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Studies on the nature of the increased monoamine oxidase activity in the rat heart after adrenalectomy

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The increased activity, induced by adrenalectomy, of the enzyme monoamine oxidase (MAO) in the rat heart was found to resemble closely that present in the hearts of control animals. No significant differences were observed in the nature of the response to heat denaturation, changes in pH or to inhibition by pargyline or clorgyline. The relative activities using the substrates tyramine, 5-hydroxytryptamine, dopamine or benzylamine were the same. No evidence was found to suggest the presence of a heat-stable or dialysable inhibitor of enzyme activity. In young rats there was an increase in the relative enzyme activity using benzylamine as substrate, compared with the activity using tyramine, in the first few days after adrenalectomy. No effect of adrenalectomy could be detected upon the MAO activity in the rat brain or liver. It is concluded that the increase in rat heart MAO following adrenalectomy cannot be due to the synthesis of an enzyme with different catalytic properties, nor to the transformation of the existing enzyme into one of increased catalytic ability but with different properties.

There is now a considerable weight of evidence which suggests that the monoamine oxidase (MAO) activity in the tissues of different animals can show widely varying properties depending on the species and upon the particular tissue (Johnston, 1968; Squires, 1968, 1972; Sandler & Youdim, 1972; Youdim, 1972). In the rat the activity of MAO in the heart can be increased by adrenalectomy of the animal (Avakian & Callingham, 1968; Callingham & Della Corte, 1971). The mechanism responsible for this increase is at present uncertain. One possibility is that adrenalectomy induces the formation of additional enzyme which has the same properties as that found in the hearts of control animals. Alternatively, an enzyme with entirely novel properties may be formed or the existing enzyme could be modified by the removal of an inhibitor or the addition of some enzyme activator.

The present experiments have been undertaken to determine if adrenalectomy modifies the properties of the MAO found in the rat heart.

MATERIALS AND METHODS

Materials

Radiolabelled materials, namely [^{3}H]dopamine and [^{3}H]5-hydroxytryptamine, for use as substrates for MAO, and [^{3}H]corticosterone for the assay of plasma steroids, were obtained from the Radiochemical Centre, Amersham. Two further substrates

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for MAO, [^{3}H]tyramine and [^{14}C]benzylamine, were obtained from New England Nuclear GMBH, Dreieichain and Mallinckrodt, St. Louis, respectively. Pargyline hydrochloride was a gift from Abbott Laboratories Chicago, and clorgyline hydrochloride (M & B 9302) was a gift from May and Baker Ltd., Dagenham. The animals used were male albino rats of a Wistar strain from A. J. Tuck & Son Ltd., Rayleigh.

Methods

The rats were adrenalectomized under ether anaesthesia and after operation were allowed free access to 0·9% sodium chloride solution. Some control animals were subjected to sham adrenalectomy; no effect on subsequent enzyme levels was seen.

Animals were killed between 09.30 and 10.30 by cervical dislocation at various times following adrenalectomy or sham operation. Blood for steroid assay was withdrawn from the inferior vena cava above the renal vein into a heparinized syringe as soon as possible after death. Tissues for MAO assay were removed, washed in 0·154 M potassium phosphate solution pH 7·8, and homogenized using a conical glass hand homogenizer in 0·001 M potassium phosphate solution, pH 7·8. Homogenates were diluted to a 1:10 or 1:20 suspension of tissue and then centrifuged at 2000 *g* for 10 min to remove large debris. The supernatants were stored for short periods at 4° or deep frozen for more prolonged storage.

Steroid assay. The method used was based on that of Corker, Naftolin & Richards (1971). Samples of plasma (20–50 μl) were diluted with water (0·2 ml) and washed with light petroleum (b.p. 40–60°) (2 ml). The mixture was deep frozen and the supernatant organic layer discarded. The plasma dilution was thawed, and extracted with diethyl ether (2 ml), which was also separated by freezing the aqueous layer. The ether extract was evaporated to dryness. Extracts and standards (0–10 ng corticosterone) were dissolved in water (0·2 ml) by warming at 37° with vigorous vortex mixing. To each tube was added 0·5 ml steroid binding solution, containing 5% male human plasma and 40 nCi ml^{-1}[^{3}H]corticosterone (36 Ci m mol^{-1}), followed by cooling in ice for 10 min. Dextran-coated charcoal suspension (0·5 ml containing 2·5 mg ml^{-1} Norit A charcoal and 25 μg ml^{-1} Dextran T40 in 0·005 M potassium phosphate solution, pH 7·4) was added, mixed and allowed to stand at 0° for 10 min. The tubes were then centrifuged in the cold; the supernatant was decanted into a scintillation vial to which was added 12 ml toluene: Triton X-100 (2:1) scintillation mixture containing 0·4% butyl-PBD. Radioactivity was measured by liquid scintillation spectrometry. The plasma content of steroid was determined from a standard curve relating [^{3}H]corticosteroid content in the supernatant to the corticosterone content of the standard solutions. The limit of sensitivity of the method was 0·5 ng corticosterone corresponding to a plasma concentration of 1–2 ng per 100 ml.

Monoamine oxidase assay. The radiochemical assay method was based on that of McCaman, McCaman & others (1965).

To each sample of homogenate (10–50 μl), made up to 50 μl, if necessary, with water, was added 50 μl of a 2 mM solution of the appropriate substrate. The substrate solution was made up in 0·2 M potassium phosphate buffer at pH 7·8, and contained 2–5 mCi m mol^{-1} of substrate.

Oxygen was blown over the mixture and the reaction tubes stoppered. Incubation was carried out over various times between 10 and 30 min. Tissue concentrations

and incubation times were chosen to ensure adequate production of metabolites without allowing the reaction to deviate from linearity.

The reaction was stopped by plunging the tubes into ice and water and adding 3N HCl (10 μl). The reaction products were extracted with 0·5 ml water-saturated benzene–ethyl acetate (1 : 1) mixture (Squires, 1972). After centrifuging to separate the layers, a 0·4 ml sample of the benzene–ethyl acetate layer was added to a scintillation vial containing 0·4% butyl-PBD in 12 ml of toluene, and the radioactivity measured by liquid scintillation spectrometry. Blank values were obtained by incubation of either substrate to which no homogenate had been added, or by incubation of substrate and homogenate in the presence of 3N HCl (10 μl). No significant difference was found in the blank values obtained by the two methods.

Samples of the substrate solutions were counted at the same time to determine their specific activities.

MAO activities are expressed in terms of n mol (of substrate consumed) (mg protein)$^{-1}$ h^{-1}, calculated as the mean values $\pm$ standard error of the mean.

No effect upon the apparent MAO activity is seen when corticosterone, cortisol or deoxycorticosterone are added to the reaction mixture *in vitro*, to give final concentrations between 10^{-8} and 10^{-4} M.

The protein contents of the tissue homogenates were determined by a micro-biuret method (Goa, 1953).

RESULTS

Effect of pH on the MAO activity in tissues from adrenalectomized and control animals

Homogenates of the hearts from 4 control and 4 adrenalectomized rats (320–420 g, and killed 5–6 months after operation) were incubated with tyramine or benzylamine as substrate and their MAO activities determined.

The mean absolute activity at pH 7·8 was 1009 $\pm$ 124 n mol (mg protein)$^{-1}$ h^{-1} for tyramine, and 18·3 $\pm$ 1·5 n mol (mg protein)$^{-1}$ h^{-1} for benzylamine in the hearts of control animals, and 1784 $\pm$ 90, and 29·5 $\pm$ 1·4 respectively, in the hearts of adrenalectomized animals.

The effect of changing the pH of the incubation medium using phosphate buffer over the range of 6·0–9·1 is shown in Fig. 1.

The effect of change of pH was the same upon the MAO activity from the hearts of either adrenalectomized or control rats, using either tyramine or benzylamine as substrate.

Brain homogenates were also prepared from these animals and tested over the same pH range with the same substrates. No difference could be seen between the homogenates obtained from the two groups of animals. The effect of pH change was similar to that seen with heart tissue, giving the same slightly increased relative activity towards tyramine compared with benzylamine over the pH range 6·3–8·0. The mean absolute activities (at pH 7·8) of the control brain homogenates were, for tyramine 264 $\pm$ 26 and benzylamine 56·5 $\pm$ 4·7, and in brains from adrenalectomized animals tyramine activity was 264 $\pm$ 26 and benzylamine 60·3 $\pm$ 6·3.

Effects of heat, enzyme inhibitors and dialysis on heart MAO activity from control and adrenalectomized animals

Rats, 200–250 g, were killed 4–6 weeks after adrenalectomy. The homogenates of hearts from a pair of animals, each consisting of a control and an adrenalectomized rat, were heated in a water bath at 55° for 30 min. Samples were removed

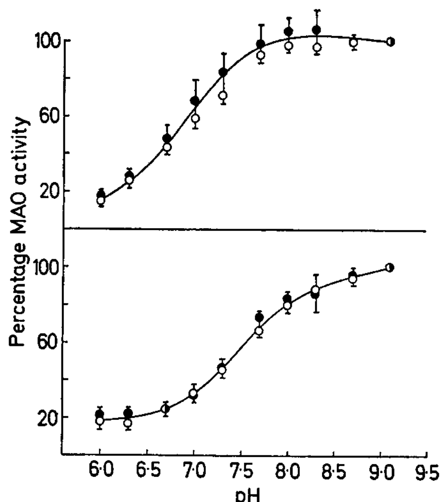

FIG. 1. The effect of change of pH of the incubation medium (0·1M phosphate buffer) upon the *in vitro* activity of MAO in homogenates of hearts from control and adrenalectomized rats. The activities are expressed as percentages of the activity of the enzyme at pH 9·1 (± standard error of the ratio n = 4): ● —— ● controls; ○ —— ○ adrenalectomized. Upper graph tyramine as substrate; lower graph bezylamine as substrate.

immediately before heating and at intervals during the heating. They were then assayed for MAO activity using tyramine as substrate. In 7 experiments there was no significant difference in the rates at which MAO activity disappeared between heart homogenates from control and adrenalectomized rats (Fig. 2).

Samples of the same homogenates were pre-incubated for 15 min at room temperature with the MAO inhibitors pargyline and clorgyline. Pargyline was added to give a final concentration in the incubation mixture between 10^{-5} and 10^{-7} M (Maître, 1967); clorgyline concentrations used were between 10^{-6} and 10^{-11} M (Hall, Logan & Parsons, 1969). There was no difference in the inhibition of MAO activity using tyramine as substrate between heart homogenates from control and adrenalectomized animals with pargyline (Fig. 3) and only at a clorgyline concentration of 10^{-8} and

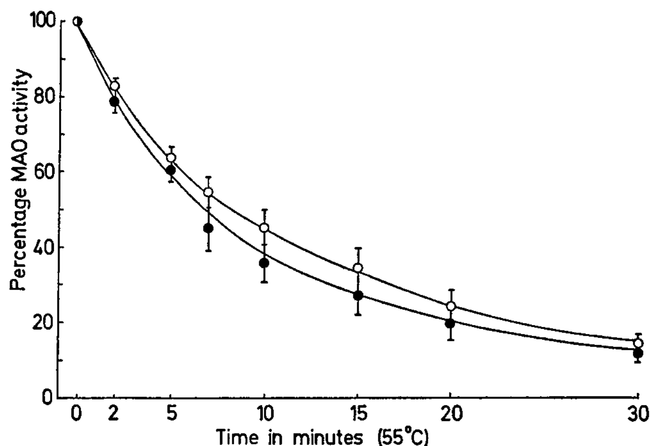

FIG. 2. The effect of prior incubation at 55° on the *in vitro* activity of MAO in homogenates of hearts from control and adrenalectomized rats. The activities are expressed as percentages of the activity of the enzyme without prior incubation (± standard error of the ratio, n = 7) using tyramine as substrate: ● —— ●, controls; ○ —— ○, adrenalectomized.

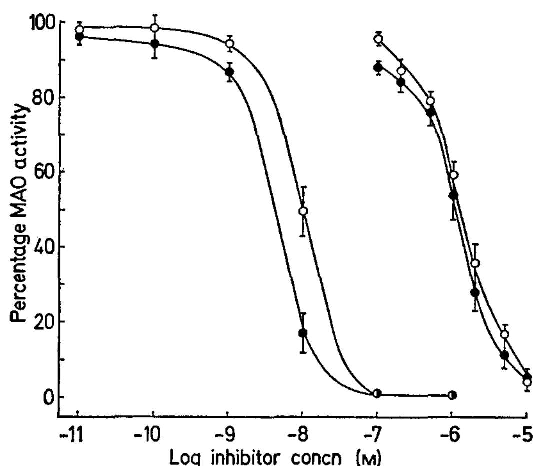

FIG. 3. The effect of the MAO inhibitors clorgyline and pargyline upon the *in vitro* activity of MAO in homogenates of hearts from control and adrenalectomized rats. The activities are expressed as percentages of the activity of untreated homogenates (± standard error of the ratio n = 7) using tyramine as substrate: ● —— ●, controls; ○ —— ○, adrenalectomized. Left hand curves, clorgyline; right hand curves, pargyline.

10^{-9} M was the percentage remaining activity significantly higher ($P < 0{\cdot}005$) in the heart homogenates from the adrenalectomized rats (Fig. 3).

The mean absolute MAO activity of the heart homogenates of the control rats was 717 ± 59 (n = 7) and of the adrenalectomized rats 1779 ± 238 (n = 7).

Samples of these heart homogenates from control and adrenalectomized animals were heated at 60° for 30 min, and were added to unheated samples of homogenates. Heat denatured homogenates had no effect on the MAO activity of heart homogenates from control or adrenalectomized rats. Dialysis of heart homogenates against 0·01 M potassium phosphate buffer (pH 7·8) or of a heart homogenate from a control rat against a heart homogenate from an adrenalectomized rat for up to 24 h at 4° had no consistent effect on the absolute MAO activity of the homogenate measured radiochemically using [^{3}H]tyramine as substrate. Thus, it would appear that there is no heat-stable or dialysable factor present in the homogenates to account for the difference between MAO activity of heart homogenates from control or adrenalectomized rats.

Substrate specificity of MAO activity of tissue homogenates from control and adrenalectomized rats

Heart, liver and brain tissues from a control and an adrenalectomized rat were homogenized and the MAO activity in the tissue from the adrenalectomized rat was expressed as a percentage of the activity from the control rat, using tyramine, dopamine, 5-hydroxytryptamine and benzylamine as substrates.

Two separate groups of experiments were performed. In experimental group A, the MAO activity was measured by the technique described in the Methods section. The brain tissue used included the mid-brain, hypothalamus, thalamus, caudate nuclei and septal regions. In experimental group B, tissues were homogenized in a modified Chappell-Perry solution (Callingham & Della Corte, 1971) and the radioactive reaction products were extracted with ethyl acetate which was then backwashed with 0·3 N HCl (Jarrott, 1971). The brain tissue used was cerebral cortex.

The results in Table 1 show that MAO activity was increased in the heart homogen-

Table 1. *MAO activity in tissues from adrenalectomized rats expressed as percentages of the activities of tissues from control rats (mean % ± standard error of the ratio).*

Substrate Tissue	Expt	Tyramine	Dopamine	5-HT	Benzylamine
Heart	A	186 ± 10	226 ± 19	202 ± 10	162 ± 19
	B	211 ± 31	219 ± 40	201 ± 26	214 ± 65
Brain	A	92 ± 7	89 ± 8	87 ± 8	99 ± 8
	B	116 ± 12	104 ± 6	103 ± 10	112 ± 9
Liver	A	97 ± 6	99 ± 10	96 ± 8	105 ± 3
	B	116 ± 11	129 ± 14	130 ± 18	100 ± 9

Expt A: 6 pairs of rats; tissue homogenized in 0·001M phosphate; brain tissue consisted of mid-brain, thalamus, and caudate regions.
Expt B: 4 pairs of rats; tissue homogenized in modified Chappell-Perry solution; brain tissue consisted of cortex.

ates from adrenalectomized animals compared with controls for all the substrates used, to approximately an equal extent. Thus, the increased MAO activity after adrenalectomy showed a similar substrate specificity to the normal MAO activity.

No increase above control values was observed in the MAO activity with any substrate in brain and liver homogenates (Table 1) or, in other experiments, the salivary glands, from adrenalectomized rats.

Steroid levels in adrenalectomized rats

In young control animals (22–45 days) the corticosterone levels in venous blood after killing ranged from 2·5 to more than 30 μg 100 ml plasma (mean 17·5 ± 1·5; n = 43), whereas in adrenalectomized rats the levels were less than 1 μg per 100 ml in all animals (n = 22) until 20 days after adrenalectomy when two higher values, 2·8 and 3·2 μg per 100 ml, suggested that some adrenal regeneration had occurred in these two animals.

In a series of older animals (250–350 g) killed at least one month after adrenalectomy, the control rats had plasma steroid levels of between 3·4 and 19·6 μg per 100 ml (mean 9·9 ± 1·0, n = 18) whereas adrenalectomized rats had a mean level of 1·71 ± 0·32 μg per 100 ml (n = 23). Only in those animals in which the steroid level was at or below 2 μg per 100 ml were the heart MAO values raised above the range of the control animals.

Increase in heart MAO activity with age and after adrenalectomy

In the rat heart, MAO activity increases with age (Horita, 1967). However, in older animals it is possible to analyse changes in MAO activity other than those due to age or body weight by dividing the MAO activity by the heart weight to give a constant MAO activity relative to age, body weight or heart weight (Callingham & Della Corte, 1972). But in young animals there is still a small residual increase in relative MAO activity as the animal grows, seen when tyramine is used as substrate but not seen with benzylamine (Fig. 4). Adrenalectomy caused a relatively more rapid increase in activity towards benzylamine 2 days after adrenalectomy whereas activity towards tyramine increased more gradually; thus adrenalectomy appears initially to increase the relative amount of benzylamine oxidase activity, so that in

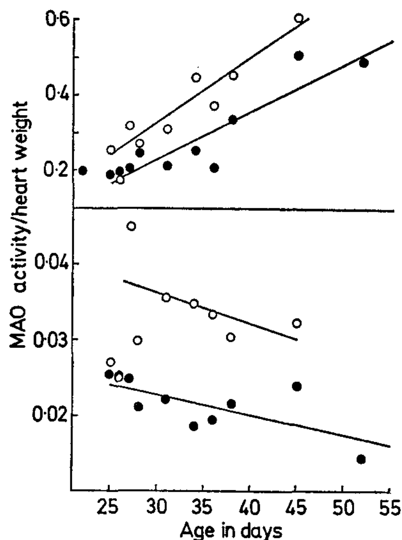

FIG. 4. The effect of age and adrenalectomy on the MAO activity of the hearts from young rats. The activity is expressed in terms of n mol (mg protein)$^{-1}$ h^{-1} (mg heart)$^{-1}$. ● —— ●, controls; ○ —— ○, adrenalectomized. Upper graph, tyramine as substrate; lower graph, benzylamine as substrate.

these young animals adrenalectomy may be changing, for the first 7 days or so only, the nature of the heart MAO activity. However, since the relative amount of this benzylamine oxidase activity is so small compared to the activity towards tyramine in the rat heart this change would make little difference to the total MAO activity.

DISCUSSION

These results show that in almost all experiments there was no difference in the nature of the MAO activity in heart homogenates from adrenalectomized rats compared with control animals. There was no difference in the response to change of pH, or heat stability, or in the nature of the response to enzyme inhibitors. If the increased activity were due to the production of a different enzyme, it might be expected that the inhibition curve, particularly with clorgyline (Johnston, 1968), could show a double sigmoid curve. This was not seen. The slight displacement of the clorgyline inhibition curve to the right is probably due to the increased amount of MAO enzyme present in heart homogenates from adrenalectomized animals. The absence of any effect of dialysis on the MAO activity of the heart homogenates would suggest that the increased activity following adrenalectomy was not due to increased dialysable or heat-stable activator or reduced inhibitor.

The absence of any change in substrate specificity in the enzyme after adrenalectomy also would suggest that the new activity is essentially the same in character as that in the control heart. Contrary to some reports (Caesar, Collins & Sandler, 1970; Holzbauer & Youdim, 1972), no increase in brain MAO activity was observed in adrenalectomized rats in these experiments. This was regardless of whether cortex or thalamus-midbrain regions were used (Table 1). This was studied on several groups of animals (see pH sensitivity results), using a variety of substrates, so that the reason for this discrepancy is not easy to see. That liver MAO does not seem to change after adrenalectomy is more universally agreed.

Experiments on very young animals suggest that adrenalectomy does increase the proportion of benzylamine oxidase activity to tyramine oxidase activity in rat heart homogenates in the first few days after adrenalectomy. In adult animals, when studied at least a month after adrenalectomy (Table 1 and pH experiments), there was a slight decrease (approx. 15%) in adrenalectomized animals, in the ratio of benzylamine oxidase activity to tyramine oxidase activity. Thus this effect may occur only in the young animal or immediately following adrenalectomy and does not explain the persistent increase in MAO activity following adrenalectomy.

In these experiments the MAO obtained from the hearts of adrenalectomized rats seemed to possess the same properties as that from the hearts of normal animals. Since, also, adrenalectomy does not alter the half-life of the enzyme (Callingham & Della Corte, 1972), this lends further weight to the suspicion that adrenalectomy leads to an increase in the synthesis of new enzyme protein. The mechanism for this effect, if it occurs, is uncertain. But the steroids may exert their action at several sites in the biosynthetic chain either directly upon the synthetic enzymes themselves or by influencing the action of non-dialysable regulating factors (Parvez & Parvez, 1973).

The problem of studying the effects of drugs or other procedures on MAO activity is complex. Each tissue and each species has its own pattern of MAO activity with various substrates (Youdim, 1972; Laverty, Browne & Callingham, 1973) so that in order to study the changes in MAO activity it is necessary to study not only one but a variety of substrates. There is no reason to suppose that the ones chosen here are necessarily to be preferred. In addition, specific complications, such as the selective increase in MAO activity in the rat heart with age (Horita, 1967; Callingham & Della Corte, 1972), make it necessary to view studies such as this, using relatively few substrates and tissues, as being limited in application and not necessarily capable of extension to other tissues, substrates or species.

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