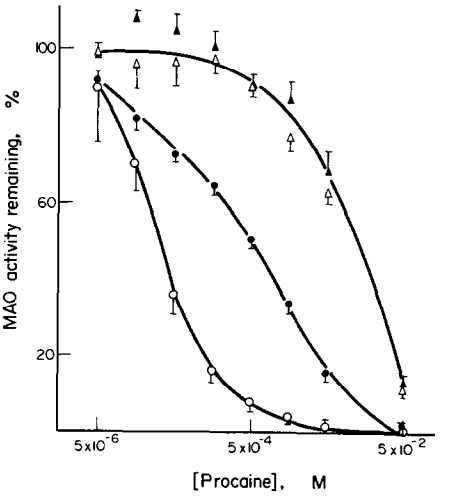

Fig. 1. The effect of procaine upon the *in vitro* activity of rat liver MAO. Vesicle fractions were preincubated with concentrations of procaine for 30 min at 37°, before addition of substrate and the appropriate amount of procaine. Each point represents the mean ± S.E.R. of triplicate determinations of activity in three groups of vesicles, each derived from the livers of two rats (body wt 365 ± 4 g), expressed as a percentage of the activity in the absence of procaine, plotted against the molar concentration of procaine. Substrates used were: 0.25 mM 5-HT (○), 0.25 mM tyramine (●), 0.05 mM β-phenethylamine (△) and 0.25 mM ^{3}H-benzylamine (▲).

The behaviour of the detergents was more complicated. Triton X-100 was found to have a much lower IC_{50} towards the oxidation of 5-HT than towards β-phenethylamine or benzylamine with or without preincubation for 30 min at 30° (Figs. 3a and b). These substrate-selective effects were considerably reduced when the vesicle fractions were preincubated with Triton X-100 for 30 min at 37° (Fig. 3c). The ionic detergents sodium deoxycholate and SDS showed no significant selectivity when prein-

Table 2. The kinetics of the inhibition of rat liver MAO activity by procaine and benzyl alcohol*

	Oxygen		Air	
	Mode of inhibition	K_i; K_i' (μM)	Mode of inhibition	K_i; K_i' (μM)
Procaine				
5-HT	Competitive	7.0 (10.5)	Competitive	6.7
Tyramine	Competitive	29.8 (26.3)	Competitive	20.7
β-Phenethylamine	Competitive	1923 (4784)	Competitive	2510
Benzylamine	Competitive	3431 (5349)	Competitive	3571
Benzyl alcohol				
5-HT	Mixed	769 ; 2912	Mixed	730 ; 2910
Tyramine	Mixed	866 ; 370	Mixed	1161 ; 583
β-Phenethylamine	Competitive	48 (77)	Competitive	58
Benzylamine	Competitive	47 (86)	Competitive	47

* For competitive inhibition, the K_i values are given, with the corresponding values calculated from the data in Table 1 by the method of Cheng and Prusoff [47] given in brackets. For mixed inhibitors, values are given as K_i; K_i' (nomenclature of Cornish-Bowden [48]). The data was determined from Lineweaver–Burk plots of the means of triplicate determinations in three groups of vesicles, each derived from the livers of two rats (body wt 399 ± 13 g), with 6 substrate concentrations, assayed under atmospheres of oxygen and air. Both inhibitors were fully reversible. The drugs were not preincubated with enzyme before assay for activity.

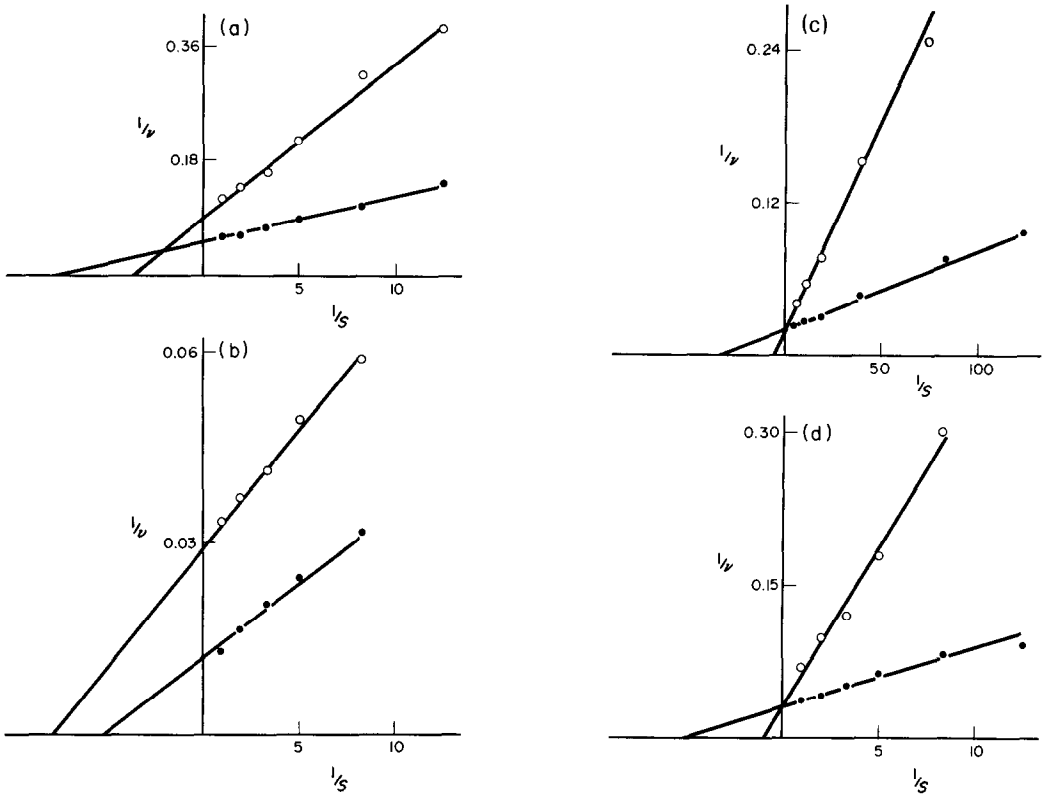

Fig. 2. Double reciprocal plots of the MAO activity in the absence and presence of benzyl alcohol. Ordinates: 1/initial velocity (in nmoles.mg protein^{-1}.min^{-1}); abscissae: 1/substrate concentration in mM. All points shown are the means of triplicate determinations of MAO activity in three groups of vesicle fractions, each derived from the livers of two rats (body wt 399 ± 13 g). No preincubation. All assays were carried out under an atmosphere of oxygen. Substrates used were: panel a: 5-HT, in the absence (●) and presence (○) of 2.2 mM benzyl alcohol; panel b: tyramine, in the absence (●) and presence (○) of 0.5 mM benzyl alcohol; panel c: β-phenethylamine, in the absence (●) and presence (○) of 0.2 mM benzyl alcohol; panel d: benzylamine, in the absence (●) and presence (○) of 0.2 mM benzyl alcohol. Lines through the points were fitted by eye.

cubated with the enzyme at 37° (Table 1). Concentrations of SDS higher than 1 mM were found to increase the value of the substrate blanks by increasing the amount of radioactive substrate in the organic layer. Dilution experiments indicated that when Triton X-100 was preincubated with the vesicle fractions for 30 min at 37°, the inhibition of the MAO towards all four substrates was irreversible.

Double-reciprocal plots of the inhibition of the MAO by Triton X-100 showed that this detergent altered both the apparent V_{max} and K_m of the MAO towards all four substrates. This type of inhibition was also found when the samples were assayed under an atmosphere of air. Preliminary experiments on a single preparation of vesicles indicated that the doses of Triton X-100 needed to cause inhibition of the oxidation of β-phenethylamine and benzylamine produced some solubilization of the MAO, and that the K_m values of the 'soluble' MAO towards these substrates (20 and 186 μM, respectively) were different from those of the MAO bound to the mitochondrial membranes (51 and 532 μM, respectively, for the Triton X-100 treated membranes, and 31 and 265 μM, respectively, for the untreated membranes).

In view of the differences between the effects of Triton X-100 when preincubated with the vesicle fractions at 30° and 37°, the effects of temperature upon the absorbance of the membrane vesicles was investigated. The absorbance at 450 nm (A_{450}) decreased considerably as the vesicles were heated from 19° to 37°, and this change was not fully reversible (Fig. 4).

DISCUSSION

Monoamine oxidase is localized predominantly in the outer membrane of the mitochondrion [34], and the preparation used in these studies produces a good yield of the enzyme with only moderate contamination with matrical, microsomal and lysosomal enzymes, and does not appear to produce any alteration in the properties of the enzyme [29].

The alcohols used in this study were all found to be MAO-B selective inhibitors. The competitive inhibition of the oxidation of β-phenethylamine and benzylamine by benzyl alcohol (Figs. 2c and d) is consistent with the suggestion of McEwen *et al.* [25] that the alcohols act as substrate analogues, and the substrate-dependent inhibition patterns found here are similar to those reported for the related compounds benzyl cyanide and 4-cyanophenol [3]. It has been reported that as the chain length of the aliphatic

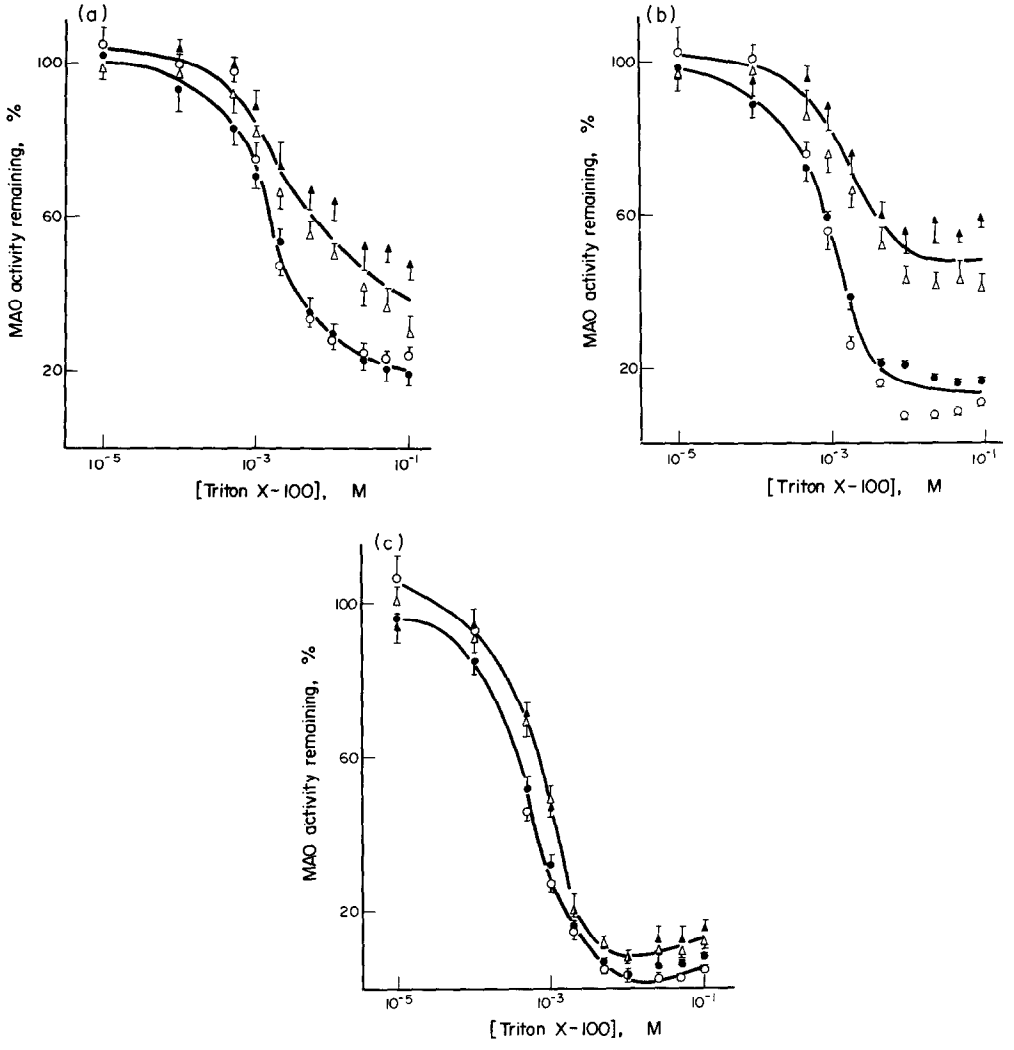

Fig. 3. The effect of Triton X-100 upon the *in vitro* activity of rat liver MAO. Panel a: Vesicle fractions and concentrations of Triton X-100 not preincubated before addition of substrate. Panel b: Vesicle fractions incubated with concentrations of Triton X-100 for 30 min at 30°. Panel c: Vesicle fractions incubated with concentrations of Triton X-100 for 30 min at 37° before addition of substrate and the appropriate concentration of Triton X-100. Each point represents the mean ± S.E.R. of triplicate determinations of MAO activity in three groups of vesicles, each derived from the livers of two rats (body weight 365 ± 4 g), expressed as a percentage of the activity in the absence of Triton X-100, plotted against the molar concentration of Triton X-100. Substrates used were: 0.25 mM 5-HT (○), 0.25 mM tyramine (●), 0.05 mM β-phenethylamine (△) and 0.25 mM ^{3}H-benzylamine (▲).

alcohols is decreased, the potency of the alcohols to inhibit MAO activity is decreased, and ethanol and methanol produce an activation of MAO [35, 36]. The reason for this is unclear, but the decreased potency with decreased chain length was also found in this investigation (Table 1).

At the concentrations of benzyl alcohol needed to inhibit the oxidation of 5-HT and tyramine, there is likely to be considerable membrane lipid disruption (see Table 1), and this could contribute to the departure of the inhibition from a simple competitive pattern. The inhibition by benzyl alcohol is not due to trace contamination with benzaldehyde, which acts as a product inhibitor of MAO [37] since studies with freshly distilled benzaldehyde indicated that at least 10 per cent of the benzyl alcohol would have had to have been converted to benzaldehyde to produce the observed inhibition. Furthermore, preincubation with NAD^+ and aldehyde dehydrogenase was without effect on the observed inhibition by benzyl alcohol.

The four anaesthetics tested were all found to be MAO-A selective inhibitors, with IC_{50} values for the inhibition of the activity of 5-HT lower than the concentrations required for local anaesthesia (Table 1). All the local anaesthetics used were tertiary amines, and thus could be acting as substrate analogues in a similar way to amphetamine [38] and mexiletine [39], both of which are MAO-A selective inhibitors. The inhibition of 5-HT oxidation by procaine was competitive and reversible, which would support this hypothesis. A competitive inhibition by procaine of rat brain kynuramine oxidation has also been reported [27]. At the concentration required

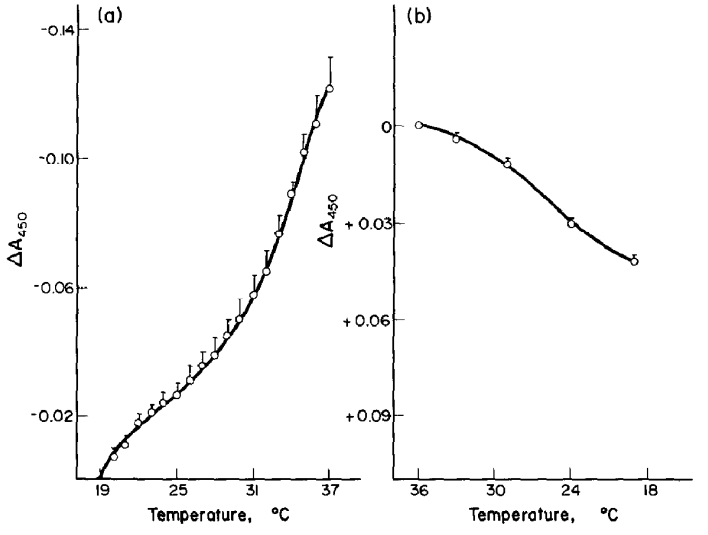

Fig. 4. Change in the absorbance at 450 nm of rat liver mitochondrial membrane vesicles at different temperatures. Samples (3 ml) were placed in a glass cuvette in a heated jacket, and the change in absorbance at 450 nm recorded as the temperature was altered. The temperature was recorded by placing a thermometer in the cuvette. The protein concentration of the vesicles was 1 mg ml^{-1}. Each point represents the mean change in A_{450} (± S.E.M.) with respect to the value of the absorbance at: a, 19°; b, 36°, for single determinations at each temperature for three groups of vesicles, each derived from the livers of two rats (body wt 399 ± 13 g). Panel a: Temperature raised from 19° to 37°. Panel b: Temperature lowered from 36° to 19°.

for the inhibition of the oxidation of benzylamine and β-phenethylamine by procaine, there must be considerable disruption of membrane lipid since the concentration of procaine is sufficient to cause anaesthesia (Table 1). The competitive inhibition found with procaine would suggest that the membrane lipids do not play a significant role in the oxidation of these MAO-B substrates, in agreement with previous work [40, 41], although lipid-depletion of rat liver mitochondrial membranes by 2-butanone extraction has been shown to change the Michaelis constants of the MAO-B towards these substrates [42]. However, the possibility that the selective action results from the local concentrations of these compounds in the region of the active centres of the enzyme forms due to lipid partition effects [43] cannot be ruled out.

Recently it has been shown that the kinetics of the inhibition of human brain β-phenethylamine oxidation by tricyclic antidepressants such as imipramine depends upon the oxygen concentration of the assay medium, consistent with the suggestion that these compounds are acting as dead-end inhibitors [44, 45]. This does not appear to be the case for the inhibition of rat liver MAO by procaine and benzyl alcohol reported here, as the mode of inhibition of MAO-A and -B activity by these two compounds was not changed when the enzyme activity was assayed under an atmosphere of air rather than oxygen.

The non-ionic detergent Triton X-100 appeared to inhibit the activity of MAO in a manner dependent upon the temperature of the preincubation of detergent and enzyme (Figs. 3 a–c). The extent of the inhibition of the activity towards 5-HT was similar with preincubation at either 30° or 37°, but the inhibition of the activity towards β-phenethylamine and benzylamine was increased greatly by preincubation at the higher temperature. A possible explanation for this behaviour is that some of the membrane lipids have to be 'melted' before the detergent can gain access to the MAO responsible for the oxidation of β-phenethylamine and benzylamine. There is a considerable decrease in the absorbance at 450 nm of the vesicle fractions as the temperature is increased from 19° to 37°, which is only partially reversible (Fig. 4). It seems, therefore, that there are significant changes in the structure of the vesicle membranes between 30° and 37°, which may account for the decrease in the selective effects of Triton X-100. After preincubation for 30 min at 37°, the ionic detergents sodium deoxycholate and SDS inhibit the activity of MAO with no discernable substrate-selectivity.

After preincubation of the vesicle fraction with Triton X-100 for 30 min at 37°, the inhibition of the MAO activity appears to be irreversible, but both V_{max} and K_m values of the remaining activity appeared to be altered. This treatment causes some solubilization of the enzyme to be found, and the soluble and membrane-bound MAO display different kinetic parameters, which may account for these changes in K_m values. It is possible that either some of the lipids that bind tightly to the enzyme are essential for activity, and that these are replaced by the detergent molecules, or that the binding of the Triton X-100 causes an irreversible change in the enzyme structure to be found, like that described for the interaction between Triton X-100 and (Na^+–K^+)–ATPase [46]. Solubilization of the enzyme with larger concentrations of Triton X-100 at lower temperatures is known to result in changes in the inhib-

itor sensitivity and kinetic mechanism [10–12], but does not lead to complete inhibition of the activity of the enzyme towards 5-HT, as might be expected from this study. It is quite possible that, at lower temperatures, the effect of Triton X-100 is reversible, and the 'melting' of the membrane components is necessary for the inhibition to become irreversible.

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