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A COMPARISON OF CARDIAC AND VASCULAR CLORGYLINE-RESISTANT AMINE OXIDASE AND MONOAMINE OXIDASE

INHIBITION BY AMPHETAMINE, MEXILETINE AND OTHER DRUGS

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Abstract—Clorgyline-resistant amine oxidase (CRAO) and monoamine oxidase (MAO) were studied in homogenates of rat heart and aorta, using benzylamine and tyramine as substrates. In heart, benzylamine at 0.001 mM was deaminated solely by CRAO. With higher concentrations of benzylamine (0.01, 0.1 and 1.0 mM), an increasing involvement of MAO-A and MAO-B became apparent in the deamination of benzylamine such that, at 1.0 mM benzylamine, deaminated products resulted equally from MAO-A, MAO-B and CRAO. In aorta, benzylamine was deaminated solely by CRAO irrespective of the concentration used. Tyramine (0.01, 0.1, 1.0 and 5.0 mM) was deaminated entirely by MAO-A in heart, whereas in the aorta both MAO-A and CRAO participated. In aorta the ratio of product formation from MAO-A and CRAO did not vary with changes in the concentration of tyramine, indicating similar K_m values for both enzymatic activities. Further studies with tyramine (0.1 mM) and clorgyline showed biphasic inhibition curves suggestive of two distinct MAO-A components in both heart and aorta. The two components showed different properties in the heart when compared with aorta. When homogenates of hearts were heated at 50° for 1 hr, their sensitivity to inhibition by clorgyline increased, while in homogenates of aorta sensitivity to clorgyline decreased. CRAO was investigated further with benzylamine as substrate. Kinetic studies gave similar K_m values for both heart and aorta (4–6 μM at pH 7.8), and these values were not altered by flushing the assay tubes with oxygen. However, flushing with nitrogen caused uncompetitive inhibition in the heart and noncompetitive inhibition in aorta. These results suggest a difference in the catalytic mechanism between CRAO of heart and aorta. In both heart and aorta, CRAO was inhibited by semicarbazide, (+)-amphetamine, phenelzine and (+)- and (−)-mexiletine, with the (+)-form being more potent. Straight-chain diamine and polyamine compounds failed to inhibit in concentrations up to 10^{-4} M. Thus, CRAO is not a typical diamine or polyamine oxidase. The results show differences between heart and aortic CRAO and MAO-A, and the possibility exists for heterogeneity within each of these two distinct forms of amine oxidase. Additionally, drugs known to inhibit MAO–(+)-amphetamine, phenelzine and mexiletine—also inhibit CRAO. However, the biological significance of this observation is not readily apparent since the physiological role of CRAO is unknown.

The cardiovascular system of animals and man is a major site for the oxidative deamination of a wide variety of amines. However, the characteristics and properties of the enzymes involved are still not fully understood.

Two forms of monoamine oxidase [MAO; monoamine:oxygen oxidoreductase (deaminating), EC 1.4.3.4.] called MAO-A and MAO-B have been described previously. The enzymes are abundant in cardiovascular tissues and are, perhaps, best defined by their differential susceptibility to inhibition by clorgyline [1, 2]. MAO-A activity is much more sensitive to clorgyline than is MAO-B. Differential classification on the basis of substrate specificity is much more difficult. Recent studies show that relative affinities, maximum velocity rates, substrate and/or product inhibition and the relative amounts of the two forms must all be taken into account [3–6]. In addition, tissue selective differences in the properties of the A and B forms cannot be excluded. For instance, benzylamine in rat brain and liver is metabolized almost entirely by MAO-B [7, 8], but in the rat heart it has been shown to possess a similar K_m for both MAO-A and MAO-B [9].

Several studies have found another amine oxidase activity in heart and blood vessels which is quite distinct from MAO [3, 4, 9–14] and for which benzylamine is also an excellent substrate. This activity is resistant to inhibition by clorgyline [9, 12] and the related acetylenic inhibitors, deprenyl and pargyline [13, 15], at concentrations which completely inhibit MAO-A and -B activity. Lewinsohn *et al.* [13] refer to this species of amine oxidase as "benzylamine oxidase", after the original work by Blaschko and co-workers on plasma amine oxidase [16, 17]. We have retained the term "clorgyline-

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resistant amine oxidase" (CRAO) [9] since it distinguishes the activity from MAO itself, and it allows for the possibility that more than one enzymatic species is involved. In rat tissues, CRAO can metabolize substrates other than benzylamine [12, 15], as in the case of plasma or serum amine oxidase [18]. Likewise, studies with inhibitors indicate that CRAO may be a copper and pyridoxal-dependent enzyme [9, 12, 13, 19]. This is quite unlike MAO which is a flavoprotein [18] and does not contain copper [20]. CRAO is widely distributed in body tissues with high activity in the aorta and other blood vessels [4, 12–15].

In the present study we have evaluated and compared the MAO and CRAO of the rat heart and aorta. The results show that both activities exhibit different properties between the two tissues.

MATERIALS AND METHODS

Male Sprague–Dawley rats (weighing 180–250 g), obtained from A. J. Tuck & Son (Raleigh, U.K.), were killed by decapitation or by a blow to the head. Heart and descending aorta were removed, rinsed thoroughly in saline [0.9% NaCl (w/v)], blotted dry, weighed and either frozen at −20° for future use or homogenized immediately. Tissues were minced with scissors and homogenized in a tissue:buffer (g/ml) ratio of 1:10 (heart) and 1:20 (aorta) in 1 mM potassium phosphate buffer, pH 7.8. Hearts were homogenized by hand in a glass–Teflon hand homogenizer (twenty strokes with a loose fitting pestle) and aortas by means of a Polytron (PT-10-35, setting 5 for four periods of 5 sec on ice). The crude homogenates were centrifuged at 600 *g* for 10 min and supernatant fractions of 1 or 2 ml were frozen in glass vials. Homogenates of aorta were diluted further (1:1 or 1:3) with 1 mM potassium phosphate buffer prior to assay. A crude mitochondrial fraction of heart was prepared by homogenizing hearts from six rats in a buffer containing 0.25 M sucrose, 0.01 M potassium phosphate, pH 7.8. The homogenate was centrifuged at 600 *g* for 10 min and the supernatant fraction was decanted and centrifuged at 12,000 *g* for 20 min. The resulting mitochondrial pellets were resuspended in homogenization buffer and stored frozen in several 3-ml portions.

Enzyme assay. Activities were determined radiochemically by the method of McCaman *et al.* [21], as modified by Callingham and Laverty [22]. Aliquots of enzyme were preincubated at 37° with or without inhibitors (usually for 20 or 30 min), then cooled on ice, and radioactive substrate (benzylamine and tyramine, 1 or 10 μCi/μmole) was added in buffer (to a final strength of 0.1 M potassium phosphate, pH 7.8, in 100 μl). After incubation at 37° under an atmosphere of oxygen, air or nitrogen, the reaction was stopped by cooling the tubes on ice and acidifying with 10 μl of 3 N HCl. Deaminated products were extracted in 600 μl of toluene–ethyl acetate [1:1 (v/v) saturated with water] and a 400-μl aliquot was taken for liquid scintillation counting with quench correction. Blank values were obtained by adding 10 μl of 3 N HCl prior to addition of substrate.

None of the drugs used altered extraction efficiency of amine metabolites by more than 7 per cent. Reported concentrations of irreversible inhibitors represent preincubation concentrations. When assays were performed in oxygen or nitrogen atmospheres, each tube was flushed for 30 sec before sealing with a rubber stopper.

K_m *and* V_{max} *determinations*. The following final concentrations of [^{14}C]benzylamine were generally used: 0.4, 0.5, 0.7, 1.0, 2.0, 4.0, 8.0 and 16 μM. Initial velocity rates were obtained by incubating homogenates of aorta for 5 min and heart for 10 min. Linearity of the reaction with time and protein concentration was ensured in all assays. K_m and V_{max} values were calculated by computer program according to the method of Wilkinson [23].

Protein assay. Protein contents were measured by the micro-biuret method of Goa [24], with bovine serum albumin (grade III) as standard.

Chemicals. Tyramine-[G-^{3}H]hydrochloride was obtained from the New England Nuclear Corp., Dreieich, West Germany, and benzylamine-[methylene-^{14}C]hydrochloride from the Radiochemical Centre, Amersham, U.K.

Clorgyline hydrochloride and (+)- and (−)-mexiletine [1-methyl-2-(2,6-xylyloxy)-ethylamine hydrochloride, Kö 1173] were gifts from May & Baker, Ltd., Dagenham, U.K., and Boehringer-Ingelheim, Ltd., Bracknell, U.K., respectively. All other reagents and chemicals were of analytical grade where possible.

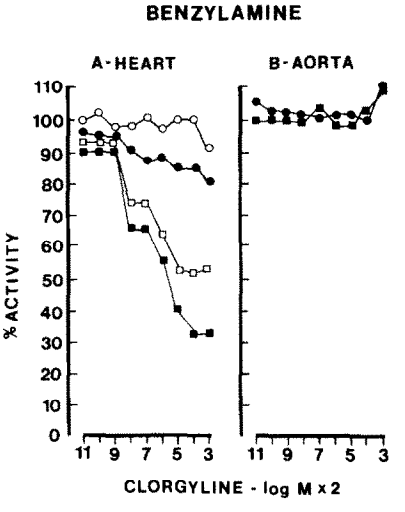

Fig. 1. *In vitro* inhibition of benzylamine deamination by clorgyline in homogenates from rat heart (A) and aorta (B). Remaining activity after preincubation with inhibitor for 20 min at 37° was assayed with benzylamine at 0.001 mM (○–○), 0.01 mM (●–●), 0.1 mM (□–□) and 1.0 mM (■–■) with an incubation time of 60 min. Each point is the mean of two or three experiments assayed in triplicate, expressed as a percentage activity of control. Control activity was determined from the means of six replicate estimations for each experiment. The standard error of the ratios (data points) did not exceed 8 per cent of the mean values.

RESULTS

The sensitivity of benzylamine deamination towards inhibition by clorgyline was determined at four different benzylamine concentrations (0.001, 0.01, 0.1 and 1.0 mM). Figure 1A shows the results obtained in the rat heart. The deamination of benzylamine (0.1 and 1.0 mM) was inhibited by increasing concentrations of clorgyline in a biphasic manner. The shape of these curves corresponds to the inhibition of MAO-A at clorgyline concentrations between 2×10^{-11} and 2×10^{-8} M, and inhibition of MAO-B between 2×10^{-7} and 2×10^{-4} M [1]. In addition, a proportion of the total benzylamine deamination was unaffected by 2×10^{-4} M clorgyline. This residual activity corresponded to CRAO (53 per cent at 0.1 mM and 33 per cent at 1.0 mM benzylamine). At 0.01 mM benzylamine, the distinction between MAO-A and MAO-B is less well defined. CRAO accounts for about 85 per cent of the total deamination. With the lowest concentration of benzylamine (0.001 mM), only CRAO activity was involved. At 2×10^{-3} M, clorgyline is no longer specific for MAO, and some slight inhibition of benzylamine deamination is seen with the 0.001 and 0.01 mM concentrations. This effect appears to depend upon the substrate concentration since no inhibition was observed with the higher concentrations of benzylamine (0.1 and 1.0 mM). Experiments done with rat aorta gave different results from those described for heart (Fig. 1B). Benzylamine (0.01, 0.1 and 1.0 mM) was deaminated solely by CRAO. (For clarity, only the 0.1 and 1.0 mM concentrations are shown in Fig. 1B.) With aorta, the highest concentration of clorgyline (2×10^{-3} M) gave a slight activation of CRAO activity rather than the inhibition as shown for heart.

Experiments with tyramine as the substrate are shown in Fig. 2. In the heart, only MAO-A deaminated tyramine, while MAO-A and CRAO were

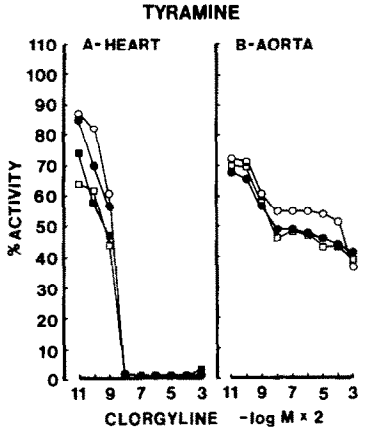

Fig. 2. *In vitro* inhibition of tyramine deamination by clorgyline in homogenates from heart (A) and aorta (B). Remaining activity after preincubation with inhibitor for 20 min at 37° was assayed with tyramine at 0.01 mM (○–○), 0.1 mM (●–●), 1.0 mM (□–□) and 5.0 mM (■–■), with an incubation time of 30 min. For other details, see legend to Fig. 1.

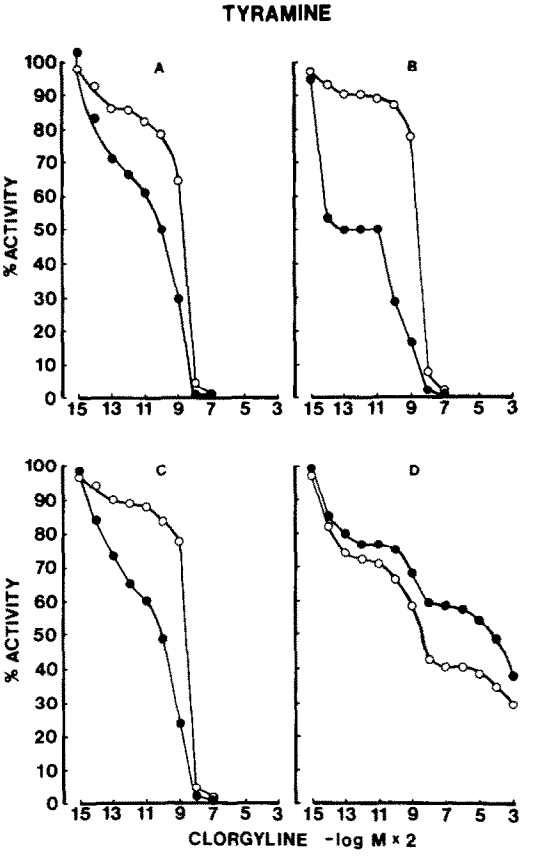

Fig. 3. *In vitro* inhibition of tyramine (0.1 mM) deamination by clorgyline in heart (A, B and C) and aorta (D). Whole homogenate (A) and crude mitochondria (B) were preheated at 50° for 1 hr prior to incubation with clorgyline at 37° for 20 min (closed circles). Control tubes were kept on ice for 1 hr prior to incubation with clorgyline (open circles). Panel C shows glass–Teflon homogenization (open circles) versus the use of a Polytron (closed circles). Two hearts were thoroughly minced over ice using a razor blade. One portion of the mince was hand homogenized using Teflon–glass (see Materials and Methods). Another portion was subjected to severe homogenization using a Polytron (4 × 15 sec periods at setting 10 over ice). Panel D shows aorta subjected to the same treatment as in A, [preheated (closed circles) and control (open circles)]. Assay time was 30 min. For other details, see legend to Fig. 1.

active in aorta. No conclusive evidence was obtained for deamination by MAO-B. Both heart and aorta showed some inhibition of tyramine deamination at 2×10^{-11} M clorgyline. Thus, tyramine (0.1 mM) was examined in the presence of lower concentrations of clorgyline. Biphasic plots were obtained with heart [Fig. 3 (A, B and C)] between 2×10^{-15} and 2×10^{-8} M clorgyline under control conditions (see legend to Fig. 3). Since complete inhibition occurred at 2×10^{-8} M clorgyline, the curves related solely to MAO-A. Other experiments showed that the inhibition with low concentrations of clorgyline (2×10^{-15}, 10^{-14}, 2×10^{-14}, 2×10^{-13} and 2×10^{-12} M) increased with the time of preincubation up to 20 min at 37°. Between 20 and 80 min no further increase in inhibition was found.

Figure 3A shows that heating heart homogenates at 50° for 1 hr prior to incubation with clorgyline increased sensitivity to clorgyline. Deaminating activity [nmoles deaminated·(mg protein^{-1}·hr)$^{-1}$] did not change (control, without clorgyline = 25.6; heated, without clorgyline = 24.8; $N = 3$ for each group). In other experiments, heating at 37° for 1 hr did not alter sensitivity to clorgyline or tyramine deaminating activity [24.6 nmoles deaminated·mg protein)$^{-1}$·hr^{-1} $N = 2$]. Heating at 50° for 3 hr increased further the sensitivity to clorgyline by about 10 per cent from than seen for 1 hr, but activity decreased to 11.6 nmoles deaminated·(mg protein)$^{-1}$·hr^{-1} ($N = 2$).

Figure 3B is identical to Fig. 3A except that a crude mitochondrial fraction of heart was used. Heating at 50° for 1 hr caused an increase in the sensitivity to clorgyline, but deaminating activity decreased by 45 per cent [control, without clorgyline = 44 nmoles deaminated·(mg protein)$^{-1}$·hr^{-1}, single experiment]. Experiments were made using two different homogenization techniques (glass–Teflon and a Polytron) (Fig. 3C). The use of a Polytron produced a similar effect to heating at 50° for 3 hr. Deaminating activity decreased from 26.1 to 13.3 nmoles deaminated·(mg protein)$^{-1}$·hr^{-1} (single experiment).

Figure 3D shows data from the aorta. Two MAO-A components are clearly visible between 2×10^{-15} and 2×10^{-8} M clorgyline. Heating at 50° for 1 hr gave a qualitatively different result from that obtained in heart. Sensitivity to inhibition by clorgyline *decreased*, and this was particularly evident for the second MAO-A component. Deaminating activity fell by about 50 per cent [10.9 to 5.8 nmoles deaminated·(mg protein)$^{-1}$·hr^{-1}, $N = 4$ control and $N = 3$ heated].

Kinetic experiments were done to characterize further the CRAO of heart and aorta. The K_m values for benzylamine deamination by this enzyme in heart and aorta were 4.39 ± 0.32 and 6.19 ± 0.70 μM, respectively, with corresponding V_{max} values of 3.78 ± 0.24 and 28.8 ± 1.3 [nmoles deaminated·(mg protein)$^{-1}$·hr^{-1}]. These values were calculated from five different homogenates of heart and four of aorta (see Figs. 6 and 7 for representative control plots). In a single experiment with heart, the Michaelis–Menten constants for benzylamine deamination were determined in the absence and presence of clorgyline (2×10^{-4} M). The calculated K_m values without and with clorgyline were 4.96 ± 0.15 and 4.45 ± 0.16 μM, respectively, with corresponding V_{max} values of 3.98 ± 0.11 and 3.61 ± 0.18 [nmoles deaminated·(mg protein)$^{-1}$·hr^{-1}]. Kinetic studies using higher benzylamine concentrations (up to 100 μM) were done in aorta only. Substrate and/or product inhibition was found between 16 and 100 μM benzylamine.

Studies were done with semicarbazide as shown in Fig. 4. In both the heart and aorta, semicarbazide inhibited the deamination of benzylamine by CRAO. Comparison with Fig. 1 shows that the percentage inhibitions are approximately the same as the activity remaining after 2×10^{-4} M clorgyline. Similarly, semicarbazide inhibited the deamination of tyramine (0.1 mM) by 30 per cent in aorta, but no inhibition

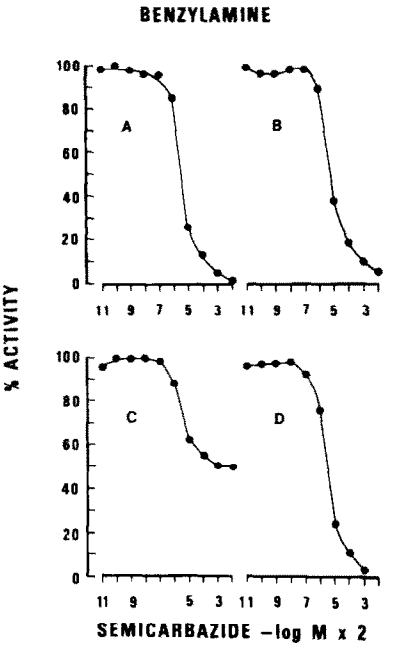

Fig. 4. *In vitro* inhibition of benzylamine deamination by semicarbazide in homogenates of heart (A, B and C) and aorta (D). Remaining activity after preincubation with inhibitor for 20 min at 37° was assayed with benzylamine at 0.001 mM (A), 0.01 mM (B), 0.1 mM (C) and 0.1 mM (D), with an incubation time of 60 min. For other details, see legend to Fig. 1.

of tyramine deamination was observed in the heart (data not shown). Again, these data are in reasonable agreement with the results obtained using clorgyline (see Fig. 2). Other experiments with semicarbazide showed this inhibitor to be a rapid, noncompetitive inhibitor of CRAO (data not shown).

CRAO was found to be inhibited by (+)-amphetamine, (+)- and (−)-mexiletine, and phenelzine in both heart and aorta (Fig. 5). Additional studies with (+)-amphetamine (10^{-5} and 10^{-4} M) showed that inhibition was time-independent and could be reversed fully upon dilution (20-fold). The type of interaction was non-competitive (Fig. 6). Replots of the $1/v$ intercepts versus inhibitor concentration gave straight lines with pH-dependent K_i values for heart and aorta of 40 and 70 μM respectively. In other experiments, (+)-amphetamine was shown to inhibit fully the deamination of tyramine (0.1 mM) in both heart and aorta (data not shown).

Several other compounds were investigated for inhibitory actions on CRAO. Unlabeled tyramine (0.1 to 3.0 mM) inhibited the deamination of [^{14}C]-benzylamine (0.01 mM) in the heart and the aorta. The amines, putrescine and spermidine, were without effect at 10^{-3} M, but spermine (10^{-3} M) produced about 40 per cent inhibition in both tissues. At 10^{-4} M, spermine was virtually inactive. The addition of pyridoxal-5′-phosphate (0.01 to 1.0 mM) to assay mixtures in the absence of inhibitors failed to increase activity.

The effects of oxygen, air or nitrogen were investigated on CRAO. Oxygen did not alter the kinetic constants compared with those obtained with air.

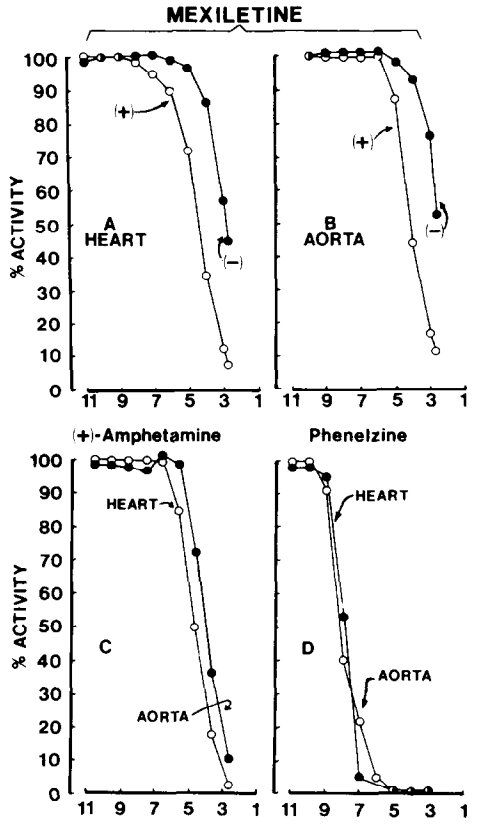

Fig. 5. *In vitro* inhibition of benzylamine (0.01 mM) deamination in homogenates of heart and aorta by (+)- and (−)-mexiletine, (+)-amphetamine and phenelzine. Phenelzine was preincubated with the homogenates for 30 min at 37° prior to the addition of benzylamine. Amphetamine and mexiletine were not preincubated. Remaining activity was assayed after 30-min incubation with substrate. Each point is the mean of three determinations expressed as a percentage of control. Control activities for each experiment were determined from the mean of six replicate determinations.

However, nitrogen produced an uncompetitive inhibition in heart and a noncompetitive inhibition in aorta (Fig. 7). A comparison of regression lines [25] gave a significant difference between slopes for the aorta ($P < 0.01$) but not in heart ($P < 0.001$). In both tissues, the difference between mean activities at each substrate concentration was significant at $P < 0.01$.

DISCUSSION

Previous studies have shown that the rat heart [9] and blood vessels [12] contain clorgyline-sensitive and clorgyline-resistant deaminating activities. This study has compared these activities in rat heart and descending aorta and has revealed certain differences in their properties.

Much of the present work concerned with substrate specificity confirms previous findings [9, 12, 13, 19]. However, by the use of more than one substrate concentration it has been possible to investigate these properties more fully. For example, in the heart, as the concentration of benzylamine was decreased from 1 mM the proportion of deamination by CRAO increased, reaching 100 per cent at 1 μM (Fig. 1A). These changes are consistent with the relative K_m values of benzylamine for the three enzymatic activities involved. Lyles and Callingham [9] reported approximate K_m values of 0.01 and 0.5 mM for CRAO and MAO (A and B) respectively. In the present study we increased the specific activity of [^{14}C]benzylamine by 10-fold and derived a K_m value of 4.45 μM for the CRAO. Thus, the K_m of benzylamine for this component is at least fifty to one hundred times less than that for MAO-A and -B. However, even with concentrations of benzylamine at or near the K_m value for CRAO some slight deamination results from MAO. At 10 μM benzylamine, about 10–15 per cent of the total deamination derives from this source. Similarly, using benzylamine concentrations of 0.4 to 8 μM, the K_m value decreased from 4.96 to 4.45 μM when MAO was

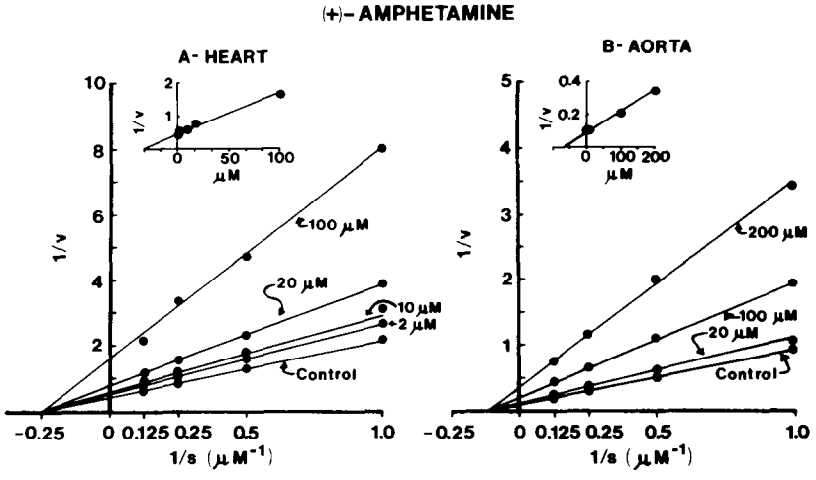

Fig. 6. Double-reciprocal plot of benzylamine deamination by CRAO in the presence of (+)-amphetamine in the concentrations shown. Abscissa: reciprocal of benzylamine concentration (μM); ordinate: reciprocal of velocity (v) of enzyme reaction in arbitrary units. (+)-Amphetamine was added together with the benzylamine. Each point is the mean of triplicate determinations. Insets: Dixon plots of $1/v$ intercept versus the concentration of (+)-amphetamine.

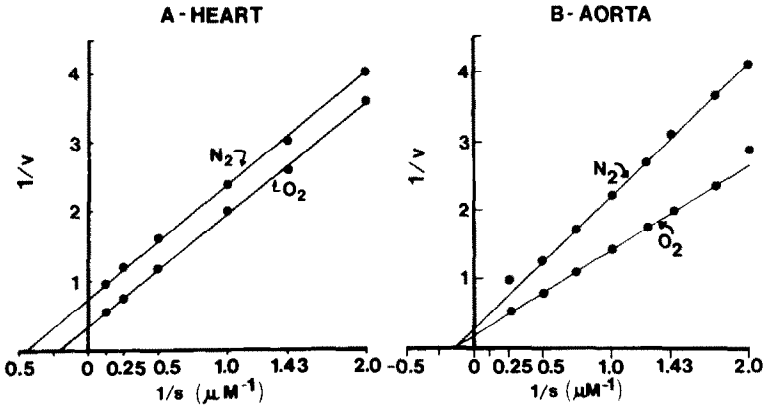

Fig. 7. Double-reciprocal plots of the deamination of benzylamine by homogenates of heart (A) and aorta (B). The homogenates were adjusted to give similar product formation following incubation. Abscissa: reciprocal of benzylamine concentration (μM); ordinate: reciprocal of velocity (v) of enzyme reaction in the same arbitrary units for each homogenate. The regression line for the heart homogenate is drawn from the means of six determinations while that from the aorta was from the mean of the three.

inhibited with clorgyline (2×10^{-4} M). Selective discrimination between MAO and CRAO on the basis of substrate concentration was only possible using 1 μM benzylamine. These results illustrate the difficulty of classifying and assaying amine oxidases on the basis of substrate specificity alone (see Introduction).

In heart the ratios of the specific activities of MAO-A and -B at 0.1 and 1.0 mM benzylamine are 1.3 and 1.06 respectively (estimated from Fig. 1A at 2×10^{-8} and 2×10^{-4} M clorgyline). These values support the claim that little difference exists between the K_m values of benzylamine for MAO-A and B in rat heart [9]. Thus, the inability of clorgyline to inhibit benzylamine deamination in aorta (Fig. 1B) provides evidence that the MAO activity in this tissue may be different from that in the heart. That MAO-A activity exists in rat aorta has been shown by others [12, 15] and by the inhibition curves to tyramine in the present study. Even at 1.0 mM benzylamine, which is in excess of its quoted K_m value for MAO-A [4, 9], no inhibitory action of clorgyline was seen. At present, in addition to the rat heart, only the human placenta and rat skeletal muscle have been reported to deaminate benzylamine by both MAO-A and MAO-B [4, 26]. However, it is possible that this phenomenon may be more widespread than is commonly supposed. Deamination of benzylamine by MAO-A may be seen with high concentrations of the substrate in tissues which contain a preponderance of the A form, or where MAO-B activity has been selectively inhibited [6].

Tyramine was deaminated by both MAO-A and CRAO in aorta, with vitrually no evidence for the involvement of MAO-B. However, in the heart tyramine was a substrate solely for MAO-A. CRAO was not involved. These findings show a clear difference between the clorgyline-resistant activities of heart and aorta. This applies to other substrates as well. Kynuramine is a substrate for CRAO in rat aorta and vena cava [15] but not in the heart [27]. Beta-phenylethylamine is reported to be a substrate for CRAO in both rat heart and mesenteric arteries [19], but others have shown only marginal deamination by CRAO in the ventricles of rat heart [3, 28].

Although tyramine was not deaminated by CRAO in heart, it was found to inhibit benzylamine deamination by this enzyme. However, whether this inhibition was caused by tyramine, or by its metabolites, is not known. In aorta, tyramine appears to show similar K_m values for both CRAO and MAO, since the relative proportional contributions of these enzymes towards tyramine deamination are unchanged at different tyramine concentrations (Fig. 2B). While changes in such curves provide a test for possible differences in K_m values, substrate and/or product inhibition of one or more of the defined activities would also influence the position and/or shape of the curves, as could non-specific effects of high concentrations of clorgyline and the physical state of the enzyme (see below).

In the present study, tyramine deamination was found to be inhibited by low concentrations of clorgyline (2×10^{-11} M). Benzylamine was less affected. With tyramine as substrate the percentage inhibition was unaffected by changes in substrate concentration in the aorta, but a possible concentration-related inhibition was found in the heart (Fig. 2). Further studies with tyramine at 0.1 mM revealed that biphasic MAO-A curves could be obtained between 2×10^{-15} M and 2×10^{-8} M clorgyline. The initial component was more pronounced in aorta than in the heart. Since physical manipulations were found to modify the relative percentages of the two MAO-A components (Fig. 3), this difference may be related to the different homogenization procedures employed for the two tissues (see Materials and Methods). It seems likely that these two components may result from possible allotropic properties of MAO-A. Catalytic activity has been shown to be dependent upon lipid attachments [29–34], and evidence is available that this same factor plays a role in the selectivity of clorgyline for MAO-A [31, 32]. The rate of inhibition of MAO-A in heart homogenates by low clorgyline concentrations was the same as that reported by

others using higher concentrations of the inhibitor [29, 35, 36]. This implies that the inhibition of the initial MAO-A component may depend upon an enhanced availability of clorgyline to its active site, rather than on a difference in enzyme structure. This notion is supported by the experiments in which whole heart homogenates were heated at 50° for 1 hr. Conversion of low to high clorgyline sensitivity was found without loss in catalytic activity. It is possible that the thermal perturbation of the lipid annulus enhanced the penetration of clorgyline to its inhibitory site. In aorta the same treatment produced opposite effects. Catalytic activity and sensitivity to clorgyline decreased. This could be construed as further evidence for differences between MAO-A of the heart and aorta. However, it cannot be excluded that the initial homogenization technique or differences in the composition of the crude homogenate are critical factors. Furthermore, in aorta it is not known whether the decreased sensitivity to clorgyline represents a greater thermal inactivation of MAO relative to clorgyline-resistant activity. Heating crude mitochondria of heart or exposing whole heart homogenates to severe homogenization decreased enzyme activity. However, unlike aorta, sensitivity to clorgyline increased. Thus, in heart the most stable form appears to be the initial MAO-A component and may possibly be explained, like thermal inactivation studies [33], upon different degrees of protection by mitochondrial lipids. It is interesting to note that other workers have observed inhibition of deamination with very low concentrations of clorgyline [37, 38]. However, in surveying the literature, this effect appears to be the exception rather than the rule.

The occurrence of two MAO-A components with clorgyline has relevance to *in vitro* titration studies with this agent for determination of the number of active sites of MAO-A [35, 39]. Absolute values may be difficult to obtain if the nature of the immediate environment modifies the available concentration of clorgyline for inhibition. Similarly, potential problems may exist when other acetylenic inhibitors, such as pargyline, are used for the same purpose [40–42].

Kinetic evaluation of CRAO activity towards benzylamine gave similar K_m values in both heart and aorta, together with substrate and/or product inhibition of the enzyme in aorta. The latter effect with CRAO has been noted previously [5, 9, 13]. The K_m values of 4.45 μM (heart, in the presence of (2×10^{-4} M clorgyline) and 6.19 μM (aorta) are somewhat lower than some reported in the literature [9, 13, 43] but are in agreement with values found in bone [5] at the same pH. Lewinsohn *et al.* [13] showed that the specific activity for benzylamine deamination by CRAO was higher in rat aorta than in the heart, an observation supported by the V_{max} values found here. It should be noted that the V_{max} value for benzylamine deamination by CRAO is at least ten times smaller than that for MAO [5, 9]. Thus, in comparison with MAO, CRAO is a low K_m low capactiy enzyme for benzylamine deamination. The kinetic properties of the enzyme at low oxygen tension revealed a difference between CRAO of heart and aorta. Unlike MAO [44, 45], at room temperature sufficient oxygen is present in buffer for maximal activity. The requirement for oxygen only becomes apparent if the assay is performed in an atmosphere of nitrogen when, in the heart, the reaction is inhibited in an uncompetitive manner. In the aorta, however, noncompetitive inhibition was seen. Uncompetitive inhibition is consistent with a double displacement or ping–pong mechanism for benzylamine deamination, as has been shown for MAO [44] and plasma amine oxidase [46, 47]. Noncompetitive inhibition is characteristic of a sequential mechanism although, at the present time, it is not known whether it proceeds in a random or ordered fashion. The ability of CRAO to deaminate at low oxygen tensions may have important physiological consequences (e.g. during hypoxia). Certainly, it would function at oxygen tensions which inhibit MAO. Since the oxygen concentration of the reaction mixture in air is 0.217 mM [48], the K_m of oxygen for CRAO can be estimated to be about 20–40 μM.

Apart from the substrate specificity and kinetic properties, few other differences between CRAO of heart and aorta were found. However, in the heart at low benzylamine concentrations, clorgyline (2×10^{-3} M) caused a slight inhibition of deamination. Conversely, a small activation was seen in the aorta. This concentration of clorgyline is beyond that required to inhibit totally MAO, and thus indicates an influence on CRAO. Houslay and Tipton [49] reported competitive inhibition of bovine plasma amine oxidase with high concentrations of clorgyline, and a similar effect in rat heart would explain the failure to observe inhibition with the two higher benzylamine concentrations. Activation of CRAO has also been observed in homogenates of bone and in amine oxidase activity contaminating several commercial samples of bovine serum albumin [5].

Semicarbazide inhibited clorgyline-resistant activity in both heart and aorta. In agreement with McEwen [50], with human plasma amine oxidase, the inhibition was rapid and noncompetitive. The percentage inhibition was similar to the percentage activity remaining after clorgyline (2×10^{-4} M) and suggests that CRAO may be pyridoxal dependent.

(+)−Amphetamine was found to be a reversible noncompetitive inhibitor of CRAO. The K_i values at pH 7.8 were 40 μM (heart) and 70 μM (aorta), with calculated pH-independent values (K_i') [43] of 0.29 and 0.53 μM respectively. These two values were determined using benzylamine concentrations of 0.001 to 0.008 mM. Thus, the lower value for heart will be influenced to a minor extent by concomitant deamination by MAO (see Fig. 1A). It is well established that (+)−amphetamine is a reversible competitive inhibitor of MAO, both *in vitro* [51, 52] and *in vivo* [53, 54], with a distinct preference for MAO-A [49, 50, 52]. K_i' values for (+)−amphetamine on rat liver MAO-A and -B are 0.037 and 1.43 μM, respectively [51, 52], with values of 0.048 and 1.24 μM for human heart [52]. Thus, the K_i' values reported here fall between those for type A and B MAO. Some of the pharmacological properties of (+)-amphetamine have been attributed to MAO inhibition [54–56], and it now seems possible that inhibition of CRAO could also play a role. These considerations might well apply to the antiarrhythmic drug mexiletine [57], which shows CNS

and cardiovascular toxicity [58]. This compound is an alpha-substituted monoamine with MAO inhibitory properties closely resembling (+)-amphetamine [59]. Like (+)-amphetamine, mexiletine was shown to inhibit CRAO. Since (+)-mexiletine was more effective than the (−) form, CRAO can recognize optical isomers, as reported previously for MAO [60, 61]. Phenelzine was also found to be an effective inhibitor of the CRAO activity. This result is consistent with the effects of other hydrazines [13, 62] and with the known ability of phenelzine to inhibit human plasma and tissue amine oxidases [4, 62].

Since hydrazines inhibit MAO as well as CRAO, hypertensive crises with tyramine [63, 64] might be more severe, or occur more frequently, with these agents than with selective MAO inhibitors. However, while tyramine has been shown to be a good substrate for human plasma amine oxidase [50], it is not known whether it is also a substrate for the tissue enzyme in man.

The physiological role of CRAO is unresolved. The present findings indicate that it is not a diamine or polyamine oxidase since straight chain di- and polyamines in concentrations up to 10^{-4} M were without effect on benzylamine deamination. Other studies appear to exclude lysyl oxidase [for references, see Ref. 13] and the copper-carrying protein caeruloplasmin [65]. While some endogenous monoamines (tyramine, kynuramine, and beta-phenylethylamine) are substrates for the blood vessel activity in rats, the endogenous substrates for human tissue amine oxidase activities have not been determined. Dopamine and beta-phenylethylamine appear not to be substrates [4, 13]. Substrates other than benzylamine have been shown for human plasma amine oxidase and include tyramine and tryptamine, although deamination rates are ten times slower [50, 62]. Furthermore, urinary tryptamine excretion depends much more upon the functional activity of MAO than plasma amine oxidase [62]. It is intriguing to speculate that benzylamine itself might be the endogenous substrate since hippuric acid, the glycine conjugate of benzoic acid, is found in urine. However, as pointed out by Lewinsohn *et al.* [13], benzylamine has not so far been identified in man or rat.

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