

THE INFLUENCE OF THYROXINE AND BARBITURATES ON  
MONOAMINE OXIDASE ACTIVITY

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THESIS

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## INTRODUCTION

When this work was started it was known that monoamine oxidase (MAO) activity decreases in the liver of hyperthyroid rats and increases in the hypertrophic heart of the same type of animals. This enzyme was reported to be localized mainly in the mitochondria and to a lesser extent also in microsomal particles. It was decided to investigate the possible influence of thyroid hormones on the subcellular distribution of MAO activity.

During preliminary experiments, in order to ascertain the hyperthyroid state of iodinated casein-fed rats their oxygen consumption was measured. To perform this measurement sodium pentobarbital was given to the animals. When twenty minutes later the thyrotoxic animals were sacrificed, the monoamine oxidase activity in the heart was extremely low in contradiction with the findings of other investigators. When the hyperthyroid rats were sacrificed without barbiturate pretreatment, the heart MAO activity was increased in agreement with previous reports.

Experiments were designed to investigate the subcellular distribution of MAO in normal and hyperthyroid rats without barbiturate pretreatment. The subcellular distribution of MAO in various tissues of pentobarbital-treated

rats was also investigated. In addition, the effect of several barbiturates on MAO activity was studied in some detail.

The results of these experiments and the conclusions in the light of the available literature are reported in this thesis.

REVIEW OF LITERATURE

PART I. THYROID HORMONE-CATECHOLAMINE INTERRELATIONSHIPS

The similarity between adrenergic activity and the symptoms of hyperthyroidism has been observed for many years. By 1865 the opinion that the cause of the disease was an overactivity of the cervical sympathetic nerves, represented a respected theory (1). In 1898, von Cyon noted that the pressor response and pulse rate increase caused by electrical stimulation of the central end of the transected depressor nerve, were blunted either by feeding iodides or by thyroidectomy (1). In 1908, Kraus and Friedenthal observed a parallelism between the effects of adrenaline and hyperthyroidism, and Eppinger, Falta and Rudinger demonstrated that the glycosuric action of adrenaline was dependent on the presence of the thyroid gland (1). In 1913, Cramer and Krause (2) noted a total disappearance of liver glycogen in cats and rats, when thyroid extract was added to the diet. In 1916, Richardson reported (3) that in hearts perfused with adrenaline the inotropic and chronotropic actions of this amine were increased after the administration of thyroid gland extract to the animals.

Following these early investigations, a large amount of controversial work has been devoted to uncover relationships between the adrenal medulla hormones and the thyroid gland. It is the purpose of this part of the review to consider separately each catecholamine-like effect of the thyroid hormones, and to present the theories implicating catecholamines in the mechanism of action of thyroid hormones.

A) THE CALORIGENIC ACTION OF THYROID HORMONES:

The close association between the oxygen consumption (i.e. basal metabolic rate, BMR) of animal species and their thyroid function, is perhaps the best known of the multiple biological actions of thyroid hormones. This property of thyroid hormones is also termed calorogenic action. Although a few authors expressed the opinion that the functional state of the thyroid gland does not influence the BMR (4), this effect of thyroid hormones has been demonstrated too often to be reasonably doubted.

The relationship between BMR and thyroid hormones is quantitative if the administration of excessive amounts of the hormones is avoided (5,6). Some interval of time elapses between the administration of the hormones and the response of the subjects (72). The overall BMR results from a complex balance between various metabolic

processes. Other hormones and many drugs (e.g. DNP, salicylates) as well as environmental and dietary factors, also affect this balance.

Adrenaline is the best example of another hormone with calorogenic activity. In all species which have been studied, it has been observed that the intravenous injection of adrenaline or noradrenaline is followed by an increase of the oxygen consumption. The subcutaneous injection has the same effect but a delay in the response is observed. The slow intravenous infusion of either amine also increases BMR (7,8).

There is a general agreement that in hyperthyroidism the increase in BMR is higher after the administration of catecholamines. The subcutaneous injection of adrenaline into thyroid-fed rats has been shown to elevate the oxygen consumption of the animals (9). The increase was reported to be higher than the elevation of oxygen consumption observed after the injection of adrenaline into normal rats (9). Similar observations were reported in thyrotoxic patients or in triiodothyronine-treated subjects (10).

In 1942, Ring studied the oxygen consumption of normal and thyroxine-treated rats after a single injection of adrenaline (11). Animals kept one week on thyroxine

exhibited the same increase in oxygen consumption, after the administration of adrenaline, as the control rats. Treatment with thyroxine for a period of four weeks resulted in 54.3% increase in the BMR. When thyroxine was given for the same period of time previous to adrenaline administration, the observed BMR increase was 94.9%. Swanson observed (12) in thyroidectomized rats a slight decrease in their oxygen consumption after the injection of adrenaline. When the thyroidectomized animals were treated with thyroid hormone, the effect of 40  $\mu$ g of adrenaline in increasing the oxygen consumption was obvious.

Noradrenaline has also been found to increase the oxygen consumption of rats but to a lesser extent than adrenaline. However in thyroidectomized animals responses to both amines are similar (13).

In contradiction, there are several reports showing that in rats adrenalectomy does not modify the calorogenic effect of thyroxine (14, 15). Ganju and Lockett have reported (16) that doses of thyroxine which were twice the amount required to increase the oxygen consumption in normal mice, failed to do so in adrenalectomized and in thyroidectomized-adrenalectomized animals.

Nevertheless there is general evidence that normal function of the thyroid gland is required in order to

elicit the calorogenic effect of catecholamines.

B) THE METABOLIC EFFECTS OF THYROID HORMONES:

Since the beginning of the century, changes in almost every major body constituent have been attributed to the absence or excess of thyroid hormones.

Some metabolic actions of thyroxine can also be produced or modified by catecholamines:

1.- Glycogenolytic action

It is generally agreed that thyroid hormones have an overall glycogenolytic effect (17, 18).

The glycogenolytic and hyperglycemic actions of adrenaline and noradrenaline are well established. It is also known that noradrenaline is much less active in producing this effect (19). After the injection of adrenaline, a simultaneous increase in blood lactate has been noted (20). Some authors have attempted to relate this lactate increase to the calorogenic effect of adrenaline (21).

Extensive investigations have been conducted on the effect of thyroid hormones on adrenaline induced hyperglycemia. Collip observed a 50% decrease in the liver glycogen of normal rats following the administration of adrenaline, whereas little reduction occurred in thyroidectomized animals (22). Houssay reported that

hyperthyroidism increases the severity of diabetes mellitus in dog and man (23). On the other hand, in thyroid-fed rabbits previously thyroidectomized, adrenaline caused a greater elevation in blood glucose than in normal animals (24). These results have been confirmed in thyroid-fed intact rabbits (25). Abbot and Vanbursbirk (26) could not find any difference between thyroid-fed and normal cats and dogs regarding their blood sugar response to adrenaline. However, in hypophysectomized rats pretreated with thyroxine, the glycogen level in striated muscle was decreased following the injection of adrenaline (27).

## 2.- Effect on the turnover of lipids

Disturbances in lipid metabolism have been associated with altered function of the thyroid gland for many years. In hypothyroidism there may be lipemia, hypercholesterolemia and fatty infiltration of the liver, while in hyperthyroidism there is a characteristic decrease in serum cholesterol (28). In thyrotoxicosis, the cholesterol biosynthesis studied by using several isotopic precursors has been shown to be enhanced (29), and most probably there is an even greater acceleration of the degradation of cholesterol by the liver (28). Hypothyroidism generally leads to a reduction of cholesterol biosynthesis (29). The synthesis

of fatty acids has been reported to increase in the liver of thyroid-fed rats and to decrease in thiouracil-fed animals (29).

On the other hand, the effects of adrenaline and noradrenaline on lipid metabolism are evident. The subject has been reviewed by Himms-Hagen and Hagen (19). The ability of adrenaline and noradrenaline to increase the hydrolysis of triglycerides in adipose tissue is well known. There is a subsequent increase in the release of free fatty acids. Both amines would achieve this effect by increasing the activity of a lipase. The infusion of noradrenaline can cause the deposition of lipids in cardiac and skeletal muscles. Following the administration of adrenaline or noradrenaline there is an increased uptake of free fatty acids in the liver, and during a prolonged noradrenaline infusion this may lead to the accumulation of triglycerides in the same organ. Finally, there is an increase of free fatty acids in blood after the injection of adrenaline or noradrenaline, and most probably this effect is the cause of the increased uptake of free fatty acids by liver following the administration of catecholamines. In addition, elevated plasma concentrations of phospholipids, cholesterol, high density lipoproteins and low density lipoproteins

have also been observed after a single injection of depot adrenaline.

The influence of thyroid hormones on the lipolytic action of catecholamines has been studied by several investigators. Hypophysectomy in monkeys abolished the response to adrenaline of plasma free fatty acids. The fatty acid levels were partially restored by giving TSH, and completely restored by triiodothyronine administration (30). Harlan et al (31) observed an increased lipolytic response to small doses of infused adrenaline and nor-adrenaline in hyperthyroid subjects. The lipolytic response to catecholamines in hypothyroidism was variable but in general reduced (31, 32). The free fatty acid content and free fatty acid release from adipose tissue were greater in hyperthyroid than in normal rats and were increased further by adrenaline administration (33, 34, 35).

Thus catecholamines may be necessary to mobilize depot fat, and thyroid hormones may potentiate this effect.

### 3.- Effect on protein synthesis

Tipton and Nixon have found that succinoxidase and cytochrome oxidase are elevated in the liver of hyperthyroid rats (36). Lardy and coworkers have reported a twenty-fold increase in the specific activity of glycerol-1-phosphate dehydrogenase in liver mitochondria after

feeding thyroid powder to the animals during 20 days (37). This enzymatic increase was abolished by ethionine (37).

Sokoloff and Kaufman (38, 39) obtained direct evidence for an effect of thyroid hormones on protein synthesis. In their experiments they used separated or recombined mitochondrial, microsomal and soluble fractions obtained from the liver of normal, hypothyroid or hyperthyroid rats. They found that when reconstituted homogenates were incubated in the appropriate medium in the presence of leucine- $^{14}\text{C}$  or valine- $^{14}\text{C}$ , the incorporation of label into a trichloroacetic acid-insoluble fraction was higher in the hyperthyroid homogenate and lower in the hypothyroid homogenate than in the same preparation from normal animals. The effect was observed after using either L-thyroxin or L-triiodothyronine to produce the hyperthyroid state in the animals. By reconstituting homogenates using fractions from normal rats mixed with fractions from treated animals, it was possible to localize this effect of thyroid hormones in the mitochondrial fraction. That is, a preparation of mitochondria from hyperthyroid rats showed elevated incorporation of the label, regardless of the source of the microsomal and soluble fractions. Nevertheless some radioactivity was also recovered in the

microsomal protein. Further studies with this system have indicated that the stimulated step is the transfer of s-RNA-bound aminoacid to proteins.

In addition, Tata et al (40, 41) have reported an increased incorporation of leucine and isoleucine into the microsomal fraction obtained from the liver and muscle of triiodothyronine-treated rats, in the absence of mitochondria. It has also been reported by the same group (43) that there is stimulation of a DNA-dependent RNA polymerase 8 hours after the administration of triiodothyronine to hypothyroid rats, and that this effect is well within the latent period for the observation of increased protein synthesis in microsomes.

Finally, Lee and coworkers (42) have observed stimulation in rats of the incorporation of thymidine methyl-<sup>3</sup>H into hepatic DNA following a single dose of triiodothyronine. The enhanced DNA synthesis reached its peak 24-48 hours after the administration of the hormone and it was sensitive to the inhibitory effects of actinomycin D and cycloheximide. They interpreted their results as an indication that the early effects of thyroid hormones on liver protein and RNA synthesis initiate DNA synthesis which in turn may lead to the increased proliferation of hepatic tissue.

The effect of catecholamines on protein synthesis has been studied to a lesser extent. Adrenaline seems to decrease protein synthesis in a variety of isolated tissues and in the intact animal. Adrenaline decreases the aminoacid levels in blood (19). The glycogenolytic action of adrenaline may in part be responsible for this reduction in the aminoacid concentration in blood (19).

C) CARDIOVASCULAR EFFECTS:

At small doses, adrenaline has a positive chronotropic and inotropic effect on the heart (44, 45). Noradrenaline does not appear to affect the heart at small doses (46). Noradrenaline has a constrictor effect on peripheral blood vessels (47). Adrenaline action varies with the organ studied. There is a marked constriction of the skin blood vessels following adrenaline injection (48), and the vessels of skeletal muscle seem to be dilated after the same treatment (49). Portal vein appears constricted following adrenaline administration, and the effect of this amine on hepatic vein is not elucidated (50, 51).

On the other hand, cardiac function is often a major consideration in clinical thyrotoxicosis. Tachycardia is a dependable diagnostic sign of clinical or experimental hyperthyroidism. Nevertheless a clear assessment of the heart sensitivity to thyroid hormones is complicated

by the action of adrenaline and the apparent synergism between both types of hormones.

The cardiovascular system has provided a convenient tool for the investigations on catecholamine-thyroid interrelations. A more prominent tachycardia has been reported in hyperthyroid dogs after giving adrenaline (52). The heart rate, cardiac output and right ventricular stroke were enhanced in thyroid-fed dogs following adrenaline or noradrenaline administration (53). When rats were injected for 2-10 weeks with increasing doses of thyroxin (0.037 to 5.88 mg) the percent increase in the cardiac output, oxygen consumption and heart weight of these animals rose in proportion with the given dose of hormone (54). In fact, this influence of hyperthyroidism on the cardiac response to adrenaline has been reported too often to be reasonably doubted (55, 56, 57, 58). However, there are several conflicting reports in the literature (59, 60).

Thyroid-catecholamine interactions are more difficult to ascertain by experimentation with hypothyroid animals. For example, after a single injection of adrenaline the response of the heart in thyroidectomized rabbits was similar to that of the heart in normal rabbits (59). In <sup>131</sup>I-treated dogs, a decrease in the blood pressure response

was found following adrenaline or noradrenaline administration (61).

Another typical manifestation of hyperthyroidism is the cardiac hypertrophy. There is experimental evidence indicating the occurrence of cardiac hypertrophy after the administration of thyroxine (62) and triiodothyronine (63). In 1962, Beznak has demonstrated (54) that the cardiac hypertrophy produced by thyroxine administration to the rat was associated with increased ventricular strength, while the hypertrophy resulting after experimental constriction of the aorta in the same type of animals was associated with a decrease in the maximum cardiac work.

Inchiosa and Freedberg (64), using rabbits which were given daily doses of thyroxine (190  $\mu\text{g}/\text{kg}$ ) during 14 days, have reported a marked cardiac hypertrophy (4.03 g of heart weight as compared to 3.10 g in the control animals). They also found that the combined ventricular weight was increased in the treated rabbits (35%).

Later on Beznak (65) has demonstrated that in hypophysectomized rats, the constriction of the aorta causes heart hypertrophy only if the animals are treated with thyroxine and growth hormone. When the same author

tried to produce the cardiac hypertrophy by means of left nephrectomy, the effect was also elicited only after the administration of the mentioned hormones (66). In the same report, thyroxine alone prevented the occurrence of cardiac atrophy, while growth hormone alone did not. Thus, it seems that both hormones must be present for the development of cardiac hypertrophy in the mentioned experimental conditions.

Recently, Minelli and Korecky (67) have studied the effects of thyroxine and growth hormone on the isometric contractile mechanics of cardiac papillary muscles, using normal and hypophysectomized rats. The maximum developed tension of the papillary muscles was decreased following hypophysectomy, was not changed after growth hormone administration, and was reversed after treatment with thyroxine. In 1968, Buccino et al (68) compared the mechanical properties of cardiac papillary muscles which were isolated from euthyroid, hyperthyroid and hypothyroid cats. Changes in thyroid activity profoundly affected the intrinsic contractile state of cardiac muscle by increasing its maximum velocity of shortening in hyperthyroid animals and by decreasing the same property in hypothyroid cats. In these experiments muscle from hyperthyroid animals were not supersensitive

to exogenous noradrenaline and the increased speed of shortening those muscles was independent of endogenous noradrenaline stores.

The significance of cardiac hypertrophy in human hyperthyroidism is uncertain. Post mortem studies have disclosed a high incidence of such hypertrophy (50% of the autopsies performed in cases of thyrotoxicosis). Nevertheless it is difficult to interpret these findings since hyperthyroidism is superimposed very often upon a different cardiac disease (69).

D) EFFECTS OF THYROID HORMONES AT THE MOLECULAR LEVEL. THEORIES IMPLICATING CATECHOLAMINES IN THE MECHANISM OF ACTION OF THYROXINE:

Explanations of how thyroid hormones act were offered even before their chemical identity was established. The number of explanations has rapidly increased as a result of the general progress in the whole field of biochemistry. Some of the most popular theories have been reviewed recently (70).

Oxidation of so many substrates was stimulated by thyroid hormones that the respiratory chain was suggested as a common denominator for all the effects of thyroxine. Intracellular concentrations of cytochrome oxidase respond to changes in the level of thyroid hormones (70). Rivlin

and Langdon (71) have proposed the regulation of hepatic levels of FAD by thyroid hormones, as an explanation for the increased activities of several flavoenzymes in hyperthyroidism. However, against the concept that thyroid hormones act on the respiratory chain is the long lag period in vivo before the response to thyroid hormones is elicited (72), and the reported direct stimulation of electron transport capacity without a concomitant change in BMR (73).

Another popular concept maintaining that thyroid hormones control cellular respiration by uncoupling of oxidative phosphorylation has been abandoned since too high concentrations of the hormones were required to produce this effect (72). For some authors defending this theory, the uncoupling of oxidative phosphorylation could be in relation with the ability of thyroxine to produce mitochondrial swelling (72).

The evidence in favor of a primary action of thyroid hormones on protein synthesis have been presented previously in this review (38, 39, 40, 41, 42, 43). This is one of the most promising theories at the present. However, Werner and Nauman (70) have considered the possibility that the effects of thyroid hormones on the transfer of acyl s-RNA to ribosomal protein, on the RNA polymerase

activity and on RNA synthesis, may reflect only one of the many cellular actions of these hormones.

Changes in the activities of many enzymes in the presence of thyroid hormones have been reported (74). Among others, mitochondrial  $\alpha$ -glycerophosphate dehydrogenase is of special interest. In rat liver, this enzyme has been shown to increase with the level of thyroid hormones (75), and it has been postulated that the elevation of this mitochondrial enzyme may increase the rate of operation of the  $\alpha$ -glycerophosphate cycle. This effect would produce an increase in the biosynthesis, mobilization and metabolism of lipids, a decrease in the NADH/NAD ratio, and a subsequent stimulation in the degradation of carbohydrates (76).

On the other hand, as we stated before one of the earliest observations in this field of research, was the parallelism between the effects of thyroid hormones and adrenaline. The following theories implicating catecholamines in the mechanism of action of thyroid hormones are worthy of consideration:

1.- The concept that the thyroid gland may exert its effects by increasing the secretion of catecholamines from the adrenal glands, was formulated in 1908 by Eppinger and coworkers (77) and subsequently received support by

the finding that the adrenals of cats contained larger amounts of adrenaline after the animals were fed thyroid extract (78). More recently, it was demonstrated, that when female mice were fed thyroid extract there was a decrease in the amount of chromaffin material of the adrenal medulla (79). When rats were given thyroxine there was a decrease of adrenaline and noradrenaline in the adrenal glands (80, 81). In another work, elevated amounts of catecholamines in the serum of thyroid-fed rats were reported (82). However, there are several contradictory reports (83, 84, 85). At the present time, it can be stated that there is no evidence to suggest a constant increase in circulating catecholamines in hyperthyroid animals.

## 2.- Thyroxine-catecholamine interactions in the sympathetic nervous system

The catecholamines have two major roles in the mammalian organism: as endocrine hormones secreted by the adrenal medulla, and as transmitter substances released at adrenergic synapses in the nervous system and in tissues with sympathetic innervation. There are several good reviews on the mechanism of action of these amines (86, 87).

The branched terminals of postganglionic sympathetic neurones contain varicosities which in turn have a large

number of small membrane-bound vesicles. These vesicles are thought to represent specialized storage structures in which noradrenaline is sequestered in some way. The presynaptic nerve terminal contains the mechanisms for the synthesis of the transmitter substance as well as for its storage. Part of the stored noradrenaline is released from the mentioned vesicles in response to nerve impulses reaching the nerve terminal according to the varying demand. The adrenergic synapse is a biochemical and physiological unit of considerable complexity. Integral part of the synapse is the area of the postsynaptic cell in apposition to the presynaptic terminal. The space between these two components of the synapse is known as the "synaptic cleft". The transmitter is released into the "synaptic cleft", and it is inactivated following its action upon the specific receptor sites which are located in the postsynaptic cell surface. The inactivation of noradrenaline at the adrenergic synapse appears to involve a reuptake of the released transmitter by the nerve terminals from which it was released.

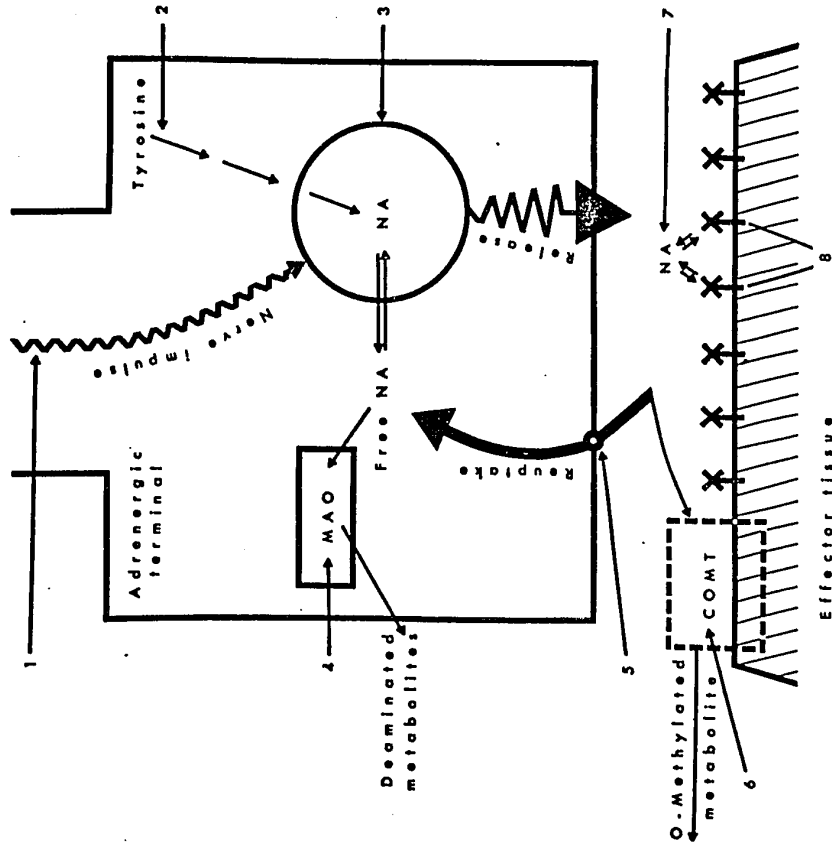
The integrated biochemical function of the adrenergic synapse can be experimentally altered at various levels by using several drugs, as can be seen in figure 1.

In 1948, Alquist (88) proposed the distinction between

Figure 1

Sites of drug action at the adrenergic synapse. Drugs may depress adrenergic transmission by (1) blocking the nerve impulse in the postganglionic sympathetic nerve (breytilium); (2) inhibiting transmitter biosynthesis ( $\alpha$ -methyl-p-tyrosine); (3) interfering with the transmitter storage mechanism (reserpine); (8) antagonizing the transmitter at postsynaptic receptor sites (phenoxybenzamine, propanolol). Drugs may enhance adrenergic transmission by (5) preventing the reuptake of the transmitter (cocaine); (4,6) preventing the metabolism of the transmitter (MAO inhibitors, COMT inhibitors); or (7) imitating the actions of the transmitter (isoprenaline, adrenaline).

(Modified from L.L. Iversen, ref.86)



two types of adrenergic receptors. The  $\alpha$ -receptors were defined as those with a greater response to noradrenaline and adrenaline than to isoproterenol and which mediate such effects as vasoconstriction and the inhibition of intestinal smooth muscle. The  $\beta$ -receptors showed a greater response to isoproterenol than to noradrenaline, mediating effects as vasodilatation, the inotropic and chronotropic responses in the myocardium and the relaxation of bronchial musculature. Later on, the decrease of cardiac glycogen and the release of free fatty acids from adipose tissue have also been considered as an index of  $\beta$ -adrenergic activity (89). The introduction of drugs which specifically block only one type of adrenergic receptors (e.g. ergotamine, phentolamine and phenoxybenzamine as  $\alpha$ -blocking agents, and pronethanol and propranolol as  $\beta$ -blocking compounds) has greatly strengthened this concept. Nevertheless, Robison et al have pointed out that the properties of  $\alpha$  and  $\beta$ -receptors are not always so clear and that very often those receptors behave in a different way in various species and tissues. The same group of investigators have considered the possibility that both  $\alpha$  and  $\beta$ -receptors may in reality be parts of an adenylyl cyclase system (90).

With the previous considerations in mind and regarding

the strong parallelism between the effects of catecholamines and thyroxine, it is easy to understand that the adrenergic system has been implied in the mechanism of action of thyroid hormones.

It was proposed 29 years ago by Aumann and Youmans (91) that probably the sensitivity of adrenergic receptors was increased by thyroid hormones. This concept has been restated recently (92).

Gafney et al (93) studied the effect of guanethidine in four volunteers in which the usual symptoms of hyperthyroidism were induced by injections of triiodothyronine. Guanethidine was found to have beneficial effects on the increased BMR and heart rate but it had no effects on the low serum cholesterol and weight loss of hyperthyroidism. The influence of guanethidine in 27 hyperthyroid patients was tested by Lee et al (94). Responses were obtained six days after the patients were given daily doses of 80 mg of this drug. In this report, a significant improvement was noted in the heart rate, blood pressure, body weight, serum cholesterol, BMR and tremor. Nevertheless the PBI and  $^{131}\text{I}$  uptake by the thyroid gland remained elevated. Reserpine has been found to produce similar effects in hyperthyroid patients (95).

The most striking effects were produced in hyperthyroid

dogs when total sympathetic block was induced by injection of procaine into the epidural space (53). In these experiments, the heart rate of thyroid-fed animals declined to normal levels and the same type of effect was observed on the cardiac index (L/min), on the left and right ventricular stroke work and on the oxygen consumption.

However, when nethalide (a potent  $\beta$ -blocking agent) was administered to thyrotoxic patients the heart rate and BMR were reported to be still high (96).

Some investigators have proposed that thyroid hormones may only change the sensitivity of  $\beta$ -adrenergic receptors (92). More recently Bray (89) has reinvestigated this possibility using thyroidectomized and normal rats which were given injections of noradrenaline or isoproterenol to elicit  $\alpha$  or  $\beta$ -adrenergic responses. He found that the adrenergic effects were reverted by specific blocking agents (phentolamine or propanolol). He also observed that thyroidectomy had little effect on the adrenergic responses mediated by  $\alpha$ -receptors, while cardiac glycogenolysis and lipolysis became much less sensitive to the administration of isoproterenol following thyroidectomy. Bray has interpreted these results as an indication of a higher sensitivity of the  $\beta$ -receptors to certain amines in the presence of thyroid hormones.

However, most authors agree that thyroid activity, as reflected by PBI levels and by  $^{131}\text{I}$  uptake by the thyroid gland remains unchanged following the administration of reserpine,  $\alpha$ -methyldopa, guanethidine, pronethalol, propranolol and other similar drugs (98). Despite the amount of work done to clarify this theory, at the present time, an interaction between adrenergic receptors and thyroxine is not considered as the primary mechanism of action of thyroid hormones.

During recent years much attention has been directed to the concept that thyroxine may decrease the inactivation (i.e. reuptake) of catecholamines by occupying the binding sites in the synapse and thus leaving a larger fraction of free active amine in contact with the receptor sites (99). This hypothesis has been tested extensively:

The heart of hyperthyroid rats was able to bind and metabolize less adrenaline than the same organ of normal rats (99). The heart level of noradrenaline following the injection of the same hormone was reduced in hyperthyroid mice (100). The in vitro uptake of noradrenaline by heart slices prepared from hyperthyroid rats was smaller than the uptake of the same amine by heart slices from control animals (101). Atrial noradrenaline and adrenaline levels were decreased in hyperthyroid rabbits, and

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the ventricular noradrenaline was increased in hypothyroid animals (102). Keswani and D'Iorio have reported that the in vitro binding and uptake of  $^3\text{H}$ -dl-adrenaline by small intestine strips of rats, were increased in thyroxine depleted tissues and were decreased when thyroxine was present in the medium (103).

However, Landsberg and Axelrod (104) have demonstrated that the turnover of cardiac noradrenaline was increased in either hypophysectomized, thyroidectomized or adrenalectomized rats. Treatment of the hypophysectomized animals with thyroxine and/or ACTH restored the turnover to normal values, the restoration produced by thyroxine being quantitatively more significant. The ganglionic blockage with chlorisondamine also restored the turnover to normal values in hypophysectomized rats. These results suggest that thyroid deficiency and, to a lesser extent, adrenal deficiency are associated with an increase in the turnover of cardiac noradrenaline and that this effect is probably mediated by an increase in the sympathetic nervous activity. Since thyroxine administration reduced the turnover and probably also the synthesis of noradrenaline, and considering that monoiodothyronine as well as diiodothyronine are potent inhibitors of tyrosine hydroxylase, the authors concluded that thyroid hormones may

inhibit noradrenaline synthesis at the rate limiting step.

Moreover, Axelrod and Tomchick (105) did not find any difference in the rate of disappearance of radioactive adrenaline between thyroxin-treated and normal mice.

In consequence, the interaction between thyroxine and noradrenaline at the adrenergic binding sites is not a satisfactory explanation for a single primary mechanism of thyroid hormones action.

Another hypothesis is now under consideration (89, 104). The enzymatic systems involved in lipolysis in adipose tissue and glycogenolysis in heart muscle are thought to be activated by the formation of cyclic 3', 5'-adenosine monophosphate (cyclic AMP). It is known that adrenaline activates adenyl cyclase, the enzyme that catalyzes the synthesis of cyclic AMP from ATP (106). It is reported in the literature that thyroid hormones may be required for the normal function and activation of adenyl cyclase (107). In vitro activation of adenyl cyclase has been observed when heart homogenates of rats were incubated with thyroxine and triiodothyronine (108). Then thyroid hormones may act primarily at the adenyl cyclase level. If this hypothesis is confirmed the selective influence of thyroid hormones on the responses to catechol-

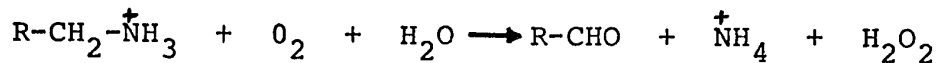
amines could be determined by those effects of noradrenaline which are mediated by cyclic AMP. This theory, however, is fairly new and it will have to stand the test of time.

3.- The effect of thyroid hormones on monoamine oxidase and catechol-O-methyl transferase

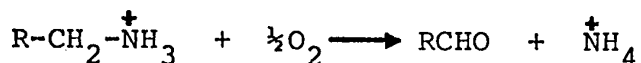
In another possibility, it has been suggested that thyroid hormones could act by inhibiting the enzymes which are involved in the degradation of catecholamines (i.e. monoamine oxidase and catechol-O-methyl transferase).

There are recent reviews on monoamine oxidase (MAO) (EC.1.4.3.4.) in the literature. This term is used to designate an enzyme, or group of enzymes, catalyzing the oxidative deamination of several primary and secondary alkyl-, aralkyl-, indolyl-, and imidazolyl-amines. MAO does not exhibit sensitivity neither to some carbonyl reagents like semicarbazide or to cyanide, while it is inhibited by octanol. These properties, together with characteristic patterns of substrate specificity and the sensitivity of this enzyme to "MAO inhibitors", enable one to distinguish MAO from diamine oxidases which are inhibited by carbonyl reagents but not by octanol.

The reaction catalyzed by this enzyme is thought to proceed according with the following general formula:



Since  $\text{H}_2\text{O}_2$  is decomposed (e.g. by catalase) to  $\text{H}_2\text{O}$  and  $\frac{1}{2}\text{O}_2$ , the following overall reaction results:



The broad substrate specificity of MAO is well known. Some of the currently most used substrates are benzylamine, tyramine, tryptamine, serotonin, adrenaline, noradrenaline and kynuramine.

MAO is widely distributed in mammalian tissues. The liver, brain and heart are good sources of the enzyme. There are quantitative differences of enzymatic activity in different species. It has been reported that MAO activity is very low in the heart of young rats while the same enzymatic activity is much higher in the same tissue of adult animals (111). Furthermore, there are sex differences, the enzyme levels in liver and heart being lower in female rats than in male animals with the same age (111).

In the cell, MAO is a membrane bound enzyme. Investigations of its intracellular distribution in rat liver have showed that mitochondria contain from 70% (112) to 76,7% (120) of the total MAO in the homogenate, whereas the remainder is mainly localized in the microsomal fraction. In brain MAO has been found to be predominantly

bound to the mitochondria (189). Recently, de Champlain et al have reinvestigated the subcellular localization of MAO in various tissues of adult rats (121). Using differential centrifugation for the separation of the cellular fractions, they found that in the liver 80% of the enzyme is located in the mitochondria and 18% of MAO is localized in the microsomal fraction. However, in the heart 50% of MAO activity was associated with the microsomal fraction and 20% of the enzyme was found in the supernatant. In the salivary glands equal amounts of the enzyme were found in both subcellular fractions. In the same publication, the mentioned results were confirmed separating the subcellular components by centrifugation in a continuous gradient of sucrose (0,25 M to 1.73 M). After spinning at 105,000 x g for one hour the 4.5 ml gradient was collected in 30 separate fractions from the bottom of the tube. In the case of liver, maximal MAO and cytochrome oxidase activities were found in the fraction number five. Following the centrifugation of the particulate fraction from the heart through the gradient, very high MAO activity was found in the 22nd fraction (microsomes) whereas only a small peak of enzymatic activity was found in the 5th fraction where the cytochrome oxidase activity was maximum.

MAO is an enzyme resisting solubilization, and it has been purified to some extent from various tissues and species (113, 114, 115). It is reported that FAD may be a coenzyme for MAO (114, 115). Tipton (115) has been able to isolate a fluorescent material from his purified brain MAO and to identify it with FAD. Furthermore, he reactivated the apoenzyme by addition of exogenous FAD. Kuzuya and Nagatsu (143) have also reported a parallelism in brain between the development of FAD and the appearance of MAO activity with the age.

The possibility of more than one kind of monoamine oxidase has been suspected for some time. MAO exhibits different specificity for various substrates in liver, brain, heart and other tissues, and this enzyme has a different susceptibility toward MAO inhibitors when assayed with different substrates (116, 117). La Motte et al (142) have reported the production of the following number of m-moles of ammonia/g of tissue/hr in the whole brain of rats: 9.25 m-moles with tyramine as the substrate, 8.40 with dopamine, 6.16 with serotonin and 4.75 with tryptamine. In the same publication, 3 hours after the intraperitoneal injection of 20  $\mu$ moles/kg of iproniazide, the whole brain exhibited 26.9% inhibition of MAO activity assayed with tyramine as the substrate,

13.2% with serotonin, 25.1% with dopamine and 35.6% with tryptamine. When La Motte et al determined the enzymatic activity in several brain areas they found that MAO activity was greater in the hypothalamus of rats and that this area of the brain showed a higher percentage of inhibition (40 to 54%) 3 hours after the administration of the same dose of iproniazide. La Motte et al also reported a similar substrate specificity for the four indicated substrates in various areas of the brain and they concluded that their results could indicate that the same type of MAO is present in different regions of brain. However, the possibility of more than one type of MAO in each area of the brain cannot be ruled out from their experiments since Johnston (117) has reported the existence of two different types of MAO in rat brain based on his findings that a plot of percentage inhibition of MAO versus different doses of M & B 9302 (a potent MAO inhibitor) did not show a simple curve, but revealed a pair of sigmoid curves joined by an horizontal section. On the other hand, the electrophoretic separation of MAO activity in several bands has also been reported in the literature (118,119). In the basis of these and other experiments it has been postulated the existence of two different enzymes in rat liver for the oxidative deamination of serotonin and tyramine (116), and the existence

of three different enzymes oxidizing tryptamine, tyramine and kynuramine (119). Recent work in this laboratory by Sierens and D'Iorio has conclusively demonstrated the presence, in rat liver mitochondria, of two different enzymes which exhibit a different kinetic behaviour toward benzylamine and serotonin (97). Nevertheless, at the present time, it is difficult to state if there are only two different enzymes in rat liver, as well as how many distinct monoamine oxidases exist in other tissues and species.

The biological role of MAO has been discussed in several reviews (86, 110).

The major biological function of MAO and catechol-O-methyl transferase (COMT) (EC.2.1.1.6) was considered for some time to be the degradation of catecholamines by acting singly or in sequence to give a variety of end products. The proposed reactions are indicated in figure 2.

COMT catalyzes the transfer of methyl groups from S-adenosyl-methionine to the 3-hydroxyl position of catecholamines and a variety of other catechol compounds.

However, the importance of MAO and COMT for the biological inactivation of catecholamines is difficult to assess. Inhibitors of MAO and COMT generally fail to produce a marked potentiation of the effects of the

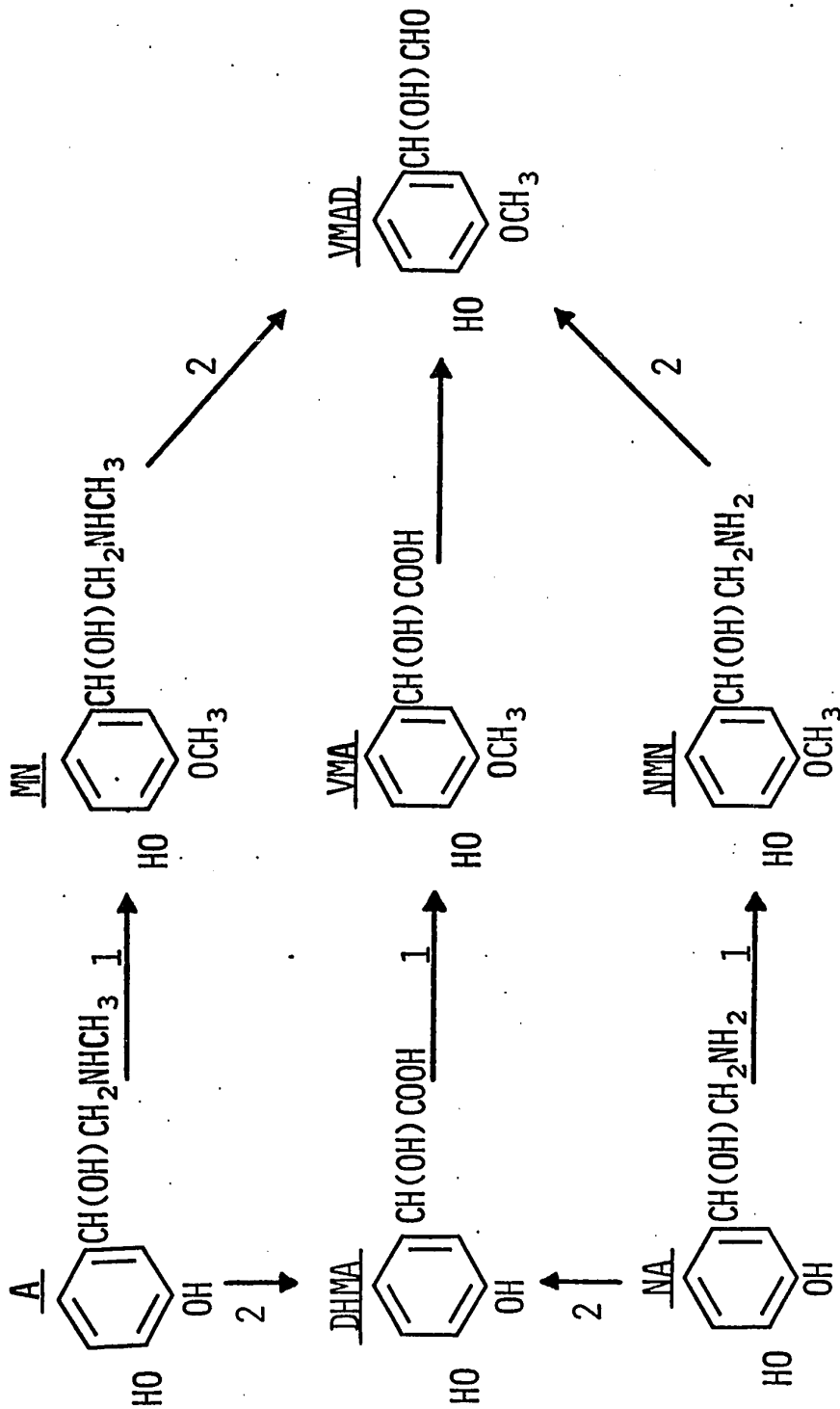


Figure 2

Metabolic degradation of adrenaline (A) and noradrenaline (NA) by COMT (1) and MAO (2). Metabolites are 3-4-dihydroxyphenylethylamine (DHMA), metanephrine (MN), normetanephrine (NMN), 3-methoxy-4-hydroxyphenylethylamine (VMA) and 3-methoxy-4-hydroxyphenylacetaldehyde (VMAD).

noradrenaline released by the stimulation of sympathetic nerves, and it is reported that monoamines accumulate in brain and other tissues only after extensive (at least 85%) MAO inhibition (109). The physiological inactivation of noradrenaline appears to involve its reuptake into the presynaptic nerve ending (86) (see fig.1).

Current theories admit that COMT mainly metabolizes circulating noradrenaline and part of this amine released into the synaptic cleft, while MAO would deaminate noradrenaline inside the presynaptic nerve ending immediately after release from adrenergic granules or after reuptake from the synaptic space (see fig 1). In this way, MAO seems to play an important role in regulating the noradrenaline concentration in the storage particles.

A more direct role seems to be played by MAO in deaminating serotonin, the amine which has been postulated to have a variety of functions in brain and other tissues (187, 188).

An important biological function of MAO and COMT concerns the detoxication of dietary amines. Pharmacologically active amines occur in abundance in certain foodstuffs. Active amines may also arise from the metabolism of the intestinal bacterial flora. However, the high activities of MAO and COMT in the liver and other tissues

provide an effective barrier which prevents these amines from reaching the general circulation before their inactivation (86).

As we pointed out earlier, the possibility of an interaction between thyroid hormones and these two enzymes has been given some consideration. Thyroxine could either affect COMT, MAO or both of them. Although the use of MAO inhibitors did not prolong the response of tissues to adrenaline or noradrenaline a concomitant decrease in the formation of metabolites was found (128, 129), and there appears to be a relationship between COMT inhibition and potentiation of adrenaline (130). On the other hand, one enzyme may compensate for the other, and the lack of effect during MAO inhibition may well be accounted for by this supplementary mechanism. This hypothesis has been investigated extensively.

Spinks and Burn, in 1952, reported the effect of thyroid feeding on the liver MAO activity of rats and rabbits. MAO was decreased by 17% in the rabbit liver after thyroid feeding. In thyroidectomized rats and rabbits, the MAO activity of the liver was increased (131). Trendelenburg also found that the liver of thyroid-fed rabbits exhibited a lower MAO activity than that of normal animals (25). Westerman was unable to confirm

these experimental findings in mice and rats. This author measured the MAO activity in the liver of animals pretreated for three to five days with L-triiodothyronine. No difference was found between the enzymatic activity in the tissue of pretreated and normal animals (133). Holtz et al confirmed Westerman's results (134).

In rats which had been fed desiccated thyroid for one month, Zile and Lardy observed a significant 50% decrease in liver MAO. Thyroxine did not have a direct inhibitory effect on the enzyme and no increase in MAO activity was found in thyroidectomized animals (82). In 1961, Novick studied the effects of both D- and L-triiodothyronine and of thyroid feeding on the heart MAO activity in rats of various ages. D-triiodothyronine had no effect. A 204% increase of the enzymatic activity in the heart of young rats was observed following the administration of L-triiodothyronine. In adult animals, this compound and thyroid feeding had the same effect in increasing heart monoamine oxidase. Neither treatment was found to have an effect on liver MAO activity (135).

Zile had previously reported that the specific activity of liver MAO was decreased in rats which had been pretreated with thyroxine, triiodothyronine, triiodothyroacetic acid or thyroxamine. In the heart or brain no

difference in enzymatic activity was observed between treated and normal animals (136).

Skillen et al have studied the effects of the thyroid gland on the heart MAO of male and female rats. A significant greater MAO activity was found in the heart of normal males compared to normal females. While thyroid feeding increased MAO activity of male heart it had no effect on male heart. On the other hand, propylthiouracil pretreatment decreased the MAO activity of male hearts but did not affect MAO in female hearts. The authors concluded that the effect of thyroid hormones on heart MAO activity is dependent upon the sex of the animals (137).

Wurtman et al studied the liver MAO activity in male and female rats as a function of their thyroid state. They found that high doses of thyroxine (500 µg/day/5 days) decreased MAO activity in the female group (99).

D'Iorio et al have investigated the interaction between COMT and thyroid hormones (138). Using protocatechuic acid as the substrate, the authors measured the formation of its methylated derivative (vanillic acid) in rat liver homogenate. In a series of in vitro experiments thyroxine was found to have no effect on the activity of COMT. Following thyroxine injection, the liver COMT activity was found significantly decreased when

compared to that of the control animals. The authors pointed out that a long pretreatment with thyroid hormones was required in order to obtain a decrease in the enzymatic activity, while the potentiation of catecholamine action by thyroid hormones seems to be immediate. Wurtman et al reported similar experiments but measuring COMT activity in a medium supplemented with S-adenosyl-methionine (99). They did not find any change in heart COMT in the hyperthyroid animals.

D'Iorio and Mavrides, working with partially purified enzyme from rat liver studied the effect, in vitro, of various thyroxine analogues on the activity of COMT (139, 140). They investigated the kinetics of this enzyme and observed that some thyroxine analogues were inhibitors of COMT. Thyroxine, at low concentrations, was without effect. Triiodothyronine was found to be a very poor inhibitor of the enzyme. These authors concluded that the potentiation, in vivo, of the physiological actions of catecholamines by thyroid hormones could not be accounted for by the inhibition of COMT.

Later on, Proulx et al confirmed the inhibition of MAO activity (25%) in the liver of iodinated casein-fed rats (141). In the same report, COMT activity was similar in the liver of thyrotoxic and control animals.

E) CARDIAC HYPERTROPHY AND MAO ACTIVITY:

It has been postulated that catecholamines could be implicated in the pathogenesis of human and experimental hypertension. A decreased accumulation of exogenous and endogenous noradrenaline in the heart of rats with experimental hypertension and cardiac hypertrophy has been reported (144). In these experiments the pathological state had been induced by treating the animals with desoxycorticosterone acetate (DOCA) and 1% NaCl solutions (144). The same observations have been reported in clinical and experimental congestive heart failure with cardiac hypertrophy (145, 146). Pool et al (147) have related this reduction in the cardiac catecholamines to the decreased tyrosine hydroxylase found in the failing heart. In addition, Krakoff et al have reported that in experimental hypertension with cardiac hypertrophy, the mechanism of storage and retention of noradrenaline by adrenergic granules is impaired (148).

On the other hand, myocardial MAO activity has been found increased in experimental cardiac hypertrophy (149). Krakoff et al (150) have studied the cardiac levels of MAO and COMT in adult cats with right ventricular hypertrophy and congestive heart failure induced by constriction of the pulmonary artery for periods of 25-32 days.

At the time of sacrifice the animals received 25 mg/kg of Nembutal (sodium pentobarbital). When MAO activity was expressed per mg of tissue, the enzyme was decreased by 34% in the right ventricle and by 31% in the left ventricle. Nevertheless, when the enzymatic activity was calculated per whole ventricle, MAO was slightly increased in the right side and again was decreased by 31% in the muscle of the left ventricle. In these experiments COMT was consistently increased. The authors concluded that an increased enzymatic degradation is not the cause of the depleted cardiac noradrenaline, and that is unlikely that the observed reduction in MAO could account for the supersensitivity of the failing heart to catecholamines.

De Champlain et al (151) have induced hypertension and cardiac hypertrophy in rats either by DOCA and NaCl or by removal of the right kidney and ligature of the left renal artery. They found that cardiac hypertrophy is associated with a marked increase in MAO activity, and they related the observed changes in MAO activity to changes in cardiac mass. They also suggested that the increased MAO activity is not related to an increased deamination of noradrenaline in the heart on the ground that during normal growth there is a continuous increase

in cardiac noradrenaline despite the progressive increase in MAO activity. Finally, these authors reported in the same publication a similar subcellular distribution of MAO in the heart of normal and hypertensive rats (i.e. mainly in the microsomal fraction).

CONCLUSION OF PART I

A large amount of work has been devoted to uncover interrelations between the thyroid hormones and catecholamines. There is a well demonstrated effect of thyroid hormones on increasing the BMR in several animal species after a latent period of several hours. Adrenaline and noradrenaline also increase the oxygen consumption. There is good evidence about the potentiating effect of both types of hormones on this calorogenic effect.

Adrenaline and thyroid hormones have been demonstrated to have glycogenolytic and hyperglycemic effects. There is no agreement about the possibility of a synergistic action of both hormones in these metabolic effects.

Catecholamines are necessary to mobilize depot fat and thyroid hormones potentiate this action.

There is definite evidence that protein synthesis increase in the liver of rats after the administration of thyroid hormones. There is no agreement whether this enhancement is localized in the mitochondrial or in the microsomal fractions. In some experiments adrenaline seems to reduce the protein synthesis.

The cardiovascular system has provided a convenient tool for the investigations on catecholamine-thyroid interrelations. There is almost general agreement that

the physiological effects of adrenaline and noradrenaline on the heart rate, cardiac output, and ventricular stroke work are potentiated in the case of clinical and experimental hyperthyroidism. In athyroid animals the experimental results are less consistent since some authors observed a decreased response to exogenous catecholamines while others obtained no response. In experimental hyperthyroidism the development of cardiac hypertrophy seems to be evident, and most probably thyroid hormone can influence the isometric contractile mechanics of cardiac muscle by increasing its maximum velocity of shortening. Nevertheless the shortening speed of cardiac muscle from hyperthyroid animals is not supersensitive to exogenous noradrenaline. The significance of cardiac hypertrophy in human hyperthyroidism is uncertain even though 50 per cent of the post mortem examinations in patients with this disease revealed cardiac enlargement.

Considering the multiple biological actions of thyroid hormones, it is difficult to explain every effect by a single primary mechanism of action at the molecular level. Some of the proposed theories have been shown to be inadequate (e.g. action at the respiratory chain level, uncoupling of the oxidative phosphorylation). There is some evidence in favor of a primary direct action of thyroid

hormones on protein synthesis, and these experiments, together with the finding of an increase in mitochondrial  $\alpha$ -glycerophosphate dehydrogenase in the presence of thyroid hormones, have lead to postulate that perhaps the primary action of thyroxine, is to increase the rate of operation of the  $\alpha$ -glycerophosphate cycle. Nevertheless present experimental evidence is not sufficient to support this hypothesis.

Among the theories implicating catecholamine in the mechanism of action of thyroxine we can find the concept that the sensitivity of adrenergic receptors to noradrenaline is increased after thyroid hormone administration. However there are several reports demonstrating that the administration of adrenergic blocking agents does not reverse the effects of thyroxine on the FBI and on the  $^{131}\text{I}$  uptake by the thyroid gland.

During recent years attention was directed to the theory that thyroxine may act by decreasing the inactivation (uptake) of noradrenaline and several reports pointed in this direction (105). However there is some evidence showing a decrease of overall catecholamine turnover in the heart after thyroxine administration suggesting a possible action of thyroid hormones at the rate limiting step in the noradrenaline synthesis.

The possibility that thyroid and adrenal medulla hormones may have a common point of action at the adenyl cyclase system level is now under serious consideration. According to this theory adenyl cyclase, which regulates the formation of cyclic AMP from ATP, would act as a second messenger under the influence of several first messengers (i.e. hormones) generating multiple biological effect at the cellular level. This mechanism has been suggested recently and has to stand the test of time.

Another concept extensively investigated is the possible action of thyroid hormones on the activity of the enzymes involved in the degradation of catecholamines (i.e. MAO and COMT). In experimental hyperthyroidism, liver MAO activity has been reported to be decreased. However in the heart of hyperthyroid rats the noradrenaline content is low (141) and in most of the reported experiments MAO activity is increased in the hyperthyroid heart. In addition, there is no significant change of COMT activity in hyperthyroidism, and at the present time the two mentioned enzymes are not considered as the main biological inactivators of catecholamines in the adrenergic synapse.

Finally several reports in the literature show a striking similarity between some biochemical characteris-

tics of the hyperthyroid heart, and some of the features found in the cardiac hypertrophy induced by other means (e.g. arterial constriction, DOCA and NaCl administration). In any of these cases the cardiac stores of noradrenaline are depleted and MAO activity is increased.

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PART II. THYROID HORMONES-BARBITURATES INTERACTIONS

The thyroid gland has been shown to exert a significant influence on the effects produced by some drugs (152). Hyperthyroid animals have been found to be more susceptible to the toxicity of some pharmacological agents (152). Hypothyroidism and hyperthyroidism delayed the metabolic removal from rat tissues of pentobarbital following its intravenous injection (153). The duration of the sleep induced by hexobarbital was markedly increased in hyperthyroid or thyroidectomized rats (154). The mortality of hyperthyroid rats after treatment with several barbiturates was much higher than the mortality observed in control animals (154). A marked increase in the lethality of hyperthyroid rats was demonstrated following the administration of a non hydrazide MAO inhibitor (152).

A) THE BIOLOGICAL FATE OF DRUGS, THE METABOLISM OF SOME BARBITURATES:

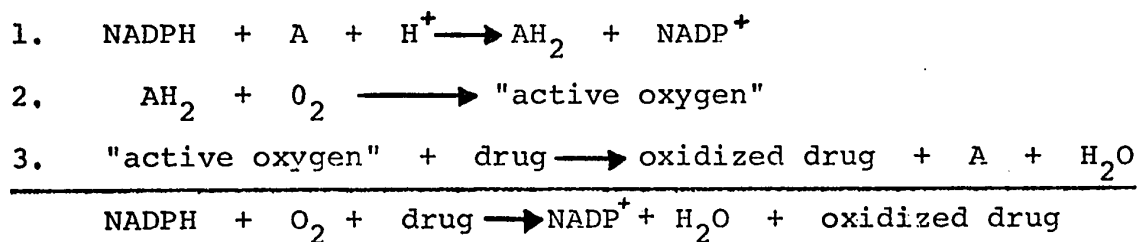
Drugs are administered to an organism to alter its function or structure. The intensity and duration of the effects of a given drug in a living system is determined by the rate of biological transformation or removal from effector sites. The physiological conditions under which a drug is administered are a major determinant in

the disposal of the drug. At the present time, there is general agreement on quantitative differences in the metabolism of drugs accounted for by variation on species, strain, age, sex, nutritional status and on hormonal balance (155). It seems that most of these factors would modify the disposal of drugs through inducing modifications in the enzymes which are responsible for the metabolism of such compounds.

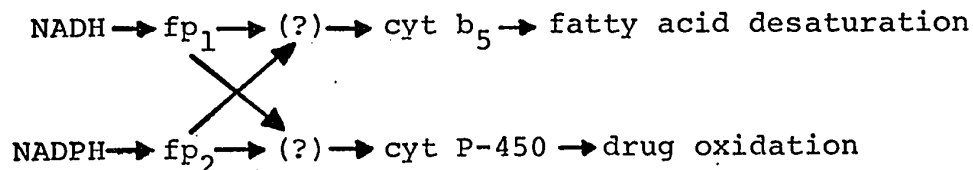
During the past several years numerous studies have shown that most drugs are oxidized or reduced by enzymes in the endoplasmic reticulum of cells. Following the administration of drugs, (e.g. barbiturates) the binding process occurs mainly in the microsomal fraction of rat liver (156). A good review on the biochemistry of drug oxidation and reduction by the microsomal enzymes is available (157).

In the endoplasmic reticulum most drugs will undergo different specific reactions according to their chemical structure. However, the more or less specific enzymatic systems catalyzing those reactions have in common several features: they are localized in the smooth-surfaced microsomal fraction of the liver and other tissues, they require oxygen and NADPH (may sometimes be replaced by NADH), and most of these enzymes can be considered as mixed function oxygenases.

According to the mixed-oxygenase mechanism, NADPH reduces a component in the microsomes that reacts with oxygen to form an "active oxygen" intermediate. The "active oxygen" is then transferred to the drug substrate.



Sato et al have postulated that this system could operate in liver microsomes by means of a special electron transport chain according to the following scheme (158):



It is known that microsomes contain a variety of flavoproteins (159), in particular a flavoenzyme termed NADH-cytochrome  $b_5$  reductase and a second flavoenzyme termed NADPH-cytochrome c reductase. It is also known that microsomes contain two hemoproteins (i.e. cytochromes P-450 and  $b_5$ ). The reduced cytochrome P-450 acts as the

oxygen activating agent in the above mentioned "mixed oxygenase" mechanism, as well as the site of substrate interaction for the oxidative transformation of various lipophilic foreign compounds and possibly also for steroids. It has been suggested a role for cytochrome  $b_5$  in the oxidative desaturation of some fatty acids. It is generally assumed that NADPH-cytochrome c reductase functions in the transfer of reducing equivalents from NADPH to cytochrome P-450 and that NADH-cytochrome  $b_5$  reductase exerts the same function between NADH and cytochrome  $b_5$ . Interaction between both flavoproteins has been postulated to occur, assuming the existence of unknown intermediates (marked as (?) in the previous scheme).

On the other hand, the wide variety of drugs which are metabolized by liver microsomes suggests that the microsomal oxidative enzymes have an extraordinary degree of nonspecificity. In this way it has been postulated the concept that either liver microsomes contain a number of enzymes each of which has P-450 as its prosthetic group, or that a common P-450 serves a number drug metabolizing enzymes (157).

A remarkable aspect of the metabolism of drugs is the effect of one drug upon the disposal of another (160). A drug may increase the biotransformation of another by

activating the enzyme which metabolizes the second compound. Barbiturates, specially phenobarbital, are remarkable activators of some microsomal enzymes. A decrease in the sleeping time produced by pentobarbital occurs following the administration of phenobarbital. A significant increase on the activities of several drug metabolizing enzymes has been reported in rat liver microsomes after the administration of 80 mg/kg of phenobarbital (161), an activation of various components of the microsomal NADPH-linked electron transport system (i.e. NADPH-cytochrome c reductase, NADPH oxidase and P-450) by the barbiturate was demonstrated in the same work. The enzymatic induction produced by some barbiturates is associated with demonstrable morphologic proliferation of the smooth-surfaced endoplasmic reticulum from which enzyme-containing microsomes are derived upon centrifugation (162).

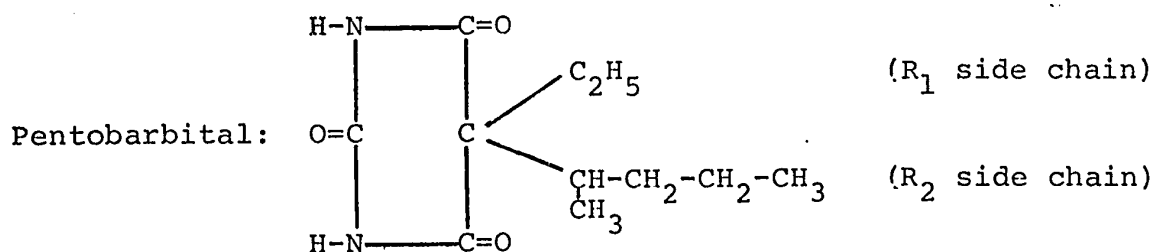
In addition, a drug may decrease the rate of biotransformation of another by inhibiting its corresponding enzymatic system. Unlike enzyme induction, the inhibition of some microsomal enzymes is often evident after a single administration of a drug (160). For example, therapeutic doses of diethyl ether inhibit the metabolism of pentobarbital (160).

Another interesting point about drugs is that metabolic transformation is not always synonymous with inactivation. A variety of barbiturates undergo metabolism to pharmacologically active compounds, and sometimes the metabolite is more active than the parent compound. For instance, N-methylphenobarbital is demethylated to phenobarbital by microsomal enzymes (160).

Finally we are going to mention in some detail the metabolism of some barbiturates.

It has been reported by Kuntzman et al (164) that the incubation of 1  $\mu$ mole of pentobarbital with rat liver homogenate, in the presence of several cofactors, at 37° C for 10 minutes, resulted in the formation of 13  $\mu$ moles of pentobarbital alcohol and 0.7  $\mu$ mole of a second metabolite (MI). In addition, while 6.5  $\mu$ moles of pentobarbital alcohol were produced in the presence of the microsomal fraction alone, little amounts of this metabolite and almost undetectable amounts of MI were produced in the presence of the other subcellular fractions. The cofactor requirements were shown to be NADPH in every experiment and both NAD and NADPH for the formation of MI. It was concluded that pentobarbital is metabolized in ~~rat~~ rat liver microsomes by an enzymatic system requiring the above mentioned cofactors. It was also concluded

that pentobarbital alcohol represents at least 50% of the resulting metabolites, and that most probably this alcohol is the precursor for the formation of the second metabolite which was not identified in this work. In a previous work by Cooper and Brodie (163) the two main metabolites formed by microsomal oxidation of pentobarbital in liver were reported to be the pentobarbital alcohol and the pentobarbital carboxylic acid. The original barbiturate and both metabolites have the following formula:



Pentobarbital alcohol: (Same structure but  $\text{R}_2$  is:  $-\underset{\text{CH}_3}{\text{CH}}-\text{CH}_2-\underset{\text{OH}}{\text{CH}}-\text{CH}_3$ )

(i.e. 5-ethyl-5-(3-hydroxy-1-methylbutyl)-barbituric acid.

Pentobarbital carboxylic acid: ( $\text{R}_2$ ,  $-\underset{\text{CH}_3}{\text{CH}}-\text{CH}_2-\text{CH}_2-\text{COOH}$ )

(i.e. 5-ethyl-5-(3-carboxy-1-methylpropyl)-barbituric acid

It has also been reported (163, 164) that the incubation of pentobarbital with homogenates of brain, heart, spleen, adrenal glands, kidneys and testes, resulted in no production of pentobarbital metabolites, and it was

postulated (164) that some endogenous inhibitor of the corresponding microsomal enzyme must be present in these organs.

Despite the classification of pentobarbital as a "short acting" barbiturate, its metabolites are excreted in urine for five days following the administration of 500 mg, and alterations in mental function persist for 14 hours after the administration of 100 mg (160). Essentially all pentobarbital is metabolized after its administration, less than 1% being excreted unchanged in the urine (160).

Phenobarbital ( $R_1, -C_2H_5$ ;  $R_2, -C_6H_5$ ) is a barbiturate of "long action" which is slowly metabolized to p-hydroxyphenobarbital (160).

In thiobarbiturates, the substitution of a sulfur atom for the oxygen in the urea portion of the barbituric acid ring, brings about a marked increase in lipid solubility and an increased ability to cross the blood-brain barrier. Thiopental is metabolized primarily in the liver and kidney by microsomal enzymes which require oxygen and NADPH (160). Thiopental is also metabolized in the brain and heart (163). It has been reported that thiopental alcohol and thiopental carboxylic acid are the main metabolites produced in the biotransformation of this barbi-

turate (163).

B) SOME METABOLIC INTERACTIONS OF THYROID HORMONES:

A number of studies have indicated that a substantial fraction of thyroid hormones after their release from the thyroid gland, are localized within tissue pools which are in a state of rapid equilibrium with thyroid hormones bound to the plasma proteins (165). The liver and kidney appear to have the highest concentrations of exchangable hormone (166).

It has been reported that 35 minutes after intravenous administration of radioactive thyroxine, most of the radioactivity (52%) is detected in the smooth elements of the microsomal fraction of rat liver (167). Thyroid hormones are mainly metabolized by a specific enzymatic system (deiodinase) which requires oxygen and NADPH and is mainly localized in the microsomes of liver, kidney and heart (168).

It is of interest to mention here the work of Kato and Takahashi regarding the effects of thyroid hormones on the activities of several drug-metabolizing enzymes and on the electron transport system of rat liver microsomes (161). When male rats were given 0.1 mg/kg of thyroxine daily during ten days, aminopyrine hydroxylase and hexobarbital hydroxylase were significantly decreased

aniline hydroxylase was increased and p-nitrobenzoic acid hydroxylase was unchanged. In female rats the four enzymatic activities were increased following the mentioned treatment. In the same series of experiments and in both sexes an increase of the activities of several enzymes related to the microsomal NADPH-linked electron transport system was demonstrated.

The levels of P-450 were decreased following the treatment with thyroxine. A decrease of NADH-cytochrome c reductase and NADH oxidase, with an increase in the levels of cytochrome b<sub>5</sub> were observed following the administration of the same hormone.

C) METABOLIC INTERACTIONS BETWEEN THYROID-HORMONES AND BARBITURATES:

The binding of the microsomal fraction of rat liver for <sup>125</sup>I-thyroxine was increased after the administration of phenobarbital (65% compared to 54% in control animals) (167). The administration of phenobarbital (100 mg/kg) resulted in an increased deiodinative capacity for thyroxine in the hepatic microsomal fraction (169). When 50 mg/kg of pentobarbital were given to thyroid-fed mice the removal of the drug from the brain, liver and plasma was slower than in euthyroid animals (153).

Finally, in the experiments reported by Kato and

Takahashi (161), when thyroxine-treated male and female rats were given 80 mg/kg of phenobarbital 72 and 48 hours before sacrifice the microsomal enzymes metabolizing aminopyrine, hexobarbital, aniline and p-nitrobenzoic acid all were significantly elevated, as well as all the enzymatic activities studied in relation with the NADPH-linked microsomal electron transport system. NADH oxidase was increased and NADH-cytochrome c reductase together with cytochrome  $b_5$  were decreased.

CONCLUSION OF PART II

From the foregoing survey the following points arise:

Most barbiturates are metabolized by more or less specific microsomal enzymes which require oxygen and NADPH. This biotransformation is postulated to operate by means of the microsomal electron transport chain. The activities of several drug metabolizing enzymes have been correlated with the P-450 chain and related enzymes (i.e. NADPH oxidase, NADPH-cytochrome c reductase, NADPH-neotetrazolium reductase). While the activities of fatty acid desaturating enzymes have been related with the other chain (i.e. cytochrome  $b_5$ , NADH oxidase, NADH-cytochrome  $b_5$  reductase).

Phenobarbital has been demonstrated to activate some drug metabolizing enzymes and most of the NADPH-linked components of the microsomal electron transport chain. Most of the NADH-linked components of this chain are inhibited by phenobarbital (161).

Some of the components of the microsomal electron transport system are known to be flavoproteins.

On the other hand, thyroxine has also been demonstrated to increase the activity of several drug metabolizing enzymes in female rats and to inhibit the same enzymes in male animals. The levels of P-450, after the adminis-

tration of thyroxine, are decreased in both male and female rats while most of the NADPH-linked enzymatic activities are increased in both sexes under the same experimental conditions. The cytochrome  $b_5$  levels are increased while NADH oxidase and NADH-cytochrome c reductase are decreased following the thyroxine treatment (161).

Therefore it is clear that there is a synergistic effect of thyroxine and barbiturates on the activities of some microsomal drug metabolizing enzymes and on the activities of some flavoproteins of the microsomal electron transport chain. A marked antagonistic effect of both types of compound on P-450, cytochrome  $b_5$  and NADH oxidase has been demonstrated.

PART III. THE EFFECT OF BARBITURATES ON THE ACTIVITY OF  
SOME FLAVOENZYMES AND ON SOME BIOGENIC AMINES

A) THE INHIBITION OF SOME FLAVOENZYMES BY BARBITURATES:

Substituted barbiturates depress cellular respiration. Amytal and other similar compounds are known to inhibit the mitochondrial electron transport from NADH to oxygen in the flavoprotein region (170, 171). Conflicting reports have indicated that the site of this inhibition might be before or after the flavoprotein step in the electron transport chain (172).

In addition, it is reported that barbiturates inhibit D-aminoacid oxidase, NADPH dehydrogenase, D-aspartate oxidase, NADH-cytochrome c reductase and other flavoenzymes (172, 173, 174, 175).

The inhibitory mechanism of various flavoenzymes by barbiturates has been studied in several works. Giuditta and Strecker demonstrated that a purified NADPH dehydrogenase was competitively inhibited by barbiturates (176). A similar mechanism of inhibition was reported for D-aspartate oxidase (173) and other flavoenzymes (175). D-aminoacid oxidase, partially purified from sheep cerebellum, was competitively inhibited (45%) in the presence of  $5 \times 10^{-3}$  M pentobarbital (174).

Giuditta and Casola (175) have studied some features of the inhibition of several purified flavoenzymes by phenobarbital. For example the  $K_m$  of NADPH dehydrogenase assayed with NADH as the substrate and ferricyanide as electron acceptor was  $1 \times 10^{-4}$  M, and with phenobarbital as inhibitor the  $K_i$  for the same enzyme was  $1.7 \times 10^{-3}$  M. The same values for D-aminoacid oxidase were  $2.2 \times 10^{-2}$  and  $4 \times 10^{-3}$  respectively, and for L-aminoacid oxidase  $2.5 \times 10^{-3}$  and  $2.9 \times 10^{-2}$ . When the same authors studied various nonflavoenzymes, also known to be inhibited by phenobarbital, the  $K_i$  values were always around  $10^{-2}$  M. In the same publication they also reported modifications of the absorption spectrum of some purified flavoenzymes after the addition of phenobarbital. They also observed modifications of the spectrum of free flavines (FMN and FAD) in the presence of the same barbiturate. In the light of this evidence they suggested that the inhibitory sites of the mitochondrial electron transport chain and of some flavoenzymes by barbiturates might be similar, and that the mechanism may be related to an interaction between barbiturates and protein-bound flavins.

On the other hand, it has been also demonstrated that the inhibition of certain flavoproteins of the microsomal electron transport system is effected by phenobarbital (161).

B) EFFECT OF THE ADMINISTRATION OF BARBITURATES OR MAO INHIBITORS ON THE LEVELS OF SOME BIOGENIC AMINES IN THE CENTRAL NERVOUS SYSTEM:

The 5-hydroxytryptamine levels (5-HT) in the brain of various species has been demonstrated to increase following the administration of barbiturates or other central depressant drugs (177, 178, 179, 180, 181).

Anderson and Bonnycastle (178) have shown in rats that following the administration of 50 mg/kg of pentobarbital the animals lost their righting reflex in about 2 minutes, at which time the brain levels of 5-HT were found to be 360 µg/g of tissue. Five minutes after the injection of the drug the brain 5-HT had increased to 754 µg/g of tissue and then the increased serotonin began to decline slowly but was still above control levels 2 hours later. Following the administration of 100 mg/kg of phenobarbital the brain 5-HT started to rise until a peak of 572 µg/g tissue was reached 2 hours later. This peak was followed by a slow decline, with serotonin levels being still above control 4 hours after the administration of the drug. After starting ethyl ether inhalation, the rats lost their righting reflex in 3 minutes, while the brain levels of 5-HT started to increase 7 minutes later.

These findings were confirmed later by Bonnycastle et al after the administration of different barbiturates (180). They concluded that the time of maximal serotonin levels in brain following the administration of barbiturates lies well within the time of maximal pharmacological action of these drugs, and that the time of appearance of the serotonin peak is in agreement with their pharmacological classification in "long and short acting barbiturates". However these workers concluded that the degree of activity of the animals following the administration of barbiturates is not related to 5-HT levels in brain.

On the other hand, the levels of serotonin and noradrenaline in brain have been studied extensively, following the administration of MAO inhibitors to experimental animals.

Chessin et al have investigated the relationship between MAO inhibition and increased serotonin levels in mouse brain (122). Inhibition of MAO activity and increased levels of serotonin were demonstrated following the intraperitoneal injection of 32 mg/kg of  $\beta$ -phenylethylhydrazine into the animals. They observed a close parallelism between the restoration of MAO activity and the decrease of serotonin to control levels. They concluded that between 50 and 100% inhibition of MAO is required in brain

in order to observe increased levels of serotonin in the same organ.

In one publication of Dubnick et al (123) it can be observed that the intravenous injection of 1 mg/kg of phenelzine into mice produced 3 hours later 46% inhibition on brain MAO activity with a concomitant increase in brain serotonin. It is also reported by Dubnick et al (123) that doses of phenelzine higher than those required to inhibit brain MAO activity by 100% gave rise to even higher serotonin levels.

Gey and Pletscher (124) reported that the administration of iproniazide increases the brain levels of serotonin and noradrenaline with a simultaneous inhibition of MAO activity in the same organ of at least 85%. They studied the effects of this irreversible MAO inhibitor on biogenic amines and MAO activity, 16 hours after the treatment of the animals with the drug.

In one report of Green and Erickson (126) it can be observed that noradrenaline is increased by 15% in the brain one hour after a single oral dose of 40 mg/kg of iproniazide. In another table of the same report it can be seen that 50% inhibition of MAO activity was obtained one hour after giving the same type of animals a single oral dose of 100 mg/kg of the same MAO inhibitor.

On the basis of these and other similar experiments it has been suggested that at least 85% inhibition of brain MAO activity is necessary in order to detect increased levels of biogenic amines in the same organ (109).

On the other hand, a number of MAO inhibitors are reported to prolong the duration of the hypnosis induced by several barbiturates (182). Hence the possibility must be considered that this potentiating effect takes place by a synergistic accumulation of serotonin in the central nervous system after treatment of experimental animals with both types of agents. In other words, barbiturates could increase brain serotonin levels by the same mechanism that MAO inhibitors exert this effect.

However it has been reported in the literature that the effect of MAO inhibitors on hexobarbital hypnosis is relatively short-acting, while the inhibition of MAO in brain after the administration of iproniazide is a long-acting effect (179). In addition, the elevation of brain 5-HT after treatment with barbiturates is relatively rapid, while the increase in this amine following the treatment with iproniazide requires more time (178).

In vitro inhibition of hexobarbital metabolizing enzymes from rabbit brain has been found in the presence of high concentrations of iproniazide (179).

With all the previous indirect evidence in mind, it has been concluded that the potentiation of barbiturate induced hypnosis by MAO inhibitors is probably due to a decreased metabolism of barbiturates in the microsomal fraction and that this potentiation has nothing to do with a possible inhibition of MAO activity by barbiturates (178, 179).

In addition, serotonin administration potentiates the hypnosis induced by hexobarbital (183) and Mahler and Humoller (181) have reported potentiation of the hypnosis produced by hexobarbital as well as increased levels of serotonin in brain mouse following the administration of 5-hydroxytryptophan. In the same experiments, when both iproniazide and the mentioned serotonin precursor were given to the animals before hexobarbital was administered, the sleep time was reduced. The authors concluded that in the presence of an intact MAO activity, the stimulation of the cerebral 5-HT metabolism produces a potentiation of the hexobarbital induced hypnosis. They postulate that some metabolite of the serotonin in the brain could play a significant role on the hexobarbital-serotonin interactions. In another publication the same workers (184) reported uncoupling of the oxidative phosphorylation in rat brain mitochondria in

the presence of serotonin or 3-indole-acetaldehyde, and elimination of this effect by blocking MAO activity with iproniazide. Nevertheless, if we consider the potentiation of the hypnosis produced by barbiturates following the administration of MAO inhibitors (182), the results of Mahler and Humoller are difficult to evaluate.

C) THE POSSIBLE INFLUENCE OF SOME BIOGENIC AMINES ON THE REGULATORY MECHANISMS OF SLEEP:

The mammalian sleeping brain successively passes through two phases which can be recognized very easily by constant electroencephalographic recording (127). The first state has been called slow wave sleep (SWS) or non-rapid eye movement sleep (NREM). In this phase the animal maintains a degree of postural tonus in some muscle groups, its eyes are closed, the pupils are miotic and the electrical activity of the cortex is characterized by spindles and slow waves. After certain interval of time this state is followed by a second phase which has been named paradoxical sleep (PS) or rapid eye movement sleep (REM) which is characterized by rapid eye movements with a total absence of electromyographic evidence of muscular activity.

Most probably these two phases of sleep are associated with two different functional states of the brain, since

PS may be selectively suppressed without altering SWS by means of treatment with various drugs and by specific cerebral lesions (125).

The possible role of serotonin and noradrenaline in the mechanism of production of these two sleep patterns has been discussed in recent reviews (125, 127, 185). The increase of the brain serotonin following the administration of 5-hydroxytryptophan leads to a suppression of PS. The injection of dihydroxyphenylserine (a direct precursor of noradrenaline) increases both SWS and PS. The specific suppression of PS and the increase of SWS have been observed in cats after the administration of MAO inhibitors. A significant correlation has been found between the decrease in SWS and the decrease in brain concentrations of serotonin after treatment of cats with p-chlorophenyl-alanine which selectively reduces the concentrations of brain serotonin through the inhibition of tryptophan hydroxylase.

This information together with some additional evidence has led several investigators to correlate the cerebral levels of serotonin and noradrenalin with the two phases of sleep (127, 125). Jouvet has postulated (125) that the mammalian brain undergoes cyclical biochemical changes from the waking state (W) which depends

upon the ascending reticular activating system, to SWS which depends upon serotonin-containing neurons of the raphe system. The SWS is followed by PS which depends upon noradrenaline-containing neurones located in the nucleus locus coeruleus, and then the cycle will be closed by passing again to the W state.

W  $\begin{matrix} \rightarrow \\ \leftarrow \end{matrix}$  SWS  $\begin{matrix} \rightarrow \\ \leftarrow \end{matrix}$  PS  $\rightarrow$  W

The first two steps of this cycle are supposed to be reversible, whereas the final step is never reversed.

The occurrence of PS may be suppressed by at least three types of compounds: MAO inhibitors, atropine and  $\alpha$ -methyl-p-tyrosine. This fact suggests that PS requires three keys in order to operate: deaminated serotonin catabolite, acetylcholine and noradrenaline. Finally, barbiturates have also been reported to reduce the duration of PS (127, 186).

CONCLUSION OF PART III

MAO inhibitors have been demonstrated to increase the cerebral levels of serotonin and noradrenaline. On the basis of some indirect evidence, it is assumed that at least 85% inhibition of brain MAO activity is required before increased levels of biogenic amines are observed.

MAO inhibitors have been shown to suppress PS. The demonstration that major alterations of the sleep patterns follow the administration of various drugs which affect the monoaminergic neurons has led Jouvett to postulate that SWS seems to depend upon serotonin levels in certain regions of brain and that PS appears related to some deaminated catabolite of serotonin, to acetylcholine and to noradrenaline.

On the other hand, MAO inhibitors have been reported to potentiate the barbiturate-induced hypnosis. Barbiturates are known to increase the cerebral levels of serotonin and this amine has been postulated to potentiate the barbiturate-induced hypnosis when the MAO activity system is intact (181, 184). In addition, it has been reported that barbiturates reduce the duration of paradoxical sleep, and also that these drugs inhibit several flavoenzymes by competition.

EXPERIMENTAL RESULTS

PART I. TOTAL PROTEINS AND MAO ACTIVITY IN VARIOUS SUB-  
CELLULAR FRACTIONS OBTAINED FROM DIFFERENT TISSUES OF  
NORMAL AND HYPERTHYROID RATS: THE EFFECT OF PENTOBARBI-  
TAL ADMINISTRATION

Even though the effect of thyroxine on MAO activity is not considered today as the primary mechanism of action of thyroid hormones, it was felt that some additional details about the effects of these hormones on MAO deserved further investigation. MAO has been thought for a long time to be a mitochondrial enzyme but it has been demonstrated that in the heart this enzyme is mainly localized in particles sedimenting with the microsomes. In view of these observations the following questions need to be answered: Is there any influence of thyroid hormones on the subcellular localization of MAO? Does thyroxine inhibit only mitochondrial MAO in rat liver or is the microsomal counterpart also inhibited?. What enzymatic activity is enhanced in the hyperthyroid heart: that localized in the mitochondria or that of microsomes?.

In addition, bearing in mind our observation in preliminary experiments of low MAO activity in the heart of thyrotoxic rats treated with pentobarbital, it was decided to investigate the effect of this barbiturate on MAO.

The following experiments were designed in an attempt to clarify the above mentioned points.

## I. MATERIALS AND METHODS

### A) MATERIALS

All the animals used in these experiments were male Sprague-Dawley rats.

The diet utilized for inducing hyperthyroidism was purchased from General Biochemicals. It had the following composition:

Corn yellow	45.75%
Soy bean meal	45.75%
Phillips-Hart salt mix	2.00%
Corn oil	5.00%
Vitamine fortification mixture	1.00%
L-cysteine	0.30%
Choline chloride	0.10%
Iodinated casein	0.10%

A control diet containing all the above ingredients except iodinated casein was obtained from the same source.

Nembutal sterile aqueous solution containing 20% propylene glycol, 10% ethanol and 50 mg/ml sodium pentobarbital was purchased from Abbott Laboratories, Montreal.

The oxygen consumption of rats was measured with a Phipps & Bird metabolism apparatus (model N. 7.2241).

A Bausch & Lomb recording spectrophotometer (Spectronic 505) was employed to determine MAO activity. Kynuramine dihydrobromide ( $C_9H_{14}N_2O.Br_2$ , mol wt 326.0), was obtained from Regis Chemical Co., Chicago, and was used as the substrate for MAO.

The readings of optical density for total protein determinations were carried out in a Coleman Junior spectrophotometer (model 6A).

Lubrol, a nonionic detergent, was supplied by C.I.L., Montreal.

## B) METHODS

### 1) Preparation of animals:

Three weeks old male rats weighing 50 grams when received, were divided into two groups. The animals from the first group received the control diet, while those from the second group received the iodinated casein diet. A greater number of rats were placed in the second group

because of the expected higher mortality rate in these animals. The rats were placed in individual cages with raised screened bottoms and the temperature of the room was controlled. All animals were allowed free access to food and fresh water, and their weights were recorded daily. The animals were kept on the diet during seven weeks. Ten per cent of the animals fed the iodinated casein diet died and these animals were discarded. At the end of this period, the oxygen consumption of the animals was measured and/or they were sacrificed in order to assay various tissues for MAO activity and total proteins.

2) Measurement of oxygen consumption:

All the rats were fasted overnight before the oxygen consumption was measured using a method already described (190). Animals were weighed to the nearest gram and were given Nembutal intraperitoneally (30 mg/kg). Ten minutes later, the rat was restrained into a cylinder made of wire mesh and then placed in the glass jar of the metabolic apparatus the bottom of which was previously covered with fresh soda lime to absorb moisture and CO<sub>2</sub>. The lid was screwed on the jar. Oxygen was bubbled into the apparatus for five minutes and another five minutes were allowed for equilibration. At the end of this period, a calibrated tube containing one drop of soap solution was inserted in

the air outlet of the chamber. The time taken for this soap bubble to travel between two existing marks in the calibrated tube (5 ml of volume), was recorded in seconds. At least five successive measurements were made on each animal. The average of the five values was taken as the time necessary for the rat to consume 5 ml of oxygen. The oxygen consumption was calculated in ml per minute per kilogram of body weight. The temperature into the chamber was recorded for every animal, and it was noted to be always  $28 \pm 0.5^{\circ} \text{C}$ .

In order to avoid the administration of pentobarbital, the oxygen consumption of the animals utilized to elucidate the influence of hyperthyroidism on the subcellular localization of MAO was not measured. Nevertheless the hyperthyroid state of this group of rats was ascertained in several animals which were discarded after the procedure.

### 3) Preparation of tissue samples:

A number of rats fed with control or iodinated casein diet were fasted overnight, but received water ad libitum. After the weight of the animals had been recorded, they were stunned, exsanguinated, and the tissues quickly removed in the following order: liver, kidneys, heart and whole brain. Immediately after removal each organ was placed in ice-cold isotonic sucrose. Then each organ in

turn was blotted on filter paper, cleaned and weighed on a torsion balance. All subsequent procedures were carried out at 0° to 3° C. Two grams of tissue from the liver, both kidneys, whole brain, and the heart from two rats, were finely minced with scissors. The nuclei and cellular debris were separated from total homogenates using the method described by de Duve et al (191). The tissues were dispersed with 3 volumes of 0.25 M sucrose (pH adjusted to 7.4) in a glass homogenizer fitted with a teflon pestle (Arthur H. Thomas Co. Philadelphia). The homogenizer, kept in cracked ice, was given three runs upwards and downwards against the rapidly rotating pestle. The resulting material was centrifuged in a Sorvall Refrigerated Centrifuge (model SS-3, rotor SM-24) for ten minutes at 1,000 x g. The sediment containing unbroken cells and nuclei, was rehomogenized in about the same volume of medium and centrifuged for ten minutes at 600 x g. After repeating this operation a second time, the nuclear sediment was discarded. The supernatants were combined, and two ml were pipetted into graduated tubes, this portion being designated cytoplasmic fraction. The remainder of the combined supernatants was further fractionated using the Spinco model L preparative ultracentrifuge (rotor number 40).

The mitochondrial and microsomal fractions were successively isolated following the method of Schneider and Hogeboom (192). The mitochondrial fraction was sedimented at 5,000 x g for 10 minutes and washed twice in a volume of 10 ml of the above mentioned medium. Following each washing the mixture was centrifuged at 24,000 x g x 10 minutes. The microsomes were sedimented at 100,000 x g x 10 minutes, washed once, and sedimented again at the same speed for 30 minutes.

The final supernatant was discarded. The final pellets, were resuspended in small volumes of the medium. They were gently homogenized and poured into graduated tubes. These fractions were designated as mitochondrial and microsomal respectively. Lubrol to a final concentration of 5% was added to the cytoplasmic, mitochondrial and microsomal fractions. This procedure partially solubilized the enzyme and facilitated the spectrophotometric readings.

All the samples were treated with 0.5 ml of 0.5 M phosphate buffer pH 7.4 and they were stored at 2° C for 48 hours before the total proteins and MAO activity were determined. The preparation of the samples from all the organs was performed simultaneously. During the procedure the following data were recorded on a "balance sheet": the total weight of each organ, the amount of organ used

in the fractionation of every tissue, the volume of the final suspension for each subcellular fraction (2.5 ml for the cytoplasmic fraction, 4.5 ml for the mitochondrial fraction, and 2.5 ml for the microsomal fraction), and the amount of tissue corresponding to each of the separated fractions.

4) Determination of MAO activity:

The enzymatic assay was performed according to Weissbach et al (193). In this method, kynuramine is used as the substrate which is oxidized by the enzyme to the corresponding aldehyde. The reaction is followed by measuring the disappearance of the substrate. This measurement was done spectrophotometrically. The decrease of absorption at the optimum wavelength of the kynuramine (360 m $\mu$ ) was taken as an index of MAO activity.

The substrate was dissolved in distilled water and refrigerated. Phosphate buffer 0.5 M, pH 7.4, was prepared by titrating a 0.5 M solution of dibasic sodium phosphate with a 0.5 M solution of sodium phosphate solution until a pH of 7.4 was reached. Both compounds were dissolved in distilled water.

The enzymatic reaction was carried out at 37° C in 1 cm quartz cuvettes. The final incubation mixture contained 200  $\mu$ moles of phosphate buffer, 0.3  $\mu$ mole of subs-

trate, enzymatic preparation, and distilled water up to 3 ml. When samples from liver and brain were assayed, 0.2 ml of cytoplasmic, fraction, 0.1 ml of mitochondrial fraction or 0.2 ml of the microsomal preparation were employed for the enzymatic assay. In the case of heart and kidney preparations 0.4, 0.2 or 0.4 ml aliquots of the respective fractions were used. A blank was set up containing the same mixture with no substrate which was substituted by the equivalent volume of distilled water. The rate of decrease of absorption at 360 m $\mu$  was measured in the spectrophotometer as described under materials. Reading of the optical density was started 1 minute after addition of the enzyme to allow temperature equilibration and recording was pursued for ten minutes. During this period of time the decrease in optical density was linear. The temperature was kept constant by means of a water jacket surrounding the cuvettes. The water was supplied and circulated from a water bath at constant temperature (37° C). In a separate set of control experiments it was shown that the Lubrol added to the samples did increase in the same proportion the enzymatic activity of the different subcellular fractions. Moreover it was shown that Lubrol did not alter the decrease of absorption of kynuramine at 360 m $\mu$  and 37° C, for ten minutes. One

unit of enzymatic activity (SP U) was considered as equivalent to the decrease in the optical density of kynuramine of 0.001 per minute.

5) Determination of total proteins:

The total protein content of each sample was estimated by a modified biuret reaction (194). The biuret reagent was prepared following the method of Gornall et al (195).

The procedure was as follows: 0.4 ml of the sample under study, and 0.4 ml of 20% trichloroacetic acid were pipetted into a conical bottom centrifuge tube and well mixed. After 10 minutes of centrifugation at 1,000 r.p.m. in a International Centrifuge, the supernatant was discarded. Then 8 ml of biuret reagent and 2 ml of distilled water were added, and the contents of the tube stirred with a glass rod at intervals until all the protein had dissolved (i.e. 5 hours). At this point, when brain samples were assayed, the protein-biuret solutions were cloudy due to a suspension of lipid material which was extracted using 2 ml of petrol-ether (b.p. 60 to 80). The blank was prepared by mixing 8 ml of biuret reagent with 2 ml of distilled water. The optical density was measured at 540 m $\mu$ . The amount of total proteins present in the aliquot was determined by referring to a standard curve constructed by using as protein standard crystalline bovine serum

albumine (Sigma Chemicals Co.). There was a linear relationship between protein and optical density with quantities of protein below 14 mg.

6) Statistical analysis:

The experimental results were analyzed for statistical significance using the student-t-test.

For comparative purposes the obtained results in control animals were assigned on hypothetic value of 100, and the increase or decrease of the corresponding parameter in hyperthyroid rats was expressed as per cent of the control.

## II. RESULTS

### A) THE BODY AND ORGAN WEIGHT AND THE OXYGEN CONSUMPTION IN CONTROL AND THYROTOXIC RATS:

In order to ascertain the effect of the employed diets on the general state and thyroid function of the animals various biological parameters were recorded.

Table 1 shows the initial and final body weights of rats under the described experimental conditions. As expected the final body weight of hyperthyroid rats was

Table 1

The total body weight in control and hyperthyroid rats

Experimental conditions <sup>1</sup>	Number of animals	Initial weight (in grams) <sup>2</sup>	Final weight (in grams) <sup>2</sup>
Control diet	30	50	321 ± 35
Hyperthyroid diet	26	50	218 ± 31 <sup>†</sup>

<sup>1</sup> The animals were fed control or thyrotoxic diet (7 weeks).  
<sup>2</sup> Means are given ± the standard deviation of the mean.  
<sup>†</sup> P < 0.001 as compared to control animals.

Table 2

The oxygen consumption of control and hyperthyroid rats

Experimental conditions <sup>1</sup>	Number of animals	Oxygen consumed (ml/min/kg body) <sup>2</sup>	Increase (%)
Control diet	12	22.65 ± 2.40	-
Hyperthyroid diet	11	48.45 ± 4.10 <sup>†</sup>	113.9

<sup>1</sup> The animals were fed control or thyrotoxic diet (7 weeks).  
<sup>2</sup> Means are given ± the standard deviation of the mean.  
<sup>†</sup> P < 0.001 as compared to control animals.

lower (34%) than that of control animals.

In table 2, it can be seen that the rats fed the iodinated casein diet were hypermetabolic since their oxygen consumption rate increased by 113.9% over those which had been given a control diet. This increase in oxygen consumption was quite constant as shown by the value of the standard deviation.

Table 3 presents the weights of various organs in control and hyperthyroid rats. The weight of brain and kidneys was similar in both groups of animals.

The liver weighed slightly less in hyperthyroid than in control rats. Another feature of hyperthyroidism, cardiac hypertrophy, was observed in the experimental animals. The heart weight of iodinated casein-fed rats was increased by 48% as compared to control animals (table 3).

B) THE EFFECT OF HYPERTHYROIDISM ON THE TOTAL PROTEINS AND MAO ACTIVITY OF VARIOUS SUBCELLULAR FRACTIONS OBTAINED FROM DIFFERENT RAT TISSUES:

Table 4 shows the total proteins and MAO activity in the cytoplasmic, mitochondrial and microsomal fractions from the liver of control rats. MAO activity is indicated in spectrophotometric units per mg of tissue or per mg of total protein. It can be observed that most of the hepatic MAO activity was confined to the mitochondrial

Table 3

The total weight of several organs in control and hyperthyroid rats

Experimental <sup>1</sup> conditions	LIVER <sup>2</sup>	HEART <sup>2</sup>	BRAIN <sup>2</sup>	KIDNEYS <sup>2</sup>
Control diet	11.95 ± 1.76 (26)	0.89 ± 0.09 (30)	1.78 ± 0.08 (25)	2.52 ± 1.3 (25)
Hyperthyroid diet	10.67 ± 1.15 (21)	1.32 ± 0.19 (26)	1.74 ± 0.07 (21)	2.40 ± 0.4 (20)
Significance	P < 0.01	P < 0.001	NS	NS

<sup>1</sup> The animals were fed control or hyperthyroid diet for seven weeks.

<sup>2</sup> Weights indicated in grams. Means are given ± standard deviation of the mean. The digits in brackets represent the number of animals.

fraction.

In table 5, it can be seen that in hyperthyroid rats the total liver proteins showed a non significant increase in the cytoplasmic fraction, a non significant decrease in the mitochondrial fraction and a significant increase of 21% in the microsomal fraction. In the same table it can be observed that in the cytoplasmic fraction MAO activity was decreased by some 53% in hyperthyroid animals regardless of whether the enzymatic activity was expressed per mg of tissue or per mg of protein.

In the mitochondrial fraction from the liver of thyrotoxic rats, the monoamine oxidase activity was approximately 50% lower than the enzymatic activity of similar fractions from control animals. In the microsomal fraction no inhibition was found when MAO activity was calculated per mg of tissue whereas a significant inhibition of 21% was demonstrated when MAO activity was expressed per mg of protein.

The MAO activity in various subcellular fraction from the heart of control rats was also measured. The microsomal fraction contained as much MAO per mg of tissue as the mitochondrial fraction as can be seen in table 6. Nevertheless the specific MAO activity in the microsomes was 7.17 while in the mitochondria it was 2.95.

Table 4

Total proteins and MAO activity in various subcellular fractions obtained from the liver of rats fed with control diet during seven weeks

RAT #	CYTOPLASMIC FRACTION <sup>1</sup>			MITOCHONDRIAL FRACTION			MICROSOMAL FRACTION		
	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)
1	13.95	0.83	5.97	4.68	0.62	13.33	3.69	0.10	4.57
2	15.18	0.82	5.43	4.21	0.59	14.00	4.21	0.08	2.97
3	14.50	0.95	6.60	4.46	0.65	14.70	4.46	0.10	3.80
4	13.65	0.94	6.92	4.62	0.67	14.69	4.28	0.11	3.99
5	14.50	0.82	5.71	5.07	0.67	13.33	3.82	0.10	4.67
6	16.31	0.84	5.18	4.37	0.68	15.60	4.40	0.09	3.54
MEAN	14.68	0.87	5.97	4.57	0.65	14.27	4.15	0.10	3.93
SDM	±0.95	±0.06	±0.67	±0.30	±0.03	±0.89	±0.31	±0.01	±0.63

<sup>1</sup> Total homogenate minus nuclei.

SPU: Spectrophotometric units as described under methods. Kynuramine was used as the substrate.  
SDM: Standard deviation of the mean.

Table 5

Total proteins and MAO activity in various subcellular fractions obtained from the liver of rats fed with thyrotoxic diet during seven weeks

RAT #	CYTOPLASMIC FRACTION <sup>1</sup>			MITOCHONDRIAL FRACTION			MICROSOMAL FRACTION		
	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)	Total protein (mg%)	MAO activity SPU/mg tissue)	MAO activity (SPU/mg protein)	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)
1	15.75	0.43	2.77	4.20	0.32	7.61	5.66	0.10	2.92
2	16.76	0.45	2.70	4.10	0.31	7.61	6.32	0.14	3.61
3	16.49	0.46	2.82	4.06	0.33	8.33	4.95	0.08	2.78
4	14.31	0.40	2.84	3.51	0.30	8.62	4.00	0.08	2.88
5	16.31	0.35	2.15	3.04	0.22	7.47	4.37	0.09	3.58
6	14.86	0.42	2.84	4.02	0.29	7.34	5.05	0.09	2.84
MEAN	15.75	0.42	2.69	3.82	0.29	7.83	5.06	0.10	3.10
SDM	±0.97	±0.04	±0.26	±0.50	±0.03	±0.51	±0.84	±0.02	±0.38
CHANGE <sup>2</sup>	7.28	-51.72	-54.94	-16.40	-55.38	-45.13	21.93	0.00	-21.12
p <sup>3</sup>	NS	<0.001	<0.001	NS	<0.001	<0.001	<0.05		<0.025

<sup>1</sup> Total homogenate minus nuclei.

<sup>2</sup> Per cent of increase or decrease as compared with the control values indicated in table 4.

<sup>3</sup> Significance as compared with control (table 4). NS, non significant change.

SPU: Spectrophotometric units as described under methods. Kynuramine was used as the substrate. SDM: Standard deviation of the mean.

In the heart of hyperthyroid rats (table 7) the total protein content was significantly increased by 15% in the cytoplasmic fraction. This increase was found to occur in the microsomal fraction (112%) whereas in the mitochondrial fraction the total proteins accused a non significant decrease. The MAO activity in the iodinated casein-fed rats was markedly increased, and though the enzymatic activity was increased in the mitochondrial fraction (39% when comparing activities per mg tissue), the more striking MAO increase was localized in the microsomal fraction (173%).

In tables 8 and 9 it can be seen that MAO is mainly localized in the mitochondrial fraction of brain tissues in control and hyperthyroid rats, and that no significant changes of this enzyme occur when the animals are fed with iodinated casein. The total proteins remained also unchanged in the brain of hyperthyroid rats. The same thing can be said when kidney samples were studied (table 10 and 11), except for an isolated increase in the total proteins of the mitochondrial fraction.

C) THE TOTAL PROTEIN AND MAO ACTIVITY IN VARIOUS SUBCELLULAR FRACTIONS OBTAINED FROM THE LIVER AND HEART OF HYPERTHYROID RATS FOLLOWING THE ADMINISTRATION OF PENTOBARBITAL:

Table 6

Total proteins and MAO activity in various subcellular fractions obtained from the heart of rats fed with control diet during seven weeks

RAT #	CYTOPLASMIC FRACTION <sup>1</sup>			MITOCHONDRIAL FRACTION			MICROSOMAL FRACTION		
	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)
1	7.70	0.167	2.17	2.83	0.081	2.85	0.96	0.063	6.54
2	7.97	0.197	2.47	2.73	0.094	3.45	1.12	0.077	6.90
3	7.88	0.213	2.70	2.69	0.081	3.00	1.62	0.109	6.77
4	7.59	0.209	2.76	3.51	0.097	2.76	1.01	0.074	7.33
5	7.70	0.197	2.55	2.69	0.074	2.76	0.98	0.079	8.05
6	7.88	0.207	2.63	2.81	0.081	2.91	1.53	0.114	7.47
MEAN	7.78	0.198	2.54	2.88	0.086	2.95	1.20	0.086	7.17
SDM	±0.14	±0.015	±0.21	±0.51	±0.008	±0.26	±0.29	±0.020	±0.55

<sup>1</sup> Total homogenate minus nuclei.

SPU: Spectrophotometric units as defined under methods. Kynuramine was used as the substrate.

SDM: Standard deviation of the mean.

Table 7

Total proteins and MAO activity in various subcellular fractions obtained from the heart of rats fed with thyrotoxic diet during seven weeks

RAT #	CYTOPLASMIC FRACTION <sup>1</sup>			MITOCHONDRIAL FRACTION			MICROSOMAL FRACTION		
	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)
1	9.03	0.377	4.18	1.77	0.094	5.33	2.63	0.246	9.17
2	8.73	0.330	3.77	2.53	0.093	3.68	2.34	0.217	9.30
3	8.47	0.285	3.36	2.21	0.089	4.05	2.46	0.188	8.09
4	8.78	0.326	3.71	2.77	0.101	3.66	2.18	0.207	9.47
5	9.45	0.449	4.77	2.50	0.150	6.04	3.46	0.328	9.48
6	9.53	0.439	4.61	3.16	0.166	5.27	2.56	0.253	9.40
7	8.92	0.380	4.26	2.45	0.148	6.04	2.27	0.212	9.55
MEAN	8.99	0.369	4.09	2.48	0.120	4.87	2.55	0.235	9.18
SDM	±0.38	±0.060	±0.50	±0.43	±0.030	±0.98	±0.42	±0.045	±0.49
CHANGE <sup>2</sup>	15.55	86.36	61.02	-13.88	39.53	65.08	112.00	173.00	28.03
P <sup>3</sup>	<0.001	<0.001	<0.001	NS	<0.02	<0.001	<0.001	<0.001	<0.001

<sup>1</sup> Total homogenate minus nuclei.

<sup>2</sup> Per cent of increase or decrease as compared with the control values indicated in table 6.

<sup>3</sup> Significance as compared with control (table 6). NS, non significant change.

SPU: Spectrophotometric units as defined under methods. Kynuramine was used as the substrate. SDM: Standard deviation of the mean.

Table 8

Total proteins and MAO activity in various subcellular fractions obtained from the brain of rats fed with control diet during seven weeks

RAT #	CYTOPLASMIC FRACTION <sup>1</sup>			MITOCHONDRIAL FRACTION			MICROSOMAL FRACTION		
	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)
1	7.42	0.216	2.92	4.64	0.153	3.29	2.46	0.035	1.45
2	8.33	0.194	2.33	6.01	0.137	2.28	2.12	0.031	1.47
3	7.79	0.181	2.32	5.68	0.124	2.19	1.93	0.044	2.30
4	7.97	0.221	2.77	5.35	0.149	2.79	2.50	0.039	1.57
5	8.88	0.190	2.14	5.79	0.129	2.24	2.10	0.032	1.55
MEAN	8.08	0.201	2.50	5.50	0.138	2.56	2.22	0.036	1.67
SDM	±0.55	±0.017	±0.33	±0.53	±0.012	±0.47	±0.24	±0.002	±0.35

<sup>1</sup> Total homogenate minus nuclei.

SPU: Spectrophotometric units as defined under methods. Kynuramine was used as the substrate.

SDM: Standard deviation of the mean.

Table 9

Total proteins and MAO activity in various subcellular fractions obtained from the brain of rats fed with thyrotoxic diet during seven weeks

RAT #	CYTOPLASMIC FRACTION <sup>1</sup>			MITOCHONDRIAL FRACTION			MICROSOMAL FRACTION		
	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)
1	9.45	0.210	2.22	6.53	0.116	1.78	2.75	0.046	1.68
2	8.01	0.168	2.10	5.15	0.121	2.35	2.06	0.038	1.85
3	9.24	0.233	2.52	6.01	0.129	2.15	2.18	0.040	1.78
4	7.59	0.168	2.22	5.90	0.123	2.08	1.95	0.034	1.75
5	9.45	0.231	2.55	6.45	0.135	2.10	1.64	0.029	1.80
MEAN	8.75	0.202	2.32	6.02	0.125	2.10	2.11	0.037	1.77
SDM	±0.88	±0.031	±0.19	±0.54	±0.007	±0.21	±0.40	±0.006	±0.06
CHANGE <sup>2</sup>	8.29	0.0	-7.20	9.40	-9.42	-17.96	-4.95	2.60	5.98
P <sup>3</sup>	NS	NS	NS	NS	NS	NS	NS	NS	NS

- 1 Total homogenate minus nuclei.
  - 2 Per cent of increase or decrease as compared with the control values indicated in table 8.
  - 3 Significance as compared with control (table 8). NS, non significant change.
- SPU: Spectrophotometric units as defined under methods. Kynuramine was used as the substrate.  
SDM: Standard deviation of the mean.

Table 10

Total proteins and MAO activity in various subcellular fractions obtained from the kidneys of rats fed with control diet during seven weeks

RAT #	CYTOPLASMIC FRACTION <sup>1</sup>			MITOCHONDRIAL FRACTION			MICROSOMAL FRACTION		
	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)
1	10.87	0.090	0.83	2.30	0.042	1.81	3.00	0.024	0.82
2	10.87	0.077	0.71	2.91	0.039	1.34	3.47	0.019	0.56
3	11.41	0.077	0.67	2.65	0.043	1.65	3.21	0.025	0.78
4	10.78	0.095	0.88	2.34	0.041	1.75	2.76	0.026	0.95
MEAN	10.98	0.085	0.77	2.55	0.041	1.64	3.11	0.023	0.78
SDM	±0.29	±0.009	±0.10	±0.28	±0.003	±0.20	±0.30	±0.002	±0.10

<sup>1</sup> Total homogenate minus nuclei.

SPU : Spectrophotometric units as defined under methods. Kynuramine was used as the substrate.

SDM : Standard deviation of the mean.

Table 11

Total proteins and MAO activity in various subcellular fractions obtained from the kidneys of rats fed with thyrotoxic diet during seven weeks

RAT #	CYTOPLASMIC FRACTION <sup>1</sup>			MITOCHONDRIAL FRACTION			MICROSOMAL FRACTION		
	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)
1	11.20	0.097	0.86	2.95	0.046	1.58	3.43	0.027	0.78
2	11.96	0.096	0.80	3.71	0.054	1.47	3.28	0.020	0.61
3	10.87	0.077	0.71	3.08	0.039	1.18	3.53	0.023	0.63
4	10.87	0.081	0.75	3.56	0.047	1.33	2.96	0.015	0.76
5	10.46	0.097	0.92	3.60	0.050	1.39	3.31	0.023	0.70
6	10.80	0.098	0.91	3.04	0.048	1.60	3.37	0.022	0.86
MEAN	11.02	0.091	0.82	3.32	0.047	1.42	3.31	0.021	0.72
SDM	±0.51	±0.009	±0.08	±0.33	±0.005	±0.15	±0.19	±0.003	±0.07
CHANGE <sup>2</sup>	0.36	5.80	6.40	30.19	11.19	-13.41	6.43	-4.34	-6.69
P <sup>3</sup>	NS	NS	NS	<0.05	NS	NS	NS	NS	NS

1 Total homogenate minus nuclei.

2 Per cent of increase or decrease as compared with the control values indicated in table 10.

3 Significance as compared to control (table 10). NS, non significant change.

SPU: Spectrophotometric units as defined under methods. Kynuramine was used as the substrate.

SDM: Standard deviation of the mean.

During preliminary experiments we had observed a very low MAO activity in the heart of hyperthyroid rats. Thus inhibition of this enzyme by the barbiturate was suspected and it was decided to investigate this possibility.

In table 12 it can be seen that the injection of 30 mg/kg of pentobarbital produced a significant inhibition of some 20% in the cytoplasmic fraction obtained from the liver of control rats. The animals were sacrificed 15 minutes after treatment. In the mitochondrial fraction the enzymatic activity was decreased by only 10% (when expressed per mg of tissue), and in the microsomal fraction MAO was inhibited by 20 or 30% when expressed per mg of protein or per mg of tissue respectively. In this group of experiments the total protein values were similar to those of control animals.

If one compares the protein contents of the various cytoplasmic fractions of liver from hyperthyroid rats treated with pentobarbital (table 13) it can be seen that these values are comparable to those observed in hyperthyroid animals without barbiturate pretreatment (table 5). Nevertheless, in the liver of hyperthyroid rats pretreated with the barbiturate the cytoplasmic MAO activity was inhibited by some 63% as compared with the enzymatic activity of euthyroid animals without barbiturate. In the mito-

Table 12

The effect of sodium pentobarbital (30 mg/kg body)<sup>†</sup> on the total proteins and MAO activity in various subcellular fractions obtained from the liver of rats fed with control diet during seven weeks

RAT #	CYTOPLASMIC FRACTION <sup>1</sup>			MITOCHONDRIAL FRACTION			MICROSOMAL FRACTION		
	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)
1	14.88	0.651	5.14	4.58	0.623	14.18	4.39	0.075	3.11
2	14.50	0.622	4.31	4.48	0.580	13.85	4.54	0.070	3.06
3	14.86	0.660	4.96	4.71	0.570	14.00	4.49	0.069	3.19
4	15.00	0.730	5.00	4.84	0.572	14.36	4.13	0.068	3.03
MEAN	14.81	0.665	4.85	4.65	0.586	14.09	4.38	0.070	3.11
SDM	±0.21	±0.045	±0.36	±0.16	±0.024	±0.22	±0.18	±0.003	±0.05
CHANGE <sup>2</sup>	0.88	-23.56	-18.76	-1.75	-10.76	-1.30	5.54	-30.00	-20.86
P <sup>3</sup>	NS	0.001	0.01	NS	0.01	NS	NS	0.001	0.05

<sup>†</sup> A single intraperitoneal injection of the barbiturate was given 15 minutes before the sacrifice

<sup>1</sup> Total homogenate minus nuclei.

<sup>2</sup> Per cent of increase or decrease as compared with the control values indicated in table 4.

<sup>3</sup> Significance as compared with control (table 4). NS, non significant change.

SPU: Spectrophotometric units as defined under methods. Kynuramine was used as the substrate. SDM: Standard deviation of the mean.

Table 13

The effect of sodium pentobarbital (30 mg/kg body)<sup>†</sup> on the total proteins and MAO activity in various subcellular fractions obtained from the liver of rats fed with thyrototoxic diet during seven weeks

RAT #	CYTOPLASMIC FRACTION <sup>1</sup>			MITOCHONDRIAL FRACTION			MICROSOMAL FRACTION		
	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)
1	15.66	0.320	2.02	3.54	0.241	6.94	4.54	0.082	2.02
2	16.07	0.381	2.38	4.02	0.263	6.95	4.81	0.085	2.23
3	15.40	0.340	2.18	3.82	0.230	6.90	4.81	0.075	2.43
4	16.31	0.292	1.78	3.73	0.232	6.50	4.77	0.070	2.38
MEAN	15.86	0.333	2.09	3.77	0.241	6.82	4.73	0.078	2.27
SDM	±0.40	±0.035	±0.25	±0.19	±0.015	±0.21	±0.13	±0.006	±0.18
CHANGE <sup>2</sup>	8.03	-61.72	-64.99	-17.50	-62.92	-52.20	13.92	-22.00	-42.23
P <sup>3</sup>	NS	0.001	0.001	0.01	0.001	0.001	0.05	0.01	0.001

<sup>†</sup> A single intraperitoneal injection of the barbiturate was given 15 minutes before the sacrifice.

<sup>1</sup> Total homogenate minus nuclei.

<sup>2</sup> Per cent of increase or decrease as compared with the control values indicated in table 4.

<sup>3</sup> Significance as compared with control (table 4). NS, non significant change.

SPU:Spectrophotometric units as defined under methods. Kynuramine was used as the substrate.

SDM:Standard deviation of the mean.

chondrial fraction obtained from the liver of hyperthyroid rats pretreated with pentobarbital MAO activity was inhibited by some 57%, and in the microsomal fraction MAO was inhibited by 22% when expressed per mg of tissue and by 42% when indicated per mg of protein.

The effect of 30 mg/kg of pentobarbital on the MAO activity in different subcellular fractions from the heart of control rats was examined and the data are presented in table 14. Fifteen minutes after the administration of the drug, the enzymatic activity was inhibited by 35% in the cytoplasmic fraction, by 21 or 30% in the mitochondrial fraction, and by 33 or 40% in the microsomal fraction when MAO activity was expressed per mg of tissue or per mg of protein respectively.

Table 15 shows the total protein and MAO activity pattern in the heart of hyperthyroid rats pretreated with the barbiturate, as compared with the observed values in control rats without barbiturate pretreatment. It can be seen that MAO activity was inhibited by 57% in the cytoplasmic fraction. In the mitochondrial fraction the enzymatic activity was decreased by 26% (per mg of tissue), and in the microsomal fraction the enzyme was inhibited by 43% (per mg of protein).

When the monoamine oxidase activity in the heart

Table 14

The effect of sodium pentobarbital (30 mg/kg body)<sup>†</sup> on the total proteins and MAO activity in various subcellular fractions obtained from the heart of rats fed with control diet during seven weeks

RAT #	CYTOPLASMIC FRACTION <sup>1</sup>				MITOCHONDRIAL FRACTION				MICROSOMAL FRACTION			
	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)	MAO activity (SPU/mg tissue)	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)	MAO activity (SPU/mg tissue)	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)	MAO activity (SPU/mg protein)
1	7.40	0.110	1.50	0.065	2.45	0.065	2.66	0.060	1.40	0.060	4.29	4.29
2	7.89	0.126	1.60	0.054	2.53	0.054	2.15	0.054	1.32	0.054	4.12	4.12
3	7.98	0.133	1.66	0.062	3.00	0.062	2.08	0.065	1.36	0.065	4.80	4.80
4	7.88	0.142	1.81	0.061	2.65	0.061	2.33	0.052	1.26	0.052	3.96	3.96
MEAN	7.78	0.128	1.64	0.060	2.66	0.060	2.31	0.057	1.34	0.057	4.29	4.29
SDM	±0.026	±0.013	±0.13	±0.004	±0.24	±0.004	±0.25	±0.005	±0.06	±0.005	±0.36	±0.36
CHANGE <sup>2</sup>	0.0	-35.35	-35.43	-30.00	-7.64	-30.00	-21.69	-33.72	11.60	-33.72	-40.16	-40.16
P <sup>3</sup>	<0.001	<0.001	<0.001	<0.001	NS	<0.001	<0.005	<0.05	NS	<0.05	<0.001	<0.001

<sup>†</sup> A single intraperitoneal injection of the barbiturate was given 15 minutes before the sacrifice

1 Total homogenate minus nuclei.

2 Per cent of increase or decrease as compared with the control values indicated in table 6.

3 Significance as compared with control (table 6). NS, non significant change.

SPU: Spectrophotometric units as defined under methods. Kynuramine was used as the substrate.

SDM: Standard deviation of the mean.

Table 15

The effect of sodium pentobarbital (30 mg/kg body)<sup>†</sup> on the total proteins and MAO activity in various subcellular fractions obtained from the heart of rats fed with thyrotoxic diet during seven weeks

RAT #	CYTOPLASMIC FRACTION <sup>1</sup>			MITOCHONDRIAL FRACTION			MICROSOMAL FRACTION		
	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)	Total protein (mg%)	MAO activity (SPU/mg tissue)	MAO activity (SPU/mg protein)
1	8.90	0.084	0.95	2.54	0.061	2.42	2.54	0.097	3.85
2	8.36	0.085	1.02	3.05	0.065	2.95	2.33	0.100	4.29
3	8.39	0.102	1.22	2.34	0.058	2.50	2.95	0.112	3.81
4	8.42	0.072	0.87	2.65	0.070	2.88	2.72	0.114	4.16
MEAN	8.52	0.085	1.02	2.64	0.063	2.68	2.63	0.105	4.02
SDM	±0.26	±0.011	±0.15	±0.30	±0.005	±0.27	±0.26	±0.008	±0.23
CHANGE	210.95	-57.08	-59.90	-8.40	-26.75	-9.20	119.16	22.09	-43.94
P <sup>3</sup>	<0.05	<0.001	<0.001	NS	<0.001	NS	<0.001	NS	<0.001

<sup>†</sup> A single intraperitoneal injection of the barbiturate was given 15 minutes before the sacrifice.

<sup>1</sup> Total homogenate minus nuclei.

<sup>2</sup> Per cent of increase or decrease as compared with the control values indicated in table 6.

<sup>3</sup> Significance as compared with control (table 6). NS, non significant change.

SPU: Spectrophotometric units as defined under methods. Kynuramine was used as the substrate. SDM: Standard deviation of the mean.

of pentobarbital-treated thyrotoxic rats was compared with the corresponding values in hyperthyroid rats without barbiturate pretreatment the inhibition was more pronounced (75% in the cytoplasmic fraction, some 45% in the mitochondrial fraction and 56% in the microsomal fraction).

The total proteins in the heart of hyperthyroid rats treated with pentobarbital was similar to those of the cardiac tissue in the thyrotoxic animals without previous administration of barbiturate.

### III. DISCUSSION

Male rats were fed an iodinated casein diet during seven weeks. At the end of this interval of time their oxygen consumption was 113.9% higher than in rats fed with a control diet. These data are a good indication that the iodinated casein-fed animals had reached a marked hyperthyroid state. In addition, in our hyperthyroid rats the final body weight was lower and the heart weight was higher than in the control animals. These results are in agreement with previous reports on rats fed with a similar diet (141).

In the oxygen consumption determination the use of an anesthetic could not be avoided. When the rat was not under complete anesthesia it moved and the readings were erratic. The metabolic apparatus was very convenient for routine measurements and the results obtained were reproducible.

After sacrifice several subcellular fractions were separated simultaneously from various tissues.

Since our main interest was the determination of MAO activity in mitochondrial and microsomal fractions, the nuclear fraction was discarded. The cytoplasmic fraction (total homogenate minus cellular debris and nuclei) was employed to determine the overall enzymatic activity of every tissue. It can be observed in the tabulated results that the amount of total proteins and MAO activity for each subcellular fraction were reproducible in separate experiments.

The decrease of liver MAO activity in several species following thyroid hormones administration had been reported (131, 132, 82, 136, 99, 141). In our experiments this inhibition of MAO activity in hyperthyroid rats was confirmed. We observed some 53% less overall MAO activity in the liver of rats fed with iodinated casein diet than in control animals. It is difficult to compare the extent

of this decrease to that found by other workers since the experimental conditions very often were different. Some differences were the method used to induce hyperthyroidism, the degree of thyrotoxicosis, the substrate employed for the enzymatic assay and the species of animals under study. Generally speaking however, it can be said that the decrease we have observed is of the order of that reported in several publications (82, 136).

When various subcellular fractions were separated it was found that in the liver of hyperthyroid rats mitochondrial MAO was inhibited by some 50%. In the microsomal fraction MAO activity was unchanged when expressed per mg of tissue and it was decreased by 21% when calculated per mg of protein. These results strongly suggest that the inhibitory effect of thyroid hormones on liver MAO is mainly exerted on the mitochondrial enzyme.

Considering the marked decrease of MAO activity in the liver of hyperthyroid rats and that MAO in liver is mainly localized in the mitochondria, the inhibition of the enzyme in this fraction was not surprising. However, we expected as well inhibition of the microsomal enzyme since it was reported that following the injection of radioactive thyroxine into rats, most of the radioactivity is localized in the microsomal fraction of liver (167).

The amount of total proteins in the liver of hyperthyroid rats was increased by a non significant 7% as compared with control animals. The microsomes of hyperthyroid rats exhibited a significant increase of 21% in protein content while in the mitochondrial fraction a non significant decrease of 16% was observed. An increased synthesis of proteins in the presence of thyroid hormones has been demonstrated (38, 39, 40, 41). Sokoloff and Kaufman (38, 39) have reported that this increase in protein synthesis takes place mainly in the mitochondria and in our experiments the total proteins of this subcellular fraction was rather decreased. Nevertheless our results are in agreement with the report of Tata et al (40, 41), who found an increased incorporation of leucine and isoleucine into the microsomal fraction obtained from the liver of triiodothyronine-treated rats.

When the homogenates from the heart of control rats were separated in various subcellular fractions it was found that the microsomal fraction contained as much MAO units per mg of tissue as the mitochondrial fraction, and that the specific enzymatic activity in the microsomes was more than twice the specific MAO activity found in the mitochondria. De Champlain et al (121) have reported that in the heart, salivary glands and vas deferens

of rats a considerable amount of MAO is localized in the microsomal fraction (50% in the case of heart). It is difficult to compare our results with those of De Champlain et al, since our method of separation of the subcellular fractions was different and their paper does not indicate if MAO activity was calculated per mg of tissue or per mg of protein. Nevertheless we can say that our results are in agreement with their report and that the subcellular distribution pattern of MAO in the heart of rats is completely different from that found in the liver.

The significance of the microsomal localization of MAO in the heart is not clear. The fact that heart MAO activity increases with the mass and growth of that organ in the rat (111) would suggest that in this organ the enzyme may be associated with the process of protein synthesis in the endoplasmic reticulum. However the microsomes are an heterogeneous fraction constituted by elements of endoplasmic reticulum, granules, vesicles and membranes of different subcellular origin, and the exact localization of MAO within these particles is difficult to establish. On the other hand, when the subcellular distribution of H<sup>3</sup>-noradrenaline and MAO was studied on the heart by means of a sucrose gradient (121), a very close association could be observed between the distribution

of the amine and that of MAO. This distribution pattern together with the observation of decreased MAO activity in the microsomal fraction of the salivary glands after denervation (121) could indicate that in these tissues a fraction of MAO might be localized within the noradrenaline storage particles. However, further investigation will be necessary in order to clarify the meaning of this different subcellular localization of MAO in various tissues.

In the heart of hyperthyroid animals (table 7) the amount of total proteins showed a significant 15.5% increase as compared with control animals (table 6). Again this protein increase was found to take place in the microsomal fraction and in this tissue the increase was more marked than in the microsomal fraction of the liver.

MAO activity was markedly increased in the heart of hyperthyroid rats, and our results are comparable to previous reports (135). This increase was reflected in both the mitochondrial and the microsomal fraction. However if we consider the total amount of MAO activity found per mg of heart tissue, it is evident that microsomal MAO experimented almost a five fold increase as compared to the increase of the enzyme in the mitochondria.

It is difficult to find a satisfactory explanation for the effects of thyroid hormones on MAO. One possibility is that in the heart of hyperthyroid rats MAO is elevated as a consequence of the overall protein increase. Nevertheless in the liver of the same type of animals MAO is inhibited and in the microsomal fraction of the same tissue the enzyme is not increased despite the evident elevation of total proteins. In consequence, this explanation is not viable at least for the inhibitory effect of thyroid hormones on MAO activity in the liver of rats.

Since FAD may be a coenzyme for MAO (114, 115, 143) and Rivlin and Langdon (71) have postulated that thyroid hormones may regulate the hepatic levels of FAD, thyroid hormones could affect MAO through their action on FAD. However, FAD decreases in the liver of hypothyroid rats and it is found in normal levels in the same tissue of hyperthyroid animals, while MAO is decreased in the liver of thyrotoxic rats. Then this possibility can also be ruled out as an explanation for the observed MAO decrease in the liver of hyperthyroid animals.

On the other hand, in the heart of hyperthyroid rats the observed MAO increase could be due to the stabilization of the enzyme against degradation rather than to enhanced

synthesis of the apoenzyme. Flavoenzymes are stabilized against degradation by the presence of their coenzymes, and the rate of formation of the coenzymes may be important in regulating enzymatic activity. It has been reported (71) that in rat liver the enzyme flavokinase (which catalyzes one step in the conversion of riboflavin to FAD) is markedly increased in hyperthyroidism. However no direct evidence is available demonstrating this hypothesis in the heart of hyperthyroid rats.

The interpretation of the possible mechanism of action of thyroid hormones on MAO is complicated by the contrary effect observed in the liver and in the heart. This double effect together with our finding of no changes of MAO in the brain and kidneys of hyperthyroid rats could be another indication of the existence of multiple forms of MAO. This enzyme in the liver is mainly localized in the mitochondria while in the heart predominates in the microsomal fraction. There are several reports suggesting the existence of various forms of MAO in the basis of the different specificity of this enzyme for various substrates in the liver, brain, heart and other tissues (116, 117, 142). There are no studies to establish whether in the same tissue the mitochondria contains the same form of MAO than the microsomes. It is possible

that in the liver of rats, thyroid hormones which are mainly metabolized in the microsomes, inhibit mitochondrial MAO through the action of some thyroxine metabolite, while in the heart the demonstrated effect of these hormones on the microsomal protein synthesis could be reflected in the observed increase of MAO activity. Nevertheless, it can be observed in our experiments some increase of enzymatic activity in the mitochondrial fraction of the hyperthyroid heart without demonstration of increased proteins on the same fraction. Evidently more experimental evidence will be necessary before the real significance of this double action of thyroid hormones on MAO can be elucidated.

On the other hand the overall increase of MAO in the hypertrophic heart of hyperthyroid rats seems to be a non-specific effect since it has been also reported increase in MAO activity in the heart hypertrophy induced by other means (151). These data would suggest again that an increased protein synthesis in the heart could give rise to an increase of MAO activity in the same organ.

Since MAO activity increases in the heart of thyrotoxic rats our results would confirm that the catecholamine-like effects of thyroid hormones cannot be accounted for by an increase in the levels of catecholamines due to in-

hibition of MAO.

Another aspect studied in this part of this thesis is the effect of pentobarbital on the monoamine oxidase activity of the liver and heart in control and iodinated casein-fed rats.

In the control animals MAO activity was inhibited by some 20% in the liver and by 35% in the heart 15 minutes after the intraperitoneal administration of 30 mg/kg of the barbiturate. In both tissues it was demonstrated that the inhibitory effect of pentobarbital was more marked in the microsomal fraction.

More marked inhibition of MAO was demonstrated in the liver of hyperthyroid rats treated with pentobarbital (63%) than in the liver of thyrotoxic animals without barbiturate pretreatment (53%). The additional inhibition was mainly reflected in the microsomal fraction.

However the more striking effect of pentobarbital pretreatment on MAO was found in the heart of hyperthyroid rats where instead of the marked increase of MAO found in rats fed with iodinated casein, some 58% inhibition was demonstrated in the cytoplasmic fraction 15 minutes after the injection of the barbiturate. The inhibitory effect was also more marked in the microsomal fraction. The inhibition of various flavoenzymes by barbiturates

is reported in the literature (172, 173, 174, 175), and our findings could be accounted for by this inhibition of several flavoproteins by barbiturates. It is worth to underline the effect of pentobarbital on MAO activity in hyperthyroid hearts. It is known that the mortality of hyperthyroid rats following the administration of barbiturates is much higher than the mortality of normal animals which have been given the same type of compounds (154). In hyperthyroid rats several microsomal drug metabolizing enzymes are increased and various components of the microsomal electron transport system are altered in one way or the other. Most probably by treating hyperthyroid rats with barbiturates one can produce further changes in a microsomal electron transport system which was already working in unfavorable conditions. Perhaps this additional changes in several flavoproteins of the system are enough to disrupt completely the unstable equilibrium producing in the heart an overall inability to cope with vital metabolic oxidations and initiating an arrest in the contraction of the cardiac muscle.

The observed inhibition of MAO by pentobarbital could be produced either by the barbiturate itself or by some metabolite produced by the oxidation of this drug in the microsomes. Some drug metabolizing enzymes are increased

in the liver microsomes of thyroxine-treated rats (161), and this could explain the striking inhibition of MAO in the thyrotoxic heart of rats pretreated with the barbiturate.

It should also be mentioned here that Krakoff et al (150) have reported a 34% inhibition of MAO activity in the hypertrophic ventricles of adults cats with congestive heart failure. They had injected 25 mg/kg of pentobarbital into the animals previous to their sacrifice. These results could indicate that cardiac MAO activity was inhibited following the administration of the barbiturate to cats with cardiac hypertrophy but probably increased previous to the administration of the drug.

PART II. STUDIES ON THE EFFECT OF SEVERAL BARBITURATES  
ON MONOAMINE OXIDASE

Considering the inhibitory effect of pentobarbital on MAO activity (part I of this thesis), it was decided to investigate to some extent the characteristics of this inhibition in various tissues of normal rats. In addition, the effect of some other barbiturates on MAO activity was also investigated.

I. MATERIALS AND METHODS

A) MATERIALS

All the animals used for these experiments were Sprague-Dawley male rats, weighing between 275 and 350 grams unless otherwise indicated.

A sterile aqueous solution of sodium pentobarbital containing 20% propylene glycol, 10% ethanol and 50 mg/ml of the barbiturate was purchased from Abbott Laboratories, Montreal. Sodium pentobarbital in crystalline form was obtained from the same source. The crystalline barbiturate dissolved in 0.25 M phosphate buffer pH 7.4, had an absorption peak at 250 m $\mu$  (37<sup>o</sup> C).

Sodium thiopental in crystalline form was also supplied by Abbott Laboratories (Penthotal). A fresh 2% solution

in sterile distilled water was prepared from the sealed ampoules before the compound was injected into the animals.

Sodium phenobarbital for injection was obtained from the same source. The sealed ampoules contained 120 mg of the barbiturate dissolved in 1 ml of propylene glycol.

Pentobarbital alcohol, 5-ethyl-1-5-(3-hydroxy-1-methylbutyl)-barbituric acid, was supplied by Aldrich chemical Co. and was dissolved before its administration to the animals in an aqueous solution containing 20% propylene glycol and 10% ethanol. For in vitro experiments, various molar concentrations of this compound were prepared in 0.5 M phosphate buffer pH 7.4.

Solutions of different substrates for MAO containing 0.6  $\mu$ mole/ml were prepared as follows:

Kynuramine dihydrobromide (Regis Chemical Co.) was dissolved in distilled water (9.58 mg/50 ml).

Serotonin (5-hydroxytryptamine creatinine sulfate from Sigma Chemical Co., mol wt 407) was also dissolved in distilled water (12.2 mg/50 ml).

Tyramine (4-hydroxyphenethylamine.HCl from Calbiochem, mol wt 173.7) was dissolved in distilled water (10.4 mg/100 ml).

Redistilled benzylamine was obtained from Eastman

Organic Chemicals (mol wt 107.6). Ten ml of 1 N H<sub>2</sub>SO<sub>4</sub> were added to 1.07 gm of this amine and the mixture was made up to 100 ml with distilled water (198). 0.6 ml of this stock solution was made up to 100 ml with distilled water and this final solution was employed in the enzymatic reaction.

The solutions of all the substrates were kept in the refrigerator.

B) METHODS

1) Preparation of animals:

All the animals had been fed regular laboratory diet and tap water ad libitum.

Several types of experiments were performed:

In some experiments, the effect of single doses of several barbiturates on the monoamine oxidase activity of various tissues of rats was investigated. Different single doses of pentobarbital, pentobarbital alcohol, thiopental or phenobarbital were injected intraperitoneally into the animals at several intervals of time before the sacrifice. When a dose equivalent to 90 mg/kg of pentobarbital was given 5% of the rats died and these animals were discarded. The control animals were injected with the solvent for the barbiturate under study (see under materials).

The animals were sacrificed by decapitation, exsan-

guinated and the tissues quickly removed.

In another set of experiments, the monoamine oxidase activity of several rat tissues was determined following the chronic administration of pentobarbital to the animals. Sprague-Dawley male rats weighing 250 grams were injected intraperitoneally 30 mg/kg of sodium pentobarbital daily during 20 days. Control animals were injected with 0.6 ml of the solvent for the barbiturate for the same period of time. The weight of the rats was recorded every two days and the dose of the barbiturate was adjusted to the change in this parameter. The 21st day the animals were weighed, a last injection of 60 mg/kg of the drug was given and 30, 120 minutes or 48 hours later the animals were sacrificed as indicated for the previous series of experiments.

2) Preparation of tissue homogenates:

The preparation of samples was carried out at 3° C. After the removal of liver, heart, brain and kidneys, each organ was washed in ice-cold isotonic sucrose, blotted in filter paper, cleaned, and finely minced with scissors. Two grams of hepatic tissue, both kidneys, the whole brain and the heart were employed for the subsequent procedure. The tissues were homogenized in an all glass homogenizer in the presence of 3 volumes of 0.25 M sucrose during one

minute. The nuclei and cellular debris were separated in a Sorvall refrigerated centrifuge at 600 x g for ten minutes. The pellet was resuspended in the same volume of 0.25 M sucrose and after a second homogenization and centrifugation at 600 x g for ten minutes both supernatants were combined. The pooled supernatants were treated with 0.5 ml of 0.5 M phosphate buffer pH 7.4 and stored at 2° C for 24 hours before the total proteins and MAO activity were determined.

3) Preparation of subcellular fractions:

The in vitro effect of pentobarbital on MAO activity was investigated using mitochondrial preparations from the liver of normal male Sprague-Dawley rats. MAO activity was also studied in mitochondrial and microsomal fractions of rat liver in the presence of pentobarbital alcohol.

The animals were fasted overnight and the mitochondrial and microsomal pellets were collected following the general schedule described in part I of this work (under methods). The pellets were resuspended in 0.25 M sucrose containing 5% of Lubrol. After the addition of 0.5 ml of 0.5 M phosphate buffer pH 7.4, the preparations were kept at 2° C, pending further determinations.

4) Determination of MAO activity:

In some experiments, the enzymatic activity of the

samples was measured by the spectrophotometric method of Weissbach et al (193) using kynuramine as the substrate. The enzymatic reaction was carried out as described in the first part of this work under methods. The incubation mixture contained 0.2 ml of the enzymatic preparation from the liver, or 0.4 ml of the preparation from the other tissues. As previously indicated, one spectrophotometric unit (SP U) of MAO activity was considered to produce a decrease in the optical density of kynuramine of 0.001 in one minute.

In some other experiments, MAO activity was assayed in several tissues of rats by the oxygen consumption method and using various substrates (kynuramine, serotonin, tyramine and benzylamine).

The incubation mixture was constituted as follows:

- 0.4 ml of phosphate buffer (0.5 M, pH 7.4).
- 0.2 ml of KCN (0.01 M solution).
- 0.2 ml of semicarbazide (0.1 M solution).
- 0.5 ml of substrate solution (0.3  $\mu$ mole).
- 0.2 ml of enzymatic preparation in the case of liver,  
or 0.4 ml in the case of other tissues.
- Boiled distilled water up to a final volume of 3 ml.

This composition is based on the method described by Creasey (199) for the assay of MAO activity using the

oxygen consumption method.

In our experiments, the oxygen consumption was followed on an oxygen monitor (model 53, Yellow Springs Instrument Co.). After the testing and calibration of the oxygen electrode, all the ingredients of the incubation mixture except the enzyme were added to the cuvette at 37° C and were equilibrated with atmospheric air for 3 minutes (while stirred with a magnetic stirrer). At that time the enzymatic preparation was added, the electrode placed in the cuvette and the measurement was started immediately. The electrode response to the decreased oxygen in the medium was recorded on a Varicord recorder (Photovolt Co.). The electrode was calibrated for 100% content of oxygen in the medium when the reaction was started. The oxygen consumption was linear at least during the first four minutes of the reaction. The velocity of the reaction was estimated by drawing the tangent to the recorded line through the origin. This velocity was read as per cent of oxygen consumed in four minutes. The blank oxygen consumption (same reaction mixture without the substrate) was subtracted in each separate determination. The rate of oxygen consumption in the 3 ml mixture was then transformed into  $\mu$ moles of oxygen consumed per hour using the appropriate factors: It was assumed that at

37° C and 760 mmHg air pressure, 1 ml of water dissolves 0.217  $\mu$ mole of oxygen (132). A previous test had shown that the solubility of oxygen in our reaction mixture was not appreciably different from the solubility of oxygen in distilled water under the same conditions. The final results were expressed in  $\mu$ liters of oxygen consumed per hour per mg of total protein.

5) In vitro experiments:

Two types of in vitro experiments were performed:

In some experiments, mitochondrial or microsomal MAO activity was assayed in the presence of various molar concentrations of pentobarbital or pentobarbital alcohol. For these experiments, different dilutions of the drug under study were prepared (see methods) in order to obtain the desired molar concentration of the compound in the final mixture after adding 1 ml of the solution.

MAO activity was assayed following the spectrophotometric method of Weissbach et al (193). The general procedure has been previously described in the first part of this work. The incubation mixture was constituted by 200  $\mu$ moles (0.4 ml) of 0.5 M phosphate buffer pH 7.4, 0.1 ml, enzymatic preparation from rat liver (0.4 ml of cytoplasmic fraction, 0.1 ml of mitochondrial fraction or 0.2 ml of microsomal fraction), 1.0 ml of distilled

water, 1.0 ml of the barbiturate solution under study, and 0.3  $\mu$ mole of kynuramine (0.5 ml). The blank was prepared in the same manner except that the substrate was substituted by the same volume of distilled water. In the control assays in the absence of barbiturates, 1.0 ml of 0.25 M phosphate buffer pH 7.4, was added to the incubation mixture instead of the barbiturate solution. The pH of the incubation mixture was tested in several occasions ( $7.40 \pm 0.05$ ). The substrate was added following the preincubation of the mixture at  $37^{\circ}$  C in a Dubnoff shaker during 15 minutes.

The decrease in the optical density of kynuramine was recorded during the first 6 minutes while the reaction was linear with time. The enzymatic activity was calculated in SP U/mg of protein. The spectrophotometric recording of incubation mixtures containing 0.3  $\mu$ mole of kynuramine and pentobarbital or pentobarbital alcohol during 30 minutes failed to show any change in optical density at 360 m $\mu$  and  $37^{\circ}$  C.

In another series of in vitro experiments, MAO activity was determined in mitochondrial preparations from rat liver using four different concentrations of kynuramine ( $10^{-4}$  M,  $0.4 \times 10^{-4}$  M,  $0.2 \times 10^{-4}$  M and  $10^{-5}$  M) in the absence and in the presence of sodium pentobarbital (final

concentration  $7.5 \times 10^{-3}$  M). The experimental set up has been described for the previous in vitro experiments. The decrease in the optical density of kynuramine was also recorded during the first six minutes of the reaction. Initial velocities were always expressed in the same type of arbitrary spectrophotometric units. In each separate assay the enzymatic activity equivalent to the mitochondrial suspension containing 2 mg of total protein was used. Three different experiments were performed to determine the initial velocity for each molar concentration of kynuramine. In order to plot  $\frac{1}{v}$  versus  $\frac{1}{S}$  ( $v$ , initial velocity, and  $S$ , concentration of the substrate) (Lineweaver and Burk plot) the least squares method (196) was used to find the best-fitting straight line through the various experimental points. One line was obtained in the absence of pentobarbital and another in the presence of the barbiturate. The slope and the intercept of both lines with the vertical axis were calculated following the general method for equations of the first degree (197).

The following general equations were considered for the calculation of  $K_m$  and  $K_i$ :

In the Lineweaver and Burk plot the equation in the absence of inhibitor would be,

$$\frac{1}{v} = \frac{K_m}{V} \frac{1}{S} + \frac{1}{V}$$

Where:  $v$ , initial velocity

$V$ , maximal velocity

$K_m$ , Michaelis constant

$S$ , concentration of the substrate

In this equation  $\frac{K_m}{V}$  is given by the slope of the line and  $\frac{1}{V}$  is the intercept of the line with the vertical axis. This concept can be expressed as follows:

$$\frac{K_m}{V} = \text{slope} \quad (\text{a}) \quad \text{and}$$

$$\frac{1}{V} = \text{intercept, or } V = \frac{1}{\text{intercept}} \quad (\text{b})$$

Substituting (b) in (a)

$$K_m = \frac{\text{slope}}{\text{intercept}} \quad (\text{c})$$

On the other hand, in the presence of a competitive inhibitor the general equation would be,

$$\frac{1}{v} = \frac{K_m}{V} \left(1 + \frac{I}{K_i}\right) + \frac{1}{V}$$

Where:  $I$ , concentration of the inhibitor

$K_i$ , inhibition constant (dissociation constant of the enzyme-inhibitor complex).

Another form of expression for this equation is,

$$\frac{1}{v} = \frac{K_p}{V} \frac{1}{S} + \frac{1}{V} \quad (d)$$

Where:  $K_p = K_m \left(1 + \frac{I}{K_i}\right)$  (e)

In equation (d),  $K_p = \frac{\text{slope}}{\text{intercept}}$  (f)

The  $K_i$  can be calculated from equation (e) after substituting  $K_p$  for its value in equation (f).

6) Determination of proteins:

The total protein content of the samples was determined following the biuret reaction indicated in the first part of this work (see under methods).

7) Statistical analysis:

The experimental results were analyzed for statistical significance following the Student-t-test.

## II. RESULTS

### A) TOTAL PROTEINS AND MAO ACTIVITY IN VARIOUS TISSUES OF NORMAL RATS FOLLOWING THE ADMINISTRATION OF SINGLE DOSES OF SODIUM PENTOBARBITAL:

In the first series of experiments different single

doses of pentobarbital were injected into the animals which were sacrificed at several intervals of time following the treatment.

The total proteins and MAO activity in the liver and heart of control rats after one injection with the solvent for the barbiturate are indicated in table 16. These animals were sacrificed 15 or 30 minutes after the injection. There was no significant difference between the values obtained for both groups.

Table 17 shows the total protein content in the liver of rats sacrificed at different intervals after the intraperitoneal injection of 30, 60 or 90 mg/kg of pentobarbital. The observed values were compared with the results obtained from control animals 30 minutes after the injection of the solvent (see table 16). The experimental results obtained 15 minutes after the administration of the barbiturate were compared with control animals sacrificed 15 minutes after the injection of the solvent for the drug. In all cases, the total proteins were not significantly different in control and pentobarbital-treated rats.

In the same experiments (table 18), the hepatic MAO activity expressed in SPU/mg protein was inhibited up to 28% following the administration of 30 mg/kg

Table 16

Total proteins and MAO activity in the liver and heart of control rats, 15 or 30 minutes after the animals were given 0.5 ml of an aqueous solution containing 20% propylene glycol and 10% ethanol

RAT #	L I V E R				H E A R T			
	Total proteins (mg%)		MAO activity (SPU/mg protein)		Total proteins (mg%)		MAO activity (SPU/mg protein)	
	15'	30'	15'	30'	15'	30'	15'	30'
1	14.12	15.12	3.98	4.18	6.38	6.83	2.91	2.44
2	13.75	15.69	4.00	4.09	7.44	8.00	2.90	2.71
3	16.73	15.69	4.08	4.30	7.00	6.50	2.50	2.31
4	16.33	15.66	4.18	4.26	7.00	5.72	2.71	2.50
5	14.25	14.68	4.04	4.14	8.49	7.85	2.59	2.76
6	13.25	14.00	3.96	4.25	6.55	7.75	2.78	2.42
7		15.18		4.11				2.45
8		15.89		4.25				2.45
MEAN	14.74	15.26	4.04	4.20	7.45	7.11	2.66	2.52
SDM	±1.43	±0.66	±0.08	±0.08	±0.84	±0.91	±0.19	±0.18

MAO activity was determined in total homogenates minus nuclei prepared as indicated under methods.  
 SPU: Spectrophotometric units as indicated under methods. Kynuramine was used as the substrate.  
 SDM: Standard deviation of the mean.

Table 17

Total proteins in the liver of rats at several intervals of time after the intraperitoneal injection of different single doses of sodium pentobarbital

TOTAL PROTEINS (mg/100 mg of tissue)

RAT #	Pentobarbital (30 mg/kg)			Pentobarbital (60 mg/kg)			Pentobarbital (90 mg/kg)				
	15'	30'	60'	15'	30'	60'	15'	30'	45'	60'	120'
1	15.32	16.41	14.84	15.94	13.78	15.18	14.51	16.77	15.18	15.18	14.31
2	15.43	16.95	15.72	15.18	15.41	15.12	14.51	12.70	12.95	16.73	15.40
3	14.70	16.41	15.76	16.12	14.98	16.45	14.68	14.40	15.95	15.67	15.58
4	13.60	16.54	14.00	15.44	14.51	16.45	15.01	16.91	14.12	16.83	14.31
5	13.44	16.24	14.86	15.01	13.83	15.60	14.51	16.50	15.27	15.52	
6	15.00	15.11	15.43	15.35	16.87	15.60	14.51	16.80	12.77	14.84	
7	15.44	15.92	15.32	16.19	15.86	15.18	15.35	12.50	12.99	15.18	
8	14.77	14.62	14.13	15.52	14.00	14.84	14.84		16.19	16.09	
MEAN	14.71	16.03	15.01	15.59	14.91	15.55	14.74	15.22	14.43	15.71	14.90
SDM	+0.79	+0.78	+0.67	+0.44	+1.10	+0.61	+0.31	+1.99	+1.40	+0.78	+0.69
CHANGE <sup>1</sup>	-0.20	5.04	-1.63	2.16	-2.94	1.90	-3.34	-0.26	-4.98	2.94	-2.35
p <sup>2</sup>	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

\* Interval of time (minutes) between treatment and sacrifice of the animals. Total proteins were determined in total homogenates minus nuclei prepared as indicated under methods.

SDM : Standard deviation of the mean.

<sup>1</sup> Per cent of increase or decrease as compared with the control values indicated in table 16

<sup>2</sup> Significance as compared with control (table 16). NS, non significant.

Table 18

MAO activity in the liver of rats at several intervals of time after the intraperitoneal injection of different single doses of sodium pentobarbital

RAT #	M A O A C T I V I T Y (SPU/mg protein)											
	Pentobarbital (30 mg/kg)			Pentobarbital (60 mg/kg)			Pentobarbital (90 mg/kg)			Pentobarbital (120 mg/kg)		
	15'	30'	60'	15'	30'	60'	15'	30'	45'	60'	120'	
1	3.49	3.07	4.32	3.39	2.61	4.22	3.37	2.30	3.56	3.49	4.30	
2	3.52	3.20	4.12	3.33	3.00	3.59	3.33	2.52	3.51	3.20	4.24	
3	3.57	2.97	4.12	3.36	2.97	3.19	3.10	2.64	3.13	3.37	4.19	
4	3.55	2.85	4.10	3.28	2.67	3.75	3.30	2.44	3.29	3.47	4.23	
5	3.33	3.18	3.93	3.37	2.68	4.00	3.26	2.17	3.51	3.48		
6	3.33	2.87	4.21	3.50	2.90	3.68	3.49	2.50	3.48	3.52		
7	3.37	2.97	4.04	3.33	2.77	3.44	2.64	2.40	3.64	3.67		
8	3.22	3.03	3.91	3.26	2.77	4.20	2.95		3.02	3.85		
MEAN	3.42	3.02	4.09	3.53	2.80	3.76	3.18	2.42	3.39	3.51	4.24	
SDM	±0.13	±0.13	±0.13	±0.04	±0.14	±0.36	±0.27	±0.15	±0.22	±0.19	±0.05	
CHANGE	15.4	-28.2	-2.5	-17.83	-33.4	-10.5	-21.5	-42.0	-19.0	-16.4	-0.9	
P2	< 0.001	< 0.001	NS	< 0.001	< 0.001	< 0.01	< 0.001	< 0.001	< 0.001	< 0.001	NS	

\* Interval of time (minutes) between treatment and sacrifice of the animals.

SPU, Spectrophotometric units as defined under methods. Kynuramine was used as the substrate. The enzymatic activity was determined in total homogenates minus nuclei prepared as indicated under methods

SDM, Standard deviation of the mean.

1 Per cent of change as compared with control values (table 16)

2 Significance as compared with controls (table 16). NS, non significant.

of pentobarbital, up to 33% after treatment with 60 mg/kg, and up to 42% when the given dose was 90 mg/kg. One consistent result was that the inhibition was already present 15 minutes after the treatment. The effect was maximal 30 minutes after the barbiturate had been given, and 60 minutes after the injection the inhibitory effect had disappeared except when 90 mg/kg had been administered. Another important feature of this effect was that a greater inhibition was observed with increasing doses of pentobarbital.

When the heart tissue was studied the total proteins were not different in control and pentobarbital-treated rats (table 19), whereas the monoamine oxidase activity was shown to be inhibited similarly to the hepatic enzyme (table 20). However, in the heart the maximal inhibitory effect was found 15 minutes after treatment, decreasing afterwards, and the observed inhibition was not markedly different with increasing doses of the barbiturate (the maximal obtained inhibition was 37% with 30 mg/kg, 41% with 60 mg/kg and 39% with 90 mg/kg).

Brain and kidneys were also investigated at several intervals of time after the injection of pentobarbital. No significant changes were detected in either the total proteins or MAO activity of these tissues as compared

Table 19

Total proteins in the heart of rats at several intervals of time after the intraperitoneal injection of different single doses of sodium pentobarbital

TOTAL PROTEINS (mg/100 mg of tissue)

RAT #	Pentobarbital (30 mg/kg)			Pentobarbital (60 mg/kg)			Pentobarbital (90 mg/kg)				
	15'	30'	60'	15'	30'	60'	15'	30'	45'	60'	120'
1	7.29	8.75	6.41	6.81	8.95	7.44	7.08	7.33	8.17	6.96	7.43
2	8.27	8.90	7.50	9.14	7.24	7.52	8.07	6.90	6.66	8.07	7.50
3	6.56	8.09	5.73	6.53	7.27	7.96	7.72	7.65	8.33	7.75	5.87
4	5.80	7.87	8.26	6.02	7.34	8.16	7.37	7.65	5.55	7.28	7.08
5	7.22	7.75	6.61	7.16	8.00	6.81	6.58	7.41	8.47	7.59	
6	8.07	8.17	6.75	7.01	7.44	7.23	9.16	7.66	6.66	7.59	
7	7.96	6.89	7.69	7.12	7.91	7.78	8.17	7.66	6.50		
8	6.33	6.33	7.07	7.00	7.75	8.68	6.91	7.05	8.00		
MEAN	7.19	7.84	7.00	7.10	7.74	7.70	7.63	7.41	7.29	7.54	6.98
SDM	±0.90	±0.87	±0.80	±0.91	±0.57	±0.58	±0.83	±0.30	±1.08	±0.38	±0.76
CHANGE <sup>1</sup>	-3.35	9.55	-1.54	-0.14	8.66	8.29	7.01	3.63	2.53	5.45	-1.82
P <sup>2</sup>	NS	NS	NS	NS	NS	NS	NS	NS	NS	MS	NS

\* Interval of time (minutes) between treatment and sacrifice of the animals. Total proteins were determined in total homogenates minus nuclei prepared as indicated under methods.  
SDM, Standard deviation of the mean.

1 Per cent of increase or decrease as compared with control values (table 16).

2 Significance as compared with controls (table 16). NS, non significant.

Table 20

MAO activity in the heart of rats at several intervals of time after the intraperitoneal injection of different single doses of sodium pentobarbital

RAT #	MAO ACTIVITY (SPU/mg protein)											
	Pentobarbital (30 mg/kg)			Pentobarbital (60 mg/kg)			Pentobarbital (90 mg/kg)			Pentobarbital (90 mg/kg)		
	15'	30'	60'	15'	30'	60'	15'	30'	45'	60'	120'	
1	1.60	1.76	2.28	1.69	2.35	2.23	1.59	1.93	2.55	2.05	2.44	
2	1.75	1.96	2.22	1.73	2.04	2.21	1.76	2.39	2.00	2.06	2.63	
3	1.51	1.94	2.29	1.35	2.45	2.24	1.84	2.55	2.50	2.45	2.17	
4	1.50	1.94	2.56	1.35	2.16	2.55	1.56	2.55	1.94	1.91	2.59	
5	1.67	2.14	2.20	1.86	2.19	2.12	1.27	1.80	2.43	2.08		
6	1.76	2.36	2.15	1.84	1.91	2.83	1.82	2.28	1.88	2.40		
7	1.67	2.17	2.00	1.33	2.24	2.83	1.60	2.50	1.79			
8	1.75	1.79	2.59	1.31	2.14	2.63	1.45	2.45	2.50			
MEAN	1.65	2.01	2.26	1.56	2.19	2.46	1.61	2.31	2.20	2.23	2.45	
SDM	±0.11	±0.20	±0.17	±0.24	±0.17	±0.29	±0.20	±0.29	±0.32	±0.24	±0.21	
CHANGE <sup>1</sup>	-37.9	-20.2	-10.5	-41.7	-13.5	-2.7	-39.9	-8.7	-13.0	-11.9	-2.7	
P <sup>2</sup>	<0.001	<0.001	<0.01	<0.001	<0.005	<NS	<0.001	NS	<0.05	<0.05	NS	

\* Interval of time (minutes) between treatment and sacrifice of the animals.

SPU, Spectrophotometric units as defined under methods. Kynuraminc was used as the substrate. The enzymatic activity was determined in total homogenates minus nuclei prepared as indicated under methods. Standard deviation of the mean.

<sup>1</sup> Per cent of change as compared with control values (table 16)

<sup>2</sup> Significance as compared with controls (table 16). NS, non significant.

with the control values. An example of the results of these experiments is given in table 21.

B) TOTAL PROTEINS AND MAO ACTIVITY IN VARIOUS TISSUES OF RATS AFTER CHRONIC TREATMENT WITH SODIUM PENTOBARBITAL:

As indicated under methods, rats weighing 250 grams were given intraperitoneally 30 mg/kg of pentobarbital daily during 20 days and the animals were sacrificed 30 minutes after the last injection which was 60 mg/kg. No significant difference was found between the final body weights of the control and experimental animals (450 ± 15 grams). The wet weights of the studied organs were also similar in both groups of rats.

It can be observed in table 22 that the monoamine oxidase activity in the liver of rats under chronic treatment with pentobarbital was a little less inhibited (26%) than in the animals which had been given a single injection of 60 mg/kg of the same barbiturate (33%)

In the same set of experiments, it was demonstrated that the cardiac MAO activity of rats treated with the barbiturate for 21 days was inhibited by 27% in comparison to the 13% inhibition observed in the heart of rats 30 minutes after the administration of a single dose of 60 mg/kg of pentobarbital (table 23).

An interesting feature was observed in the brain

Table 21

Total proteins and MAO activity in the brain and kidneys of rats 20 minutes after the injection of 0.5 ml of an aqueous solution containing 20% propylene glycol and 10% ethanol (control) or 20 minutes after the administration of 60 mg/kg of sodium pentobarbital (pentob.)

RAT #	B R A I N				K I D N E Y			
	Total proteins (mg%)		MAO activity (SPU/mg protein)		Total proteins (mg%)		MAO activity (SPU/mg protein)	
	Control	Pentob.	Control	Pentob.	Control	Pentob.	Control	Pentob.
1	8.50	9.44	1.70	1.75	10.93	10.39	0.61	0.47
2	8.88	7.81	1.80	1.78	10.50	9.57	0.54	0.54
3	8.11	8.33	1.53	1.68	10.35	10.18	0.59	0.56
4	8.35	8.53	1.68	1.53	11.30	11.45	0.52	0.41
5	9.91	8.25	1.56	1.50	11.23		0.48	
6	8.11	9.15	1.52	1.79	11.37		0.53	
7	8.75	8.11	1.75	1.44	11.10		0.48	
8		7.98		1.46				
MEAN	8.66	8.45	1.65	1.61	10.97	10.40	0.54	0.49
SDM	+0.63	+0.57	+0.11	+0.14	+0.40	+0.78	+0.005	+0.07
CHANGE%		-2.42		-1.30		-5.19		-9.25
SIGNIFICANCE		NS		NS		NS		NS

MAO activity and total proteins were determined in total homogenates minus nuclei. SPU, Spectrophotometric units as defined under methods. Kynuramine was used as the substrate. SDM, Standard deviation of the mean. NS, Non significant change.

Table 22

Total proteins and MAO activity in the liver of rats after chronic treatment with sodium pentobarbital\*

RAT #	TOTAL PROTEINS (mg%)			MAO ACTIVITY (SPU/mg protein)		
	Control		Pentobarbital	Control		Pentobarbital
	30'	30'	120'	30'	30'	120'
1	15.11	14.29	14.29	4.30	2.91	4.52
2	15.11	14.94	15.27	4.19	3.15	4.68
3	14.62	15.27	15.11	3.78	3.09	4.19
4	15.43	14.94	15.43	4.11	3.04	4.00
5	15.11		13.97	4.52		4.18
6	15.59		14.94	3.96		4.56
7	14.87		14.62	3.77		4.22
8	15.86		15.27	4.53		4.25
MEAN	15.20	14.86	14.86	4.12	3.05	4.30
SDM	±0.38	±0.41	±0.52	±0.27	±0.10	±0.22
CHANGE%		-2.23	-2.23		-25.97	4.36
pl		NS	NS		<0.001	NS

\* The animals were injected i.p. 30 mg/kg of pentobarbital daily during 20 days and the last injection was of 60 mg/kg. Control animals were injected 0.6 ml of pentobarbital solvent during the same period. The rats were sacrificed 30 or 120 minutes after the last injection. Total proteins and MAO activity were determined in total homogenates minus nuclei.

SDM, Standard deviation of the mean.  
SPU, Spectrophotometric units as defined under methods. Kynuramine was used as the substrate.

1, Significance as compared with control values. NS, non significant

Table 23

Total proteins and MAO activity in the heart of rats after chronic treatment with sodium pentobarbital\*

RAT #	TOTAL PROTEINS (mg%)				MAO ACTIVITY (SPU/mg protein)				
	Control		Pentobarbital		Control		Pentobarbital		
	30'	120'	30'	120'	30'	120'	30'	120'	
1	7.00	7.34	8.12	2.32	1.97	2.59	2.32	1.97	2.59
2	7.79	7.32	7.20	2.64	1.63	2.35	2.64	1.63	2.35
3	7.00	7.46	8.59	2.68	1.95	2.82	2.68	1.95	2.82
4	7.23	7.32	8.12	2.54	1.67	2.78	2.54	1.67	2.78
5	7.22		6.88	2.31		2.40	2.31		2.40
6	7.77		8.33	2.32		2.67	2.32		2.67
7	7.91		7.41	2.45		2.60	2.45		2.60
8	7.43			2.71			2.71		
MEAN	7.42	7.36	7.81	2.49	1.80	2.60	2.49	1.80	2.60
SDM	±0.36	±0.07	±0.64	±0.17	±0.18	±0.20	±0.17	±0.18	±0.20
CHANGE%		-0.80	5.25		-27.71	4.41		-27.71	4.41
P		NS	NS		<0.001	NS		<0.001	NS

\* Control and experimental animals were treated as indicated in table 22. The rats were sacrificed 30 or 120 minutes after the last injection. Biochemical assays were performed in total homogenates minus nuclei.

SDM, Standard deviation of the mean.  
SPU, Spectrophotometric units as defined under methods. Kynuramine was used as the substrate.

P, significance as compared with control values. NS, non significant change.

of the animals after chronic treatment. In this tissue, 30 or 120 minutes after the last injection of the barbiturate, MAO was decreased by a significant 13% as compared to the control animals, and this inhibition was even higher (21%) 48 hours after the treatment was completed (table 24).

In these experiments, MAO activity was decreased by 18% in the kidneys of barbiturate- treated rats 30 minutes after the sacrifice (table 25).

It can also be observed that for every tissue the total proteins were not significantly different in the control and experimental animals.

C) IN VITRO INHIBITION OF MITOCHONDRIAL MAO BY SODIUM PENTOBARBITAL:

To find out whether pentobarbital has any effect on MAO activity in vitro, a series of experiments were performed using mitochondrial preparations from rat liver. The experimental set up has been described under methods.

The figure 3 represents the inhibition (%) of mitochondrial MAO activity in the presence of different molar concentrations of sodium pentobarbital. It can be seen that the enzymatic activity was already inhibited when the concentration of the barbiturate in the medium was  $10^{-6}$  M. Nevertheless marked inhibitory effect was

Table 24

Total proteins and MAO activity in the brain of rats after chronic treatment with sodium pentobarbital\*

RAT #	TOTAL PROTEINS (mg%)				MAO ACTIVITY (SPU/mg protein)			
	Control		Pentobarbital		Control		Pentobarbital	
	30'	50'	120'	48 hr	30'	50'	120'	48 hr
1	10.72	9.92	9.74	9.09	1.67	1.58	1.50	1.34
2	9.74	9.13	9.74	9.09	1.58	1.35	1.45	1.54
3	9.58	8.86	9.99	9.91	1.69	1.58	1.58	1.06
4	9.42	8.71	9.24	8.44	1.72	1.44	1.46	1.53
5	9.74		10.07	9.09	1.66		1.53	1.33
6	9.91		9.74	9.26	1.48		1.41	1.14
7	9.42		9.42		1.72		1.29	
8	8.93		9.74		1.64		1.41	
MEAN	9.68	9.15	9.77	9.15	1.64	1.43	1.43	1.29
SDM	±0.51	+0.54	±0.20	±0.47	±0.08	±0.10	±0.10	+0.16
CHANGE%		-5.47	0.92	-5.47		-12.8	-12.8	-21.34
P		NS	NS	NS		<0.005	<0.005	<0.001

\* Control and experimental animals were treated as indicated in table 22. The rats were sacrificed 30, 120 minutes or 48 hours after the last injection. Biochemical assays were performed in total homogenates minus nuclei.

SPU, Spectrophotometric units as defined under methods. Kynuramine was used as the substrate.

SDM, Standard deviation of the mean.

P, Significance as compared with control values. NS, non significant change.

Table 25

Total proteins and MAO activity in the kidneys of rats after chronic treatment with sodium pentobarbital

RAT #	TOTAL PROTEINS (mg%)						MAO ACTIVITY (SPU/mg protein)					
	Control			Pentobarbital			Control			Pentobarbital		
	30'	30'	48 hr	30'	120'	48 hr	30'	30'	50'	120'	48 hr	
1	10.98	10.26	9.67	9.07	0.61	0.45	0.50	0.53				
2	10.03	10.73	11.38	10.04	0.52	0.44	0.49	0.51				
3	9.26	10.15	10.34	10.48	0.50	0.46	0.58	0.56				
4	9.00	10.03	9.92	10.93	0.57	0.47	0.59	0.57				
5	9.45		9.75	9.55	0.56		0.51	0.51				
6	9.91		9.87	8.71	0.59		0.57	0.49				
7	10.03		10.12		0.52		0.49					
8	9.80				0.54							
MEAN	9.81	10.29	10.15	9.80	0.55	0.45	0.53	0.53				
SDM	±0.60	±0.30	±0.59	±0.84	±0.04	±0.01	±0.04	±0.03				
CHANGE%		4.89	3.46	-0.10	-18.18	-3.63	-3.63	-3.63				
P		NS	NS	NS	< 0.001	< 0.001	NS	NS				

Control and experimental animals were treated as indicated in table 22. The rats were sacrificed 30, 120 minutes or 48 hours after the last injection. Biochemical assays were performed in total homogenates minus nuclei.

SPU, Spectrophotometric units as defined under methods. Kynuramine was used as the substrate.

SDM, Standard deviation of the mean.

P, Significance as compared with control values. NS, non significant change.

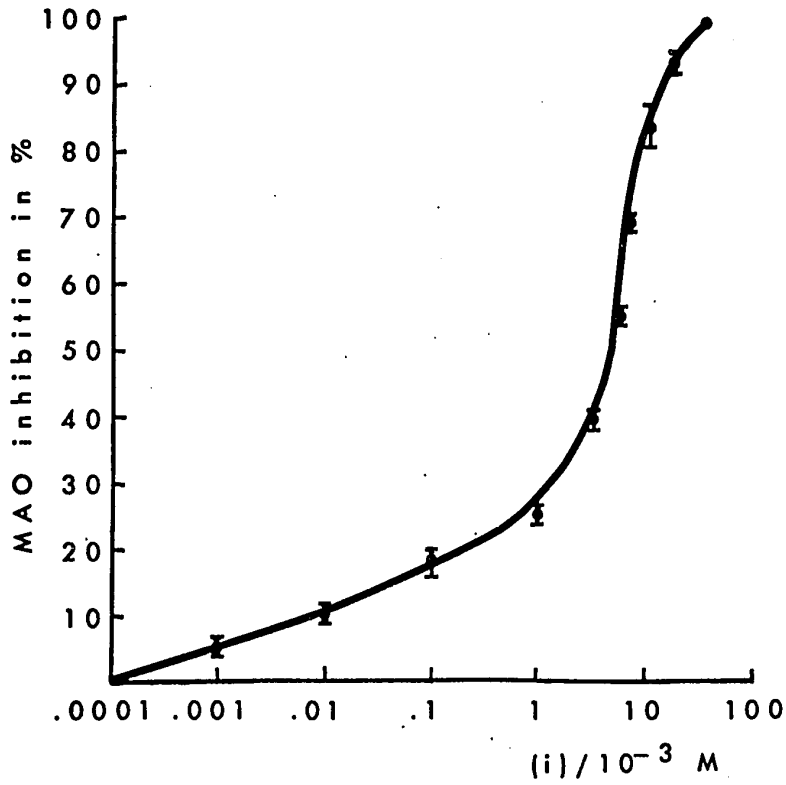


Figure 3

In vitro inhibition of rat liver mitochondrial MAO in the presence of increasing molar concentrations of sodium pentobarbital. Five different experiments were performed for each concentration of the barbiturate. The mean  $\pm$  the standard deviation of the mean is indicated for each point.

only observed with molar concentrations higher than  $10^{-4}$  M. Inhibition by 50% was reached at  $6 \times 10^{-3}$  M, and no enzymatic activity at all was detected when the concentration of the barbiturate in the medium was  $2 \times 10^{-2}$  M.

In order to determine the mechanism through which the inhibition was achieved, the enzymatic activity of mitochondrial suspensions was assayed using various molar concentrations of kynuramine in the presence and in the absence of sodium pentobarbital. The details of the procedure has been indicated under methods. The results are shown in figure 4. When the barbiturate was absent the slope was 0.34, the intercept was 20.70, and the calculated  $K_m$  was  $0.16 \times 10^{-4}$  M. In the presence of pentobarbital the calculated values were: slope 2.31, intercept 20.05, and  $K_i$   $12.1 \times 10^{-4}$  M. It can be observed from this Lineweaver and Burk plot that these results are in agreement with a competitive type of inhibition.

D) EFFECT OF PENTOBARBITAL ADMINISTRATION ON THE RAT LIVER AND BRAIN MAO ACTIVITY ASSAYED BY THE OXYGEN CONSUMPTION METHOD:

In order to corroborate the previous results, some of the in vivo experiments were repeated using a different method for the assay of MAO. As in the previous experiments, kynuramine was also employed as the substrate

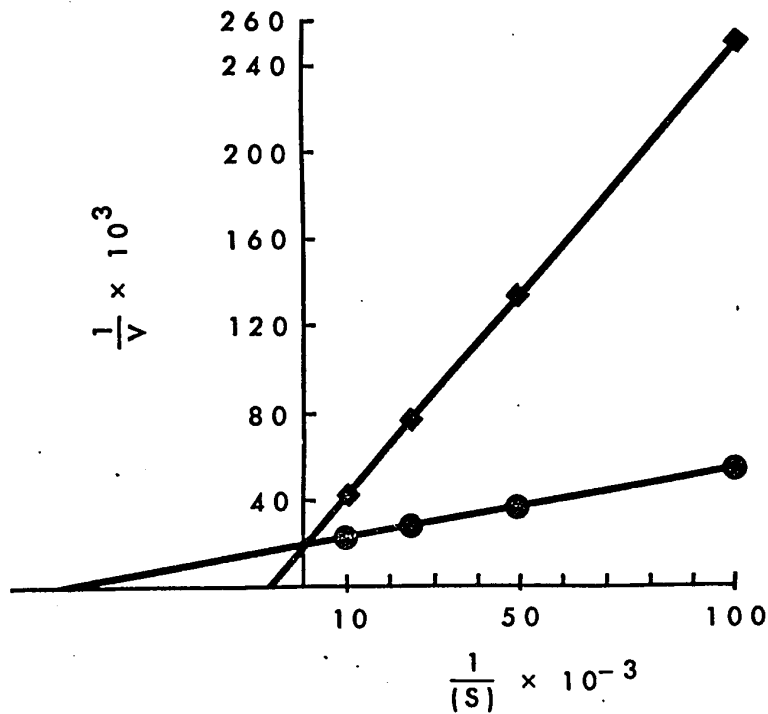


Figure 4

Lineweaver-Burk plot for rat liver mitochondrial MAO assayed with kynuramine. ● In the absence of pento-barbital. ■ In the presence of the barbiturate ( $7.5 \times 10^{-3} M$ ). Three different experiments were performed for each point. The least squares method was used.

and the enzymatic activity was determined following the oxygen consumption procedure as described under methods.

It can be seen in table 26 that in these experiments MAO activity was inhibited by 58% in the liver of rats following the intraperitoneal injection of 90 mg/kg of pentobarbital. In the same table, it can also be seen that MAO was not inhibited in the brain of rats submitted to the same treatment.

E) THE IN VIVO AND IN VITRO EFFECT OF PENTOBARBITAL ALCOHOL ON MAO ACTIVITY ASSAYED WITH KYNURAMINE:

These experiments were undertaken in order to investigate the possibility of MAO inhibition by some metabolite of pentobarbital.

It can be seen in table 27 that we were unable to demonstrate any enzymatic inhibition in various tissues of rats following the intraperitoneal administration of 40 mg/kg of pentobarbital alcohol dissolved as described under materials.

Table 28 shows that MAO activity was unchanged in various subcellular fractions of rat liver following their incubation with different molar concentrations of pentobarbital alcohol.

F) THE EFFECT OF PENTOBARBITAL OR PENTOBARBITAL ALCOHOL ON MAO ACTIVITY ASSAYED WITH SEVERAL SUBSTRATES:

Table 26

The effect of one i.p. injection of pentobarbital (90 mg/kg) on MAO activity in the liver and brain of rats, using the oxygen consumption method and kynuramine as the substrate

EXPERIMENTAL CONDITIONS	L I V E R		B R A I N	
	Total proteins (mg%)	MAO activity( $\mu$ l O <sub>2</sub> cons/hr/mg protein)	Total proteins (mg%)	MAO activity( $\mu$ l O <sub>2</sub> cons/hr/mg protein)
CONTROL	14.46 $\pm$ 0.83 (8)	11.02 $\pm$ 1.50 (8)	8.41 $\pm$ 0.31 (4)	2.15 $\pm$ 0.10 (4)
PENTOBARBITAL	14.22 $\pm$ 0.80 (8)	4.57 $\pm$ 0.89 (8)	8.37 $\pm$ 0.21 (4)	2.12 $\pm$ 0.19 (4)
INHIBITION %	1.66	58.53	0.48	1.40
SIGNIFICANCE (P)	NS	<0.001	NS	NS

Sodium pentobarbital was given 30 minutes before the sacrifice. Biochemicals assays were performed in total homogenates minus nuclei. MAO activity was determined as indicated under methods (oxygen consumption). The digits in brackets represent the number of animals sacrificed. The means are given  $\pm$  the standard deviation of the mean.  
NS, non significant change.

Table 27

Total proteins and MAO activity in various tissue of rats, 30 minutes after one i.p. injection of 5-ethyl-1-5-(3-hydroxy-1-methylbutyl)-barbituric acid (40 mg/kg)

TISSUE	C O N T R O L *		E X P E R I M E N T A L	
	Total proteins (mg%)	MAO activity (SPU/mg protein)	Total proteins (mg%)	MAO activity (SPU/mg protein)
LIVER	14.77 ± 0.75 (4)	4.34 ± 0.08 (4)	14.58 ± 1.16 (4)	4.31 ± 0.09 (4)
HEART	7.47 ± 0.25 (4)	2.14 ± 0.10 (4)	7.37 ± 0.59 (4)	2.26 ± 0.16 (4)
BRAIN	8.65 ± 0.49 (4)	1.58 ± 0.05 (4)	8.56 ± 0.49 (4)	1.54 ± 0.08 (4)
KIDNEYS	10.80 ± 0.17 (4)	0.53 ± 0.04 (4)	10.75 ± 0.18 (4)	0.54 ± 0.03 (4)

\* Control animals were injected 1 ml of the solvent for the drug (see methods).

Biochemical assays were performed in total homogenates minus nuclei prepared as indicated under methods. MAO activity was determined by the spectrophotometric assay described under methods and kynuramine was used as the substrate. The digits in brackets represent the number of animals sacrificed. The means are given ± the standard deviation of the mean.

SPU, Spectrophotometric units as described under methods.

Table 28

MAO activity in various subcellular fractions of the liver of normal rats, in the absence (control) and in the presence of different concentrations of 5-ethyl-1-5-(3-hydroxy-1-methylbutyl)-barbituric acid

SUBCELLULAR FRACTION	M A O A C T I V I T Y (SPU/mg protein)	FINAL CONCENTRATION OF THE DRUG IN THE INCUBATION		
		CONTROL (No drug)	10 <sup>-5</sup> M	10 <sup>-4</sup> M
CYTOPLASMIC	6.26 ± 0.32 (4)	6.22 ± 0.25 (4)	5.92 ± 0.26 (4)	6.21 ± 0.18 (4)
MITOCHONDRIAL	15.63 ± 0.48 (4)	15.21 ± 0.52 (4)	15.42 ± 0.58 (4)	15.62 ± 0.30 (4)
MICROSOMAL	4.05 ± 0.30 (4)	3.98 ± 0.13 (3)	4.10 ± 0.25 (3)	3.90 ± 0.12 (3)

Several subcellular fractions were prepared from rat liver homogenates as indicated under methods. The total homogenate minus nuclei was considered as the cytoplasmic fraction. The mixture was preincubated at 37°C during 15 minutes before the substrate was added. The spectrophotometric method was used for MAO assays and kynuramine was employed as the substrate. The composition of the final incubation mixture is indicated under methods. SPU, spectrophotometric units as defined under methods. The digits in brackets represent the number of animals. Means are given ± the standard deviation of the mean.

Since it has been reported that MAO exhibits a different sensitivity to various inhibitors when the enzymatic activity is assayed using different substrates (116,117), it was decided to investigate whether this was also applicable for pentobarbital. It was also decided to elucidate whether pentobarbital alcohol produces inhibition of MAO assayed with different substrates.

Both drugs were administered intraperitoneally to the animals and the enzymatic activity was assayed using the oxygen consumption procedure described under Methods. Serotonin, tyramine and benzylamine were employed as different substrates.

Table 29 presents the results when the liver MAO was assayed. It can be seen that MAO activity was also inhibited by pentobarbital when assayed with serotonin, tyramine or benzylamine. A marked inhibition of MAO was obtained with every substrate after the administration of high doses of the barbiturate. Comparing these results with those shown in table 26, it can be observed that following the injection of 90 mg/kg of pentobarbital MAO activity was inhibited in the liver of rats by 58% when assayed with kynuramine, by 50% with serotonin, by 44% with tyramine and by 32% when assayed

Table 29

MAO activity assayed with various substrates in the liver of normal rats after treatment with single doses of sodium pentobarbital or 5-ethyl-1-5-(3-hydroxy-1-methylbutyl)-barbituric acid

EXPERIMENTAL CONDITIONS	TOTAL PROTEINS (mg%)		SEROTONIN		TYRAMINE		BENZYLAMINE	
	MAO activity	Inhibition %	MAO activity	Inhibition %	MAO activity	Inhibition %	MAO activity	Inhibition %
CONTROL (8)	14.56±0.71	4.23±0.38	-	-	4.88±0.50	-	3.20±0.13	-
PENTOBARBITAL (60 mg/kg) (4)	14.51±0.48	3.52±0.35*	16.78	18.64	3.97±0.47*	18.64		
PENTOBARBITAL (90 mg/kg) (4)	14.73±0.28	2.08±0.36 <sup>Δ</sup>	50.82	44.26	2.72±0.66 <sup>Δ</sup>	44.26	2.17±0.53 <sup>†</sup>	32.18
METABOLITE (40 mg/kg) (4)	14.50±0.80	3.29±0.16 <sup>Δ</sup>	22.22	27.45	3.54±0.44 <sup>Δ</sup>	27.45		

Both compounds were injected i.p. into the rats. Control animals were given 1 ml of the solvent for the drugs. Biochemical assays were performed in total homogenates minus nuclei. MAO activity was determined by the oxygen consumption method (see methods). The enzymatic activity is indicated in  $\mu$ l of oxygen consumed/hr/mg of protein. The digits in brackets represent the number of animals sacrificed. Means are given  $\pm$  the standard deviation of the mean. The animals were injected 30 minutes before the sacrifice.

\*, P < 0.05

<sup>Δ</sup>, P < 0.001

<sup>†</sup>, P < 0.01

with benzylamine.

In table 29 it can also be seen that pentobarbital alcohol which in some of our previous experiments did not exhibit any inhibitory effect on liver MAO assayed with kynuramine, produced a 22% inhibition of the enzyme assayed with serotonin and a 27% decrease with tyramine. In our experiments, the employed dose of this pentobarbital metabolite (40 mg/kg) was unable to induce hypnosis in the animals.

It can be observed from table 30 that in the heart of pentobarbital-treated rats MAO activity was also inhibited when assayed with the three mentioned substrates. The enzyme was inhibited by some 47% with serotonin as the substrate, by 36% with tyramine, and by 16% with benzylamine following the administration of 90 mg/kg of pentobarbital. Comparing these results with those of table 20, it can be observed that the heart MAO activity assayed with kynuramine was inhibited by some 39% after the administration of the same single dose of the barbiturate.

The experimental results in the case of brain are indicated in table 31. It can be observed in this table that MAO activity was markedly inhibited in this tissue following the administration of single doses of pento-

with benzylamine.

In table 29 it can also be seen that pentobarbital alcohol which in some of our previous experiments did not exhibit any inhibitory effect on liver MAO assayed with kynuramine, produced a 22% inhibition of the enzyme assayed with serotonin and a 27% decrease with tyramine. In our experiments, the employed dose of this pentobarbital metabolite (40 mg/kg) was unable to induce hypnosis in the animals.

It can be observed from table 30 that in the heart of pentobarbital-treated rats MAO activity was also inhibited when assayed with the three mentioned substrates. The enzyme was inhibited by some 47% with serotonin as the substrate, by 36% with tyramine, and by 16% with benzylamine following the administration of 90 mg/kg of pentobarbital. Comparing these results with those of table 20, it can be observed that the heart MAO activity assayed with kynuramine was inhibited by some 39% after the administration of the same single dose of the barbiturate.

The experimental results in the case of brain are indicated in table 31. It can be observed in this table that MAO activity was markedly inhibited in this tissue following the administration of single doses of pento-

Table 30

MAO activity assayed with various substrates in the heart of normal rats after treatment with single doses of sodium pentobarbital

EXPERIMENTAL CONDITIONS	TOTAL		SEROTONIN		TYRAMINE		BENZYLAMINE	
	PROTEINS (mg%)	MAO activity	Inhibition %	MAO activity	Inhibition %	MAO activity	Inhibition %	
CONTROL (4)	7.46±0.76	2.49±0.45	-	1.76±0.14	-	0.94±0.08	-	
PENTOBARBITAL (60 mg/kg) (4)	7.41±0.30	1.46±0.17 <sup>†</sup>	41.36	1.12±0.17 <sup>Δ</sup>	36.36			
PENTOBARBITAL (90 mg/kg) (4)	7.48±0.19	1.31±0.29 <sup>Δ</sup>	47.39	1.12±0.16 <sup>Δ</sup>	36.36	0.78±0.11	16.82	

Both compounds were injected i.p. into the animals 30 minutes before the sacrifice. Control rats were given 1 ml of the solvent for the drugs. Biochemical assays were performed in total homogenates minus nuclei. MAO activity was determined by the oxygen consumption method (see methods). The enzymatic activity is indicated in  $\mu$ l of oxygen consumed/hr/mg of protein. The digits in brackets represent the number of animals sacrificed. Means are given  $\pm$  the standard deviation of the mean.

<sup>†</sup>, P < 0.01

<sup>Δ</sup>, P < 0.001

Table 31

MAO activity assayed with various substrates in the brain of normal rats after treatment with single doses of sodium pentobarbital or 5-ethyl-1-5(3-hydroxy-1-methylbutyl)-barbituric acid

EXPERIMENTAL CONDITIONS	TOTAL		SEROTONIN		TYRAMINE		BENZYLAMINE	
	PROTEINS (mg%)	MAO activity	MAO activity	Inhibition %	MAO activity	Inhibition %	MAO activity	Inhibition %
CONTROL (8)	8.45±0.55	1.89±0.12	-	-	1.91±0.13	-	1.26±0.13	-
PENTOBARBITAL (60 mg/kg) (4)	8.86±0.32	1.48±0.18 <sup>Δ</sup>	21.69	15.70	1.61±0.15*	15.70		
PENTOBARBITAL (90 mg/kg) (4)	8.42±0.33	1.14±0.08 <sup>Δ</sup>	39.68	16.75	1.59±0.18*	16.75	0.66±0.08 <sup>Δ</sup>	47.61
METABOLITE (40 mg/kg) (4)	8.56±0.50	1.09±0.14 <sup>Δ</sup>	42.32	18.32	1.56±0.08 <sup>∇</sup>	18.32		

Both compounds were injected i.p. into the animals 30 minutes before the sacrifice. Control rats were given 1 ml of the solvent for the drugs. Biochemicals assays were performed in total homogenates minus nuclei. MAO activity was determined by the oxygen consumption method (see methods). The enzymatic activity is indicated in  $\mu$ l of oxygen consumed/hr/mg of protein. The digits in brackets represent the number of animals sacrificed. Means are given  $\pm$  the standard deviation of the mean.

\*, P <0.02  
<sup>Δ</sup>, P <0.001  
<sup>∇</sup>, P <0.005

barbital. The enzyme was inhibited up to 39% when assayed with serotonin, up to 47% with benzylamine, and up to 16% with tyramine.

An interesting feature when MAO activity was determined in brain tissue using different substrates, was the demonstration of enzymatic inhibition following the administration of pentobarbital alcohol (see also table 31). The maximal inhibition was obtained with serotonin as the substrate (42%). With tyramine the observed inhibition was 18%.

In the same series of experiments MAO activity was also inhibited in the kidneys of rats following the administration of pentobarbital (table 32). In this tissue the maximal inhibition was observed using tyramine as the substrate (20%) while the enzyme was inhibited by a non significant 10% when assayed with serotonin.

G) THE EFFECT OF THIOFENTAL OR PHENOBARBITAL ADMINISTRATION ON THE MONOAMINE OXIDASE ACTIVITY OF VARIOUS TISSUES OF RATS:

Since increased levels of serotonin in the brain of rats have been reported following the administration of pentobarbital as well as after the injection of some other barbiturates (178,180), it

Table 32

MAO activity assayed with various substrates in the kidneys of normal rats after treatment with single doses of sodium pentobarbital

EXPERIMENTAL CONDITIONS	TOTAL		SEROTONIN		TYRAMINE		BENZYLAMINE	
	PROTEINS (mg%)	MAO activity	MAO activity	Inhibition %	MAO activity	Inhibition %	MAO activity	Inhibition %
CONTROL (8)	10.05±0.78	0.73±0.25	-	-	0.84±0.12	-	0.39±0.08	-
PENTOBARBITAL (60 mg/kg) (4)	9.63±0.85	0.65±0.26	10.90	20.33	0.67±0.04*			
PENTOBARBITAL (90 mg/kg) (4)	10.63±0.22	0.66±0.09	9.58	19.04	0.68±0.11*		0.41±0.07	-4.32

Both compounds were injected i.p. into the rats, 30 minutes before the sacrifice. Control animals were given 1 ml of the solvent for the drugs. Biochemical assays were performed in total homogenates minus nuclei. MAO activity was determined by the oxygen consumption method (see methods). The enzymatic activity is indicated in  $\mu$ l of oxygen consumed/hr/mg of protein. The digits in brackets represent the number of animals sacrificed. Means are given  $\pm$  the standard deviation of the mean.

\*, P<0.05

was decided to investigate the in vivo effect of thiopental and phenobarbital on the monoamine oxidase activity of various tissues of rats. Kynuramine was used as the substrate.

The effect of a single intraperitoneal injection of sodium thiopental (60 mg/kg) on MAO activity in the liver and heart of rats can be observed in table 33. In the hepatic tissue the enzymatic activity was inhibited by 24% ten minutes after the treatment, by 21% twenty minutes after the injection and by 11% thirty minutes after the administration of the barbiturate. In the heart, 39, 44, and 28% inhibition was observed ten, twenty, and thirty minutes respectively after the drug had been given to the animals.

It can be seen in table 34 that MAO activity was also inhibited in samples prepared from the brain of thiopental-treated rats. A significant inhibition was observed ten minutes after treatment (17%) as well as thirty minutes after the administration of the drug (10%).

It can also be seen in table 34 that the enzymatic activity in the kidneys of rats injected with 60 mg/kg of thiopental was significantly inhibited (28%) twenty minutes after treatment.

The monoamine oxidase activity of several rat tissues

Table 33

Total proteins and MAO activity in the liver and heart of rats at several intervals of time after a single injection of sodium thiopental (60 mg/kg)

EXPERIMENTAL CONDITIONS	LIVER			HEART		
	Total protein (mg%)	MAO activity (SPU/mg prot)	MAO Inhib (%)	Total protein (mg%)	MAO activity (SPU/mg prot)	MAO Inhib (%)
CONTROL (10 minutes)	14.61±0.50(6)	4.31±0.08(6)	-	6.74±1.21(6)	2.21±0.16(6)	-
THIOPENTAL (10 minutes)	14.93±0.98(6)	3.24±0.10(6) <sup>Δ</sup>	24.82	7.38±0.65(6)	1.33±0.33(6) <sup>Δ</sup>	39.81
THIOPENTAL (20 minutes)	15.34±0.71(5)	3.38±0.07(5) <sup>Δ</sup>	21.57	7.56±0.92(5)	1.23±0.16(5) <sup>Δ</sup>	44.33
THIOPENTAL (30 minutes)	14.44±0.92(6)	3.80±0.34(6) <sup>†</sup>	11.83	7.41±0.94(6)	1.58±0.15(6) <sup>Δ</sup>	28.50

Control animals were given i.p. 1 ml of the solvent for thiopental. The interval of time between the injection and sacrifice is indicated for every type of experiment. The biochemical assays were performed in total homogenates minus nuclei. MAO activity was determined by the spectrophotometric method previously indicated. Kynuramine was used as the substrate. SPU, spectrophotometric units as described under methods. The digits in brackets represent the number of animals that were sacrificed. The means are given ± the standard deviation of the mean.

Δ, P < 0.001

†, P < 0.01

Table 34

Total proteins and MAO activity in the brain and kidneys of rats at several intervals of time after a single injection of sodium thiopental (60 mg/kg)

EXPERIMENTAL CONDITIONS	BRAIN			KIDNEYS		
	Total protein (mg%)	MAO activity (SPU/mg prot)	MAO Inhib (%)	Total protein (mg%)	MAO activity (SPU/mg prot)	MAO Inhib (%)
CONTROL (10 minutes)	8.73±0.69(6)	1.59±0.05(6)	-	9.91±1.18(6)	0.53±0.08(6)	-
THIOPENTAL (10 minutes)	8.71±0.25(6)	1.31±0.06(6) <sup>Δ</sup>	17.61	10.41±0.79(6)	0.44±0.10(6)	16.98
THIOPENTAL (20 minutes)	8.11±0.55(5)	1.46±0.08(5)	8.17	10.26±0.90(5)	0.38±0.10(5) <sup>*</sup>	28.30
THIOPENTAL (30 minutes)	8.50±1.03(6)	1.43±0.05(6) <sup>†</sup>	10.06	10.65±1.16(6)	0.49±0.10(6)	7.54

Control animals were given i.p. 1 ml of the solvent for thiopental. The interval of time between the injection and sacrifice is indicated for every type of experiment. The biochemical assays were performed in total homogenates minus nuclei. MAO activity was determined by the spectrophotometric method previously indicated. Kynuramine was used as the substrate. SPU, spectrophotometric units as defined under methods. The digits in brackets represent the number of animals that were sacrificed. The means are given ± the standard deviation of the mean.

Δ , P < 0.001

\* , P < 0.02

† , P < 0.01

following the intraperitoneal administration of sodium phenobarbital (120 mg/kg) is reported in tables 35 and 36. In the liver the greatest inhibition (27%) was obtained 60 minutes after treatment. At the same time a similar inhibition was observed in the heart, but in this tissue the inhibition was already present 30 minutes after injecting the barbiturate.

No enzymatic inhibition was observed in the brain and kidneys of rats following the administration of phenobarbital (table 36).

Finally, it can also be observed in tables 33, 34, 35, and 36 that the total protein content of the various tissues was not significantly altered by the administration of thiopental or phenobarbital.

### III. DISCUSSION

After the intraperitoneal administration of single doses of sodium pentobarbital. MAO activity (assayed with kynuramine) exhibited a significant inhibition in the liver and heart of rats. The enzymatic activity was inhibited by 28% in the liver and by 37% in the heart after the injection of 30 mg/kg of the drug. More inhi-

Table 35

Total proteins and MAO activity in the liver and heart of rats at several intervals of time after a single injection of phenobarbital (120 mg/kg)

EXPERIMENTAL CONDITIONS	LIVER			HEART		
	Total protein (mg%)	MAO activity (SPU/mg prot)	MAO Inhib (%)	Total protein (mg%)	MAO activity (SPU/mg prot)	MAO Inhib (%)
CONTROL (30 minutes)	14.72±0.62 (8)	4.34±0.15 (8)	-	7.53±0.41 (4)	2.34±0.15 (4)	-
PHENOBARBITAL (30 minutes)	14.55±0.77 (8)	4.00±0.15 (8) <sup>Δ</sup>	7.83	7.42±0.31 (4)	1.70±0.16 (4) <sup>Δ</sup>	27.35
PHENOBARBITAL (60 minutes)	14.72±0.42 (8)	3.15±0.17 (8) <sup>Δ</sup>	27.41	7.39±0.49 (4)	1.72±0.35 (4) <sup>Δ</sup>	26.49
PHENOBARBITAL (2 hours)	14.70±0.55 (8)	4.06±0.19 (8) <sup>†</sup>	6.45	7.48±0.14 (4)	2.30±0.21 (4)	1.70

Control animals were given 0.35 ml i.p. of the solvent for phenobarbital. The interval of time between the injection and sacrifice is indicated for every type of experiment. Total homogenates minus nuclei of both tissues were employed in these experiments. MAO activity was determined by the spectrophotometric method previously indicated. Kynuramine was used as the substrate. SPU, spectrophotometric units as defined under methods. The digits in brackets represent the number of animals sacrificed. The means are given ± the standard deviation of the mean.

Δ, P < 0.001

†, P < 0.01

Table 36

Total proteins and MAO activity in the brain and kidneys of rats at various intervals of time after a single injection of phenobarbital (120 mg/kg)

EXPERIMENTAL CONDITIONS	BRAIN			KIDNEYS		
	Total protein (mg%)	MAO activity (SPU/mg prot)	MAO Inhib (%)	Total protein (mg%)	MAO activity (SPU/mg prot)	MAO Inhib (%)
CONTROL (30 minutes)	8.64±0.30 (8)	1.63±0.06 (8)	-	10.38±0.59 (8)	0.53±0.07 (8)	-
PHENOBARBITAL (30 minutes)	8.33±0.61 (8)	1.66±0.11 (8)	-1.84	10.40±0.56 (8)	0.53±0.08 (8)	0.0
PHENOBARBITAL (60 minutes)	8.74±0.47 (8)	1.62±0.14 (8)	0.62	10.63±0.21 (8)	0.52±0.04 (8)	1.88
PHENOBARBITAL (2 hours)	8.60±0.20 (8)	1.65±0.11 (8)	-1.22	10.45±0.45 (8)	0.55±0.08 (8)	-3.77

The experimental conditions and procedures were as indicated in table 35.

bition was detected in the liver with increasing doses of the barbiturate. The maximal inhibition observed was 40% for both tissues, and in both cases it was shown that this effect was reversible in a short period of time (less than two hours in the liver and less than one hour in the heart).

A high dose of the drug was required in order to inhibit the enzyme by 40% in the liver. However, when only 30 mg/kg were injected a marked inhibition was obtained in this tissue and the enzymatic activity was decreased by 37% in the heart.

The effect of pentobarbital administration on the monoamine oxidase of rat liver was corroborated using the same substrate (kynuramine) and a different method for the enzymatic assay (oxygen consumption method).

Nevertheless, following the administration of pentobarbital MAO activity assayed with kynuramine was not inhibited in rat brain. These results were also confirmed utilizing the oxygen consumption method for the enzymatic assay.

In order to explain our initial failure to demonstrate inhibitory effect of pentobarbital on the brain MAO, the following hypotheses were considered:

- a) It has been reported in the literature that

MAO from different tissues exhibits a different susceptibility toward some inhibitors when assayed with different substrates (116, 117, 142) and in the basis of these results it has been postulated the existence of multiple forms of monoamine oxidase. In our initial experiments MAO activity was assayed using only kynuramine as the substrate. In consequence the possibility of MAO inhibition in the brain of rats by pentobarbital was not excluded for other substrates.

b) One could also postulate that the inhibitory effect of the barbiturate is exerted indirectly after being metabolized to some active compound. Compatible with this hypothesis is the report in the literature that some barbiturates are metabolized to pharmacologically active intermediates, the metabolite being sometimes more active than the parent compound (160). In addition, it is also reported in the literature that pentobarbital is not metabolized in the presence of brain homogenates (163, 164).

c) Finally, in our experiments brain MAO was determined only 20 or 30 minutes after the injection of the barbiturate. Probably the concentration of pentobarbital in the brain following the administration of single doses of the drug was insufficient to inhibit the enzyme,

or perhaps the time course of the enzymatic inhibition was different in the nervous tissue.

In order to test the last hypothesis, experiments were undertaken to see if after chronic treatment of rats with pentobarbital MAO activity was significantly inhibited in the brain. As previously indicated, after treating the animals with the barbiturate during 3 weeks MAO activity was inhibited in the cerebral tissue. However, only 21% inhibition was observed and the maximum effect was detected 48 hours after the last injection. This delayed effect could be also compatible with an inhibition of the enzyme produced by some pentobarbital metabolite rather than by the barbiturate itself. This conclusion was supported by the report in the literature that the pentobarbital metabolites are excreted in the urine even five days after the parent compound has been given (160).

Before performing more experiments to elucidate some additional details on the in vivo inhibition of MAO by pentobarbital, it was decided to investigate the in vitro effect of the barbiturate on the liver enzyme.

Since purified preparations of MAO are not available, mitochondrial suspensions prepared from rat

liver were used for the in vitro experiments. These suspensions are known to be a good source for this enzyme.

As previously indicated, MAO activity was inhibited in the presence of sodium pentobarbital. However, a high molar concentration of the barbiturate ( $6 \times 10^{-3}$  M) was required in order to obtain 50% inhibition of the mitochondrial MAO. These results could be accounted for by the reported low binding capacity of liver mitochondrial fractions for barbiturates (156). In an alternative explanation, if the in vivo inhibition was originated at least in part through some pentobarbital metabolite produced in the microsomes, the in vitro inhibition of mitochondrial MAO by pentobarbital could be reduced in the absence of microsomal fraction.

On the other hand, our results were in agreement with previous workers reporting in the literature that some other flavoenzymes are inhibited in vitro by high molar concentrations of barbiturates (174).

The Lineweaver and Burk plot obtained in another set of in vitro experiments suggests that the inhibition of liver mitochondrial MAO proceeds through a competitive mechanism which is similar to the reported type of inhibition of L-aminoacid oxidase, D-aspartate oxidase and other flavoenzymes by some barbiturates (173, 174, 176).

The kinetic constants that we obtained for liver mitochondrial MAO assayed with kynuramine ( $K_m$ ,  $0.16 \times 10^{-4}$  M) and in the presence of pentobarbital ( $K_i$ ,  $12.1 \times 10^{-4}$  M) would indicate that this barbiturate is a poor inhibitor of MAO in vitro. However, here again it can be observed that these values are comparable to those reported after the in vitro inhibition of some flavoenzymes by various barbiturates (175).

Since our initial experiments pointed to the possibility of MAO inhibition (at least in part) by some metabolic product of pentobarbital, the next step in this work was directed to elucidate whether the pentobarbital alcohol either by injection or in vitro was able to produce any effect on MAO activity. As previously reported under results, no inhibition of the enzyme assayed with kynuramine was demonstrated in the liver, heart, brain, or kidneys of rats following the administration of 40 mg/kg of pentobarbital alcohol, and it was impossible to show in vitro any inhibitory effect of this pentobarbital metabolite on hepatic MAO. Nevertheless, it is difficult to rule out only with these experiments the above mentioned possibility since it is known that pentobarbital can be degraded enzymatically to at least a second metabolite (pentobarbital carboxylic acid).

Unfortunately this metabolite was not available for experimentation.

In another series of experiments reported in this thesis, MAO activity was determined with different substrates after the administration of pentobarbital or pentobarbital alcohol. It can be observed from these experiments that:

a) In the liver, MAO activity was inhibited for all the substrates employed in the procedure. A similar inhibition was obtained for serotonin and kynuramine (50 and 58%), less inhibition was obtained for tyramine (44%), and much less inhibitory effect was observed for benzylamine (32%). The pentobarbital alcohol produced inhibition only on MAO activity assayed with serotonin or tyramine (22 or 27%).

b) A similar inhibitory pattern was found in the liver and heart after giving pentobarbital to the animals.

c) While brain MAO activity measured with kynuramine as substrate did not appear to be inhibited after a single dose of pentobarbital or pentobarbital alcohol, it was substantially inhibited when assayed with other substrates. A marked inhibition for serotonin and benzylamine was observed after the administration of either drug. In these experiments, a lesser inhibition was found when

the enzymatic activity was determined with tyramine.

d) Pentobarbital inhibited renal MAO only when assayed with tyramine.

The different susceptibility of MAO toward its inhibitors when assayed with different substrates has been previously reported (142). This property has been regarded as an indication of the existence of multiple forms of MAO. In this direction the possibility of two or three types of MAO has been postulated (116, 117, 118, 119). The pattern of inhibition of MAO by pentobarbital demonstrated in our experiments is in agreement with these reports. However, it is difficult to conclude how many types of MAO exist in the different tissues of rats. To clarify this point on the basis of this type of experiment, a screening assay of MAO activity for all the possible biological substrates would be required, and obviously this is not an easy task.

The following general interpretation could be given to the results reported in this part of our work:

The injection of pentobarbital into rats produces inhibition of the hepatic and cardiac MAO activity measured with kynuramine. In the brain and kidneys the enzyme assayed with the same substrate is susceptible

to the mentioned barbiturate only after chronic treatment of the animals. This inhibitory effect is more marked in the microsomal than in the mitochondrial fractions (see tables 12 and 14). The preferential inhibition of MAO activity in the microsomal fraction is not surprising since barbiturates are reported to be mainly bound and metabolized in the microsomes (156, 164). Upon oxidation by microsomal enzymes pentobarbital originates at least two metabolites (163). In the light of our experiments it seems clear that MAO activity measured with kynuramine is not inhibited by one of the pentobarbital metabolites (pentobarbital alcohol).

Following the administration of single doses of pentobarbital or pentobarbital alcohol MAO activity assayed with serotonin as the substrate was inhibited in various tissues of rats including the brain. These findings could be accounted for by the existence of a different form of MAO responsible for the oxidation of serotonin. This type of MAO could be present in the brain of rats as well as in the liver and heart. The inhibitory effect of pentobarbital on this form of MAO could be exerted through the pentobarbital alcohol following its formation in the liver of the animals. This interpretation would explain the inhibition of

brain MAO when assayed with serotonin and the absence of inhibition of the enzyme in the same tissue when assayed with kynuramine. "Kynuramine oxydase" was also inhibited in the brain of rats after chronic treatment with pentobarbital and this result could indicate that this type of MAO is also susceptible to pentobarbital alcohol when high concentrations of this metabolite are reached in the cerebral tissue.

MAO activity assayed with tyramine showed a similar susceptibility to pentobarbital than when assayed with serotonin. Nevertheless, the inhibition in the brain was higher when the second substrate was used. Here again the inhibitory effect seems to be produced through the action of pentobarbital alcohol.

The inhibitory pattern of MAO by pentobarbital changed with benzylamine as the substrate. This result would confirm the demonstration by Sierens and D'Iorio of two different forms of rat mitochondrial MAO for serotonin and benzylamine (97).

On the other hand, it has been discussed in the previous part of this thesis how this inhibitory effect could be accounted for by the reported inhibition of several flavoenzymes in the presence of some barbiturates (172, 173, 174, 175). It has also been discussed

how MAO inhibition could have biological significance in thyrotoxic rats where the barbiturate exerts a much more marked inhibition of the enzyme.

The inhibition of brain MAO following the administration of pentobarbital could have some other biological implications.

As indicated in the review of the literature, it has been postulated that the SWS phase in the mammalian sleeping brain depends upon increased levels of serotonin in certain brain areas and that the PS phase depends upon noradrenaline containing neurons(125). It has also been postulated that some metabolic step requiring the enzymatic catalysis of MAO could play an important role in the transition between SWS and PS (125). In addition, barbiturates have been reported to reduce the duration of the PS phase (127, 186). These observations together with our results demonstrating a marked MAO inhibition in the brain of pentobarbital-treated rats, suggest that the decrease in PS after the administration of barbiturates could be accounted for by the inhibition of this enzyme.

The following points would favor this hypothesis:

a) A marked increase in brain serotonin has been demonstrated following the administration of several

barbiturates (177, 178, 179, 180, 181).

b) It can be observed in our experiments that MAO is inhibited in the brain 30 minutes after the administration of single doses of pentobarbital and this time course lies well within the time of brain serotonin elevation in rats submitted to the same treatment ( 5 minutes to 2 hours) (178).

c) More inhibition was demonstrated in our experiments when MAO activity was measured with serotonin as the substrate.

d) It has been reported in the literature that a number of MAO inhibitors prolong the duration of the hypnosis produced by several barbiturates (182). The possibility must be considered that barbiturates increase brain serotonin levels through the same mechanism that MAO inhibitors exert this effect.

Against the mentioned interpretation of our results one can offer the following observations:

a) It has been concluded by other investigators that the potentiation of barbiturate-induced hypnosis by MAO inhibitors is probably due to a decrease in the metabolism of barbiturates by microsomal enzymes, and it has been postulated that this potentiation has nothing to do with a possible inhibition of MAO by barbiturates (178, 179).

These conclusions are based on the demonstrated inhibition of microsomal drug metabolizing enzymes in the presence of MAO inhibitors, as well as on the observation that the effect of MAO inhibitors on hexobarbital hypnosis is short-acting while the inhibition of brain MAO activity after the administration of iproniazide is long-acting. In addition it was observed that the increase of brain serotonin was more rapid following the administration of barbiturates than following iproniazide. Nevertheless, iproniazide is a long-acting MAO inhibitor and, still more important, the MAO activity in the brain of the experimental animals was never reported. On the other hand, serotonin itself produces potentiation of the hypnosis induced by hexobarbital (183) and Mahler and Humoller have postulated that some serotonin metabolite could play a key role in the brain regarding the hexobarbital-serotonin interactions. In consequence, these experiments cannot be considered as a direct evidence against our hypothesis.

b) Since the maximal inhibition of brain MAO demonstrated in this work was 47%, another argument against this hypothesis could be the opinion that at least 85% inhibition of brain MAO is required in order to detect increased levels of biogenic amines

in the same tissue (109).

Following the review of the literature, this concept cannot be considered as a general rule (122, 123, 124, 126). For example, Chessin et al (122) concluded that between 50 and 100% inhibition of brain MAO was required to observe increased levels of serotonin in this tissue. In the same line, Dubnick et al (123) have reported elevation of brain serotonin 3 hours after giving phenelzine, MAO activity being inhibited at the same time by 46%.

In addition, it is known that MAO activity is more concentrated in the hypothalamus than in other areas of the brain (142), and that iproniazide produces more inhibition of MAO in the hypothalamus than the enzymatic inhibition detected in the whole brain (142). Since in our experiments MAO activity was assayed in the whole brain it is difficult to conclude, at this time, the degree of enzymatic inhibition in the hypothalamus after the administration of pentobarbital. Further investigations could be of great value to clarify this point.

Thiopental was also demonstrated to produce in vivo a significant inhibition of MAO activity measured in various rat tissues. After the administration of 60 mg/kg of this barbiturate a greater inhibition was observed in the heart than in the liver. In both tissues

the percent of inhibition was comparable to the inhibitory effect produced after the administration of a similar single dose of pentobarbital. However, in the liver, the maximal inhibition was observed 10 minutes after the treatment with thiopental while the maximal effect of pentobarbital was observed 30 minutes after treatment.

Perhaps the more important difference between the effect of both barbiturates is the observation of MAO inhibition in the brain for kynuramine following the administration of a single dose of thiopental.

There are two main differences between both barbiturates which could explain their different effect on "kynuramine oxidase" at the cerebral level:

a) Pentobarbital is not metabolized in the brain (163, 164) while thiopental can be oxidized by the microsomal enzymes of the same tissue. In consequence, brain MAO activity assayed with kynuramine could be inhibited by some thiopental metabolite. This explanation is supported by our results demonstrating MAO inhibition for kynuramine after chronic treatment of rats with pentobarbital.

b) Another difference is that thiobarbiturates have a greater ability to cross the blood-brain than oxibarbiturates (60). Then following the administration

of thiopental the obtained levels in brain could be greater than the respective levels after the injection of pentobarbital.

In addition, the administration of 120 mg/kg of phenobarbital was also demonstrated to inhibit MAO in the liver and heart of rats.

Our results in the experiments dealing with thiopental and phenobarbital would support the hypothesis that barbiturates could reduce the PS phase of sleep through inhibition of MAO.

SUMMARY AND CONCLUSION

(Claims to original contributions are marked with asterisk)

A) The total proteins and MAO activity of various subcellular fractions obtained from several tissues of control and hyperthyroid rats have been investigated in this work (part I). The results can be summarized as follows:

- 1) The total proteins were increased in the microsomal fraction of the liver and heart of thyrotoxic animals.
- 2) MAO activity was mainly localized in the mitochondrial fraction obtained from the liver brain and kidneys of control and hyperthyroid rats, while the specific enzymatic activity was much higher in the microsomal fraction isolated from the heart of the same type of animals.
- 3) The overall MAO activity was decreased by 53% in the liver of hyperthyroid rats.
- \* 4) This inhibition was mainly detected in the mitochondrial fraction.
- 5) The enzymatic activity was increased by 86% in the heart of hyperthyroid rats.

- \* 6) The increase in cardiac MAO activity was reflected to a greater extent in the microsomal than in the mitochondrial fraction.
- 7) MAO activity was unchanged in the brain and kidneys of thyrotoxic rats.

These results which interpretation is complicated by the possible existence of several forms of MAO have been discussed. It appears that the observed increase of MAO in the heart of hyperthyroid rats is a non-specific effect which could be accounted for by the observed protein increase. Perhaps the thyroid hormones produce a more direct effect on the hepatic MAO activity of hyperthyroid rats. Our experiments demonstrating that in the liver of thyrotoxic animals the enzyme is mainly inhibited in the mitochondrial fraction could suggest that MAO is inhibited by some metabolite of the thyroid hormones.

B) The effect of pentobarbital on the liver and heart MAO has also been investigated using control and iodinated casein-fed rats.

- \* 1) The intraperitoneal injection of 30 mg/kg of this barbiturate produced 20% inhibition of MAO in the liver of control rats. The enzyme was inhibited by 35% in the heart of the same

type of animals following a similar treatment. For both tissues, the inhibition exerted by pentobarbital was reflected to a greater extent in the microsomal than in the mitochondrial fraction.

- \* 2) The hepatic MAO activity was more inhibited in hyperthyroid rats treated with pentobarbital than in thyrotoxic animals without barbiturate pretreatment. The additional inhibition was mainly detected in the microsomal fraction of the hepatic tissue.
- \* 3) The more striking effect of pentobarbital was observed in the heart of hyperthyroid rats where a 57% inhibition of MAO was detected instead of the 86% increase demonstrated in thyrotoxic animals without barbiturate pretreatment.

These findings together with previous works in the literature reporting alterations of several flavoenzymes by some barbiturates could offer an explanation for the increased mortality observed in rats treated with barbiturates.

C) Some other details about the inhibitory effect of pentobarbital on MAO activity were studied in several tissues of normal rats (part II).

- \* 1) When kynuramine was employed as the substrate

for MAO, more inhibition of the enzyme was observed in the liver with increasing single doses of the barbiturate. The highest inhibition was 58% in the liver, and 40% in the heart. High doses of the barbiturate were required to inhibit the enzyme by 58% in the liver. However, when 30 mg/kg were injected a marked inhibition was obtained in the hepatic tissue and MAO activity was decreased by 37% in the heart. The inhibitory effect was reversible in a short period of time for both tissues.

- \* 2) When the enzymatic activity was also assayed with kynuramine, no MAO inhibition was demonstrated in the brain and kidneys of rats following the administration of single doses of pentobarbital. Nevertheless, the enzyme was inhibited by 21% in the brain of the animals which had been treated with the barbiturate during three weeks.
- \* 3) Mitochondrial MAO from rat liver was inhibited also in vitro by sodium pentobarbital. High concentrations of the barbiturate were required to inhibit the enzyme by 50% ( $6 \times 10^{-3}$  M).
- \* 4) The Lineweaver and Burk plot obtained suggests that the in vitro inhibition of mitochondrial

MAO from rat liver by sodium pentobarbital proceeds through a competitive mechanism

- \* 5) MAO activity measured with kynuramine was neither inhibited in vivo or in vitro by a pentobarbital metabolite (pentobarbital alcohol).
- \* 6) MAO activity assayed with three other substrates was also inhibited in various tissues of rats which had been treated with single doses of pentobarbital:
  - a) In the liver and heart the enzymatic activity was more inhibited for serotonin and tyramine than for benzylamine.
  - b) Pentobarbital inhibited brain MAO assayed with these three substrates and the effect was more marked with serotonin and benzylamine.
  - c) MAO activity was also inhibited in the kidneys when measured with tyramine.
- \* 7) MAO activity was also determined with serotonin or tyramine as the substrates in the liver and heart of rats which had been treated with 40 mg/kg of pentobarbital alcohol. The effect of this metabolite was parallel to that shown after giving the parent compound.

These results are compatible with the existence of at least three forms of MAO in the liver and brain of rats. However, it is difficult to conclude on the basis of these experiments how many different types of MAO are present in every tissue. Our results would also suggest that pentobarbital directly inhibits "kynuramine oxidase" while its effect on "serotonin oxidase" and "tyramine oxidase" seems to be achieved, at least in part, through the action of the pentobarbital alcohol.

On the other hand, the inhibition of MAO activity in the brain of pentobarbital-treated rats could suggest that the observed reduction of the paradoxical phase of sleep following the administration of barbiturates is produced through the inhibition of this enzyme.

C) The in vivo effect of thiopental and phenobarbital on MAO activity measured with kynuramine has also been presented in the last section of this work.

\* 1) MAO activity was inhibited in the liver, heart, brain and kidneys of rats after the animals were treated with single doses of thiopental.

\* 2) Phenobarbital was shown to inhibit MAO activity only in the liver and heart of the animals.

Our results in this section of the thesis would support the postulated reduction of paradoxical sleep.

by barbiturates through the inhibition of brain MAO.

In conclusion, we do not think that our results (part I) provide the answer for the mechanism underlying the interrelationships between thyroid hormones and catecholamines. However, some details concerning the interactions between thyroid hormones and MAO at the subcellular level have been added to the current knowledge in the field.

We hope that our experiments dealing with MAO and barbiturates may lead to additional experiments in order to clarify some of the effects of these drugs on the central nervous system.

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