Biochemical Pharmacology, Vol. 28, pp. 2337–2343.

0006-2952/79/0801–2337 $02.00/0

DEVELOPMENTAL ASPECTS OF RAT HEART MONOAMINE OXIDASE

DAVID J. EDWARDS, KOON YAN PAK and MARK C. VENETTI

Department of Psychiatry, Western Psychiatric Institute and Clinic, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, U.S.A.

(*Received* 11 *September* 1978; *accepted* 4 *January* 1979)

Abstract—Rat heart mitochondria were demonstrated to contain two forms of monoamine oxidase which resemble the A and B forms found in other tissues on the basis of the effects of clorgyline, thermostability, and kinetic characteristics. The relative amounts of these two enzyme forms underwent a marked change between 3 and 15 weeks of age. In the adult, only the form resembling type A MAO could be demonstrated.

Rat heart monoamine oxidase (MAO: EC 1.4.3.4) exhibits the unusual property of increasing in specific activity with increasing body weight for the entire lifespan of the animal [1]. This property appears to be specific to the rat and is not observed in other species such as the mouse, rabbit or cat [1]. Although other tissues also show increases in MAO activity during development, unlike the heart the activity in these tissues reaches a plateau in early life and does not continue to increase with growth of the animal [1].

Rat heart MAO has been reported to differ in its enzymatic properties from MAO in other species and tissues. In tissues such as brain and liver, two types of MAO can be distinguished by the effects of the inhibitor, clorgyline; the type A form is inhibited by low concentrations of clorgyline, and the type B form is inhibited only by high concentrations of the inhibitor [2]. Thus, if tyramine, a nonspecific substrate, is used, double sigmoidal clorgyline inhibition curves are observed if both types A and B MAO are present. However, clorgyline produces identical, sigmoid inhibition curves for rat heart MAO whether tyramine or serotonin (an A specific substrate) is used [3]. Even phenylethylamine, a specific substrate for type B MAO in other tissues [4], has been reported to produce a single, sigmoid activity [5, 6]. On the other hand, biphasic clorgyline inhibition curves have been observed for the substrate, benzylamine, which is another specific substrate for type B MAO in other tissues [5].

In the present report, the characteristics of rat heart mitochondrial MAO were studied at various developmental stages. Our results indicate that rat heart contains two forms of MAO which are similar to the types A and B enzymes in other tissues. Only the type A enzyme shows a marked increase in activity with age. The age-related change in the proportions of the two enzyme activities may account for many of the discrepancies reported previously.

MATERIALS AND METHODS

Materials. [2-^{14}C]Serotonin creatinine sulfate (49 mCi/m-mole), [1-^{14}C]phenylethylamine hydrochloride (48 mCi/m-mole), and [1-^{14}C]tyramine hydrochloride (12.4 mCi/m-mole) were obtained from New England Nuclear (Boston, MA). Phenylethylamine hydrochloride, tyramine hydrochloride and serotonin creatinine sulfate were purchased from K & K Laboratories (Plainview, NY), the Aldrich Chemical Co. (Milwaukee, WI) and the Sigma Chemical Co. (St. Louis, MO) respectively. Ultrapure sucrose was obtained from Schwarz/Mann, Orangeburg, NY. Protosol was obtained from New England Nuclear. All other reagents were obtained from the Fisher Scientific Co. (Pittsburgh, PA). Clorgyline was a gift from May & Baker, Ltd., Dagenham, U.K. Deprenyl was kindly provided by Professor J. Knoll, Semmelweis University, Budapest, Hungary. [^{3}H]Pargyline (6.75 Ci/m-mole) was synthesized by New England Nuclear. Male Sprague–Dawley rats were supplied by Zivic–Miller Laboratories (Pittsburgh, PA).

Preparation of rat heart mitochondria. Rats were decapitated and the hearts were homogenized in 9 vol of cold 0.32 M sucrose using a Teflon–glass tissue grinder. A crude mitochondrial fraction was obtained by a modification of the procedure of Autilio *et al.* [7]. After removing the crude nuclear fraction by centrifugation at 1000 *g* for 10 min in a Sorvall RC-2B centrifuge, the resulting supernatant fraction was centrifuged at 14,000 *g* for 20 min. The pellet was washed in 0.32 M sucrose and the final pellet was resuspended in 0.2 M potassium phosphate buffer, pH 7.4, except where indicated for the thermal inactivation studies, in which the pellet was resuspended in 0.32 M sucrose, pH 7.4.

MAO assay. MAO was assayed by radiochemical procedures based on the method of Wurtman and Axelrod [8]. Serotonin (a type A specific substrate), phenylethylamine (a type B specific substrate) and tyramine (a nonspecific substrate) were used as substrates. All assays were carried out in Beckman Microfuge tubes in a final volume of 100 μl, containing 0.2 M potassium phosphate buffer (pH 7.4). The reaction was initiated by the addition of the substrate solution and was carried out with shaking at 37° for 30 min. The reaction was stopped by the addition of 20 μl of 2 N HCl and the deaminated products were extracted with vortex mixing into 300 μl toluene (when phenylethylamine was the substrate) or ethyl acetate (when serotonin or tyramine was the substrate). After centrifuga-

tion for 30 sec in a Beckman Microfuge B, 200 μl of the organic phase was removed for scintillation counting, using a mixture containing 2,5-diphenyloxazole (PPO) and 1,4-bis-2-(5-phenyloxazolyl)benzene (POPOP) in toluene. When ethyl acetate was used as the extracting solvent, Triton X-100 (1:2, v/v) was added to the mixture.

The stock substrate solutions were prepared by dissolving the radioactive and the nonradioactive substrates in 0.001 N HCl and washing 3 times with toluene (for the phenylethylamine substrate) or ethyl acetate (for serotonin and tyramine) in order to minimize the blanks. The washed substrates were then lyophilized and redissolved in 0.001 N HCl. Aliquots were stored at −20° until needed. All of the stock substrate solutions contained 2.5 μCi/ml. The concentrations of the substrate were chosen to give final concentrations in the assay of 100 μM serotonin, 20 μM phenylethylamine or 500 μM tyramine.

Protein concentrations were determined by the method of Hartree [9], using bovine serum albumin as standard.

Drug inhibition studies. Inhibition curves were obtained by adding clorgyline or deprenyl to the assay tubes containing the enzyme and buffer and preincubating at room temperature for 20 min before the substrate was added.

Thermal inactivation of rat heart MAO. Rat heart homogenates (1:10, w/v, in 0.32 M sucrose) and crude mitochondrial fractions in 0.32 M sucrose (3.5 mg protein/ml) were incubated at 50° for 30 min and assayed for MAO activity. Control samples were kept at room temperature for 30 min.

SDS gel electrophoresis of MAO labeled with [^{3}H]pargyline. Crude mitochondria (containing 180 μg protein) isolated from rat heart, liver or brain were incubated for 1 hr at 25° with 4×10^{-6} M [^{3}H]pargyline (6.75 Ci/m-mole). The labeled mitochondria were centrifuged for 30 sec in a Beckman Microfuge B and the pellet was washed 4 times with 200 μl of 6% perchloric acid to remove the unbound pargyline. The final pellet was resuspended in 100 μl of 50 mM Tris buffer, pH 7.4, containing 1% SDS, 10 mM mercaptoethanol and 0.003% bromophenol blue. One drop of glycerol was added and the samples were then incubated at 60° for 40 min. Each sample (50 μl) was loaded onto a 7.5% SDS polyacrylamide slab gel and subjected to electrophoresis at 20 mA for 2 hr according to the procedures of Weber and Osborn [10]. Each gel was sliced into 2.5 mm sections with a razor blade. Individual gel slices were incubated overnight at 25° in glass scintillation vials with 5 ml of a PPO–POPOP–toluene mixture containing 4% Protosol. Radioactivity was determined in a Packard model 3390 liquid scintillation spectrometer equipped with an Absolute Activity Analyzer to correct for quenching. Nonspecific binding of [^{3}H]pargyline was corrected for by pretreating identical samples with 2 mM pargyline for 40 min before the addition of [^{3}H]pargyline. Protein bands were stained in unsliced gels by using 0.05% Coomassie Blue in 10% acetic acid–25% 2-propanol. The gels were destained in 7% acetic acid. The molecular weight of the labeled subunit was determined by comparing its mobility with that of the following standard marker proteins: bovine serum albumin (68,000), aldolase (40,000), alcohol dehydrogenase (37,000), trypsin (23,300), lysozyme (14,300) and cytochrome *c* (11,700).

RESULTS

Effect of clorgyline on phenylethylamine deamination. Figure 1 shows the effects of clorgyline on the deamination of phenylethylamine by rat heart mitochondria from 3-, 8- and 15-week-old animals. In 3-week-old rats, phenylethylamine deamination was relatively unaffected except by concentrations greater than 10^{-6} M (Fig. 1A).

In contrast, the clorgyline inhibition curves obtained with phenylethylamine as substrate for 4-week- (data not shown) and 8-week-old rats (Fig. 1B) were clearly biphasic, indicating that phenylethylamine is deaminated in these mitochondria by at least two forms of MAO. The I_{50} values for the two sigmoid portions of the curves were approximately 4×10^{-10} M and 2×10^{-5} M. These values are in close agreement with those reported for types A and B MAO in other tissues [2]. The relative amounts of the two enzymes in rat heart are difficult to estimate since the position of the plateau in the inhibition curve is dependent upon the amount of enzyme included in each assay tube. The exact cause for this shift in the plateau is not clear but is consistent with previous work by Lyles and Greenawalt [11], who carefully studied the effect of varying the protein content on the inhibition by clorgyline of the two forms of MAO in rat liver. These investigators observed that the apparent sensitivity of both forms of the enzyme toward clorgyline decreased as the concentration of protein in the assay was increased. This effect is presumably related to the rather complex nature of the interaction between clorgyline and the enzyme active sites.

The phenylethylamine-deaminating activity remaining, even after pretreatment with 1 mM clorgyline (Fig. 1, A and B) is due presumably to the clorgyline-resistant amine oxidase present in rat heart [5]. The relative contribution of this enzyme was much greater in whole tissue homogenates.

In rat heat mitochondria obtained from 15-week-old rats, phenylethylamine deamination was totally blocked by 10^{-8} M clorgyline (Fig. 1C). Clorgyline produced a single sigmoid inhibition curve with an I_{50} of approximately 10^{-9} M, regardless of the amount of enzyme present.

The specific activity of the heart mitochondria MAO was determined with the substrates, serotonin and phenylethylamine. With serotonin as substrate, the specific enzymatic activities were 1.6, 55 and 80 nmoles/hr/mg of protein for the 3-, 8- and 15-week-old rats respectively. Thus, the MAO activity toward this type A substrate increased by more than 30-fold in only 5 weeks. The total deamination of phenylethylamine increased from 0.70 to 4.9 to 11.1 nmoles/hr/mg of protein at 3, 8 and 15 weeks of age respectively. However, as is evident from Fig. 1, phenylethylamine is deaminated by two forms of MAO. Most, if not all, of this increase in deamination is apparently due to an increase in the clorgyline-sensitive enzyme. At 15 weeks of age, it was impossible to detect any phenylethylamine deamination by the clorgyline-insensitive enzyme, since clorgyline produced total inhibition at a

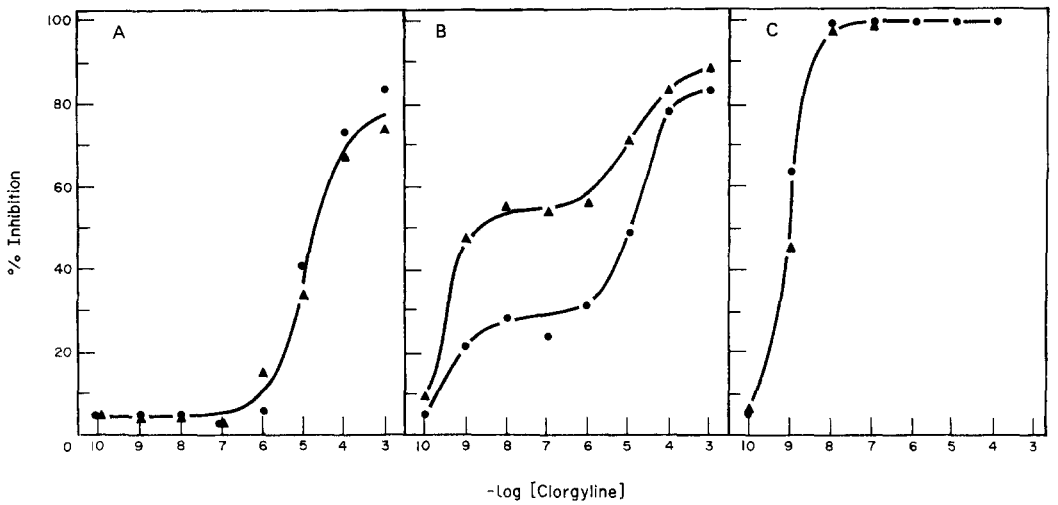

Fig. 1. Inhibition by clorgyline of rat heart mitochondrial MAO using phenylethylamine as substrate for (A) 3-week-old rats, (B) 8-week-old rats or (C) 15-week-old rats. Mitochondria were isolated by homogenizing six rat hearts in 9 vol of cold 0.32 M sucrose, centrifuging at 1000 *g* for 10 min to remove the cellular debris (P_1) and then centrifuging at 14,000 *g* for 20 min to obtain the mitochondrial pellet (P_2). After washing the P_2 pellet in 0.32 M sucrose, the mitochondria were resuspended in 0.2 M potassium phosphate buffer (pH 7.4). This suspension was diluted with buffer to give a final concentration of 2.5 mg protein/ml. To each assay tube was added either 10 μl (25 μg, ●) or 20 μl (50 μg, ▲) of the mitochondrial suspension, sufficient buffer (0.2 M potassium phosphate, pH 7.4) to bring the total volume of buffer and mitochondria to 70 μl, and 10 μl of either water (for control samples), 2 mM pargyline (for blanks), or a solution containing clorgyline in a concentration between 10^{-2} M and 10^{-9} M. After preincubating the enzyme with the inhibitor for 20 min at room temperature, the enzyme reaction was initiated by the addition of 20 μl of [^{14}C]phenylethylamine (2.5 μCi/ml, 100 μM) to give a final substrate concentration of 20 μM and 0.05 μCi of radioactivity in each assay tube. The assays were carried out at 37° for 30 min. After the reaction was stopped by the addition of 20 μl of 2 N HCl, the deaminated products were extracted into 300 μl toluene and the radioactivity was determined by scintillation counting. The assay was linear up to at least 50 μg protein. The specific enzymatic activities for the control samples were 0.7, 4.85 and 11.1 nmoles phenylethylamine deaminated/hr/mg of protein for the mitochondria from 3-, 8- and 15-week-old rats respectively. Each point represents the mean of duplicate determinations.

concentration of 10^{-7} M. Thus, if any clorgyline-insensitive MAO is present, it is masked by the large excess of the clorgyline-sensitive enzyme.

During the course of these experiments, we observed that the rat heart mitochondrial MAO activity from 3-week-old rats measured with either phenylethylamine or tyramine as substrate was relatively unstable in phosphate buffer when stored at either 4° or at −20°. In contrast, MAO from 8- and 15-week-old rats was relatively stable under these conditions. Therefore, the clorgyline inhibition studies for rat heart mitochondria of 3-week-old rats were repeated using mitochondria resuspended in 0.32 M sucrose, pH 7.4. Under these conditions, the MAO activity remained relatively unchanged after storage for 2 days at 4°. Results obtained with 50 μg protein/assay were similar to those shown in Fig. 1A except that the curve was now distinctly biphasic.

Effects of deprenyl on phenylethylamine deamination. The effects of deprenyl on phenylethylamine deamination by rat heart MAO are shown in Fig. 2. With mitochondria from either 8- or 15-week-old rats, almost identical sigmoidal inhibition curves were observed, with an I_{50} of approximately 2×10^{-6} M. Mitochondria from 3-week-old rats resulted in a distinctly different inhibition curve. This curve appeared to be biphasic or possibly triphasic.

Effects of clorgyline on rat heart mitochondrial MAO with tyramine as substrate. The clorgyline inhibition curve for rat heart mitochondria from 3-week-old animals was a biphasic curve when tyramine was used as substrate with I_{50} value of 1.8×10^{-9} M and 3.6×10^{-6} m (Fig. 3).

Apparent K_m of rat heart MAO for phenylethylamine as substrate in 4- and 15-week-old animals. Kinetic studies reveal two active sites of MAO which have markedly different K_m values for phenylethylamine. The double reciprocal plot for phenylethylamine deamination by heart mitochondria from 4-week-old rats results in two linear portions of the curve, indicating the presence of a high K_m and a low K_m site (Fig. 4). This is compatible with the data shown in Fig. 1B, which shows that at this age phenylethylamine is deaminated by two forms of MAO. Preincubation of these mitochondria with 10^{-7} M clorgyline to abolish all type A MAO activity results in a straight line plot which gives an apparent K_m of 8 μM.

The data obtained for rat heart mitochondria from 15-week-old rats yield a straight line double reciprocal plot with an apparent K_m of 114 μM (Fig. 5). These data, in agreement with the clorgyline inhibition curves (Fig. 1C), show no evidence for more than one form of MAO activity, although again a small amount of another enzyme form could be masked by the large

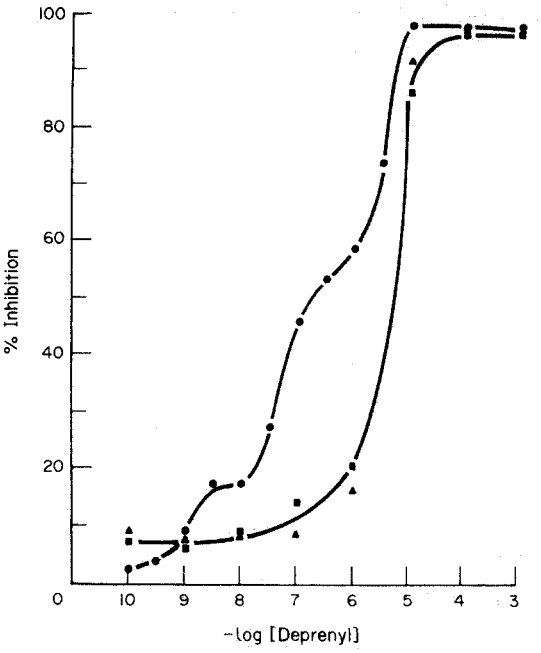

Fig. 2. Inhibition by deprenyl of rat heart mitochondrial MAO using phenylethylamine as substrate for 3-week-old (●), 8-week-old (▲) and 15-week-old (■) animals. This experiment was carried out as described in the legend to Fig. 1, except that deprenyl was used as the inhibitor instead of clorgyline. For clarity, only data obtained using 50 μg protein/assay tube are shown. The specific enzymatic activities of the control samples are given in the legend of Fig. 1.

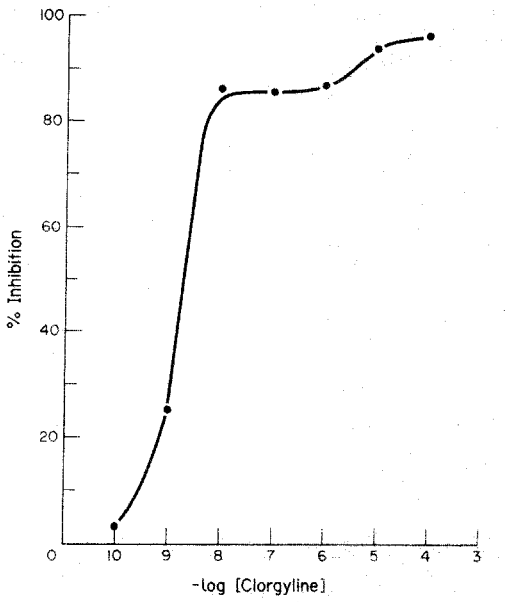

Fig. 3. Inhibition by clorgyline of heart mitochondrial MAO from 3-week-old rats using tyramine as substrate. Hearts were removed from six 3-week-old weanling rats and the mitochondria were isolated as described in the legend of Fig. 1. The final mitochondrial pellet was resuspended in 0.2 M potassium phosphate buffer (pH 7.4) using 3 ml/g of original tissue. Then, 20 μl of the homogenate was preincubated for 20 min at 37° with 50 μl of buffer and 10 μl of a solution of clorgyline to give a final concentration of the inhibitor over the range of 10^{-4} to 10^{-10} M. The assay was initiated by the addition of 20 μl of a stock solution of [^{14}C]tyramine (1 μmole/μCi, 2.5 mM). The reaction was carried out for 60 min at 37° and stopped by the addition of 20 μl of 2 N HCl. The amount of enzyme activity in the control tubes was 2.05 nmoles tyramine deaminated/hr.

activity of this one form. The high apparent K_m for phenylethylamine calculated from these results is approximately 14-fold higher than was the low K_m site in the 4-week-old animals.

Similar results were obtained with phenylethanolamine, which is also a selective substrate for type B MAO [12]. Using mitochondria from 4-week-old rat hearts, the clorgyline inhibition curves were biphasic and parallel to the ones obtained with phenylethylamine as substrate. As when phenylethylamine was used as substrate, the double reciprocal plots had two linear portions, except when the mitochondria was preincubated in the presence of 10^{-7} M clorgyline. The apparent K_m of phenylethanolamine measured in the presence of clorgyline (10^{-7} M) was 5 μM.

Thermal inactivation of rat heart MAO. Heating of rat heart homogenates from 3-week-old animals at 50° resulted in a differential loss of MAO activity, when measured with serotonin and phenylethylamine as substrates (Table 1). The activity remaining after 30 min of heating was 90 per cent for the substrate serotonin and 55 per cent for the substrate phenylethylamine. This is comparable to results obtained for rat brain homogenates in which the corresponding activities were 98 and 44 per cent respectively [13]. Similar results were also

Table 1. Thermostability of MAO in heart homogenates and mitochondria of 3- week- and 18-week-old rats

Enzyme	Age (weeks)	Substrate (per cent activity) Serotonin	Phenylethylamine
Rat heart homogenate (1:9 in 0.32 M sucrose)	3	90	55
Rat heart mitochondria Resuspended in 0.32 M sucrose	3	82	51
	18	100	97
Resuspended in 0.2 M potassium phosphate*	3*	42	42
	18	44	29

* Inactivation was carried out for 15 min; all other data were obtained after 30 min of inactivation at 50°.

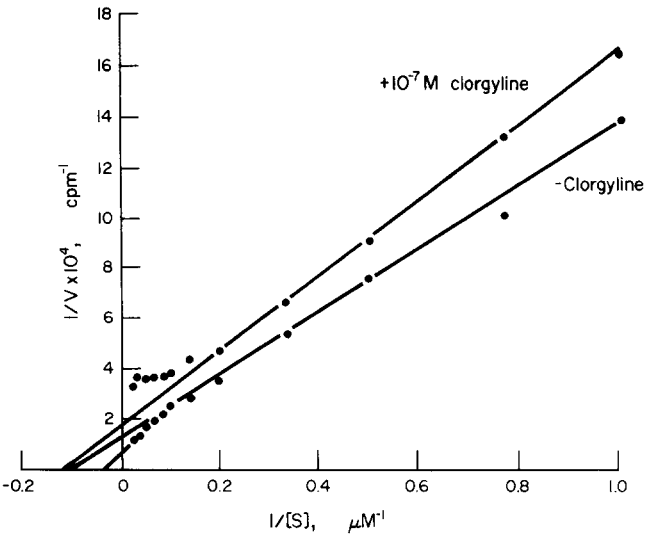

Fig. 4. Double reciprocal plots of MAO from rat heart mitochondria from 4-week-old animals using phenylethylamine as substrate with preincubation for 20 min at room temperature, either in the presence or absence of 10^{-7} M clorgyline. Mitochondria were prepared as described in the legend of Fig. 1 and resuspended in 0.32 M sucrose (3 ml/g of original tissue). Varying amounts of substrate were added to each assay tube to give final concentrations in the assays over the range of 1–40 μM, and sufficient 0.001 N HCl was added to bring the total volume of substrate plus 0.001 N HCl to 20 μl. Then, to each assay tube was added 40 μl of 0.2 M potassium phosphate buffer (pH 7.4), and the enzyme reaction was initiated by the addition of 40 μl of enzyme suspension, which had been mixed previously (3:1) with either water (for controls), 10^{-3} M clorgyline (for blanks), or 10^{-6} M clorgyline and preincubated at room temperature for 20 min. Enzyme activity was determined as described in the legend of Fig. 1 using a 60-min incubation time. At the standard substrate concentration (20 μM), the enzyme activity of the mitochondrial suspension added to each assay tube was 0.18 nmoles/hr for the control sample and 0.084 nmoles/hr in the presence of 10^{-7} M clorgyline. All points represent the mean of duplicate determinations.

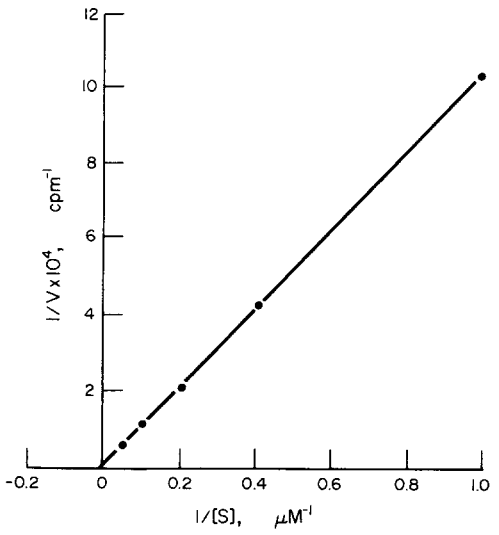

Fig. 5. Double reciprocal plot of MAO from rat heart mitochondria from 15-week-old animals using phenylethylamine as substrate. Mitochondria were prepared as described in the legend of Fig. 1. The final substrate concentration varied over the range of 1–20 μM. The amount of enzyme added to each assay tube had an activity of 19.3 nmoles/hr when measured at a substrate concentration of 20 μM. The apparent K_m calculated by least mean squares was 114 μM.

obtained from crude mitochondria resuspended in 0.32 M sucrose. In contrast, both the phenylethylamine- and serotonin-deaminating activities were stable at 50° when the mitochondria were isolated from 18-week-old rats and resuspended in sucrose. Data obtained for mitochondria resuspended in phosphate buffer show that this medium causes a decreased thermal stability of the enzyme activity, particularly toward serotonin as substrate. As a consequence, no differentiation in the loss of activity toward the two substrates was observed when rat heart mitochondria of 3-week-old animals were resuspended in phosphate buffer.

Estimation of molecular weights of rat heart, liver and brain MAO subunits by SDS gel electrophoresis. When the crude mitochondria preparations obtained from either heart, liver or brain homogenates were labeled with [^{3}H]pargyline, the bulk of the radioactivity migrated on the SDS slab gel by electrophoresis as a single band with a molecular weight in each case estimated to be 60,000 daltons. The radioactivity in this band was almost completely abolished by pretreatment with 2 mM pargyline. An additional band of radioactivity which migrated slightly ahead of the tracking dye was apparently due to nonspecific binding of the [^{3}H]pargyline to a low molecular weight substance. McCauley [14] observed a similar binding in rat liver mitochondria membranes and suggested that this was due to the binding of pargyline by lipids.

DISCUSSION

Our results demonstrate that rat heart mitochondria contain two forms of MAO which can be distinguished on the basis of their sensitivity to the inhibitor, clorgyline, using phenylethylamine as substrate. The most striking finding was the marked change in the relative proportion of these enzyme forms during development. Thus, phenylethylamine was deaminated primarily by a clorgyline-insensitive MAO in the 3-week-old rat heart, by both the clorgyline-sensitive and the clorgyline-insensitive enzyme in 8-week-old animals and exclusively by the clorgyline-sensitive enzyme in the 15-week-old animals.

These developmental changes may explain discrepancies previously reported for the properties of rat heart MAO. Fuentes and Neff [6] found that, when phenylethylamine was used as substrate, the clorgyline inhibition curve was sigmoidal, with an I_{50} identical to that obtained with norepinephrine as substrate. From those results, they concluded that rat heart contains a single form of MAO which has properties similar to those of the type A enzyme found in other tissues. However, in those studies, only 200–250 g Sprague–Dawley rats were used. Their results, therefore, appear consistent with the results we obtained for 15-week-old animals. Lyles and Callingham [5] observed biphasic clorgyline curves when benzylamine, but not when phenylethylamine, was used as substrate. Although the ages of the animals were not stated, on the basis of our present results these differences could be explained if animals of different ages had been used. Dial and Clarke [15] have also shown developmental changes in rat heart MAO by clorgyline inhibition curves using kynuramine as substrate.

The changes in the pattern of occurrence of multiple forms of MAO during development may be very important in trying to understand the effects of hormones. For example, in young rats with a weight of about 100 g, thyroxine produced an increase in cardiac MAO when measured with serotonin as substrate but a decrease when measured with benzylamine [16]. In adult rats (200 g), there was no change in cardiac MAO when measured with either substrate. Lyles and Callingham [17] also found a differential effect on rat heart MAO of thyroxine, which was substrate dependent. In their studies, MAO activity was increased with both tyramine and benzylamine, although the relative change was greater with tyramine as substrate. Adrenalectomy of adult rats has also been found to increase rat heart MAO but there were no differences in relative changes when measured with various substrates, including tyramine, serotonin and benzylamine [18]. In contrast, adrenalectomy did produce in young rats a greater increase in the MAO activity with benzylamine as substrate than with tyramine [18]. Again, these results may be explained by the presence of at least two forms of cardiac MAO in young rats and the predominance of a single form of MAO in adult animals, as is suggested by the results of our present study.

The developmental changes in rat heart MAO appear to be a result of a changing ratio of the two MAO forms rather than to a change in their enzymatic properties. The two forms of MAO which were demonstrated in the hearts of rats 8 weeks of age or younger exhibited many properties in common with the types A and B enzymes in other tissues. For example, the clorgyline inhibition curves obtained with phenylethylamine as substrate indicated that this substrate was deaminated by both a clorgyline-sensitive and a clorgyline-insensitive enzyme having I_{50} values comparable to those reported for the A and B enzymes in other tissues. Moreover, in the young rat heart the phenylethylamine-deaminating activity was more thermolabile than was the serotonin-deaminating activity. This is consistent with the properties of the A and B enzymes observed in other tissues, in which the B enzyme was found to be more thermolabile than the A enzyme [4].

The kinetic studies show further that the clorgyline-resistant enzyme has a high affinity of phenylethylamines with an apparent K_m of 8 μM. This is in close agreement with the apparent K_m of phenylethylamine for MAO in human platelets ($K_m = 3$ μM), in which the only the B form of MAO is present [19]. In the absence of clorgyline, the double reciprocal plots for MAO from rat heart mitochondria of 4-week-old animals were nonlinear, indicating the presence of a high K_m active site, in addition to the low K_m active site deaminating phenylethylamine. The double reciprocal plots for rat heart MAO of 15-week-old animals show that only the high K_m site is detectable at this age. Taken together, these data suggest that two forms of MAO having properties similar to the A and B enzymes are present in the hearts of young rats but that the activity of only the form resembling type A MAO increases with age. By 15 weeks of age, only the latter form of MAO could be detected in rat heart. From our data, it is impossible to say whether the form resembling type B MAO is unchanged (and thus masked by the large excess of the other enzyme form) or whether it has actually decreased in activity.

When tyramine was used as substrate, the presence of A and B forms of MAO could be demonstrated according to criteria originally used by Johnston [2] in rat heart mitochondria from 3-week-old animals (Fig. 3) but not from older animals, even at the age in which the inhibition curves using phenylethylamine as substrate were clearly biphasic. This is presumably due to the lack of sensitivity by which A and B forms may be detected using tyramine as substrate when one of the forms strongly predominates. Thus, as we have suggested previously [20], the use of specific substrates instead of nonspecific one is a far more sensitive method for the detection of multiple forms of MAO, particularly when the amount of one is small, compared to the other.

In spite of many similarities between the two forms of MAO in rat heart and the A and B forms in other tissues, the question of whether the multiple forms of MAO in heart are identical to those in other tissues cannot yet be answered with certainty. Our data do suggest that there may be some significant differences in the properties of the multiple forms of MAO in rat heart and other tissues. For example, deprenyl appeared to show less selectivity between the two forms of MAO in rat heart than is observed for the A and B enzymes in other tissues. The possibility of a triphasic inhibition curve suggests that there may even be two or more B-like forms of MAO in rat heart. Finally, we have noted that the activity of rat heart MAO of 3-week-old animals is relatively unstable and considerable activity is lost upon simply freezing and thawing the homoge-

nates. In order to compare more precisely the properties of MAO in rat heart and other tissues, further studies will be required in which the age of the animal is carefully controlled.

The use of thermal inactivation studies to demonstrate the presence of multiple enzyme forms has been criticized since the differential thermal stabilities toward different substrates appear to lessen or disappear when isolated mitochondria are used instead of whole tissue homogenates [21]. Our experiments show the importance of the buffer which is used to resuspend the mitochondria. Thus, when the mitochondria were resuspended in 0.32 M sucrose, the stability of MAO toward both serotonin and phenylethylamine was similar to that for the whole tissue homogenate which was in the same medium. Under these conditions, differential thermal stabilities toward the two substrates were apparent for both the whole homogenate and the isolated mitochondria of 3-week-old rats. However, when the mitochondria were resuspended in phosphate buffer, the MAO activity toward both substrates showed a greater loss in activity upon heating as compared to mitochondria resuspended in 0.32 M sucrose. The effect of the phosphate was greater on the serotonin-deaminating activity and consequently the thermal stability of MAO toward these two substrates was no longer distinguishable.

The molecular basis for the multiple forms of MAO in rat heart or in other tissues remains unresolved. The two or more forms of MAO could represent separate proteins or a single protein that differs by post-translational modifications, such as the binding of lipids or other membrane components. The question of the molecular basis of the multiple enzyme forms is particularly important in trying to understand the mechanism of their genetic control. For example, the age-dependent selective rise in the clorgyline-sensitive form of MAO in rat heart could be due to an increase in synthesis in the corresponding gene product (if two or more genes are involved) or to an increase in the binding to the MAO apoenzyme of lipids or other components necessary for the clorgyline-sensitive activity. Using [^{3}H]pargyline to label the active site of MAO, a single band of radioactivity was observed on SDS gel electrophoresis corresponding to the same molecular weight regardless of the tissue or whether A and/or B activities were present. We, therefore, infer that the A and B sites of MAO occur either on the same subunit or on separate subunits of similar molecular weight. These data are consistent with, but do not prove, the notion that the multiple forms of MAO contain the same apoenzyme.

Acknowledgements—We thank Cathy Rupp for typing. Clorgyline was a gift from May & Baker, Ltd. Deprenyl was kindly provided by Professor Knoll. This work was supported in part by a grant from the Health Research and Services Foundation.

REFERENCES

1. A. Horita, *Nature, Lond.* **215**, 411 (1967).
2. J. P. Johnston, *Biochem. Pharmac.* **17**, 1285 (1968).
3. M. C. Lowe, D. D. Reichenbach and A. Horita, *J. Pharmac. exp. Ther.* **194**, 522 (1975).
4. H.-Y. T. Yang and N. H. Neff, *J. Pharmac. exp. Ther.* **187**, 365 (1973).
5. G. A. Lyles and B. A. Callingham, *J. Pharm. Pharmac.* **27**, 682 (1975).
6. J. A. Fuentes and N. H. Neff, *Biochem. Pharmac.* **26**, 2107 (1977).
7. L. A. Autilio, S. H. Appel, P. Pettis and P.-L. Gambetti, *Biochemistry* **7**, 2615 (1968).
8. R. J. Wurtman and J. Axelrod, *Biochem. Pharmac.* **12**, 1439 (1963).
9. E. F. Hartree, *Analyt. Biochem.* **48**, 422 (1972).
10. K. Weber and M. Osborn, *J. biol. Chem.* **244**, 4406 (1969).
11. G. A. Lyles and J. W. Greenawalt, *Biochem. Pharmac.* **26**, 2269 (1977).
12. D. J. Edwards, *Life Sci.* **23**, 1201 (1978).
13. A.K. Student, *Thesis*, University of Pittsburgh (1975).
14. R. McCauley, *Biochem. Pharmac.* **25**, 2214 (1976).
15. E. J. Dial and D. E. Clarke, *Fedn Procs Fedn Am. Socs exp. Biol.***37**, 510 (1978).
16. J. H. Tong and A. D'Iorio, *Endocrinology* **98**, 761 (1976).
17. G. A. Lyles and B. A. Callingham, *J. Pharm. Pharmac.* **26**, 921 (1974).
18. B. A. Callingham and R. Laverty, *J. Pharm. Pharmac.* **25**, 940 (1973).
19. D. J. Edwards and S.-S. Chang, *Life Sci.* **17**, 1127 (1975).
20. D. J. Edwards and C. W. Malsbury, *Biochem. Pharmac.* **27**, 959 (1978).
21. M. D. Houslay and K. F. Tipton, *Life Sci.* **19**, 467 (1976).