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The effects of thyroid hormones on monoamine oxidase in the rat heart

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The administration of thyroxine to young male rats produced an increase in the specific activity of their cardiac monoamine oxidase (MAO). A reduction in the circulating concentrations of thyroid hormones, brought about by 2-thiouracil, led to a decrease. The relative change in activity produced was greater with tyramine than with benzylamine as substrate. By following the time-course of the return of enzyme activity, with tyramine as substrate, after a single injection of pargyline *in vivo*, it was concluded that both excess and lack of thyroid hormones cause their effects on MAO activity by changing the rate of synthesis of the enzyme and not its degradation rate constant. The degradation rate constant did change with the age of the animal. The MAO activity, which increased towards tyramine as substrate in hyperthyroid rat hearts, behaved in the same way as that of controls to heat treatment, irreversible inhibition by pargyline or by clorgyline and also in K_m determinations. The pattern for benzylamine oxidation was similar, except for the effect of the inhibitor clorgyline which shifted the plateau region of the double sigmoid inhibition curve significantly using enzyme from hyperthyroid rat hearts. The plateau region was also shown to be affected by the age of the animal. The possibility is discussed that the increased cardiac MAO activity produced by thyroid hormones and by the growth of the animal is mediated by that form of the enzyme primarily responsible for the oxidation of tyramine. Mixed substrate experiments suggested that tyramine oxidation could be inhibited competitively by benzylamine.

Although it has been reported that thyroid hormones do not influence rat heart monoamine oxidase (MAO; E C 1.4.3.4.), (Zile, 1960), later workers have been able to show that some relation does exist. Usually, thyroid hormones appear to cause an increase in rat heart MAO activity, especially in young animals (Novick, 1961; Utley, 1964). Ho-van-Hap, Babineau & Berlinguet (1967) have found that adult female rats respond in the same way as the young animals, but adult males show a decrease in cardiac MAO after thyroid treatment.

The following experiments were designed to examine some of these conclusions by the use of rats in which the concentrations of circulating thyroid hormones had been modified.

Preliminary results of this work have been published (Callingham & Lyles, 1974).

MATERIALS AND METHODS

Materials

The radioactive substrates for MAO [^{3}H]tyramine and [^{14}C]benzylamine were

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obtained from New England Nuclear GMBH, Dreieichain and Mallinckrodt, St. Louis, respectively. Pargyline hydrochloride was a gift from Abbott Laboratories, Queenborough, Kent, and clorgyline hydrochloride (M & B 9302) was a gift from May & Baker Ltd., Dagenham, Essex. (−)-Thyroxine (free acid) and 2-thiouracil were obtained from Sigma London, Kingston-upon-Thames, Surrey. Male Albino Wistar rats, weighing 50–70 g at the start of each experiment, were obtained from A. J. Tuck & Son, Rayleigh, Essex.

Methods

Rats were made hyperthyroid by daily subcutaneous injections of (−)-thyroxine (600 μg kg^{-1}) for at least 16 days. The hormone was dissolved in NaOH (pH 8·5) and diluted with 0·9% NaCl solution (saline). Control animals for these experiments received daily injections of saline only.

Rats were made hypothyroid by feeding them with a ground meal diet *ad libitum* containing 2-thiouracil (2 g kg^{-1} of meal) (Barker, 1949). Control animals in these experiments received ground meal diet without drug.

Animals were killed, after weighing, by cervical dislocation. Hearts were excised, blotted and larger blood vessels dissected away. After individual weighing, the hearts were then washed in saline and homogenized in 0·001M potassium phosphate buffer, pH 7·4, using a conical glass homogenizer. Homogenates were diluted to a 1 to 10 (w/v) suspension of tissue in buffer, and then centrifuged at 600 g for 10 min to remove debris. The supernatants were decanted and stored on ice for immediate MAO assay or deep-frozen for longer storage. The deep-frozen homogenates were used, after thawing, for heat sensitivity, inhibitor sensitivity, K_m determinations and mixed substrate experiments since the character of the MAO was not changed by deep-freezing.

The radiochemical assay for MAO used was modified from that of McCaman, McCaman & others (1965), as described by Callingham & Laverty (1973). [^{3}H]-Tyramine and [^{14}C]benzylamine were used as substrates. MAO values are expressed as specific activities, i.e. in n mol (of substrate consumed) (mg protein)$^{-1}$ h^{-1}, calculated as mean values ± standard error of the mean.

Protein contents of the homogenates were estimated by the micro-biuret method (Goa, 1953).

Statistical significance between groups was determined by the non-parametric Wilcoxon Rank Test (Goldstein, 1967).

RESULTS

Effect of thyroid hormones on cardiac MAO activity

Rats subjected to thyroxine treatment showed typical effects of hyperthyroidism—a slower rate of weight gain and also a marked cardiac hypertrophy. This represented an increase in the growth of heart tissue, and was not due to an increase in the water content of the hearts. On the contrary, there was a small but significant reduction in the water content of the hearts of these animals when compared with controls. The water content (as a percentage of total wet weight of the heart) of the hyperthyroid rats was 75·9 ± 0·3%, and of the control rats was 77·1 ± 0·1%, for groups of 6 animals ($P < 0·05$).

MAO activity was significantly increased in the thyroxine-treated group, compared with control animals, using both tyramine and benzylamine as substrates; but the

percentage increase was larger with tyramine (Table 1). As reported by Okamoto (1971), thyroid hormones had no effect on the activity of MAO when added to the assay system *in vitro* in concentrations of 5 and 10 μg ml^{-1}.

Table 1. *Effect of (−)-thyroxine on rat cardiac MAO activity. Animals were treated for at least 16 days with (−)-thyroxine (600 μg kg^{-1}). MAO activity was assayed in each heart for both substrates. All values are expressed as mean ± s.e., n = 18 for both groups assayed in quadruplicate.*

Group	Mean body weight (g)	Mean heart weight (mg)	MAO activity [n mol (mg protein)$^{-1}$ h^{-1}] Tyramine	Benzylamine
Hyperthyroid	114 ± 3	597 ± 17	173 ± 12**	9·5 ± 0·5*
Control	133 ± 3	455 ± 12	118 ± 7	7·9 ± 0·3

* $P < 0.05$ (19% increase compared with control); ** $P < 0.01$ (47% increase compared with control).

Rats made hypothyroid with 2-thiouracil suffered the expected stunting of growth. These animals grew slowly up to about 100 g and thereafter grew very little. Control animals gained weight steadily throughout the experiments. MAO activity was significantly lower in the hypothyroid group (Table 2).

Table 2. *Effect of 2-thiouracil on rat cardiac MAO activity. Animals were treated with 2-thiouracil for 6 weeks (0·2% in the diet). MAO activity was assayed in each heart for both substrates. All values are expressed as mean ± s.e., n = 20 for both groups assayed in quadruplicate.*

Group	Mean body weight (g)	Mean heart weight (mg)	MAO activity [n mol (mg protein)$^{-1}$ h^{-1}] Tyramine	Benzylamine
Hypothyroid	97 ± 3	315 ± 7	165 ± 10**	10·3 ± 0·4*
Control	207 ± 5	576 ± 13	233 ± 20	12·5 ± 0·7

* $P < 0.05$ (19% decrease compared with control); ** $P < 0.01$ (29% decrease compared with control).

Effect of thyroid hormone on the half-life of cardiac MAO

The recovery of enzyme activity after inhibition with pargyline was investigated in both hyperthyroid and hypothyroid rats. Pargyline produces an irreversible inhibition of MAO (Oreland, Kinemuchi & Yoo, 1973). *In vivo* recovery of enzyme activity after inhibition with pargyline is believed to be due to *de novo* synthesis of enzyme protein. The kinetics of this process have been shown by others to be consistent with a zero-order rate of synthesis and a first-order rate of degradation (Goridis & Neff, 1971), since the semi-log plot of enzyme activity against time after inhibition gives the predicted straight line. The slope of this line is dependent upon the first-order degradation rate constant and is not related to the rate of synthesis.

At the end of 16 days treatment with thyroid hormone or saline, half of the hormone treated and half of the control animals received a single subcutaneous injection of pargyline hydrochloride (25 mg kg^{-1}). The other half of each group received a corresponding saline injection. The four groups of animals thus produced were:

a. Thyroid hormone treated + pargyline;
b. Thyroid hormone treated + saline;
c. Control + pargyline;
d. Control + saline.

A similar experimental design was used for hypothyroid animals. In this case the pargyline injection was given after 6 weeks of treatment with 2-thiouracil.

Hormone or thiouracil treatment, where appropriate, continued throughout the whole course of the experiment. Three animals were killed from each of the four groups at various time intervals after the injection of pargyline. The mean value for the MAO activity from groups b, and d, was used as the steady state level for hormone treated and control animals respectively. The mean activity of MAO in the pargyline-treated animals at each time interval, was expressed as a percentage of the corresponding steady-state level. Percentage inhibition of MAO was plotted on a log scale against time after pargyline injection. The curve for the recovery of the MAO activity was fitted to the data by the method of "least squares".

Fig. 1 shows the recovery of MAO activity in hyperthyroid rat hearts, and in their corresponding controls. Fig. 2 shows the results obtained with hypothyroid rats. Fig. 3 illustrates the results obtained when another group of hypothyroid animals were used, which were older at the start of the thiouracil treatment, when they weighed 80–100 g. The final retardation of growth was less than that seen in the younger animals.

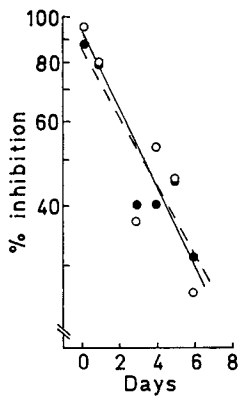

FIG. 1

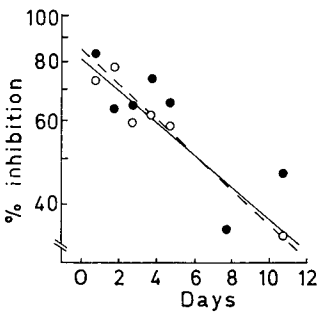

FIG. 2

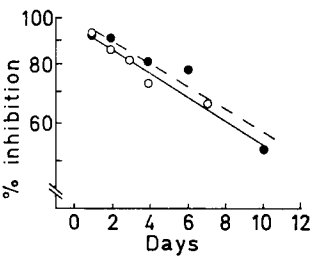

FIG. 3

FIG. 1. The recovery of cardiac MAO activity after irreversible inhibition *in vivo* with pargyline hydrochloride (25 mg kg^{-1} s.c.) in control and hyperthyroid rats aged approximately 6 weeks at the beginning of the experiment. The MAO activity is expressed as percentage inhibition on a log scale compared with the steady-state value and is plotted against the time in days after injection of pargyline. Tyramine was used as substrate. ○——○, controls; ●— —●, hyperthyroid. Each point represents the mean of 4 assays on each of 3 animals.

FIG. 2. The recovery of cardiac MAO activity after irreversible inhibition *in vivo* with pargyline hydrochloride (25 mg kg^{-1} s.c.) in control and hypothyroid rats aged approximately 10 weeks at the beginning of the experiment. The MAO activity is expressed as percentage inhibition on a log scale compared with the steady-state value and is plotted against the time in days after injection of pargyline. Tyramine was used as substrate. ○——○, controls; ●— —●, hypothyroid. Each point represents the mean of 4 assays on each of 3 animals.

FIG. 3. The recovery of cardiac MAO activity after irreversible inhibition *in vivo* with pargyline hydrochloride (25 mg kg^{-1} s.c.) in control and hypothyroid rats aged approximately 13 weeks at the beginning of the experiment. The MAO activity is expressed as percentage inhibition on a log scale compared with the steady-state value and is plotted against the time in days after injection of pargyline. Tyramine was used as substrate. ○——○, controls; ●— —●, hypothyroid. Each point represents the mean of 4 assays on each of 3 animals.

No difference was found between recovery rates for treated animals and their controls in each experiment. This suggests that neither thyroid treatment nor thiouracil treatment, although producing changes in MAO activity, affects the rate constant for the degradation of the enzyme.

Callingham & Della Corte (1972) showed that the degradation rate constant of rat cardiac MAO decreased as the animal grew older (i.e. the half-life of the enzyme increased). This effect was also seen here. Approximate ages of the animals in Figs 1, 2 and 3 were 6 weeks, 10 weeks and 13 weeks respectively. Table 3 summarizes the data for the half-lives of the MAO in the hearts of the animals used in these experiments.

Table 3. *MAO half-life in control, hyperthyroid and hypothyroid rats.*

Data from:	Group	Mean body weight (g)	Mean heart weight (mg)	MAO half-life (days)
Fig. 1	Control	133	445	3·7
	Hyperthyroid	114	592	4·2
Fig. 2	Control	208	580	8·8
	Hypothyroid	96	313	8·4
Fig. 3	Control	242	636	12·0
	Hypothyroid	176	446	12·3

The nature of the increased cardiac MAO activity in hyperthyroid rats

Heat treatment. Samples from control and hyperthyroid rat heart homogenates were subjected to a temperature of 55° in a water bath for various time periods. They were then assayed for MAO activity using both tyramine and benzylamine as substrates (Fig. 4). There was no difference in the pattern of inactivation between control and hyperthyroid heart homogenates for either substrate. However, benzylamine oxidation appeared to be more resistant to heat than tyramine oxidation.

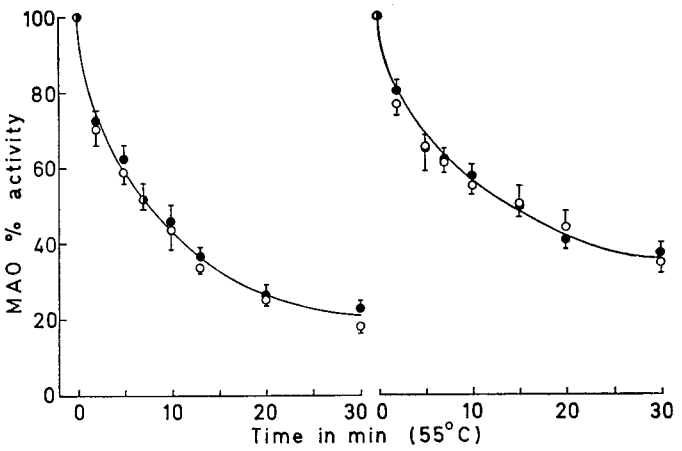

FIG. 4. The effect of prior incubation at 55° on *in vitro* activity of MAO in homogenates of hearts from control and hyperthyroid rats. Left hand curve, tyramine as substrate; right hand curve, benzylamine. Activities are expressed as percentages of the activity of the enzyme without prior incubation (± standard error of the ratio, n = 4 for each group, assays performed in duplicate). ○——○, controls; ●——●, hyperthyroid.

Effects of pargyline and clorgyline. MAO activity in control and hyperthyroid heart homogenates was assayed in the presence of the inhibitors pargyline and clorgyline. Final pargyline concentrations in the incubation mixture ranged from 5×10^{-8} to 5×10^{-4}M, and final clorgyline concentrations were from 5×10^{-11} to 5×10^{-4}M. Benzylamine and tyramine were used as substrates.

No difference was observed between inhibition patterns of tyramine oxidation for both control or hyperthyroid rat hearts, whether pargyline or clorgyline was used

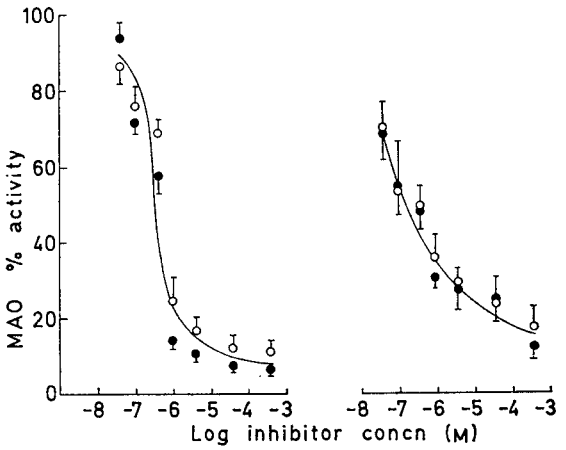

FIG. 5. The effect of the irreversible inhibitor pargyline upon the *in vitro* activity of MAO in homogenates of hearts from control and hyperthyroid rats. Left hand curve, tyramine as substrate (n = 4, for each group, assays performed in duplicate); right hand curve, benzylamine as substrate (n = 6, for each group, assays performed in duplicate). Activities are expressed as percentages of the activity of untreated homogenates (± standard error of the ratio). ○——○, controls; ●- -—●, hyperthyroid.

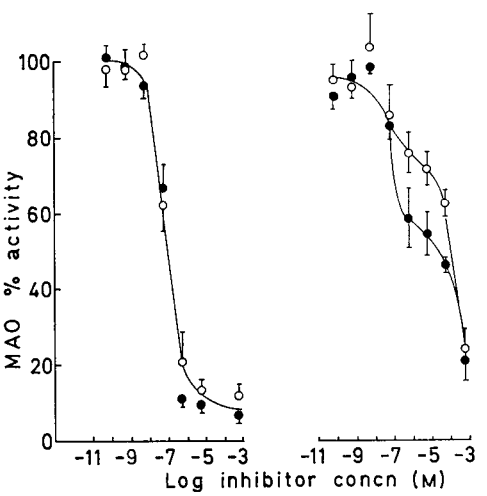

FIG. 6. The effect of the irreversible inhibitor clorgyline upon the *in vitro* activity of MAO in homogenates of hearts from control and hyperthyroid rats. Left hand curve, tyramine as substrate (n = 4, for each group, assays performed in duplicate); right hand curve, benzylamine as substrate (n = 6 for each group, assays performed in duplicate). Activities are expressed as percentages of the activity of untreated homogenates (± standard error of the ratio). ○——○, controls; ●——●, hyperthyroid. With benzylamine as substrate the plateau regions of the curves (extending between clorgyline concentrations of 5×10^{-7} and 5×10^{-5}M) are significantly different ($P < 0.05$).

as the inhibitor. Similarly, the inhibition of benzylamine oxidation by pargyline was the same for both groups of animals. There was however, a significantly greater inhibition of hyperthyroid cardiac MAO in the clorgyline concentration range 5×10^{-7} to 5×10^{-5}M, using benzylamine as substrate than was found in the hearts of control animals. These results are shown in Figs 5 and 6.

It should be noted, however, that differences do exist between the inhibition patterns for the two substrates used. Tyramine oxidation was more resistant to the lower pargyline concentrations, while with clorgyline, a single sigmoid curve was seen for tyramine oxidation and a double sigmoid curve, with a plateau, for benzylamine oxidation. The position of this plateau differed between the groups of animals. For example, Fig. 7 illustrates the effects of age. The plateau for the hearts of normal rats weighing 414 g was significantly lower than that for rats weighing 151 g ($P < 0{\cdot}005$).

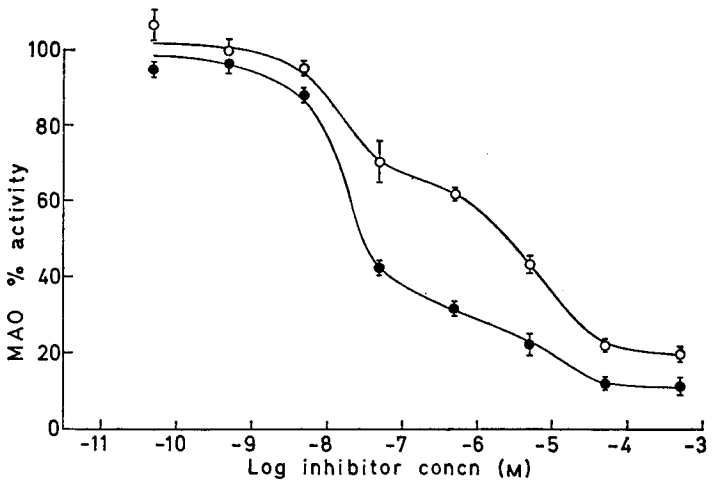

FIG. 7. The effect of clorgyline upon the *in vitro* activity of MAO in homogenates of hearts from young (151 g body weight, n = 3, assays performed in duplicate), and adult rats (414 g body weight, n = 3, assays performed in duplicate). Benzylamine was used as substrate. Activities are expressed as percentages of the activity of untreated homogenates (± standard error of the ratio). ○——○, young rats; ●——●, adult rats. The plateau regions of the curves extending between clorgyline concentrations of 5×10^{-7} and 5×10^{-5}M are significantly different ($P < 0{\cdot}005$).

The possibility that the plateau in the clorgyline inhibition curve was due to contamination with blood was excluded. There was no detectable MAO activity using tyramine as substrate in red cells, plasma or buffy coat. There was a barely detectable activity towards benzylamine in the buffy coat but none in red cells or plasma.

Michaelis constants for cardiac MAO. K_m values for cardiac MAO were determined in heart homogenates from control and hyperthyroid rats. Samples from each homogenate were assayed with final substrate concentrations (for benzylamine and tyramine) of 62·5, 125, 250 and 500 μM, at incubation times of 5, 10, 20 and 30 min (for tyramine), and 10, 20, 40 and 60 min (for benzylamine). Initial reaction velocities were calculated by computer program from the reaction progress curves obtained. K_m values were found by computer program designed to produce the line of best fit to a Lineweaver-Burk plot. The K_m values determined in this way showed no significant difference between control and hyperthyroid animals for either substrate. (Tyramine $\times 10^4$: control, 1·14 ± 0·07, hyperthyroid, 0·97 ± 0·13; benzylamine ×

10^5: control 3·12 ± 0·79, hyperthyroid 4·35 ± 1·04. Means ± s.e. Initial velocities were calculated from progress curves derived from duplicate assays on groups of 4 rats.)

K_m values for tyramine oxidation were also determined in the presence of two concentrations of non-radioactive benzylamine (0·1 and 0·2 mM, final concentrations). Table 4 shows the K_m and V_{max} values obtained. The effect of the second substrate upon the oxidation of tyramine was to alter significantly the K_m, without influencing the V_{max}. This situation would indicate competition between the two substrates for the enzyme.

Table 4. *Effect of benzylamine on the kinetic constants of tyramine oxidation by MAO in control and hyperthyroid rat hearts. n = 4 for controls and 3 for hyperthyroid rats, all assays performed in duplicate. Non-radioactive benzylamine was added to the tyramine-MAO reaction mixture to give the final concentrations below. In all other respects the reaction was carried out as described in the text.*

Constant	Group	Benzylamine (final concentration) 0	0·1mM	0·2mM
K_m (× 10^4)	Control	0·80 ± 0·08	2·39 ± 0·17	3·24 ± 0·57
	Hyperthyroid	1·06 ± 0·20	2·12 ± 0·26	3·05 ± 0·57
V_{max} [n mol (mg prot.)$^{-1}$ h^{-1}]	Control	125 ± 26	128 ± 24	129 ± 33
	Hyperthyroid	259 ± 29	228 ± 17	231 ± 15

DISCUSSION

These results confirm that thyroid hormones may affect the concentration of MAO in the heart of the young male rat. Excess thyroxine causes an increase in the specific activity of MAO, and hypothyroidism, induced by the antithyroid drug, 2-thiouracil, causes a reduction, compared with control animals of the same age. MAO exists both extraneuronally and intraneuronally; in the rat vas deferens, for example, roughly 50% is found within the nerves (Jarrott & Iversen, 1971). However, in the rat heart, by far the major proportion of MAO activity is extraneuronal, since treatment of rats with 6-hydroxydopamine fails to reduce the activity of the MAO in the heart (Horita & Lowe, 1972). It has not been established, at present, whether the effects of thyroid hormone reported here, represent a selective effect on either site of the enzyme. But if the effect were mediated on the intraneuronal site, the change would need to be very great indeed.

The present results confirm those obtained by Ho-van-Hap & others (1967), who used young male rats weighing 100 g at the beginning of their experiments. These authors also treated male rats weighing 400 g with thyroid hormone and found, in contrast, a decrease in cardiac MAO activity. At the present time there appears to be no explanation for this difference in response. Possibly a male rat of over 400 g is past the prime of life and the fall in MAO may represent stimulation of the degrading enzymes rather than of the synthetic enzymes for MAO. A similar situation may result from adrenalectomy in the adult male rat. Here the consequent rise in cardiac MAO activity has almost disappeared in animals weighing 260 g, while in animals weighing about 100 g the MAO activity doubled (L. Della Corte; personal com-

munication). It is probable that it is the age of the animal rather than its weight that is the controlling factor in these responses.

The specific activity of rat cardiac MAO increases with the age of the animal (Horita, 1967). It has been shown that this process can be explained by a decreasing degradation rate constant of the enzyme as the animal grows (Callingham & Della Corte, 1972), and this effect has been observed in the present experiments. The effects of thyroid treatment seemed, at a superficial level, to be similar to those of ageing, that is, an increase in heart weight together with an increased MAO activity. However, these results indicate that after *in vivo* inhibition of the enzyme with pargyline, the rate of recovery of enzyme activity is not affected by the thyroid status of the animal, and hence, the degradation rate constant is unchanged. This suggests that thyroid hormones may alter MAO activity in the rat heart by affecting the rate of synthesis of the enzyme. Similar conclusions have been drawn in studies of the influence of thyroid hormone on rat salivary gland MAO (Goridis & Neff, 1973). This effect in the heart may be due to a selective increase in the synthesis of MAO protein, which probably takes place on the ribosome, the formed enzyme then being transported to the mitochondrion where it is bound (Erwin & Simon, 1969). The site of the binding is on the mitochondrial outer membrane (Schnaitman, Erwin & Greenawalt, 1967). Thus it may also be necessary for there to be an increased growth of the mitochondrial outer membrane to accommodate this newly synthesized MAO, unless sufficient binding sites are always available. An alternative explanation may be that increased synthesis of the outer membrane or some change in its conformation may expose previously hidden enzyme active centres.

MAO is a flavoprotein enzyme (Youdim & Sourkes, 1972). It is also a possibility that the thyroid hormones produce their effects on MAO by altering cofactor metabolism. Rivlin & Langdon (1966) have shown that the activity of flavokinase, an enzyme involved in converting riboflavine to flavine mono-nucleotide, is reduced in the hypothyroid rat liver, as, in addition, are several other flavoprotein enzymes, such as mitochondrial α-glycerophosphate dehydrogenase and NADPH-cytochrome C reductase. Thus it is possible that flavoprotein enzyme activities may be regulated by the amount of cofactor available for incorporation into the apoenzyme.

Other methods for producing cardiac hypertrophy have different effects on MAO. Hypertrophy resulting from experimental hypertension (induced either by desoxycorticosterone acetate and sodium loading, or by renal artery stenosis) is accompanied by an increase in the specific activity of MAO (de Champlain, Krakoff & Axelrod, 1968). However, hypertrophy induced by isoprenaline treatment of rats produces no significant change (Mueller, de Champlain & Axelrod, 1968). Recovery of enzyme activity after *in vivo* inhibition with pargyline has only been studied in the latter case, and no difference in recovery rate was observed between control and treated animals (Planz, Quiring & Palm, 1972).

The experiments on heat sensitivity, inhibitor sensitivity and determination of K_m values, suggest that whatever the mechanism responsible for increasing the MAO activity, the properties of the enzyme are largely unchanged. The only significant difference occurred with the biphasic inhibition curve, obtained with clorgyline as inhibitor and benzylamine as substrate. Previous workers have suggested that a biphasic curve of this type is indicative of two forms of the enzyme, with different sensitivities to the inhibitor clorgyline (Johnston, 1968). The more sensitive form has been called "enzyme A" and the less sensitive form "enzyme B". In these

experiments, the increase in the oxidation of benzylamine brought about either by increasing age or by hyperthyroidism, appears to be predominantly mediated by a change in enzyme A, while tyramine oxidation is exclusively by this enzyme. This could explain the greater percentage changes in cardiac MAO activity seen with tyramine as substrate than with benzylamine following thyroid treatment.

The heat sensitivity and inhibitor sensitivity experiments revealed marked differences between the effects of these treatments on tyramine oxidation and benzylamine oxidation. These studies have been cited in the past as evidence of multiple forms of MAO (Squires, 1972; Sandler & Youdim, 1972). Tipton, Houslay & Garrett (1973) have produced evidence that these effects may result from interactions between the membrane lipid environment and substrate binding to the active site of the enzyme. The results reported here, using mixed substrates, suggest that despite behaving differently to heating and inhibitors, the same active site may be involved in the oxidation of all of the tyramine and some if not all of the benzylamine.

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