demonstrated that the furanocoumarins and the pyranocoumarin exhibited significant inhibitory effect on HB hydroxylation and AP *N*-demethylation at concentrations of 5×10^{-5} and 2×10^{-5} M respectively, whereas other coumarins did not affect enzyme activity as expected (Table 1).

The presence of a double bond in a furan or pyran ring attached to the coumarins seems to be essential for the manifestation of the activity, since the furanocoumarins and the pyranocoumarin with a double bond on the heterocyclic rings showed a significant activity while the other coumarins such as dihydrofurano- and dihydropyranocoumarins did not.

As disclosed by the direct comparison of the enzyme inhibitory potency of the coumarins by IC_{50} values (Table 2), imperatorin (XI) and isoimperatorin (XII), which possess an isoprenyl side chain, were found to elicit the strongest inhibitory potency on both HB hydroxylation and AP *N*-demthylation, which was approximately equipotent to that of SKF-525A. The inhibitory potency was decreased in the order of oxypeucedanin (XIV), isooxypeucedanin (XV) and oxypeucedanin methanolate (XVI), indicating that the polarity of the side-chain moiety is associated with a depression of the enzyme inhibitory potency. Xanthyletin (IX), which is devoid of any side chains, showed the least activity.

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Shift in double-sigmoid MAO inhibition patterns in oxygen-saturated reaction mixture

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Stimulation of the reaction velocity of monoamine oxidase (MAO, EC 1.4.3.4) by high oxygen tensions reported long ago [1, 2] has been observed in recent years to be substrate-selective [3–6]. Recently we have demonstrated that oxygen stimulation of rat brain MAO is type-selective [7], type B MAO being much more strongly stimulated than type A MAO. The biphasic inhibition patterns of MAO using common substrates like tyramine by selective inhibitors like clorgyline and deprenyl are often studied to get an approximate idea of the ratio of the two forms of MAO in a particular tissue preparation [8]. In most of these studies, however, little consideration has been given to the concentrations of the substrate amine employed, even though the amine concentrations used in various studies [9–11] can be perceived to have considerable influence on the inhibition patterns obtained. The concentration of the second substrate, i.e. oxygen, has not been given any consideration in these studies even though, in view of the type-selective nature of oxygen stimulation of MAO, oxygen tension in the incubation mixture may be expected to influence the biphasic MAO inhibition patterns considerably.

In the present study the expected shift in the double-sigmoid MAO inhibition curves with clorgyline, deprenyl and harmaline effected by oxgen saturation of the incubation medium was demonstrated. The crude mitochondrial preparations of rat brain and liver were used as the enzyme source. The preparation of crude brain mitochondria and the assay method for MAO activity using tyramine as substrate were as described earlier [12]. The enzyme was allowed 30 min pre-incubation with clorgyline and deprenyl, and 15 min pre-incubation with harmaline in an otherwise complete incubation mixture at 37° prior to the addition of substrate, and the product formed was measured after 15 min incubation. One set of incubation mixtures was equilibrated with air at 37° and was frequently shaken during incubation. The other set was flushed with 100% oxygen through a gas manifold and capillary tubes 5 min prior to the addition of amine substrate. A moderate flow of oxygen was maintained during the incubation period to ensure that the assay mixture remained saturated with oxygen.

Figure 1 shows the dose–response curves of the inhibition of tyramine oxidation by three selective MAO inhibitors, deprenyl, clorgyline and harmaline, in incubation mixtures equilibrated with air and 100% oxygen using the rat brain crude mitochondrial preparation. With each of the three inhibitors there was a noticeable shift in the inhibition curve obtained in the oxygen-saturated assay mixture from the one plotted with air as the gas phase and this shift was most marked in the plateau region. In each case, however, at higher inhibitor concentrations, the two inhibition curves converged again.

Figure 2 shows the results of similar experiments performed with rat liver mitochondrial preparation. The patterns obtained are qualitatively very similar to the patterns

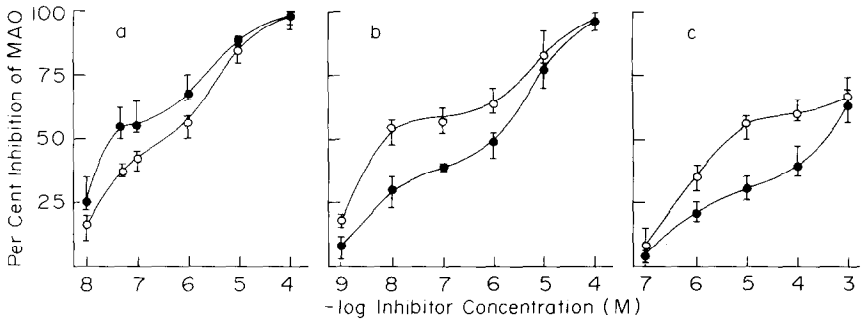

Fig. 1. *In vitro* inhibition of tyramine deamination by rat brain mitochondrial MAO by (a) deprenyl, (b) clorgyline and (c) harmaline in incubation mixtures equilibrated with air (○) and 100% oxygen (●), respectively. Each point is the average of four determinations and the bar encompasses all results.

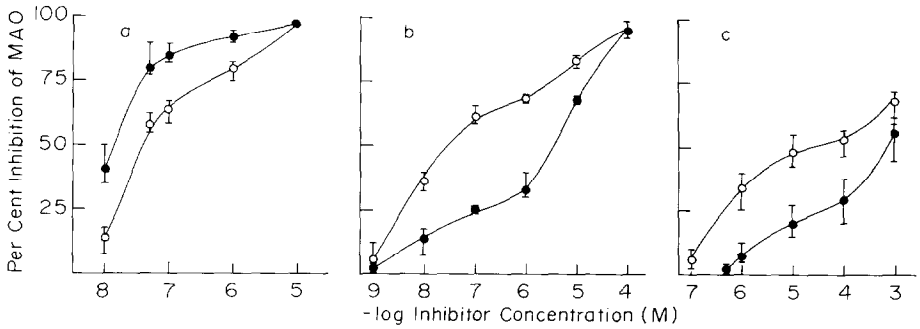

Fig. 2. *In vitro* inhibition of tyramine deamination by rat liver mitochondrial MAO by (a) deprenyl, (b) clorgyline and (c) harmaline in incubation mixtures equilibrated with air (○) and 100% oxygen (●), respectively. Each point is the average of four determinations and the bar encompasses all results.

obtained with the rat brain preparations described above. The shift in oxygen-saturated media is more remarkable with this tissue preparation. With both tissue preparations the shift in the deprenyl dose–response curves is the least striking. The incubation mixtures were flushed with oxygen after the enzyme had been allowed 25 min pre-incubation with clorgyline and deprenyl—when the inhibition by these inhibitors can be assumed to have almost reached completion. With suicide inhibitors the oxygen tension of the reaction mixture may or may not influence the binding of the inhibitors with MAO during pre-incubation, which would complicate the picture. Harmaline, being a reversible MAO inhibitor [13, 14], does not really require pre-incubation with the enzyme preparation. However, a shorter pre-incubation was allowed because in some tissue preparations it was observed that brief pre-incubation does increase the degree of MAO inhibition by harmaline [15] to some extent.

The shift in the biphasic MAO inhibition patterns is quite predictable on the basis of the differential stimulation of reaction velocities of the two MAO types of rat brain by oxygen with respect to the reaction rates in the presence of air [7]. This difference in the characteristics of the two MAO types also holds in the case of rat liver. Rather in this tissue the difference in the amount of oxygen activation of the two MAO forms is somewhat greater than in rat brain. The position of the plateau of a biphasic dose–response curve in the per cent inhibition scale is a measure of the A/B ratio of the tissue preparation for the oxidation of a particular amine at the concentration employed. This ratio is understandably altered in oxygen-saturated reaction mixtures on account of the difference in the K_o values of the two enzyme forms [7].

Summarizing, the contributions of the two forms of MAO in a particular tissue preparation towards the oxidation of a common substrate amine, as indicated by the position of the plateau in the inhibition dose–response curves of selective MAO inhibitors, is considerably altered in oxygen-saturated reaction mixtures compared to those obtained with air as the gas phase.

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Effects of a chemical sympathectomy on cardiac muscarinic receptors in normotensive (WKY) and spontaneously hypertensive (SHR) rats

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The rate and contractility of the heart are regulated by the parasympathetic system through muscarinic receptors. Muscarinic receptors tested in binding studies with a labelled antagonist behave as a homogeneous class of receptors but can be distinguished into subclasses with respect to their affinity for agonist molecules [1]. Yamada *et al.* [2] suggested that, at least in rat, most of these cardiac muscarinic cholinergic receptors are located postsynaptically and are not related to adrenergic neurons. Sharma and Banerjee [3] have concluded, however, that a large number of these muscarinic receptors are located presynaptically on noradrenergic nerve endings, based on the observation that the number of these receptors decreases markedly following 6-OH dopamine* administration, a treatment that selectively destroys nerve endings taking up catecholamines [4]. This interpretation is supported by the pharmacological demonstration of a reduction, by muscarinic agonists, of the stimulation-evoked release of norepinephrine in heart [5, 6].

Spontaneously hypertensive (SHR) rats from the Okamoto strain exhibit hyperactivity of the norepinephrine pathway of the autonomic system [7, 8] that leads to a decrease in the number of β-adrenergic receptors in the heart [9–11]. In the same tissue, the total number of muscarinic receptors is not modified [11] but the relative proportion of high- and low-affinity binding sites for agonists, as well as the balance of pre- and postsynaptic muscarinic receptors, has not yet been documented.

We recently described a methodological approach [12, 13] for the determination in heart membranes of the relative densities of high- and low-affinity binding sites for muscarinic agonists based on the use of two radioactive ligands: the antagonist L-[^{3}H]QNB and the agonist [^{3}H]oxo-M. In the present study, we used this method to compare the distribution of muscarinic cholinergic receptor subclasses in the heart from normotensive (WKY) and spontaneously hypertensive (SHR) rats before and after chemical sympathectomy with 6-OH dopamine.

Experiments were conducted on male SHR rats of Okamoto strain, 15 weeks of age at the beginning of the experiment, and age-matched with normotensive Wistar–Kyoto (WKY) rats. Animals were injected intravenously with 6-OH dopamine, administered in two doses of 50 mg/kg body weight at a 24-hr interval. Control animals received an equivalent volume of the vehicle. The rats were sacrificed 3 weeks after the first injection. Each heart was dissected out, rinsed with 0.15 M NaCl, weighed and stored in liquid nitrogen until use.

The schedule utilized for 6-OH dopamine injections was exactly that proposed by Yamada *et al.* [2]. The norepinephrine content of heart was not measured in the present study but the efficacy of the treatment was indirectly demonstrated by increased 10^{-4} M D,L-isoproterenol-stimulated adenylate cyclase activity in heart membranes (+30% in WKY rats and +45% in SHR rats, on average).

Membranes were prepared from thawed hearts by homogenization (5%, w/v homogenate) at 4° in a buffer consisting of 20 mM Tris–HCl (pH 7.4), 2 mM dithioerythritol and 5 mM $MgCl_2$. After filtration through two layers of medical gauze, the homogenate was centrifuged at 520 *g* for 10 min. The crude particulate extract was treated as previously described [14] and membranes obtained were tested for muscarinic receptors.

Heart membrane proteins (90–110 μg) were incubated for 30 min at 25° with increasing concentrations of L-[^{3}H]QNB (specific radioactivity 40 Ci/mmole) or [^{3}H]oxo-M (specific radioactivity 84 Ci/mmole), obtained from New England Nuclear Corporation (Dreieich, FRG) in 1.2 ml of 50 mM sodium phosphate buffer (pH 7.5) enriched with 1 mM $MgCl_2$, and in the absence or presence of 1 μM atropine (in order to determine the non-specific binding). Membrane-bound radioactivity was separated from free radioactivity by filtration through glass-fibre filters GF/C (Whatman, Maidstone, U.K.) and washed three times with ice-cold buffer. The methodology used has been previously detailed [12, 13]. Under these experimental conditions, L-[^{3}H]ONB bound to all muscarinic receptors whereas [^{3}H]oxo-M bound only to high-affinity receptors for muscarinic agonists.

The main characteristics of the animals are detailed in Table 1. 6-OH dopamine treatment was without effect on body weight increase, heart rate and blood pressure. The cardiac hypertrophy of SHR rats was also not altered by the drug.

The total number of muscarinic sites measured by L-[^{3}H]QNB binding, the proportion of high-affinity and low-affinity sites for the agonist [^{3}H]oxo-M, and the dissociation constants (K_D) of the receptors for both ligands were identical in normotensive (WKY) and hypertensive (SHR) rats (Table 2 and Fig. 1).

In WKY rats, neither the total number of muscarinic receptors nor the number of high-affinity agonist binding sites was modified following 6-OH dopamine treatment (Table 2 and Fig. 1, left panel). These negative data are in line with those of Story *et al.* [15] and Yamada *et al.* [2] (the latter group reported a significant increase in muscarinic receptor density only in the atria and not in the ventricles).

In SHR rats, the total number of muscarinic receptors was unaltered by 6-OH dopamine treatment but the density of the high-affinity binding sites for agonists showed a 63% increase. The number of low-affinity binding sites for agonist (i.e. the total number of receptor minus the number

* Abbreviations used: [^{3}H]oxo-M, [methyl-^{3}H]oxotremorine acetate; L-[^{3}H]QNB, L-[benzilic-4,4′-^{3}H]quinuclidinylbenzilate; 6-OH dopamine, 6-hydroxydopamine.