THYROID HORMONES AND BRAIN MONOAMINE OXIDASE ACTIVITY: AN IN VITRO STUDY

A Dissertation

Presented to

the Faculty of the Department of Biophysical Sciences

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

---

by

Magdi Mikhaeil Asaad

June 1976

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ABSTRACT

In vitro experiments were made to study the effect of L-thyroxine and L-triiodothyronine upon the activity of whole rat brain monoamine oxidase.

Using tryptamine as the substrate, evidence was obtained for the presence of a preformed endogenous inhibitor of monoamine oxidase. The activity of the inhibitor was suppressed by the thyroid hormones so as to increase monoamine oxidase activity and indoleacetic acid formation. This effect of L-thyroxine and L-triiodothyronine was concentration related, temperature dependent, reversible, stereochemically independent (obtained with D and L forms), and was critically dependent upon pre-incubation of the hormones with the homogenate prior to substrate addition. Additionally, the effect of the thyroid hormones was absent in a washed crude preparation of brain mitochondria but could be restored by replacing certain of the removed cellular components.

No evidence of inhibitor activity was found using kynuramine as the substrate. Neither L-thyroxine nor L-triiodothyronine influenced product formation (4-hydroxyquinoline). However, mixed substrate experiments revealed that tryptamine could inhibit kynuramine deamination but the extent of this inhibition was not modified by prior incubation with the thyroid hormones. Thus, L-thyroxine and L-triiodothyronine appear to modulate the activity of a tryptamine sensitive monoamine oxidase which is not involved with the deamination of kynuramine.

Studies made in whole tissue homogenates from liver, kidney and heart revealed an organ-related selectivity in the ability of the thyroid hormones to enhance indoleacetic acid production from tryptamine; cardiac

monoamine oxidase activity being hardly affected. Thus, the effectiveness of thyroid hormones upon monoamine oxidase activity is characteristic of the organ. From studies made with brain, it was shown that the activity of the thyroid hormones toward monoamine oxidase activity did not vary with a progressive increase in animal weight and thus appears age-independent.

The present study has described, for the first time, (1) the presence of a tissue inhibitor or inhibitors for a tryptamine-sensitive monoamine oxidase and (2) the suppression of inhibitor activity by the thyroid hormones. These new *in vitro* findings may have relevance to thyroid control mechanisms on monoamine oxidase activity *in vivo*.

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I. STATEMENT OF THE PROBLEM

Current interest in factors which modify monoamine oxidase (MAO) activity is increasing rapidly. MAO plays a vital role in the deamination of certain endogenously occurring amines and deviations in the activity of the enzymes have been connected with several pathological conditions in man. Investigators have shown that a variety of exogenous and endogenous factors can serve to modulate the activity of MAO, including endocrine secretions. Among the latter, the thyroid hormones have been shown to alter the MAO activity of heart, liver and kidney, as well as that of certain other organs.

The mechanism or mechanisms by which L-thyroxine and L-triiodothyronine produce their effects upon MAO are ill-defined. Both increases (heart) and decreases (liver) in enzyme synthesis have been suggested. However, preliminary studies made in our laboratories (Moonat *et al.*, 1975) have indicated rapid and possible direct actions of the hormones, perhaps upon MAO itself, and/or modulating substances. These studies were made in the kidneys of rats, but no information was obtained on other organs. Since MAO of the brain is believed to play a key role in neurogenic function and in the extra-neuronal metabolism of monoamine neuro-transmitters, studies were undertaken to examine the effect of both L-thyroxine and L-triiodothyronine upon whole brain MAO activity.

The work to be described was conducted entirely *in vitro* in order to more specifically determine the effects of the hormones under conditions of limited variability. This approach is particularly important when dealing with the thyroid hormones because of their diverse physiological functions under *in vivo* conditions.

The broad objective of the study pertains to elucidating possible control mechansims serving to regulate the deaminating activity of MAO. A close interplay of neurogenic function and endocrine secretions serves to maintain internal homeostasis. Peripheral and central interactions between these two controlling systems provides fine cellular, organ and inter-organ physiological regulation and integration. A point of intimate interplay may be located at the level of MAO, particularly for the thyroid hormones and their interaction with monoaminergic mechanisms.

II. LITERATURE SURVEY

1. An introduction to monoamine oxidase.

In 1928 Hare identified a rat liver enzyme which oxidized tyramine and since monoamine oxidase was unknown at that time, the enzyme was named tyramine oxidase. Later, it was recognized that the same enzyme catalyzes amines other than tyramine (Blaschko et al., 1937; Pugh and Quastal, 1937a and 1937b). Zeller (1951) was the first to term this enzyme monoamine oxidase to distinguish it from diamine oxidases. Monoamine oxidase (MAO)(monoamine:oxygen oxidoreductase (deaminating) E.C.1.4.3.4) catalyzes the oxidative deamination of monoamines such as dopamine, 5-hydroxytryptamine and norepinephrine, which are considered to be neurotransmitters in the central nervous system (Sandler and Youdim, 1972; Tipton, 1972). Beside tryptamine and tyramine, many other primary and secondary amines not occurring naturally in animal organisms are substrates for MAO (Zeller, 1951; Blaschko, 1952; Davison, 1958; Gorkin, 1966). The catalytic product of MAO is the corresponding amine aldehyde as shown below:

$$R - CH_2NH_2 + O_2 + H_2O \longrightarrow R - CHO + NH_3 + H_2O_2$$

MAO differs from other mammalian amine oxidases in being insensitive to inhibition by carbonyl reagents such as cyanide and semicarbazide (Blaschko, 1963). Blaschko (1963) attributed such a difference to the coenzymes employed by the different types of oxidases, since MAO has been shown to be a flavoprotein, while diamine oxidase and spermine oxidase are both pyridoxal-phosphate dependent. An additional difference is that MAO is active towards N-methylated amines, while the other

mammalian amine oxidases will not oxidize these compounds (Blaschko, 1963).

The substrate specificity of MAO has been reviewed in considerable detail (Blaschko, 1952; Tipton, 1973). MAO has a high affinity for phenylethylamine derivatives which lack a beta-hydroxyl substitutent and which have monophenolic or catechol substituents in the aromatic ring (Blaschko et al., 1937; Blaschko, 1952; Pratesi and Blaschko, 1959). Phenylethylamine derivatives with an alpha-methyl substituent (amphetamine, ephedrine, methamphetamine, alpha-methyl norepinephrine) are not substrates for the enzymes, but instead act as competitive inhibitors (Blaschko et al., 1937). In most species, the enzyme shows no stereochemical specificity for the optical enantiomers of epinephrine and norepinephrine (Giachetti and Shore, 1966).

Although MAO has been demonstrated to be localized on or in the outer mitochondrial membrane (Schnaitman et al., 1967), there is no evidence that MAO is synthesized in the mitochondria. Erwin and Simon (1969) postulated a precursor pool relationship between the microsomal and mitochondrial enzyme, whereby MAO originates in the microsomes and is subsequently transferred to the mitochondria. In more recent studies Erwin and Deitrich (1971) confirmed their previous findings that under total irreversible enzyme inhibition, the rate of return of newly synthesized enzyme was more rapid in the microsomal fraction than in the mitochondria. They showed that the half-life of MAO in the outer mitochondrial membrane is approximately 1.8 to 3.5 days, whereas that located in microsomal membrane is approximately twenty-four hours.

It is now generally accepted that the enzyme contains one mole of flavin co-factor per 120,000 g protein, identified as flavin adenine dinucleotide (FAD) (Erwin and Hellermann, 1967; Tipton, 1968). Most of the purified preparations of MAO show flavin fluorescence and this fluorescence is greatly reduced by the presence of substrate; this implies that the flavin is reduced by the amine (Tipton, 1973a). The strength of attachment of FAD to the enzyme appears to be species dependent. For instance, in beef kidney and liver it is tightly bound (Erwin and Hellerman, 1967; Igaue et al., 1967), while the FAD appears to be more loosely bound in pig brain (Youdim and Sourkes, 1972).

Nara et al., (1966) suggested that MAO may be a copper containing protein. However, this suggestion has not received wide support because purified preparations of MAO have been found to contain very little copper (Akopyan et al., 1971; Oreland, 1971; Youdim and Sourkes, 1966) and copper deficiency in animals does not affect enzyme activity (Shih and Eiduson, 1969; Youdim and Sourkes, 1966). However, it now appears that rat liver MAO may depend on a nutritional requirement of iron for its activity (Symes et al., 1969; 1971). The presence of about one atom of iron per 300,000 g of protein has been reported in purified preparations of rat liver MAO (Youdim and Sourkes, 1966), but considerably lower concentrations of this metal have been reported to be present in preparations of the enzyme from other sources. Iron has also been detected in the pig liver enzyme, but some fully active preparations contained only 0.5 atoms of iron per mole of enzyme (Oreland, 1971).

2. Multiple forms of monoamine oxidase.

There have been many suggestions that MAO exists in more than

one form and it has been postulated that such multiple forms of MAO might have different physiological roles in the deamination of the various biogenic amines (Gorkin, 1966; Eidusen, 1972; Sandler and Youdin, 1972). For instance, Alles and Heegaard (1943) tested a large number of substrates using extracts of liver enzyme from different species. They reported marked species differences, as judged by the relative rates of oxidation. Also in 1955, Satake suggested that MAO may be a mixture of enzymes that have different substrate specificities and that each tissue may have a different distribution of the constituent enzymes.

More recently, much additional evidence for multiple forms of MAO has accumulated. Youdim and Sandler (1967) were among the first to report that the MAO of rat tissues could be separated into several bands of activity by polyacrylamide gel electrophoresis. Using this technique, several forms of MAO have been found by other investigators (Kim and D'Iorio, 1968; Gorkin, 1969) in various rat tissues and in other species. One form of the enzyme found on electrophoresis has been designated as "dopamine MAO" because dopamine deamination occurred at a faster rate than that of other amines, and the Km for dopamine was lower than for other substrates (Youdim, 1973). The thermal stability characteristics (Youdim and Sourks, 1965; Jarrott, 1971) and pH optima (Barbato and Abood, 1963; Youdim and Sourks, 1965) of mitochondrial MAO preparations also vary with the substrate employed, and have similarly been used as criteria for enzyme multiplicity (Gorkin and Romanova, 1959;; Gorkin, 1966).

Further evidence for the existence of multiple forms of MAO stems from the work of Johnston (1968). Using a new MAO inhibitor, clorgyline,

(M & B 9302, N-methyl-N-propargyl-3-(2,4-dichloro phenoxy propylamino hydrochloride), he showed that a double sigmoidal inhibition curve resulted when tyramine was employed as the substrate. Pre-incubation with low concentrations of clorgyline resulted in a progressive, concentration-dependent inhibition of tyramine deamination in rat brain homogenate. The inhibition curve became invarient with increasing inhibitor concentration at about 50% inhibition, but then progressed to completion at much higher clorgyline concentrations. In order to account for this phenomenon, Johnston (1968) postulated that there were two forms of MAO in brain; an enzyme sensitive to clorgyline, which was designated enzyme A, and a more resistant form, which was designated enzyme B. Neff and Goridis (1972) termed these two forms Type A and Type B enzymes, suggesting two distinct classes of enzymes with similar characteristics rather than two single enzymes. Johnston (1968) also found that serotonin was a preferred substrate for Type A MAO and Hall et al., (1969) found that benzylamine was a preferred substrate for Type B MAO. One endogenously occurring preferred substrate for the Type B enzyme is beta-phenylethylamine (Yang and Neff, 1973). Tryptamine and tyramine are good substrates for both enzyme types (Johnston, 19680. Hall et al., (1969) showed that clorgyline could be used to demonstrate the presence of multiple enzyme forms in several species, but not in all, while McCauley and Racker (1973) reported that they could distinguish between MAO A and MAO B by their antigenic properties.

The following table shows the preferred substrates and specific inhibitor drugs for the two types of MAO, as summarized by Neff and Yang (1974).

	Monoamine Oxidase	
	Type A	Type B
Preferred Substrates	Serotonin Norepinephrine Normetanephrine	Benzylamine β-phenylethylamine
Specific Inhibitor Drugs	Clorgyline Lilly 51641 Harmaline	Deprenyl
Common Substrates	Dopamine, Tyramine and Tryptamine	
Non-specific Inhibitor Drugs	Pargyline, Phenelzine, Iproniazid, Isocarboxazid, Tranylcypromine, Nialamide Pheniprazine	

The whole problem of the multiple forms of MAO was made controversial when Houslay and Tipton (1973) proposed that the multiple enzymes found on polyacrylamide gel electrophoresis might be artifacts that result during the preparation of the enzyme. For instance, they showed that the MAO reported by Youdim et al., (1969) which remained on the surface of the gel occurred only when the enzyme preparation was mixed with Sephadex G200 before electrophoresis. Houslay and Tipton (1973) proposed that this form of the enzyme was an artifact of the loading method and was probably due to aggregation or precipitation of the enzyme when it was mixed with dry Sephadex. Moreover, they found that treatment of a

partially purified enzyme preparation with perchlorate, to remove phospholipid before electrophoresis, resulted in a single band of enzyme activity. Furthermore, they were unable to detect the specific "dopamine MAO" as reported by Youdim et al. (1969). As a consequence of these studies, Houslay and Tipton (1973) concluded that the multiple forms of MAO may represent a single enzyme protein with different amounts of attached phospholipid and that the phospholipid may govern the enzyme's mobility during electrophoresis. However, it is very important to note that Houslay and Tipton (1973) lost more than half of the MAO activity during their purification procedure which indicates that labile forms of the enzyme were lost during preparation and thus their conclusion that there is only a single species of enzyme must remain problematical. However, Veryoukina et al., (1964) suggested that multiple forms of MAO could be due to the attachment of membranous debris or varying amounts of phospholipid. These ideas would support the observation of Tipton (1973a) that no separable bands of activity can be found when rat liver MAO is subjected to polyacrylamide gel electrophoresis in Triton-X-100 (presumably due to the removal of the phospholipid component). Other workers have explained the concept of multiple forms of MAO upon the basis of conformational differences (Collins et al., 1970b; Youdim and Collins, 1971) and polymerization of enzymatically active subunits (Gomes et al., 1969).

Irrespective of the precise nature of the enzyme or enzymes, credibility for the existence of the two major types of MAO has been recently obtained in vivo. Following the administration of clorgyline to rats, brain serotonin metabolism was decreased, whereas phenylethylamine oxidation was unaffected. The reverse situation prevailed

following the administration of Deprenyl (Neff et al., 1974). Similar results were obtained by Green and Youdim (1975); however, they pointed out that both Type A and Type B MAO seems to act as an integrated system. The rise in brain serotonin levels was maximal after inhibition of both types, suggesting that when MAO Type A is non-functional, serotonin metabolism may still proceed via the Type B enzyme. In other studies, blockade of MAO with Clorgyline reversed the amine depletatory action of reserpine (Neff et al., 1974; Fuentes and Neff, 1975). In line with the dual affinity of dopamine for both MAO Types A and B in vitro, it was also found that both clorgyline and Deprenyl could increase brain dopamine levels (Neff et al., 1974). More recently, Bakhle and Youdim (1976) have shown that serotonin metabolism in the perfused rabbit lung can be inhibited by clorgyline, while that of phenylethylamine is unaffected. Furthermore, when both amines were perfused concomitantly, no interference in deamination rates was obtained, indicating separate enzyme activities against the two substrates. These data, taken in conjunction with in vitro studies using cell homogenates, provide strong evidence for at least two major forms of MAO which seem to posess differing substrate and inhibitor specificities and physiological roles.

3. Localization and distribution of monoamine oxidase.

MAO is present in most organs of the body (Blaschko, 1952; Davison, 1958). It is either absent or present in very low amounts in skeletal muscle and erythrocytes, although it does occur in platelets (Collins and Sandler, 1971) and a variant form of soluble MAO exists in the plasma (McEwen and Cohen, 1963). The brain, liver, kidney, intestine, stomach and aorta are considered to be rich sources of the enzyme (Tipton, 1973b).

Intracellularly, MAO is localized almost exclusively in the mitochondria and is associated with the outer mitochondrial membrane (Ernster and Kuylenstierna, 1970; Schnaitman et al., 1967). Jarrott and Iversen (1968) have indicated that the relatively small amount of MAO activity, previously reported to be in the microsomal fraction of the cell, seemed to have been derived from the mitochondria as a result of translocation occurring during the cell fractionation procedure. However, in the liver, there is good evidence that a small fraction of MAO is associated with the microsomes (Erwin and Deitrich, 1971) and that this may represent the precursor pool for the mitocondrial enzyme.

The observation that homogenization could release MAO from the mitocondria of some tissues and not others implied that mitochondria are probably not homogeneous and indeed, there is evidence for heterogenity. Mitochondria from glial cells and from neuronal cells of rabbit brain have different buoyancies (Hamberger et al, 1972). Furthermore, the activities of several enzymes varies in different populations of mitochondria (Blokhius and Veldstra, 1970), including the activities of Type A and Type B MAO (Yang and Neff, 1973). In actual fact, Yang and Neff (1973) indicated that more Type B MAO activity is associated with mitochondria of high buoyancy than with mitochondria of low buoyant density.

Several studies have shown that MAO exists both extra-neuronally and intra-neuronally, the latter being located within monoaminergic neurons. In general, the proportion of extra-neuronal MAO to the intra-neuronal enzyme is very much higher, although specific exceptions are to be found. In the central nervous system, MAO has been shown to be present

in various types of glial cells (Silberstein et al., 1972), as well as in synaptosomes (Rodriguez de Lores Arnaiz and De Robertis, 1962). However, the proportion of the total MAO located inside brain and spinal cord neurons is very small, since no detectable fall in enzyme activity has been observed following wide-spread denervation studies (Goridis et al., 1972). Similarly, the vast majority of cardiac MAO in the rat (Horita and Lowe, 1972; Lowe et al., 1975) and that found in the rabbit ear artery (DeLa Lande et al., 1970) is extra-neuronal in locus. Results from immunosympathectomized rats (Klingmann, 1966) and rats in which sympathetic nerve degeneration has been achieved with 6-hydroxydopamine (Jarrott, 1971; Clarke and Sampath, 1976) have indicated the preponderance of extra-neuronal MAO. Thus, kidney and liver MAO seems virtually 100% extra-neuronal (Jarrott, 1971; Clarke and Sampath, 1976) and about 70-80% of that found in the spleen and salivary glands is located outside of the nervous innervation (Jarrott and Iversen, 1971). Conversely, the rat vas deferens exhibits a very high proportion of neuronal MAO since a 50% reduction in total activity occurs upon denervation (Clarke and Sampath, 1972). Quantitative estimates of the proportion of intraneuronal and extraneuronal MAO which are based upon denervation experiments may be open to doubt, since a fall in the activity of an extraneuronal enzyme might be expected if it depended on an intact nerve supply for full activity (Marsden et al., 1971). However, the clear and consistent fall in MAO activity following denervation of the vas deferens (Jarrott, 1971; Clarke and Sampath, 1972; 1976) the pineal gland (Snyder et al., 1965) and the iris (Waltman and Sears, 1964) has been interpreted to indicate that at least part of the MAO activity in peripheral tissues

is associated within sympathetic nerves. Several other lines of evidence, although mainly indirect, strongly support this contention. Biochemical studies, utilizing MAO inhibitor drugs (Pletscher, 1958; Clarke and Sampath, 1973) and histochemical studies on the repletion of noradrenergic nerves following reserpine depletion (Malmfors, 1965) have provided convincing evidence for an intraneuronal locus of MAO. Acidic and alcoholic metabolites have been recovered from tissues in which the nerves have been previously loaded with radio-labeled transmitter (Langer, 1974). These studies were carefully designed so as to preclude the possibility of extra-neuronal deamination. Finally, one of the most convincing and direct studies was made by Roth and Stone (1968). They used pure bovine splanchnic nerve pieces in vitro and showed the formation of deaminated metabolites of both dopamine and norepinephrine following incubation with ^{14}C-tyrosine.

This experiment serves to illustrate the vital importance of intraneuronal MAO in the physiology of the neuron, even though it most often represents only the minority of the total enzymatic activity of any given tissue.

Interestingly, it has been shown that the amine found in tissues may in part govern the presence or absence of MAO, or the presence of a particular type of MAO. For instance, Consolo et al., (1968) reported that there was no MAO in cholinergic neurons, whereas the sympathetic nerves that innervate the pineal gland (Goridis and Neff, 1971), and the mesenteric arteries (Coquil et al., 1973) contain primarily MAO Type A, the enzyme that metabolizes norepinephrine (Goridis and Neff, 1971). However, the presence of a particular amine in a tissue is not always

an indication that the "proper" MAO will be present in that tissue. For instance, serotonin is found in the rat pineal gland where it is an intermediate for the synthesis of 5-methoxy-N-acetyl tryptamine (melatonin)(Lerner et al., 1958). Lerner et al., (1958) have shown that the predominant MAO in the pineal gland is the Type B enzyme which is not active toward serotonin; thereby assuring that serotonin will remain available for the formation of melatonin. Meanwhile, Willner et al., (1974) indicated that the highest brain concentration of beta-phenylethylamine, which is a preferred substrate for Type B MAO, is found in the pineal gland. The physiological significance of these observations is not, as yet, clarified.

An interesting observation stemmed from recognizing different localizations for MAO, the presence of Type A MAO in sympathetic neurons and its apparent absence at some post-synaptic sites, such as the pineal parenchymal cells. This observation is consistent with the hypothesis that primary oxidative deamination of norepinephrine takes place within neurons and to a lesser extent at post-synaptic sites (Kopin and Axelrod, 1963).

4. The functional role of monoamine oxidase.

The physiological role of MAO is not fully understood despite the fact that it has been extensively discussed in several publications (Zeller, 1959; Kopin, 1964; Axelrod, 1965). One probable function is the destruction of potentially toxic amines that are ingested in foodstuffs, and the particularly high levels of MAO activity in the liver, stomach and intestine may be important in this respect. Support for such a protective function of MAO comes from the severe hypertensive

reaction which can occur when patients who have been treated with MAO inhibitors, like tranylcypromine or pargyline, eat cheese or other foods with a high tyramine content (Cuthill et al., 1964; Glazner et al., 1964; Knoll and Magyar, 1972). Under these conditions the primary metabolic route of tyramine is inhibited, and excessive amounts of norepinephrine are released from sympathetic nerve endings, causing intense cardiac stimulation and wide-spread vasoconstriction. Other amines are likewise involved, such as dihydroxyphenylalanine (high concentration in kidney beans) and serotonin (high concentration in bananas).

The circulating concentration of several amines found to occur endogenously in the body is also critically dependent upon MAO activity. For instance, following MAO inhibition, urinary tryptamine levels are increased (Sjoerdsma et al., 1959a and 1959b). Peripheral MAO activity has been shown to govern the brain levels of beta-phenylethylamine (Borison et al., 1975). Hyperactivity and CNS stimulation following the use of MAO inhibitors may, in part, be related to this phenomenon. On the other hand, circulating levels of the sympathetic neuro-transmitter norepinephrine and the hormone epinphrine, appear to be largely independent of MAO activity despite the fact that they are good substrates for the enzymes. O-methylation by catechol-o-methyl transferase (COMT) appears to be the primary metabolic route, which may then be followed by deamination via MAO (Molinoff and Axelrod, 1971; Iverson, 1973).

Historically, Zeller (1959) had proposed that O-methylation of catecholamines could either precede or follow oxidation and suggested that both pathways may function in the kidney and liver. However, Tipton

(1972) showed that the Km value of rat liver MAO for metanephrine (the O-methylated metabolite of epinephrine) was some ten-fold lower than the Km value for epinephrine itself; indicating that MAO would be more efficient at oxidizing low concentrations of the O-methylated amine derivatives. Circulating catecholamines must be taken up by tissues prior to metabolism due to the intra-cellular location of catechol-o-methyl transferase and MAO. The extra-neuronal uptake of norepinephrine and epinephrine by a variety of tissues is well established (Gillespie, 1973) and includes cardiac and smooth muscle cells (Clarke et al., 1969; Gillespie and Hamilton, 1966), fibroblasts (Jacobowitz and Brus, 1971) and the glial cells of the brain (Hendley et al., 1970). The primary metabolic product is the O-methylated derivative (Iversen and Salt, 1970) either because of substrate availability and/or because of affinity differences (see Tipton, 1972, above). However, both MAO and COMT must be inhibited concomitantly to prevent metabolism (Iversen and Salt, 1970), thus explaining why MAO inhibition or inhibition of COMT alone fails to potentiate the effects of circulating catechols.

In addition to extraneuronal metabolism, uptake of circulating epinephrine and norepinephrine by sympathetic nerve terminals seems to represent a most important mechanism for terminating the activity of these amines (Iversen, 1967), although the relative importance of this process appears to be highly organ selective (Burnstock and Costa, 1975). Whereas extra-neuronal MAO appears to play a secondary role to COMT, the MAO found inside monoaminergic neurons seems to function as the primary metabolic enzyme. In fact, only sparse evidence exists for neuronal localization of COMT (Guldberg and Marsden, 1975). The functional role

of intra-neuronal MAO is still under debate. Neuronal MAO has long been implicated in the physiological control of norepinephrine levels, as well as dopamine and serotonin, but the precise site at which this controlling influence is mediated is still uncertain. For instance, in noradrenergic neurons, MAO might preferentially deaminate newly synthesized amine (Roth and Stone, 1968; Clarke and Sampath, 1973) or that which, once manufactured, becomes accumulated within intra-neuronal vesicles (Axelrod et al., 1961; Kopin and Gordon, 1963). Alternatively, the intra-neuronal role of MAO might be concerned with norepinephrine that is removed from the extracellular space by means of the neuronal uptake process (Tarlov and Langer, 1971; Langer et al., 1972; Langer, 1974). Malmfors (1969) speculated that norepinephrine might undergo intra-neuronal deamination subsequent to vesicular release by nerve impluses whereas Weiner and Bjur (1972) and Trendelenburg et al., (1972) proposed that MAO limits excessive accumulation of "free" or cytoplasmic norepinephrine so as to release tyrosine hydroxylase from end-product inhibition. MAO inside noradrenergic neurons appears to deaminate dopamine (Roth and Stone, 1968) and therefore may function indirectly to control norepinephrine levels by limiting the availability of its precursor substance for beta-hydroxylation (Clarke and Sampath, 1973). Overall, a multifunctional role for intra-neuronal MAO seems probable, where the enzyme serves as one of several important processes contributing to the homeostatic mechanisms of neuronal regulation.

More recently it has become apparent that the amine metabolites resulting from intra-neuronal deamination, and their subsequent biochemical interactions, may be of physiological importance for optimal

neuronal function. Langer (1974) has shown that intra-neuronal aldehyde reductases catalyze the conversion of 3,4-dihydroxyphenylglycolic aldehyde to 3,4-dihydroxyphenylglycol (major intra-neuronal metabolite of norepinephrine) with the concomitant formation of $NADP^+$. $NADP^+$ levels are the limiting factor for the oxidation of glucose via the pentose shunt (Wenner and Weinhouse, 1956). Langer (1974) considers that stimulation of the pentose shunt would lead to high intra-neuronal levels of NADPH, which in turn, are required to form dihydrobiopteridine, the co-factor of tyrosine hydroxylase. Thus, the intra-neuronal metabolism of norepinephrine by MAO seems once again to represent an important line in the regulation of amine synthesis.

Although the above discussion has centered upon the importance of MAO in catechol monoamine metabolism, the enzyme also controls the intra-neuronal and extra-neuronal levels of indole amines. Tryptamine oxidation and serotonin metabolism in the rat brain (Neff and Tozer, 1968) and periphery (Neff and Yang, 1974) is primarily through deamination by MAO. Actually histochemical and biochemical studies have shown that MAO inhibition has a far greater effect on brain serotonin levels than on the endogenous levels of dopamine and norepinephrine. Again, it is presumed that intra-neuronal MAO serves to keep "free" or cytoplasmic levels of serotonin low, so as to give rise to optimal neuronal function. However, unlike noradrenergic neurons, these levels are generally thought not to be critical for synthesis control. Tryptophan hydroxylase, the rate-limiting step in serotonin synthesis, does not seem to undergo significant end product feed-back inhibition, under physiological

conditions, although under pharmacological manipulations such inhibition has been observed (Hamon and Glowinski, 1974).

Other functions have been suggested for MAO. For instance, beef thyroid gland MAO appears to be inactive towards epinephrine and norepinephrine, but tyramine is a good substrate. It has been suggested that the thyroid enzyme may function as a hydrogen peroxide generating system for iodothyronine synthesis (Fischer et al., 1966). It has also been proposed that the metabolites produced by MAO may function as regulators of cellular oxidation reactions (Gorkin and Orekhovitch, 1967), and the suggestion that the aldehydes produced by the action of MAO may be involved in the mechanism of sleep (Jouvet, 1969) may again support the implication that the function of the enzyme is not solely degradative. The still unexplained effects of MAO inhibitors on carbohydrate metabolism in vivo (Cooper and Keddie, 1964; Praag and Leijnse, 1965) are probably due to the interference of MAO inhibitors with some unknown function of MAO or may represent non-specific effects of this group of drugs.

5. Neurogenic control of monoamine oxidase.

Intraneuronal MAO plays an important role in regulating transmitter levels and neurogenic activity. Thus, it is reasonable to assume that neurogenic activity may likewise serve to regulate either the activity or levels of intraneuronal MAO itself. However, only a few investigators have given attention to this potentially important interrelationship. Clarke and Sampath (1973) used indirect methods for assessing in vivo neuronal deaminating activity in rats. They proposed a close relationship between neurogenic discharge rates and the activity

of neuronal MAO. In both central and peripheral noradrenergic neurons the intra-neuronal deaminating activity was geared to the frequency of neuronal discharge. When the rate of impulse discharge was high, deamination was considerable, but it fell toward zero in quiescent neurons. No absolute change in the *in vitro* activity of MAO was found over the six hour period of study, irrespective of whether neurons were inactive or subjected to high discharge rates. The findings agree with those of Anden *et al.*, (1967) who sectioned the spinal cord of rats and studied the acute effect of MAO inhibition. The rise in spinal cord norepinephrine obtained distal to the lesion was only one-third of that obtained in the proximal areas. These results suggest that the intra-neuronal availability of substrate may, in the long term, regulate not only the minute-to-minute activity, but also the steady-state level of MAO activity in nerves. However, specific experimental data in support of this contention is lacking. Spector *et al.*, (1972) administered L-DOPA (400-800 mg/kg) to rats for 5 days and found a graded increase in the MAO activity of the heart and mesenteric vasculature, but whether these increases occurred inside or outside neurons was not investigated. The question as to whether nerve impulses activate intra-neuronal MAO by way of changes in ion concentrations and/or availability also remains unknown. It has been suggested that the influx of calcium ions can serve to activate tyrosine hydroxylase during neuronal firing (Morgenroth *et al.*, (1973). Since the activity of this enzyme and that of neuronal MAO appear to work in concert (Clarke and Sampath, 1973), calcium may also function to activate MAO. However, the amount of MAO activity in the superior cervical ganglion of rats is not altered by chronic surgical

denervation (10 days)(Clarke and Sampath, 1975) indicating that trans-synaptic trophic influences, which would include ionic changes, are not essential to maintain the steady-state activity of cell body MAO.

Although changes in MAO activity have been observed in situations where high noradrenergic activity is chronically present (following reserpine treatment, in spontaneously hypertensive rats and rats subjected to environmental stressors)(Spector et a ., 1972; Maura et al., 1974), once again the experimentors have failed to determine whether selective influences were exerted upon the intra-neuronal enzyme. At present therefore, little is known concerning the specific effects of neurogenic influences on intra-neuronal MAO; the limitations in techniques for measuring this relatively small component of total organ enzyme presents a severe limitation on such studies. Furthermore, the physiology of peripheral and possibly central noradrenergic neurons, may vary (Clarke et al., 1974). Likewise, in vivo, it is difficult to divorce direct nerve-induced changes from those mediated by concomitant disturbances in other interrelated, but influencing systems (e.g., endocrine secretions).

6. Hormonal influences on monoamine oxidase.

Evidence is accumulating that the activity of MAO can be influenced profoundly by hormonal secretions. One area in which rapid advances have been made involves the steroid sex hormones. Variation in uterine MAO activity using histochemical techniques was originally noted in the human endometrium during the menstrual cycle (Cohen et al., 1965) and was confirmed later by Southgate et al., (1968). Changes in MAO activity during the estrous cycle have been reported to occur in a

number of organs, including the brains of rats (Holzbauer and Youdim, 1973), the adrenal glands and ovaries (Holzbauer et al., 1972; Holzbauer and Youdim, 1973; Youdim, 1975) and in plasma (Klaiber et al., 1974) and blood platelets (Belmaker et al., 1974). Evidence is available that estrogens inhibit MAO activity while progesterone increases deamination. In the human endometrium up to ten-fold increases in MAO activity were observed between the 19th and 21st day of the menstrual cycle (Southgate et al., 1968), the maximal increase coinciding with peaks in progesterone concentration. In the rat, injections of progesterone increased uterine MAO activity (Collins and Southgate, 1970), whereas estradiol inhibited the enzyme in vivo and in vitro (Collins et al., 1970a, Collins and Southgate, 1970).

Southgate (1972) found that after progesterone treatment, uterine MAO activity was more enhanced when dopamine was used as the substrate than when benzylamine or tyramine was used. In contrast, estradiol seemed without effect using dopamine as the substrate, whereas the activity towards benzylamine was inhibited. Thus, it seems as though the different forms of MAO are differently affected by estrogens and progesterones. Using kynuramine, Holzbauer and Youdim (1973) showed that estradiol could decrease uterine ovarian and adrenal gland MAO of rats, whereas progesterone increased activity. Liver, heart and kidney MAO were not affected by either hormone, demonstrating a selectivity of effect. Klaiber et al., (1974) measured the effect of estrogen on plasma MAO activity of amenorrhaic women and found a dramatic decline. This result is in line with an earlier report that contraceptive-estrogenic steroids can lower serum MAO activity (Tryding et al., 1969).

The cyclic variation in MAO activity of the plasma (Klaiber et al., 1974) and platelet (Belmaker et al., 1974) enzyme in humans appears to be closely related to corresponding changes in blood estrogen and progesterone concentrations. In rats, Holzbauer and Youdim (1973) obtained an indirect index of cyclic variations in progesterone production by measuring the progesterone content of both the adrenal and ovarian hormone. The sum of the amounts of progesterone contained in the ovaries and adrenal glands ran parallel (adrenal glands) or slightly in front of the cyclic changes in the MAO activity of the uterus and hypothalamus. Thus, a lag period seems required to affect the latter tissues, whereas adrenal MAO is quickly altered, due presumably, to the high association of progesterone with adrenal mitochondria (Youdim et al., 1974). The extent of changes in tissue MAO activity during the estrus cycle also depends upon the light-dark cycle, especially during estrus and metestrus (Holzbauer and Youdim, 1973). A considerable fall in uterine and hypothalamic MAO occurred in only a few hours upon changes from light to dark conditions. These findings imply that lowered MAO activity during this period is associated with activity and wakefulness, but studies correlating these two phenomena have not been made.

The observed variations in MAO activity are consistent with observations that the metabolism of monoamines undergoes changes during the estrus cycle. Cyclic variations in tissue catecholamines and serotonin have been reported consistently (Stefano and Donoso, 1967; Sandler, 1968; Kurachi and Horita, 1969; Greengrass and Tonge, 1971) and may occur as a consequence of changes in MAO. In turn, these changes have been linked with the variations in mood seen during the pre-menstrual phase of the

menstrual cycle, during mixed contraceptive pill administration in certain patients or during the menopausal syndrome (Klaiber et al., 1971). Further recent support has been obtained for these suggestions since Klaiber et al., (1974) have reported that estrogen therapy significantly lowered plasma MAO, which is exceedingly high in depressed women, with a corresponding elevation of the depressive symptoms. Recorded EEG responses to photic stimulation were also changed by estrogen therapy toward a situation that indicates enhanced central adrenergic activity. Depressed men were also found to have elevated levels of plasma MAO, which could be restored to normal by testosterone (Klaiber et al., 1974). In this latter respect, it has been reported previously that the ontogenic development of MAO is influenced by androgenization. Eiduson (1972) and Muntzing (1971) showed that castration of male rats increased MAO activity in the prostrate, the effect being reversed by the administration of testosterone.

The mechanism by which the steroid sex hormones alter MAO activity is not understood, although changes in the physical properties of the enzyme molecule may be involved. Yielding and Tomkins (1960) found that estrogens can inhibit crystaline glutamate dehydrogenase by disaggregating the enzyme molecule from a polymeric to a monomeric form. Similar results have been reported by Collins and Southgate (1970) for uterine MAO and this effect might well explain changes in substrate specificity. However, Youdim et al., (1974) favors the idea that the steroids might cause conformational alterations in the outer mitochondrial membrane with resultant permeability changes. MAO itself may also be affected, since the enzyme is an integral part of the outer mitochondrial membrane (Schnaitman et al., 1967).

Beside the sex steroids, other steroids have been shown to influence MAO activity.

Avakian and Callingham (1968) first showed that bilateral adrenalectomy could increase markedly the MAO activity of the rat heart, an observation which has been widely confirmed (Caeser et al., 1970; Callingham and Corte, 1972; Westfall and Osada, 1969; Bhagat, 1969; Sampath et al., 1972). In addition, the MAO activity of the spleen, vas deferens, superior cervical ganglion and the hypothalamus is also increased but no change was found in the liver, kidney or rest of the brain (Clarke and Sampath, 1976). Thus, the effects of bilateral adrenalectomy is organ specific. No change in organ MAO resulted after bilateral adrenal demedullation and all the effects of complete adrenalectomy could be prevented by replacement doses of dexamethasone (Clarke and Sampath, 1975). Other experiments revealed that pituitary influences, by way of adrenocorticotrophic hormone, were not involved (Sampath and Clarke, 1972; Clarke and Sampath, 1975). Callingham and Laverty (1973) found that the nature of the raised MAO activity of the rat heart following bilateral adrenalectomy closely resembled that present in the hearts of control rats. No significant changes were observed in the response to heat denaturization, pH changes or to inhibition by pargyline or clorgyline. Additionally, the relative activities using tyramine, 5-hydroxytryptamine, dopamine or benzylamine as substrates were the same. They conluded that bilateral adrenalectomy increased the synthesis of MAO in the rat heart and that this enzyme(s) did not differ in its properties from that normally found in the myocardium. Clarke and Sampath (1976) noticed that bilateral adrenalectomy increased MAO activity

only in organs in which there was a dense monoaminergic innervation. Using surgical denervation and chemical sympathectomy, they showed that bilateral adrenalectomy increased predominantly neuronal MAO with little or no effect upon the extra-neuronal enzyme (Sampath and Clarke, 1972; Clarke and Sampath, 1973; 1976). The relationship of an increased neuronal MAO to physiological regulatory processes within the neuron has not been fully established. However, in neonatal rats, plasma corticosteroid levels fall to extremely low levels three to fifteen days after birth (Sze et al., 1976), being equivalent to those found after adrenal gland removal. Such an event could trigger the synthesis of neuronal MAO as part of ontogenetic neuronal development. Indeed, during this time period, tryptophan hydroxylase activity undergoes a marked increase which is absolutely dependent upon steroid insufficiency (Sze et al., 1976). Some minor evidence has been presented (Clarke and Sampath, 1976), that bilateral adrenalectomy in adult rats appears to reverse the aging process. Furthermore, bilateral adrenalectomy has been shown to be far less effective at increase the MAO activity in "old" rats than in younger animals (Callingham and Laverty, 1973).

From the foregoing discussion, it might be concluded that hypophysectomy would likewise cause an increase in MAO activity, since plasma corticosterone levels would be markedly diminished. However, this is not the case. Hypophysectomy either causes no change (heart, hypothalamus, rest of the brain, vas deferens, liver, superior cervical ganglion)(Landsberg and Axelrod, 1968b; Clarke and Sampath, 1976) or a decrease (kidney, spleen, adrenal)(Clarke and Sampath, 1976). It appears that the pituitary gland can exert both facilitatory and

inhibitory effects on MAO and that hypophysectomy removes opposing hormonal influences to leave the enzyme largely unaffected (Clarke and Sampath, 1976). For instance, cardiac MAO is unaffected by hypophysectomy but replacement therapy with adrenocorticotrophic hormone causes a marked decrease. A speculative interpretation is that hypophysectomy resulted in the loss of a hormone or hormones which function to enhance cardiac MAO (e.g., thyroid hormones). Thus, studies devoted to pituitary infleunces on MAO are exceedingly complex due to its central role in endocrine regulation. Further investigations utilizing hypophysectomy and hormonal replacement therapy are required in order to fully elucidate the functional modulating influences on MAO by this complex endocrine organ.

Thyroid influences on MAO were first revealed in 1952 by Spinks and Burn. They showed that liver MAO activity was reduced by L-thyroxine administration to rats. The observation was quickly confirmed by several investigators (Trendelenburg, 1953; Zile and Lardy, 1959; Zile, 1960; Novick, 1961). Also, the MAO activity of other organs was found to be altered by L-thyroxine. Levine et al., (1962) showed a decreased MAO activity in the human jejunal mucosa, whereas the cardiac MAO of rats was usually found to be augmented (Novick, 1961; Utley, 1964; Ho-Van-Hap et al., 1967; Lyles and Callingham, 1974). However, other workers found no change (Zile, 1960) or a decrease in adult male rats (Ho-Van-Hap et al., 1967). Moonat et al. (1975) showed that the directional change in cardiac MAO of the rat depended upon the dose of L-thyroxine administered. "Low" doses (10 and 30 μg/kg) decreased cardiac MAO activity, whereas a high dose (300 μg/kg) increased MAO activity by 63%. Kidney MAO was increased

by all dose levels in a concentration-dependent fashion and liver MAO was reduced, the extent of the reduction also being related to the dose administered. As shown previously (Zile, 1960; Skillen et al., 1961), the brain enzyme was unaffected, as was that of the vas deferens, adrenal and thyroid glands.

Thus, once again the effect of a hormone was shown to be organ specific and additionally, the influences were found to be reversible upon cessation of treatment (Moonat et al., 1975).

Lyles and Callingham (1974) conducted a detailed study concerning the effects of L-thyroxine upon cardiac MAO of the rat. Unfortunately, however, they used only one dose of L-thyroxine (600 g/kg) which is more likely to simulate overt hyperthyroidism and its associated toxicity than give indications as to the physiological role of the hormone. Nevertheless, they concluded that L-thyroxine increased the synthesis of cardiac MAO and was without effect upon the degredation rate constant of the enzyme. Some slight difference in substrate specificity was noted for benzylamine but for tyramine the enzyme responded in the same way as that of the controls to heat treatment, irreversible inhibition by clorgyline or by pargyline and also in Km determinations. Hypothyroidism, induced by 2-thiouracil, led to a decrease in cardiac MAO. In contrast to the notion expressed by Lyles and Callingham (1974), Okamoto (1971) suggested a decrease in enzyme synthesis to explain the effect of L-thyroxine on the rat liver enzyme. Thus, the way in which L-thyroxine modulates MAO activity is not resolved and may vary from one organ to another. Moonat et al., (1975) found a rapid rise in kidney MAO activity after only six hours of treatment with L-thyroxine. Since the half-life

of MAO is in the order of several days, such an increase implies that protein synthesis may not be the only means by which L-thyroxine modifies MAO activity *in vivo*. However, certain investigators (Okamoto, 1971; Lyles and Callingham, 1974) have failed to observe any effects of L-thyroxine on MAO activity *in vitro*, thus a direct action on the enzyme(s) seems highly unlikely. MAO is a FAD-linked enzyme and it has been suggested that the thyroid hormones may regulate the production of this co-factor for incorporation into the apoenzyme (Lyles and Callingham, 1974).

In general, studies with L-triiodothyronine have yielded similar results to those stated for L-thyroxine, however, the number of investigations has been less numerous.

Goirdis and Neff (1973) showed that L-triiodothyronine increases the MAO activity of rat salivary gland in both normal and thyroidectomized animals. Furthermore, the rate of recovery of MAO after irreversible inhibition with pargyline was accelerated. The results again suggest that thyroid hormones play an important role in regulating the rate of formation of MAO, since the degradation rate constant was unaltered. The thyroid hormones have a pronounced effect on the activities of mitochondrial enzymes and the synthesis of mitochondrial protein (Freeman *et al.*, 1963; Coulson *et al.*, 1968), thus the concept of increased MAO synthesis is highly reasonable. In fact, as postulated for L-thyroxine (Lyles and Callingham, 1974), the noted increase in cardiac MAO and weight with L-triiodothyronine (Novick, 1961; Utley, 1964) probably reflects protein synthesis. However, just as with L-thyroxine, L-triiodothyronine decreases the activity of hepatic MAO (Utley, 1964) and that of the human

jejunal mucosa (Levine et al., 1962). It is possible that within the same animal thyroid toxicity may be evident at certain sites (e.g., liver and gut) while protein synthesis remains stimulated at others (e.g., heart). However, Moonat et al., (1975) still found a small decrease in rat liver MAO activity following L-thyroxine even when utilizing a dose level (30 μg/kg, s.c. daily for five days) which is well below the toxic range.

7. Interrelationship between thyroid hormones and catecholamines.

Historically the interactions between the sympathetic nervous system and the thyroid gland have been noticed as early as 1786 (Parry, 1825). During the 19th and the early part of the 20th centuries, physicians realized that many manifestations of the thyroid hormones are very similar to those of the sympathetic activity. For instance, many of the clinical features of hyperthyroidism, such as tachycardia, sweating and tremor, were similar to those produced by stimulation of the sympathetic nervous system (Leak, 1970). Historical aspects of the interactions between thyroid hormones and the sympathetic nervous system have been discussed in detail by Waldstein (1966).

Studies by Rosenquist et al., (1971) seem to indicate that the lipolytic effect of norepinephrine is reduced in hypothyroid states in man, apparently due to an altered balance in sensitivity of the alpha- and beta-adrenergic receptors in the subcutaneous adipose tissue. Similarly, it has been shown that the concentration of free fatty acids in plasma is reduced in hypothyroidism, but is increased in hyperthyroidism (Rich et al., 1959) or following treatment of experimental animals with thyroid hormones (Goodman and Bray, 1966). Treatment with triiodothyronine enhances the sensitivity of adipose tissue to the lipolytic effects

effects of catecholamines in less than three hours (Challoner, 1969). Swanson (1956) has also shown that the general calorigenic effects of epinephrine are almost completely abolished by thyroidectomy and are potentiated by treatment with thyroxine. These findings support evidence (Ellis, 1956; Harrison, 1964) indicating that thyroid hormones affect those processes that are mediated by beta-adrenergic receptors at the cell membrane but have perhaps little or no effect on those mediated by alpha-adrenergic receptors.

Numerous investigators have implicated cyclic-AMP as a secondary messenger through which thyroid hormones may influence the lipolytic effect of catecholamines. Yet the precise mechanism by which thyroid hormones alter adipose cell cyclic-AMP is still under debate. For instance, Krishna et al., (1968) indicated that hypothyroid rats showed diminished adenylyl cyclase activity in response to catecholamines. They drew the conclusion that this is due to a primary effect of thyroid hormones on adenylyl cyclase synthesis. This suggestion was not widely accepted. For instance, Armstrong et al., (1974) have shown that fat cell adenylyl cyclase activity is normal in hypothyroid rats and that it can be activated as usual by epinephrine. Thus, contrary to previous beliefs, fat cell catecholamine receptors, and the coupling to adenylyl cyclase, appears to be normal. Armstrong et al., (1974) postulated that thyroid hormones might exert a modulating influence on cyclic-AMP-mediated responses by regulating the activity of a membrane bound cyclic-AMP phosphodiesterase. At this time, it was well established that a close coupling of adenylyl cyclase and lipase activity existed in fat cells (Manganiello et al., 1971). Recently, Van Inwegen et al., (1975) has

supported the postulate of Armstrong et al., (1974). They showed that a low Km, membrane bound, cyclic-AMP phosphodiesterase is activated in hypothyroidism, thus diminishing the lipolytic effect of epinephrine. Thyroid hormone replacement therapy reversed this effect. Thus, it was concluded that the thyroid hormones modify catecholamine-induced responses by inhibiting phosphodiesterase, thus allowing intracellular cyclic-AMP accumulation.

In the adrenal medulla, abundant evidence indicates that the concentration of epinephrine in medullary tissues of rats, sheep and guinea pigs is significantly reduced by treatment with thyroid hormones (Leak, 1970). Conversely, hypothyroidism is associated with an increase in the content of norepinephrine and epinephrine in the adrenal medulla (Hokfelt, 1951). However, Haggendal and Svedmyr (1966) have shown that blood levels of epinephrine remain unchanged.

With regard to the cardiovascular actions of catecholamines, many investigators have suggested that excess of thyroid hormones can enhance these effects (Goetsch, 1918; Schneckloth et al., 1953; Brewster et al., 1965; Harrison, 1964; Waldstein, 1966; Prange et al., 1968). Furthermore, Wurtman et al., (1963) proposed that the increased cardiovascular sensitivity to catecholamines demonstrated in hyperthyroidism was not due to changes in the activities of the enzymes that metabolize catecholamines (MAO and COMT) but rather to a decreased ability of the hyperthyroid heart to take up as much catecholamines per unit weight as control hearts. However, these authors reported that a few minutes after a dose of tritiated epinephrine to hyperthyroid rats, the cardiac content of all the main metabolites decreased, while an increase was found in hypothyroid

states. In contrast, other studies negated the assumption that a hypersensitivity to sympathomimetic amines occur in the hyperthyroid states. For example, it was reported that the cardiovascular responses to epinephrine and norepinephrine were not augmented (VanderSchoot and Moran, 1965), that the response to sympathetic nerve stimulation was normal (Margolius and Gaffney, 1965; Cairoli and Crout, 1967), and that blood pressure and heart rate responses to tyramine were essentially the same as those in control animals (Benfey and Varma, 1963). However, there is a possibility that such controversy is due to the fact that the animal experiments were made under anesthesia, while in human studies anesthetics were not employed.

While there have been considerable controvery concerning the response to sympathomimetic amines following thyroid treatment, biochemical studies show a clear interaction between the peripheral sympathetic nervous system and thyroid function. For instance, Lipton et al., (1968) has shown that there is a marked acceleration in the synthesis of ^{14}C-norepienphrine from the ^{14}C-tyrosine in the heart, spleen and adrenals of hypothyroid rats. Similarly, Landsberg and Axelrod (1968a) found that the accelerated discharge of norepinephrine observed in the heart of hypophysectomized rats was due primarily to the lack of thyroid hormone. Additionally, the increased sympathetic activity in hypophysectomized rats was prevented by the long-lasting ganglionic blocking agent, chlorisondamine, again indicating an increased turnover of norepinephrine in these animals. Prange et al., (1970) have reported that treatment of rats with thyroxine for 10 days diminished the rate at which ^{14}C-tyrosine was incorporated into labeled catecholamines. All these findings may at first seem

paradoxical, since overtly hyperthyroid animals seem "hyperadrenergic" whereas hypothyroid animals show signs of diminished adrenergic activity. In general, such data have been interpreted to suggest that changes in neuronal activity occur to compensate for the change in hormonal function (Landsberg and Axelrod, 1968b; Prange et al., 1970).

In the central nervous system there are corollaries to these alterations in norepinephrine turnover in hypo- and hyperthyroid situations seen in the peripheral nervous system. For instance, Emlen et al., (1972) have demonstrated increased tyrosine hydroxylase activity in the midbrain of thyroidectomized rats, but no change in thyroxine-treated animals. Similarly, Lipton et al., (1968); Prange, et al., (1970) reported that the conversion of labeled tyrosine to norepinephrine is decreased in the brain of hyperthyroid rats and is increased in hypothyroid rats. The effect of thyroid hormones on catecholamine turnover in the central nervous system has implications in psychiatric abnormalities where catecholamines are generally believed to play an important role. This view received some support by the finding that low doses of triiodothyronine can potentiate the antidepressant action of imipramine (Wilson et al., 1974). However, the mechanism of this interaction is, at present, unknown (Breese et al., 1974).

III. METHODS AND MATERIALS

1. Animals and Animal Housing

All experiments were made using male albino rats which were Sprague-Dawley descendants obtained from Texas Inbred, 305 Almeda-Genoa Road, Houston, Texas 77047. In the majority of the experiments, the animals weighed between 200 and 350 g, except in specific studies where the effect of age on monoamine oxidase activity was investigated.

The animals were housed in pairs in animal quarters maintained at 22-24° C. Food and water were given ad libitum and the food used was in the form of pellets obtained from the Purina Company, St. Louis, Missouri.

2. Monoamine Oxidase Determinations

Two separate substrates, tryptamine and kynuramine, were used for the assay of monoamine oxidase (MAO).

a. Tryptamine Assay

Since MAO exists in more than one molecular form, with differing substrate specificities, the choice of substrate is obviously of great importance. Extensive use was made of the tryptamine assay because this substrate is known to interact with the two general groups of MAO, type A and type B (Neff and Yang, 1974). Furthermore, tryptamine is an endogenous constituent of the mammalian central nervous system (Martin et al., 1971; Saavedra and Axelrod, 1972; Horn and Snodgrass, 1973) and is also present in peripheral tissues (Saavedra and Axelrod, 1972) and in human urine (Rodnight, 1956). Thus, tryptamine is a physiological substrate for the enzyme.

The assay depends on the measurement of indoleacetic acid (IAA) formation from tryptamine, as first described by Lovenberg et al., (1962). The immediate product of tryptamine deamination by MAO is indoleacetaldehyde which can be recovered quantitatively as IAA in the presence of excess aldehyde dehydrogenase (AD) and nicotinamide adenine dinucleotide (oxidized NAD). Small amounts of formed IAA can be easily detected since the endogenous tissue level is very small and is below the sensitivity limits of the assay. The fluorescent properties of IAA permit detection of microgram quantities and provides the final means by which product formation can be measured.

Assay Procedure: Rats were stunned and killed by cervical dislocation. The required tissues were removed, washed free of blood in N saline, blotted and either used immediately or were frozen in glass vials by placing in a dry ice-acetone mixture. The latter tissues were stored in a freezer at -5° C until used. Under these conditions, MAO activity is stable for at least one month. Frozen, or freshly removed tissues, were homogenized in ice-cold 0.25 M sucrose using a Tissumizer homogenizer (Tekmar Company, P. O. Box 37202, Cincinnati, Ohio 45222). Whole tissue homogenates containing 100 mg (wet weight)/ml for brain, heart and kidney were used, while a 50 mg (wet weight)/ml homogenate of liver was utilized. The composition of the entire incubation mixture was:

1.60 ml, distilled water

0.40 ml, phosphate buffer, pH 7.4, 1 M

0.50 ml, nicotinamide, 100 μMole

0.70 ml, nicotinamide adenine dinucleotide, oxidized form (NAD), 23.2 mg/ml.

0.40 ml, aldehyde dehydrogenase preparation (see below)

1.00 ml, tissue homogenate

0.40 ml, tryptamine (14 μMoles)

The various components of the incubation mixture were added in the order listed above, and were contained in a 25 ml Erlenmeyer flask, pre-chilled on ice. At least duplicate incubations were made for each sample. Incubation was conducted under air at 37° C in a Dubnoff Metabolic Shaking Incubator. After a 10 minute pre-incubation period, which allowed the contents of the beaker to reach 37° C, the reaction was initiated by the addition of 0.40 ml tryptamine. After various times, usually 20 minutes, 10 ml of 0.5N HCl was added to the incubating mixture to stop the reaction. Aliquots of 3 ml were removed and placed in tapered screw-capped, teflon lined 50 ml centrifuge tubes containing approximately 4.5 g of sodium chloride. After the addition of 15.0 ml of toluene, the tubes were agitated for 15 minutes using a mechanical shaker. The tubes were then centrifuged at 2000 rpm. for 5 minutes. Ten ml of the organic layer, which contained the IAA, was transferred to another screw-capped centrifuge tube containing 1.5 ml of 0.5 M phosphate buffer, pH 7.0. The tubes were again shaken for 15 minutes to extract the IAA into the aqueous phase and were then centrifuged for 10 minutes (2000 rpm.) to clearly separate the organic from the aqueous phase. The toluene was removed by aspiration and the fluorescence of the extracted IAA in buffer was then measured using an Aminco-Bowman Spectrofluorometer at uncorrected wavelengths of 280 mμ (excitation) and 364 mμ (emission).

To correct for traces of MAO activity present in the AD preparation, control assays were performed by substituting 0.25 M sucrose for the tissue homogenate, incubating and then carrying the aliquots of this incubation mixture through the entire extraction procedure.

Appropriate blanks for tissue and other components of the reaction mixture were carried out to permit direct calculations of the results. The recovery of IAA from aliquots of the incubation mixture, added before toluene extraction, ranged between 60-75%. The recoveries were found to vary within the given range depending on the batch of spectroanalyzed toluene used. Internal standards were made with each assay and external standards of IAA, made in Sørensens phosphate buffer, 0.5 M, pH 7.0, were also utilized.

Tryptamine did not affect the recovery of IAA nor did it increase the tissue blanks. The reaction was shown to proceed at a linear rate for 30 minutes; a relationship that will be discussed in the Results section.

The external standard curves were used to determine the IAA concentration (μg) in each 1.0 ml aliquot from the incubation medium. The specific activity (SA) of MAO in μMoles of IAA/gram tissue/hour was calculated using the following formula:

$$SA = IAA\ (\mu g) \times \frac{1000}{mg\ tissue} \times \frac{60}{20} \times 5^{*} \times \frac{3^{*}}{2} \times \frac{1}{175} \times \%\ \frac{100}{recovery}$$

*Correction for dilution.

$\frac{1}{175}$ = Conversion to μMoles, where 175 is the molecular weight of IAA.

Source of Aldehyde Dehydrogenase: Male guinea pigs were injected with the long lasting MAO inhibitor tranylcypromine, 20 mg/kg, intraperitoneally. After 18-24 hours the animals were sacrificed by cervical dislocation and their kidneys were dissected out immediately. They were washed with ice-cold (0.25 M) sucrose solution and the surrounding capsule and connecting tissues were removed quickly. The kidneys were blotted, weighed and homogenized in 0.25 M sucrose solution (4 ml/gram tissue) at 4° C. The homogenate was centrifuged for 1 hour at 78000 x g. The AD was obtained from the supernate and was tested for residual MAO activity. The enzyme was transferred to glass vials and stored at -5° C for periods up to 2 weeks, during which time there was no deterioration in activity. The AD preparation, when added to tissues already containing sufficient endogenous AD (e.g., liver), did not reduce their MAO activity, indicating no significant amount of residual tranylcypromine. Additionally, a routine check was made for any inherent MAO activity in the preparation.

To ascertain the reliability and maximum efficiency of the enzyme assay, the following investigations were conducted:

(i) The recoveries of IAA from aliquots of incubation mixtures were found to be reliably consistent from 60-75%. Lovenberg et al. (1962) reported comparative recoveries of IAA based upon the recovery of IAA in the presence of tissue as compared with that from aqueous standards. They obtained a figure of 95-100% recovery, but this procedure does not take into account extraction losses (i.e., actual recovery). Klingman and Klingman (1966) reported actual recoveries of added IAA (47-52%). The present investigator found that by adding 4.5 g sodium chloride to

the aliquots of the acidified incubating mixture, a single toluene extraction will yield a 60-75% recovery of added IAA. The sodium chloride, by a salting-out procedure, allows a more efficient extraction of the non-ionized IAA from the acid medium into the toluene.

(ii) The activation and emission spectra if IAA (authentic) were measured and found to occur maximally at 280 mμ (excitation) and 364 mμ (emission). These values were taken as the uncorrected wavelengths for each assay.

(iii) The linear relationship between IAA (μg/ml of 0.5 M phosphate buffer, pH 7.0) and relative intensity of fluorescence was established.

(iv) The amount of substrate used was based upon experiments made with both liver (highest MAO activity/g, wet weight) and heart. The optimal substrate concentration was found to be 2.8 μMoles/ml (14 μMoles/incubating mixture) for 50 mg liver and also for 100 mg heart.

(v) The reaction was found to be linear over 30 minutes for a fixed enzyme concentration. An incubation time of 20 minutes was selected therefore as being submaximal.

(vi) Since MAO is an oxygen dependent enzyme system, the effect of aeration with 95% O_2 + 5% CO_2 was compared to that of exposure to air. Figure 1 illustrates that the incubation medium possessed an optimal level of O_2 and substitute aeration did not increase the enzyme activity. Therefore, all enzyme determinations reported were carried out in air.

(vii) The same figure illustrates that at 37° C the reaction proceeds linearly for 30 minutes, while at 4° C is reduced to approximately 15% of the optimum.

Figure 1. Effect of incubation temperature and aeration (95% O_2 + 5% CO_2) on indoleacetic acid production from tryptamine at different reaction times, using rat liver homogenate (10 mg/ml).

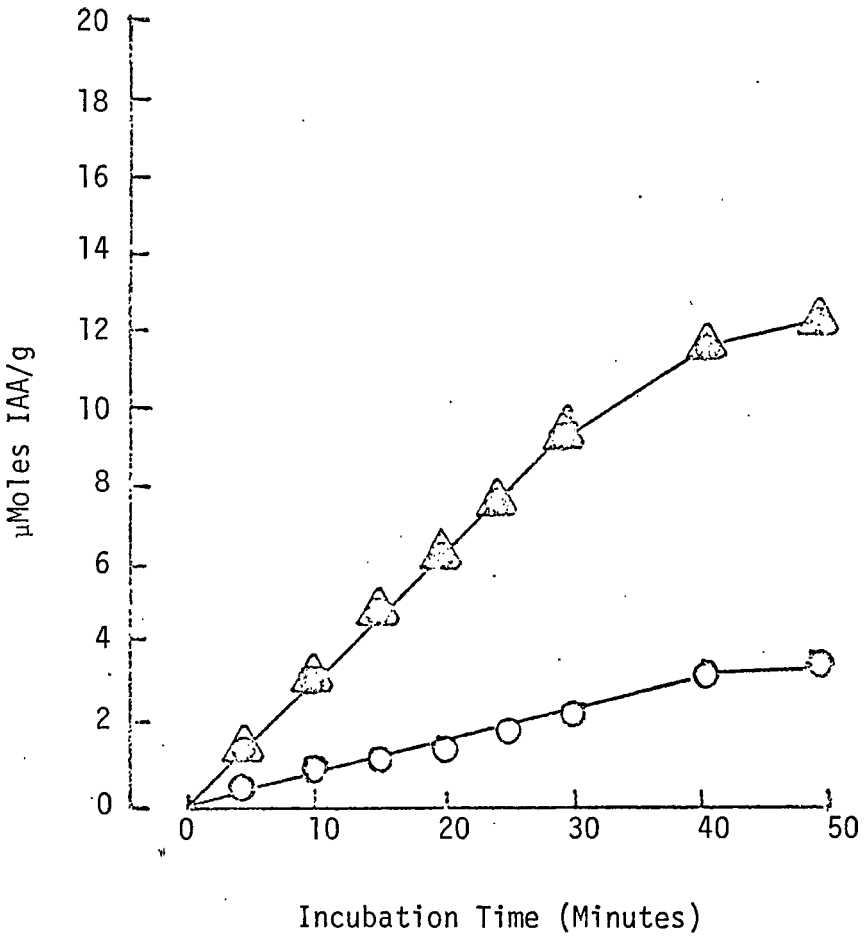
Indoleacetic acid (IAA) production is expressed in μMoles IAA/g tissue.

Shown are the mean values of at least two determinations.

Incubation at 37° C in air (●——●)

Incubation at 4° C in air (○——○)

Incubation at 37° C + (95% O_2 + 5% CO_2) (△——△)


20
18
16
14
12
10
8
6
4
2
0
µMoles IAA/g
0
10
20
30
40
50
Incubation Time (Minutes)

(viii) The oxidative deamination of tryptamine to indoleacetaldehyde and then to IAA requires AD for the second stage. Therefore, to ensure that the amount of AD used would not be a limiting factor in the reaction, the optimal level of AD was determined and found to be 0.08 ml/ml (0.4 ml/incubation mixture).

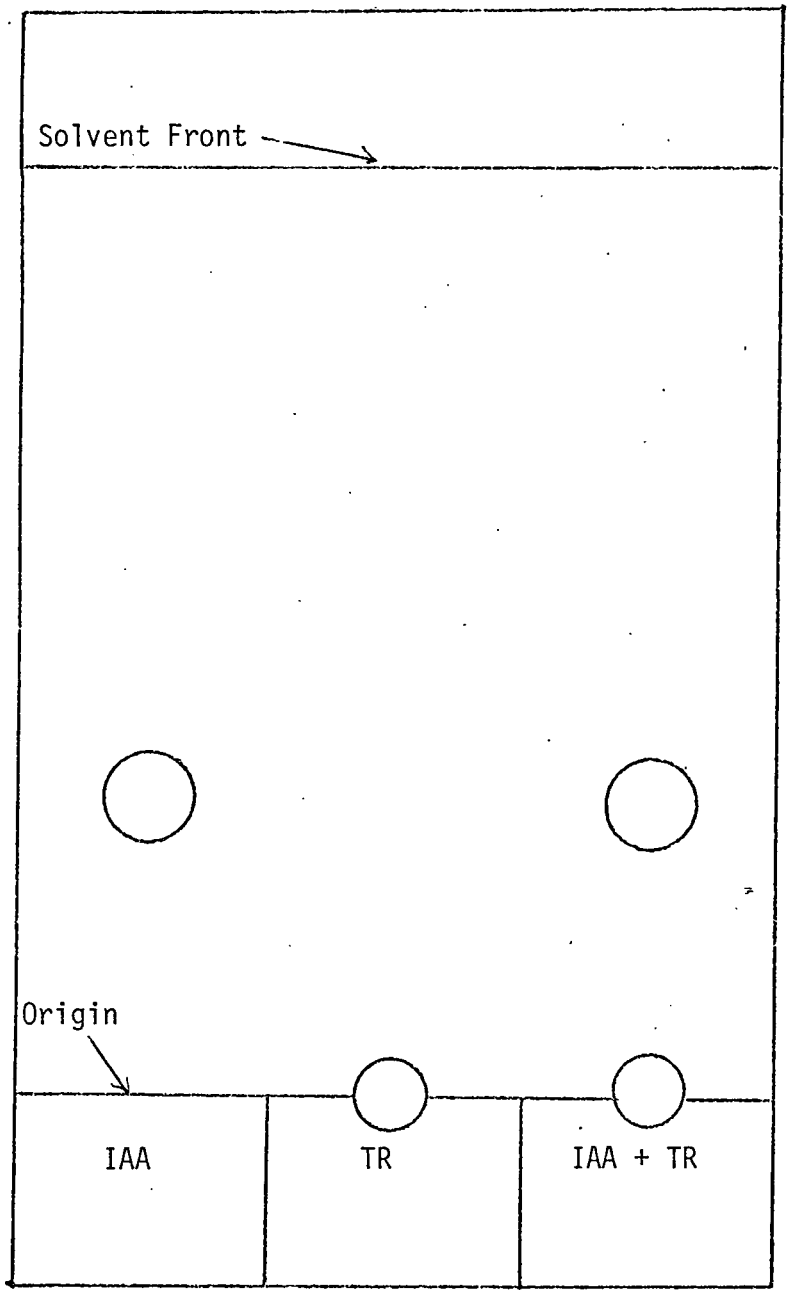
(ix) Oxidized NAD (nicotinamide adenine dinucleotide) which is added to the incubation medium as the hydrogen acceptor, was also studied for optimal concentration and was found to be 4.9 μMole/ml (24.5 μMoles/ incubating mixture).

(x) To demonstrate that the assay system used was indeed measuring MAO activity, a known MAO inhibitor, pargyline, was added to the incubating mixture in a concentration of 60 μg/ml. Complete inhibition of IAA production occurred.

(xi) Paper chromatography was used to verify that IAA was indeed the product formed from incubating tryptamine with the tissue, and was carried out as follows. In experiments with tryptamine, incubation mixtures were deproteinized with 0.5N HCl, centrifuged and the supernatant fractions from four incubation flasks were pooled, made alkaline to pH 8.0 by the addition of solid sodium carbonate, and extracted with two volumes of diethyl ether. The remaining aqueous phase was rendered acidic with 5N HCl and again extracted with two volumes of diethyl ether. Both etheral layers were pooled, evaporated to dryness in a Rotory Film Evaporator, and the residue dissolved in a small volume of distilled water. Tryptamine and IAA contained in this aqueous phase were separated by descending paper chromatography (Whatman Paper, No. 1). The solvent was the toluene layer of a two-phase system containing toluene, acetic

Figure 2. Identification and separation of indoleacetic acid (IAA) and tryptamine (TR) by paper chromatography (see Methods Section).

Shown are the authentic standards both separately and in combination.


Solvent Front
Origin
IAA
TR
IAA + TR

acid and water in a proportion of 4:1:5, the aqueous layer serving as the stationary phase. After 2.5 hours, the chromatograms were dried and the indole compounds were identified by spraying with Ehrlich's reagent. Authentic tryptamine and IAA were used and were compared with the indoles from the assay. Figure 2 clearly demonstrates the ability of the system to separate tryptamine from IAA.

(xii) To ensure adequate homogenization so that availability of the substrate to the enzyme is not made a limiting factor, an experiment was designed where tissues were homogenized either in distilled water or in a 0.2% aqueous solution of Triton X-100. The IAA production was compared with that in 0.25 M sucrose. No significant differences were found, demonstrating complete availability of the mitochondrial enzyme when homogenization was made in 0.25 M sucrose.

b. Kynuramine Assay

As explained previously, MAO exists in more than one molecular form, thus the use of more than one substrate may provide additional information with regard to activity changes in the various enzymes. Although kynuramine is not an endogenous substrate for MAO, it is a good substrate which allows for an easy and rapid determination of enzyme activity.

The assay depends on measuring the rate of 4-hydroxyquinoline (4-OHQ) formation from kynuramine as described by Kraml (1965). The method is based on the original observation of Weissbach et al. (1960) that kynuramine is deaminated by MAO to an unstable amino aldehyde, which by intramolecular condensation (nonenzymatic), is rapidly and quantitively converted to a highly fluorescent product, 4-OHQ. This compound exhibits

fluorescent peaks at 315 mμ (excitation) and 380 mμ (emission) at pH 11.0 - 12.0. This method of MAO determination has the advantage that the product formation involves no enzymatic step except deamination by MAO.

Assay Procedure: Tissue homogenization was carried out in glass distilled water using the homogenizer mentioned previously. The tissue concentrations used were 10 mg/ml liver and 20 mg/ml for the kidney, brain and heart. The composition of the incubation mixture was as follows:

0.5 ml, phosphate buffer, 0.5 M, pH 7.40

1.0 ml, water

1.0 ml, tissue homogenate

0.5 ml kynuramine (0.31 μMoles)

A pre-incubation time of 10 minutes at 37° C was employed before the addition of the substrate. The various components of the incubation mixture were added in the order listed above and the contents were contained in tapered glass 15 ml centrifuge tubes, pre-chilled on ice. Incubation was conducted under air at 37° C in a Dubnoff Metabolic Shaking Incubator. Duplicate incubation were carried out for each sample. After 30 minutes incubation, 2 ml of 10% trichloroacetic acid was added to terminate the reaction. Precipitated proteins were separated by centrifugation. One ml of the clear supernatant was transferred to a Silica Cuvette which contained 2 ml of 1N NaOH. The cuvette was then covered with parafilm and thoroughly shaken. The fluorescence was read on an Aminco-Bowman Spectrofluorometer at the uncorrected wavelengths given above. Reagent blanks, tissue blanks, and 4-OHQ standards were carried through the entire procedure. The external 4-OHQ standard curves were constructed so as to determine the concentration of product in the aliquot of the incubation mixture.

The Specific Activity (S.A.) of MAO in mμ moles of 4-OHQ/g tissue/hour was calculated using the following formula:

$$\text{S.A.} = \text{4-OHQ (m}\mu\text{ Moles)} \frac{1000}{\text{mg tissue used}} \times \frac{60}{30}$$

The following investigations were carried out to ensure the reliability and ascertain the specificity of the assay system in measuring MAO activity.

(i) Pargyline (2 μg/ml), when added to the incubation mixture, completely inhibited the formation of 4-OHQ.

(ii) Kynuramine did not affect the recovery of 4-OHQ nor did it interfere with the fluorescence of the compound.

(iii) 4-OHQ standard curves are linear over a wide concentration range.

(iv) For all tissues, the reaction was shown to proceed at a linear rate with both time and tissue concentration, so that the initial velocity rate of the reaction was being measured.

3. Mixed Substrate Experiments

Mixed substrate experiments were made using a combination of tryptamine and kynuramine. Tryptamine was added to the incubating mixture in an equimolar concentration to that of kynuramine and the rate of 4-OHQ production was measured.

The following criteria were established prior to commencing these studies.

1. Tryptamine was found not to interfere with the fluorescence of 4-OHQ either in the presence or absence of tissue.

2. Tryptamine, when present together with kynuramine, was found not to interfere with 4-OHQ fluorescence.

3. Tryptamine alone, or in the presence of kynuramine or homogenate, did not alter the blank values.

4. Preparation of Rat Brain Mitochondria

A crude fraction of rat brain mitochondria was prepared in accordance with the procedure described by Goridis and Neff (1971).

Brains were removed from rats and were pooled and homogenized in 0.25 M cold sucrose in a mechanical glass homogenizer fitted with a loose teflon pestle. Homogenization was performed at 0-4° C. The homogenate was then centrifuged at 700 x g for 10 minutes at 4° C. The sediment of cell membranes and nuclei was retained for subsequent use. The supernatant was then centrifuged at 12,000 g for 20 minutes at 4° C. The resultant supernatant was retained and the mitochondrial pellets were washed once in 0.25 M cold sucrose and recentrifuged at 12,000 x g for 20 minutes before use.

5. Statistical Methods

The standard error of the mean was calculated using the following formula:

$$\sqrt{\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{N}}{n(n-1)}}$$

P = Probability level as determined by student-"t" test:

$$\frac{\bar{x}_1 - \bar{x}_2}{S_d \sqrt{\frac{1}{N_1} + \frac{1}{N_2}}}$$

where S_d = standard deviation.

6. Sources and Preparative Procedures for Drugs and Chemicals

(i) Disodium Phosphate (Na_2HPO_4,$2H_2O$, Mallinckrodt).

(ii) Hydrochloric acid (Fisher). 42.70 ml of HCl diluted in GDW to 1000 ml to yield a 0.50 N solution.

(iii) 4-Hydroxyquinoline Trihydrate (Aldrich Chemical Co., Inc.). A 1000 mμM/ml solution was made in GDW (Glass distilled water) and stored in opaque bottle under refrigeration.

(iv) Indole-3-Acetaldehyde Sodium Bisulfite (Regis Chemical Co.). A 5.3 mg/ml solution was made in GDW. This concentration is equivalent to 20 μMoles/ml.

(v) Indoleacetic Acid (Indole-3-Acetic Acid, $C_{10}H_9NO_2$, Aldrich Chemical Co., Inc.). A 200 μg/ml stock solution was made in GDW and stored in opaque bottle under refrigeration.

(vi) Kynuramine Dihydrobromide (Nutritional Biochemical Corp.). A 200 μg/ml stock solution was made in GDW and stored in opaque bottle under refrigeration.

(vii) NAD Oxidized (Cozymase, Coenzyme I, Ehtanol Free, Nutritional Biochemical Corp.). A 23.20 mg/ml solution in GDW was made just prior to use.

(viii) Nicotinamide (Nicotinic Acid Amide-Niacinamide 3-Pyridine Carboxylic Amide, Nutritional Biochemical Corp.). A 24.40 mg/ml solution was mde in GDW and stored in opaque bottle under refrigeration.

(ix) Potassium Phosphate (KH_2PO_4, primary monobasic, Fisher).

(x) Propylthiouracil (Robinson). A 0.851 mg/ml solution was made by dissolving the powder in the least amount of 0.01 N NaOH, then diluting to the required concentration with GDW. This concentration is equivalent to 5 μMoles/ml.

(xi) Sodium Chloride (NaCl, Granular, Fisher). A 0.90% W/V solution was made in GDW.

(xii) Sodium Hydroxide (NaOH, Pellets, reagent grade, Fisher).

1 N solution: 10 g was dissolved in GDW and volume adjusted to 250 ml.

(xiii) Sørensens Phosphate Buffer, 0.50 M, pH 7.

A. 33.88 g of KH_2PO_4 was dissolved in sufficient GDW to bring the volume up to 500 ml.

B. 44.33 g of $Na_2HPO_4.2H_2O$ was dissolved in sufficient GDW to bring the volume up to 500 ml.

39.20 ml of solution A and 60.80 ml solution B were mixed together giving a buffer of 0.50 M, pH 7.

(xiv) Sørensens Phosphate Buffer, 0.50 M, pH 7.40.

A. 33.88 g of KH_2PO_4 was dissolved in sufficient GDW to bring the volume up to 500 ml.

B. 44.33 g of $Na_2HPO_4.2H_2O$ was dissolved in GDW to bring the volume up to 500 ml.

35 ml of solution A and 65 ml of solution B were mixed to give a buffer of 0.50 M at pH 7.40.

(xv) Sørensens Phosphate Buffer, 1 M, pH 7.40.

A. 67.76 g of KH_2PO_4 was dissolved in sufficient GDW to bring volume up to 500 ml.

B. 88.66 g of $Na_2HPO_4.2H_2O$ was dissolved in sufficient GDW to bring the volume up to 500 ml.

35 ml of solution A and 65 ml of solution B were mixed giving a buffer of 1 M at pH 7.40.

(xvi) Sucrose (Saccharose, Fisher). 0.25 M solution: 85.575 g was dissolved in sufficient GDW to make 1000 ml and stored under refrigeration.

(xvii) D-Thyroxine (Sodium Dextro Thyroxine, Flint Laboratories). Solution was prepared the same as that of L-Thyroxine.

(xviii) L-Thyroxine (Synthroid, Sodium Levo Thyroxine, Flint Laboratories, 500 µg vials for injection). Desired concentration was made in normal saline.

(xix) Toluene (Spectranalyzed, $C_6H_5CH_3$, Fisher).

(xx) Tranylcypromine Sulphate (ISO #132294, Smith, Kline and French Laboratories). A 20 mg/ml solution was made in normal saline.

(xxi) Trichloroacetic Acid (Reagent ACS, CCl_3COOH, Eastman Kodak Co.). A 10% solution was made in GDW and stored in opaque bottle.

(xxii) D-Triiodothyronine (D-3,5,3'-Triiodothyronine, USV Pharmaceutical Co.). Solution was prepared the same as that of L-triiodothyronine.

(xxiii) L-Triiodothyronine (Liothyronine Sodium, Smith, Kline and French Laboratories). A 500 µg/ml solution was made as follows: 10 mg Liothyronine sodium was dissolved in 5 ml 0.01 N NaOH. After solution was complete, 15 ml of normal saline was added. The solution was stored in opaque bottle under refrigeration for no more than seven days.

(xxiv) Tryptamine Hydrochloride (98% $C_{10}H_{12}N_2$.HCl, Aldrich Chemical Co., Inc.). A 5.60 mg base/ml or 6.80 mg salt/ml solution was made in GDW and stored in opaque bottle under refrigeration.

IV. EXPERIMENTS AND RESULTS

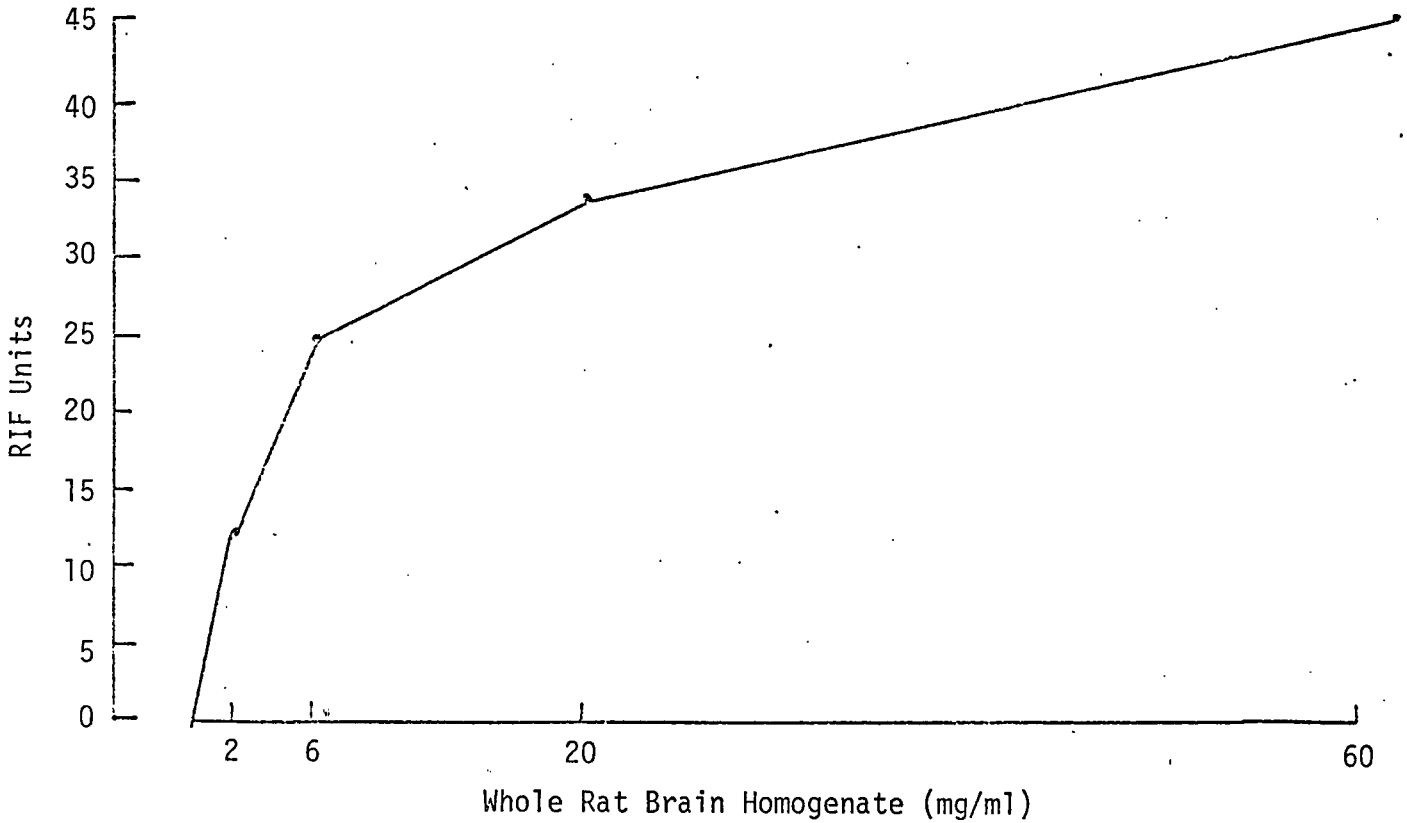
1. Relationship between product formation with enzyme concentration and reaction time.

Substances which interfere with enzymatic reactions may do so by interacting with the enzyme itself or by influencing modulating factors, such as substrate availability, activators and inhibitors. Certain of these latter factors often escape detection by employing *in vitro* assay systems. For instance, purified enzyme preparations lack endogenous modulators and preclude the production of influencing factors due to the lack of available cellular constituents. Even with crude homogenates, the tissue is usually diluted markedly so as to obtain linear reactions with respect to both time and differing homogenate concentrations. Thus, again the influence of endogenously occurring modulators is strictly limited or completely diluted out. In view of these considerations, it was decided to investigate the effects of the thyroid hormones over a wide range of enzyme concentrations and to employ whole brain homogenates rather than mitochondrial fractions or more purified preparations.

Figure 3 shows the relationship obtained between differing concentrations of rat brain homogenate and the indoleacetic acid formed from tryptamine. The curve deviates markedly from linearity beyond the 6 mg/ml concentration. However, for any given homogenate concentration the rate of product formation was linear over a 30 minute time period. This is illustrated for the 20 mg/ml homogenate concentration in Figure 4.

Figure 3. Relationship between indoleacetic acid formation from tryptamine and whole rat brain homogenate concentration.

The indoleacetic acid formed is expressed in relative intensity of fluorescence units (RIF). Each value is the mean of at least two determinations. Reaction time = 20 minutes.


45
40
35
30
25
20
15
10
5
0
RIF Units
2
6
20
60
Whole Rat Brain Homogenate (mg/ml)

2. Effect of L-thyroxine (T_4) and L-triiodothyronine (T_3) on the formation of indoleacetic acid from tryptamine.

Figure 5 shows that both T_3 and T_4 (10 µg/ml) enhanced indoleacetic acid formation when added to the incubating mixture 15 minutes prior to the addition of tryptamine. The effect varied from no change at the lowest homogenate concentration to a marked increase at concentrations of 20 and 60 mg/ml. The 20 mg/ml concentration was selected for rate studies (Figure 4). In this experiment both T_3 and T_4 (10 µg/ml) markedly enhanced indoleacetic acid formation in a linear manner, such that a two to three-fold increase in product formation occurred after 30 minutes of incubation with tryptamine.

3. Effect of lowered tryptamine concentrations upon the formation of indoleacetic acid.

Although Figures 4 and 6 indicate that substrate insufficiency was not a factor with regard to the non-linearity of the homogenate concentration vs. product curves (Figures 3 and 5), experiments were made, using lower tryptamine concentrations, in order to further define this point.

Figure 7 shows that 14 µMoles of tryptamine represents a greater than 10-fold substrate excess. Even at 1.4 µMoles of tryptamine, T_3 (10 µg/ml) still caused the same increase in indoleacetic acid production

Figure 4. Relationship between indoleacetic acid formation from tryptamine and reaction time.

The indoleacetic acid formed is expressed in relative intensity of fluorescence units (RIF). Each value is the mean of at least two determinations. Whole rat brain homogenate (20 mg/ml).

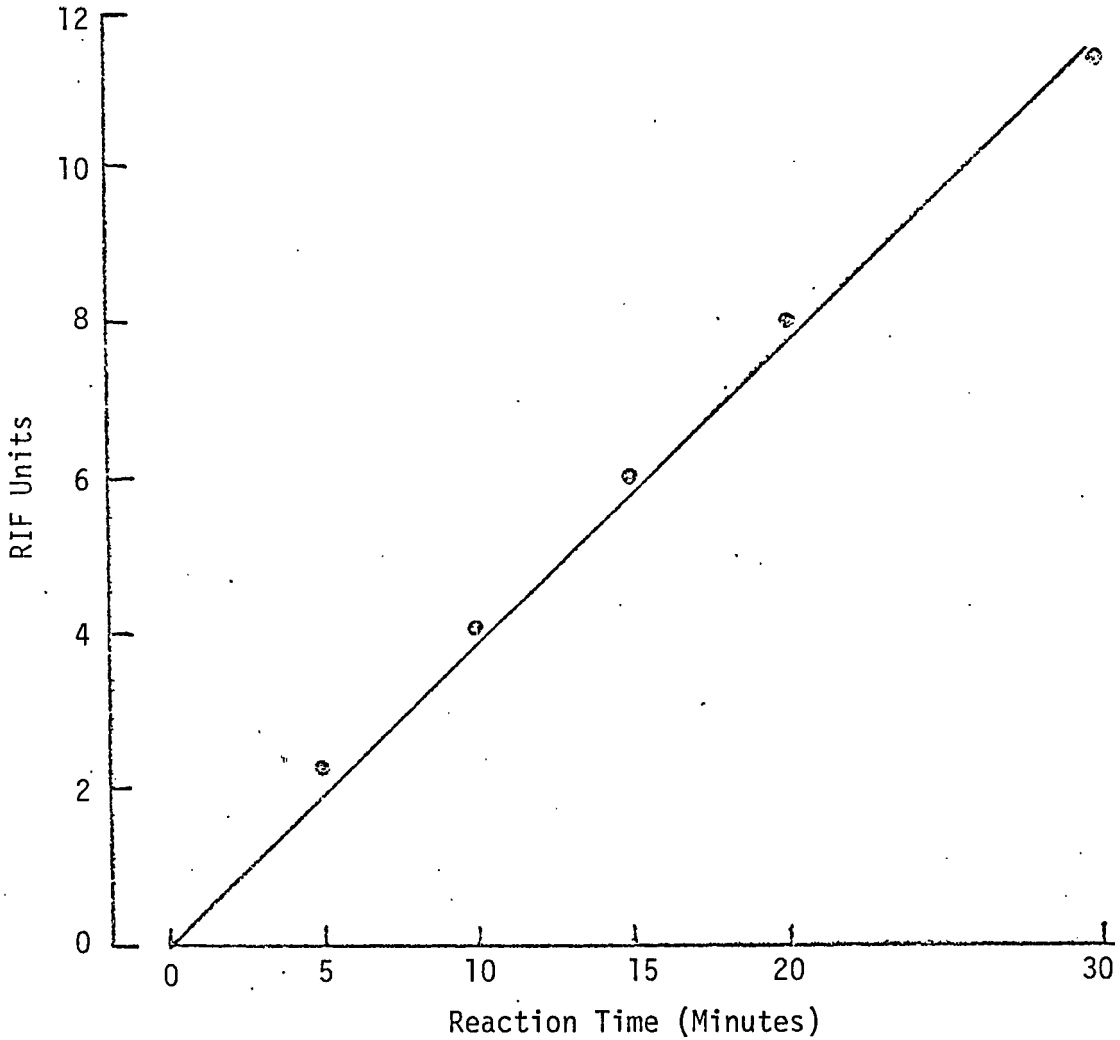

12
10
8
6
4
2
0
RIF Units
0
5
10
15
20
30
Reaction Time (Minutes)

Figure 5. Effect of L-throxine (Δ) and L-triiodothyronine (o)(both 10 μg/ml) on indoleacetic acid formation from tryptamine using different whole rat brain homogenate concentrations (control, no hormone = ●).

The indoleacetic acid formed is expressed in relative intensity of fluorescence units (RIF). Each value is the mean of at least two determinations. The hormones (10 μg/ml) or the vehicle was added 15 minutes prior to the addition of tryptamine. Reaction time = 20 minutes.

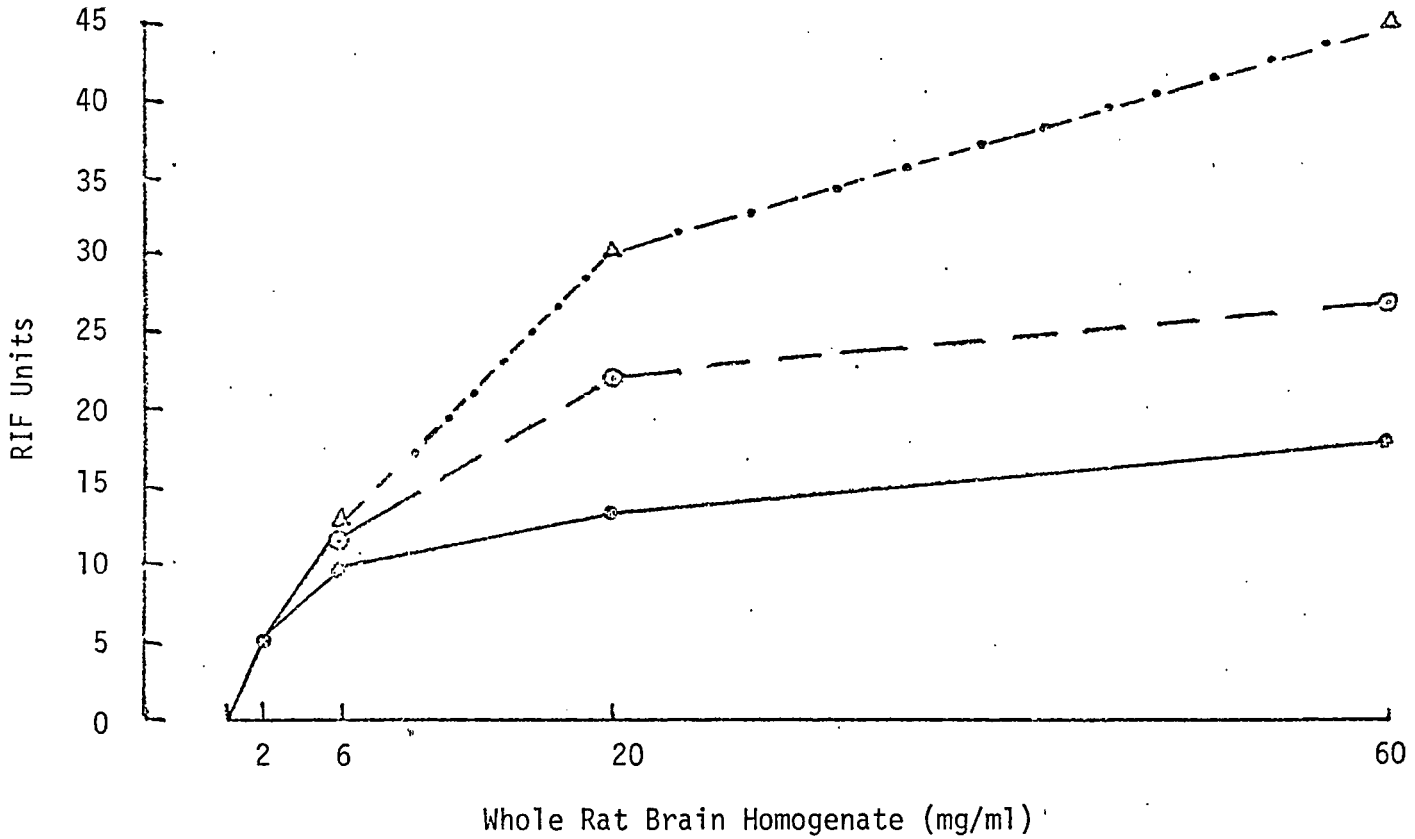

45
40
35
30
25
20
15
10
5
0
RIF Units
2
6
20
60
Whole Rat Brain Homogenate (mg/ml)

Figure 6. Effect of L-thyroxine (Δ) and L-triiodothyronine (o)(both 10 µg/ml) on indoleacetic acid formation from tryptamine using different reaction times (control, no hormone = ●).

The indoleacetic acid formed is expressed in relative intensity of fluorescence units (RIF). Each value is the mean of at least two determinations. Whole rat brain homogenate (20 mg/ml).

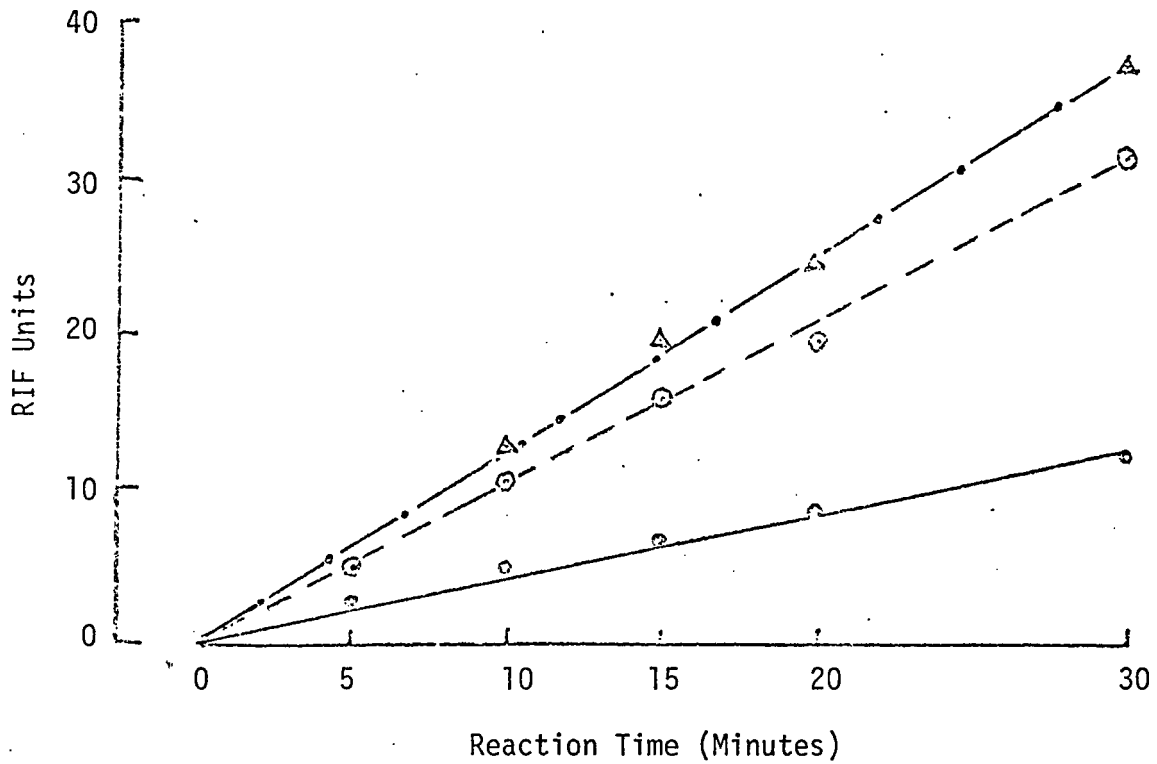
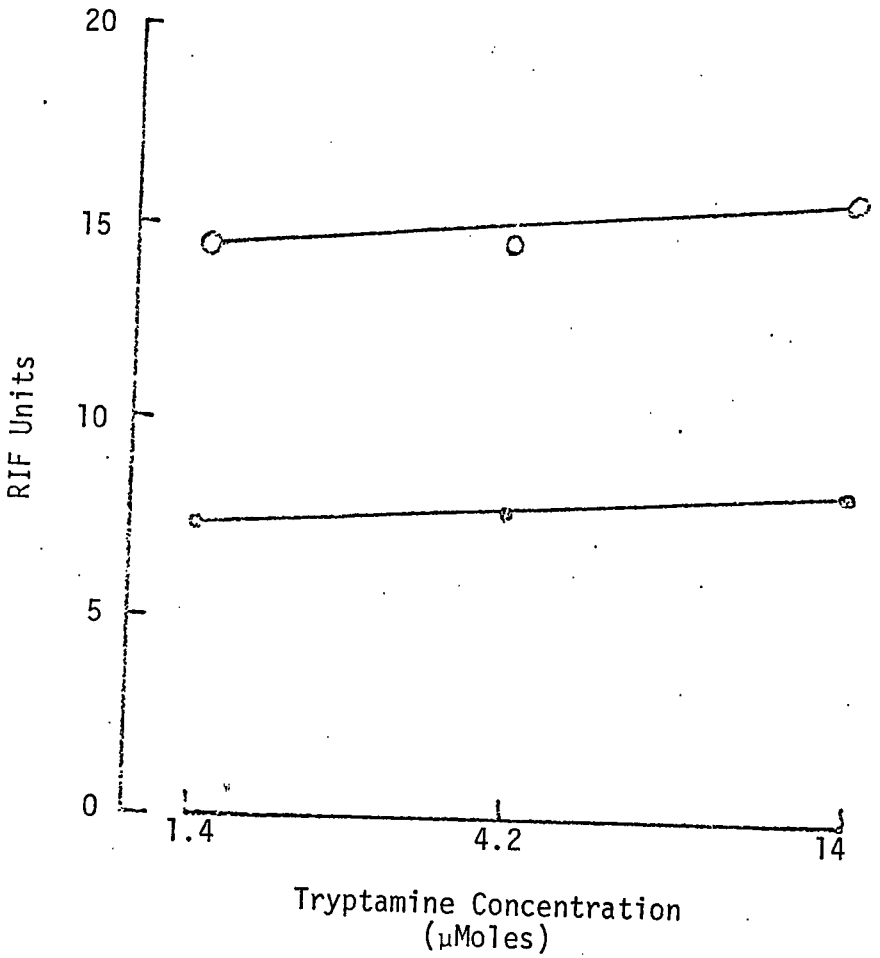

40
30
20
10
0
RIF Units
0
5
10
15
20
30
Reaction Time (Minutes)

Figure 7. Effect of L-triiodothyronine (o)(10 µg/ml) on indoleacetic acid formation in the presence of lowered tryptamine concentrations (control, no hormone = ●).

The indoleacetic acid formed is expressed in relative intensity of fluorescence units (RIF). Each value is the mean of at least two determinations. Whole rat brain homogenate (20 mg/ml). Reaction time = 20 minutes.


20
15
10
5
0
RIF Units
1.4
4.2
14
Tryptamine Concentration
(µMoles)

as that occurring with 14 μMoles of tryptamine. Lower concentrations of tryptamine, 0.07 and 0.014 μMoles, revealed a decline in indoleacetic acid production (Figure 8). The former concentration giving rise to about 50% of the indoleacetic acid production seen with 14 μMoles of tryptamine at an homogenate concentration of 20 mg/ml.

4. Effect of L-triiodothyronine (T_3) on indoleacetic acid production at a submaximal substrate concentration (0.07 μMoles, tryptamine).

Figure 9 shows that T_3 (10 μg/ml, pre-incubated for 15 minutes) increased the rate of formation of indoleacetic acid. Evidence of substrate insufficiency is seen between 15 and 20 minutes of incubation.

The result clearly shows that the effect of T_3 upon indoleacetic acid formation is not dependent upon substrate excess.

5. Effect of varying concentrations of L-thyroxine (T_4) and L-triiodothyronine (T_3) upon indoleacetic acid formation from tryptamine.

Various concentrations of either T_3 or T_4 were pre-incubated for 15 minutes with 20 mg/ml of brain homogenate. Figure 10 shows a near typical sigmoidal relationship between the concentration of T_3 and the product formed. At or below 0.3 μg/ml T_3, no effect was seen, but approximately a three-fold increase in indoleacetic acid production resulted at concentrations of 30 μg/ml or above. Similarly, the effect of T_4 upon indoleacetic acid formation was concentration-related (Figure 11). However, from this experiment, T_4 appears less active than T_3 at the higher

Figure 8. Effect of lowered tryptamine concentrations on indoleacetic acid formation.

The indoleacetic acid formed is expressed in relative intensity of fluorescence units (RIF). Each value is the mean of at least two determinations. Whole rat brain homogenate (20 mg/ml). Reaction time = 20 minutes.

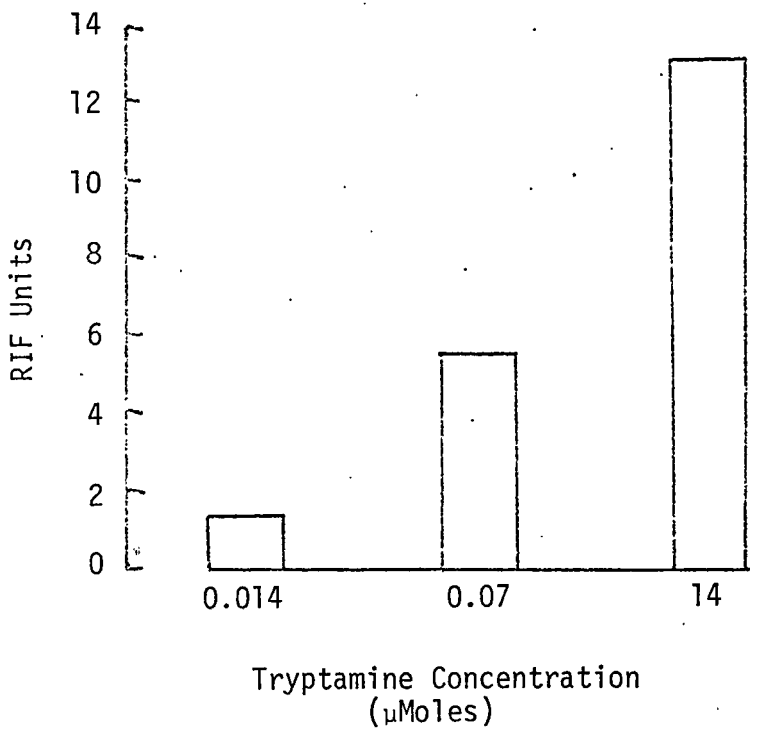

14
12
10
8
6
4
2
0
RIF Units
0.014
0.07
14
Tryptamine Concentration
(μMoles)

Figure 9. Effect of L-triiodothyronine (o)(10 µg/ml) on indoleacetic acid formation from a submaximal concentration of tryptamine (0.07 µMoles) using different reaction times (control, no hormone = ●).

The indoleacetic acid formed is expressed in relative intensity of fluorescence units (RIF). Each value is the mean of at least two determinations.

Whole rat brain homogenate (20 mg/ml).

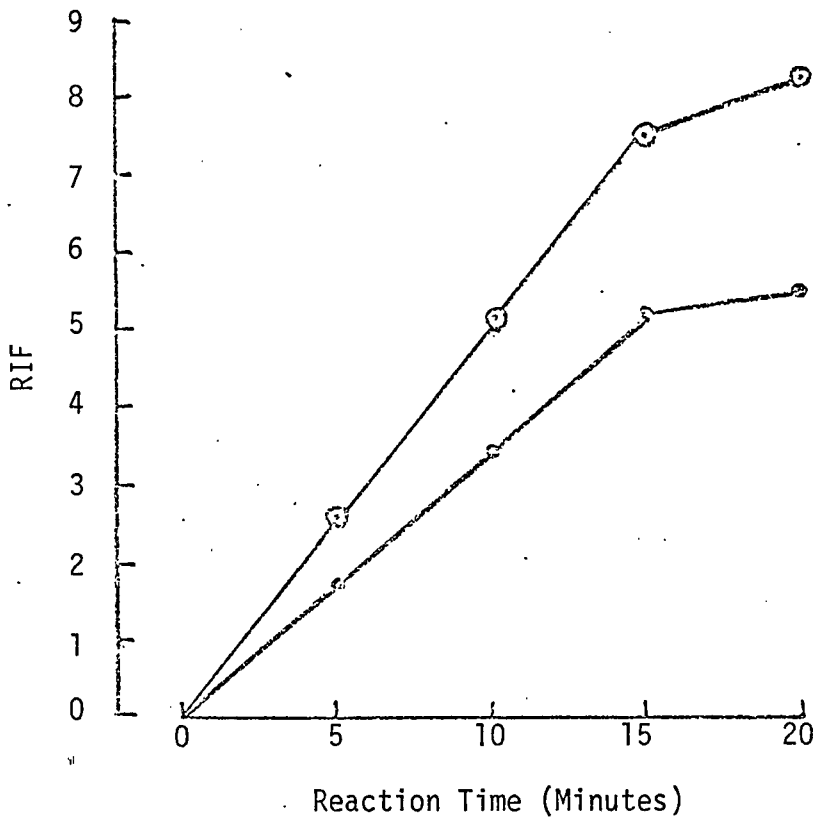

RIF
9
8
7
6
5
4
3
2
1
0
0
5
10
15
20
Reaction Time (Minutes)

Figure 10. Effect of various concentrations of L-triiodothyronine upon indoleacetic acid formation from tryptamine.

The indoleacetic acid (IAA) formed is expressed as a percentage of the control specific activity (2.4 μMoles IAA/g/hr = 100%). Each value is the mean of at least three determinations. Whole rat brain homogenate (20 mg/ml).

Reaction time = 20 minutes.

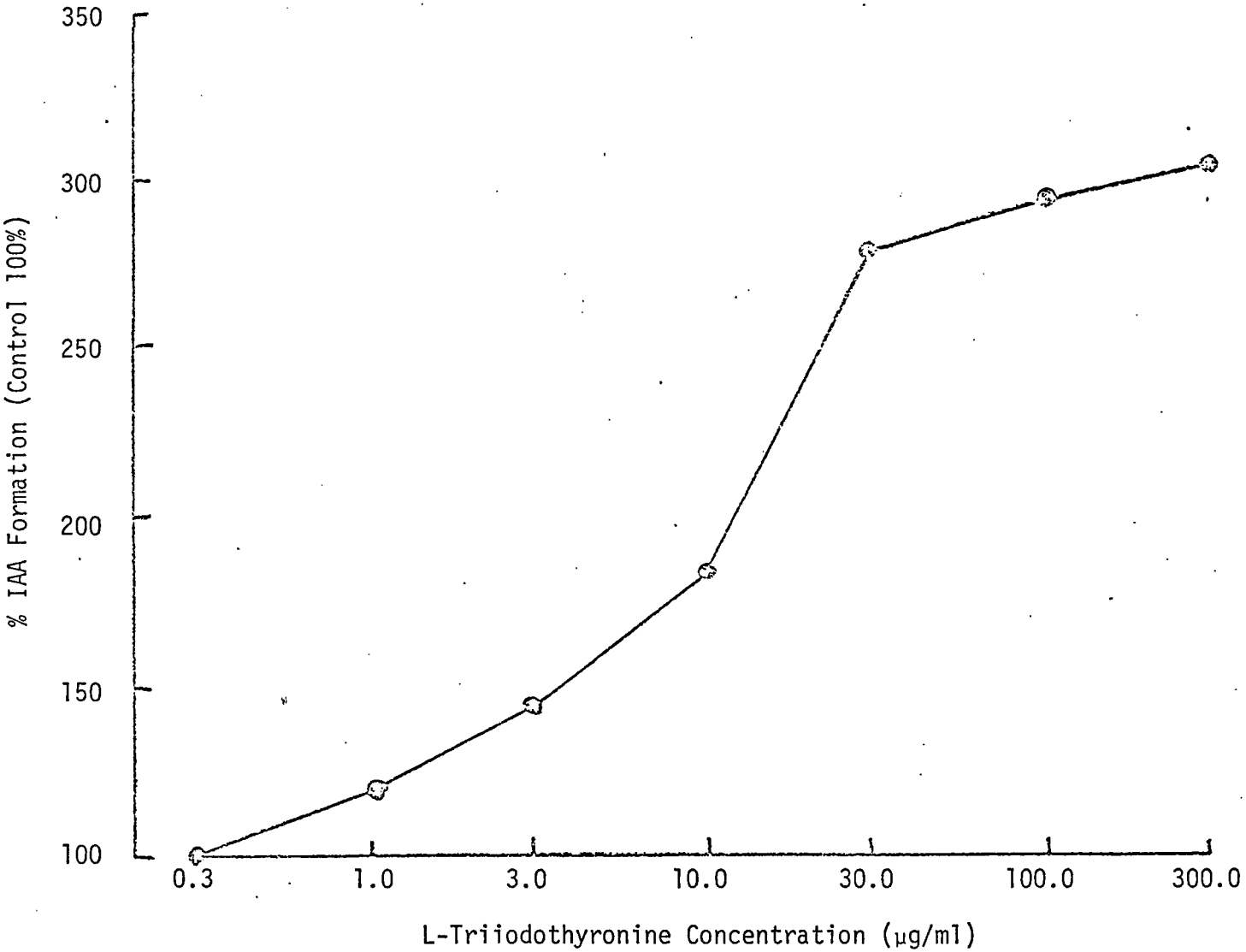
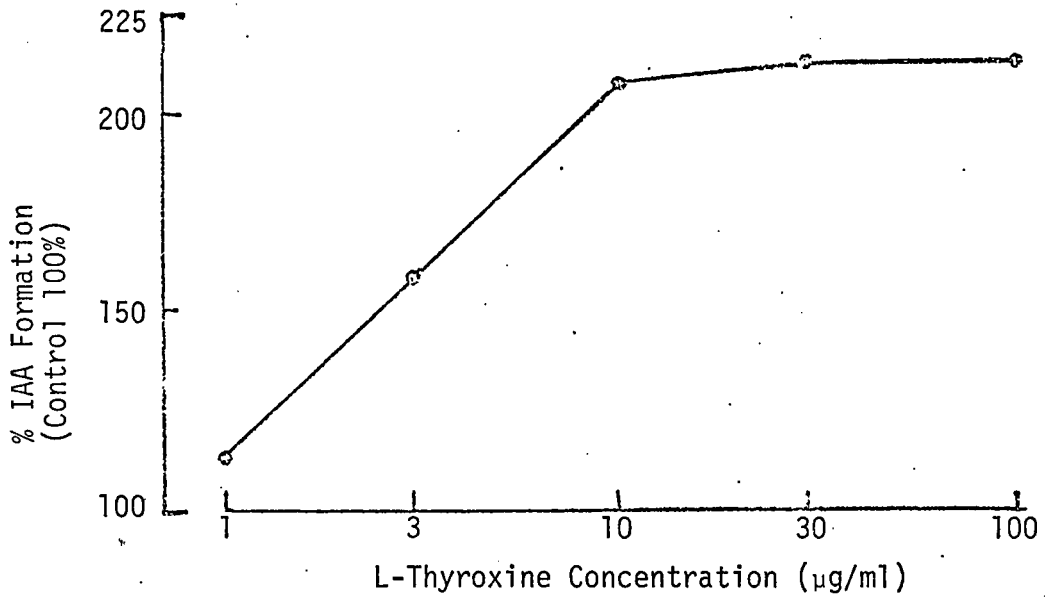

350
300
250
200
150
100
% IAA Formation (Control 100%)
0.3
1.0
3.0
10.0
30.0
100.0
300.0
L-Triiodothyronine Concentration (µg/ml)

Figure 11. Effect of various concentrations of L-thyroxine upon indoleacetic acid formation from tryptamine.

The indoleacetic acid (IAA) formed is expressed as a percentage of the control specific activity (4.9 μMoles IAA/g/hr = 100%). Each value is the mean of at least two determinations.

Whole rat brain homogenate (20 mg/ml). Reaction time = 20 minutes.


225
200
150
100
% IAA Formation
(Control 100%)
1
3
10
30
100
L-Thyroxine Concentration (µg/ml)

concentrations and the maximal response occurred earlier (compare Figure 10 with Figure 11).

However, strict comparisons of relative activity between the two experiments cannot readily be drawn since the percentage response to T_4 is dependent upon the absolute specific activity of the homogenate. Figure 12 shows that there is a good correlation between the absolute specific activity of the homogenate and the percentage increase in indoleacetic acid formation for T_4 (10 μg/ml). Interestingly, no such correlation exists for T_3. Thus, at low specific activities, T_4 is more active than T_3, whereas at higher specific activities, in the region of 5.0 μMoles indoleacetic acid/g/hr, it is approximately of equal potency. Because of this relationship it is likely that the position of the entire concentration-effect curve for T_4 will vary according to the level of inherent activity present in any particular homogenate. It should be pointed out, however, that for any particular homogenate, variation in the effect of either T_4 or T_3 was negligible, as was day-to-day variation when assaying from the same homogenate.

6. Effect of varying the pre-incubation time and temperature on the thyroid-induced stimulation of indoleacetic acid production.

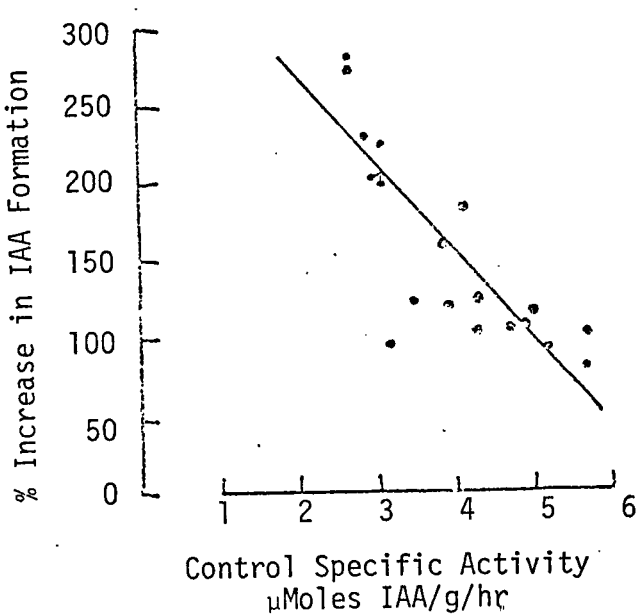
When either L-thyroxine (T_4) or L-triiodothyronine (T_3) were added to the homogenate at the same time as tryptamine no increase in indoleacetic acid formation resulted. Figure 13 shows clearly that pre-incubation of the hormones with the homogenate, prior to the substrate addition, is an absolute pre-requisite for the "activation." In view of

Figure 12. Correlation between the specific activity of monoamine oxidase in rat brain homogenates (20 mg/ml) and the percentage increase in indoleacetic acid formation when L-thyroxine (n=20)(Panel A) and L-triiodothyronine (n=31)(Panel B) were added to the incubating mixture, 15 minutes prior to the addition of tryptamine. Each value is the mean of at least two determinations. Reaction time = 20 minutes.

Correlation Coefficient (Panel A) = 0.88

Correlation Coefficient (Panel B) = -0.10

(A)


% Increase in IAA Formation
300
250
200
150
100
50
0
1
2
3
4
5
6
Control Specific Activity
µMoles IAA/g/hr

(B)

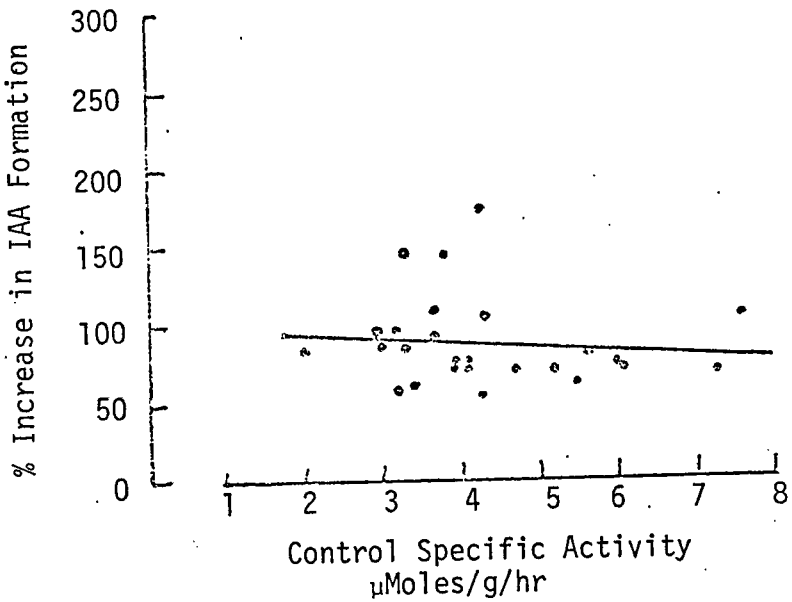
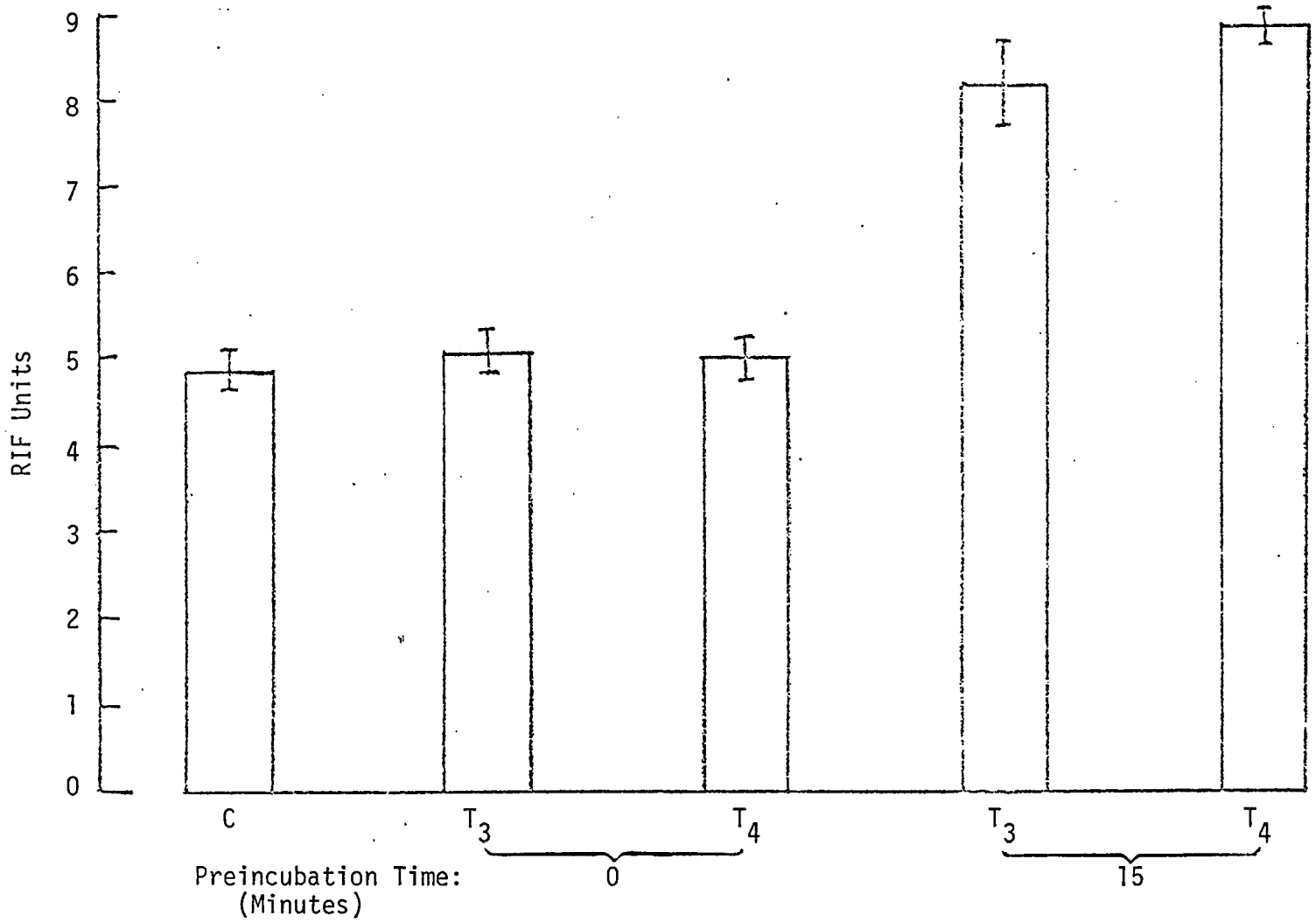

% Increase in IAA Formation
300
250
200
150
100
50
0
1
2
3
4
5
6
7
8
Control Specific Activity
µMoles/g/hr

Figure 13. Effect of pre-incubation on the thyroid-induced stimulation of indoleacetic acid formation from tryptamine.

Shown are mean values ± SEM for at least 3 experiments. The hormones (10 μg/ml) or vehicle were added either with the tryptamine (0 minutes) or 15 minutes prior to the addition of tryptamine. Pre-incubation for 15 minutes with the vehicle did not change the control values. C = control, no hormone; T_3 = L-triiodothyronine; T_4 = L-thyroxine. Whole rat brain homogenate (20 mg/ml). Reaction time = 20 minutes.


RIF Units
0
1
2
3
4
5
6
7
8
9
C
T_3
T_4
T_3
T_4
Preincubation Time: (Minutes)
0
15

this finding, an experiment was made to study the effect of varying the pre-incubation time. Figure 14 illustrates the results obtained. Both T_3 and T_4 (10 g/ml) produced a maximal stimulation of indoleacetic acid formation after only 5 minutes of pre-incubation. The efficacy of T_4 remained constant over the various pre-incubation times studied, but T_3 showed a marked decline in effectiveness at 30 and 45 minutes. This decline was not due to heat instability since T_3 incubated for 45 minutes in the absence of tissue was found to retain full activity when subsequently added to the homogenate 5 minutes prior to tryptamine. Thus, the decline in activity of T_3 was intimately related to the presence of the tissue homogenate.

Figure 14 also shows the effect of pre-incubation with T_3 at 4° C instead of 37° C. The substrate was added at the termination of the pre-incubation period and the flasks were immediately transferred to a Dubnoff metabolic incubator maintained at 37° C. Under conditions of "pre-incubation" at lowered temperature, both T_3 and T_4 failed to increase the formation of indoleacetic acid denoting the clear temperature dependence of the interaction.

7. Effect of propylthiouracil (PT) upon the thyroid-induced increase in indoleacetic acid formation.

A major metabolic route for L-thyroxine (T_4) is deiodonation to form L-triiodothyronine (T_3) which, in turn, can be further deiodonated. Indeed, several reports suggest that the physiological functions of T_4 are mediated indirectly by way of this enzymatic conversion to give the

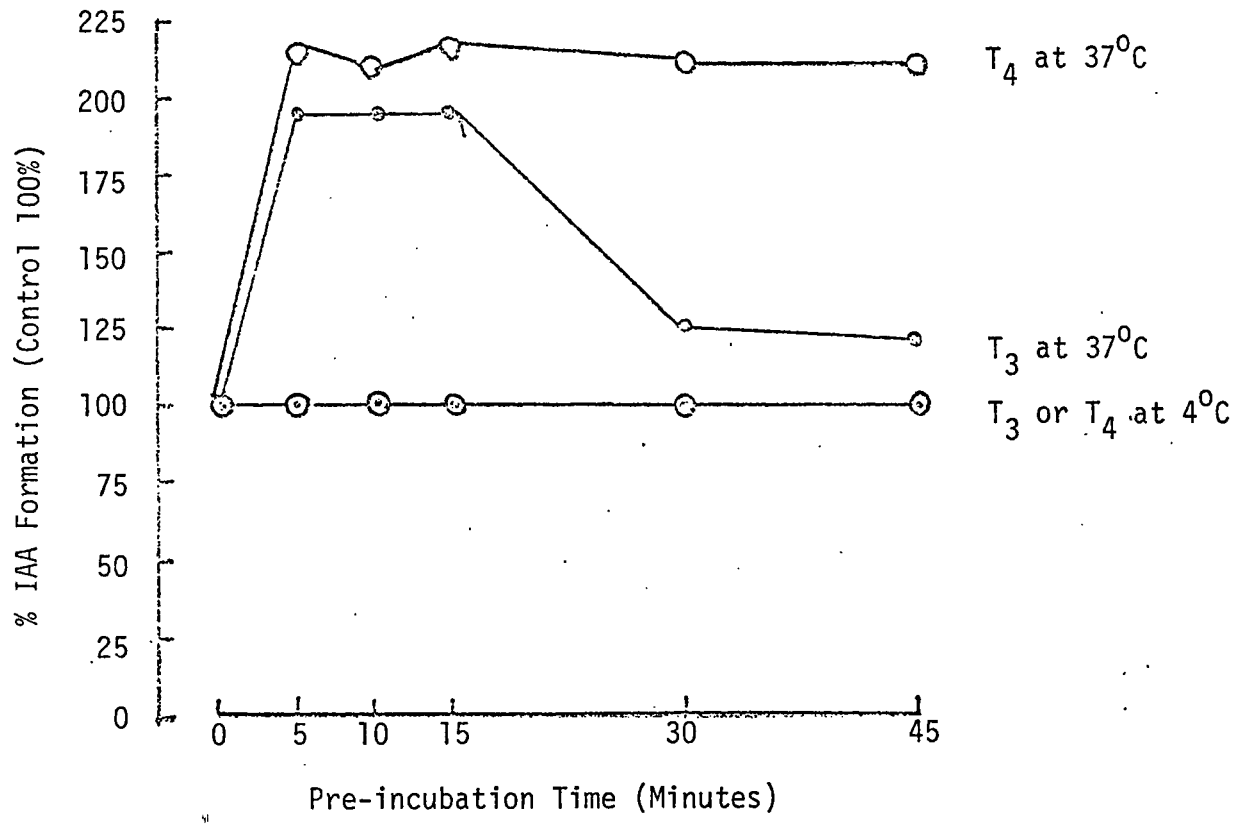
Figure 14. Effect of different pre-incubation times at 37° C and at 4° C on the thyroid-induced stimulation of indoleacetic acid formation from tryptamine.

The indoleacetic acid formed is expressed as a percentage of the respective control specific activity (control, no hormone = 100%) for the various times shown. Each value is the mean of at least two determinations. L-thyroxine = O and L-triiodothyronine = ● (both 10 μg/ml).

Control homogenates showed a decline in specific activity (μMoles indoleacetic acid/g/hr) during incubation at 37° C. Control values for L-thyroxine decreased from 5.0 to 3.7 over 45 minutes (26% decrease) and control values for L-triiodothyronine decreased from 3.3 to 2.35 (29% decrease).

In the experiment made at 4° C, "pre-incubation" with or without the hormones was made over an ice-water mixture and tryptamine was added immediately prior to heating at 37° C. No change in control values occurred over the 45 minutes at 4° C.

Whole rat brain homogenate (20 mg/ml). Reaction time = 20 minutes.


225
200
175
150
125
100
75
50
25
0
% IAA Formation (Control 100%)
0
5
10
15
30
45
Pre-incubation Time (Minutes)
T4 at 37°C
T3 at 37°C
T3 or T4 at 4°C

physiologically active T_3. Such a mechanism for the stimulation of indoleacetic acid formation would not be inconsistent with the findings of previous experiments (see #6 above). Thus, since propylthiouracil has been reported to inhibit the thyroid deiodonase enzyme, an experiment was made to investigate the effect of this drug upon the thyroid-induced increase in indoleactic acid formation.

Initial experiments made with brain homogenate revealed that PT (1 μMole/ml) itself, was a potent inhibitor of indoleacetic acid formation (86% decrease). A ten times lower concentration also revealed marked inhibitory properties leaving little remaining activity upon which to study the effect of the thyroid hormones. In an attempt to circumvent this problem, studies were made using liver tissue since this organ exhibits a higher endogenous deaminating activity. The results using a 50 mg/ml homogenate of rat liver are shown in Table 1.

PT (0.1 μMoles/ml) decreased indoleacetic acid production from a control value of 19.0 μMoles/g/hr. to 12.2 μMoles/g/hr. AS found previously for brain tissue, both T_3 and T_4 enhanced indoleacetic production. However, this enhancement was not markedly altered by the presence of PT.

8. Effect of sodium and potassium iodide on indoleacetic acid formation.

Using similar reasoning to that outlined in #7 above, it was argued that deiodonation of the thyroid hormones might be accompanied by the

Table 1. Effect of propylthiouracil upon the thyroid-induced increase in indoleacetic acid (IAA) formation in whole rat liver homogenate.

Shown is the specific activity and percentage change from control for at least two determinations. T_3 = L-triiodothyronine, T_4 = L-thyroxine (both 10 µg/ml, pre-incubated for 15 minutes prior to the addition of tryptamine) and PT = propylthiouracil (0.1 µMole/ml, pre-incubated for 15 minutes prior to the addition of tryptamine. Homogenate concentration = 10 mg/ml and reaction time = 20 minutes.

	EXPERIMENTAL	SA µMoles IAA/g/hr	% change
CONTROL	Control + Vehicle	19.0	
	Control + T_3	35.3	+ 85
	Control + T_4	36.2	+ 95
TEST	Control + PT + Vehicle	12.2	
	Control + PT + T_3	23.3	+ 90
	Control + PT + T_4	25.0	+105

subsequent indonation of monoamine oxidase and/or influencing factors. In order to check this possibility, studies were made using sodium and potassium iodide.

Figure 15 shows that neither compound caused an increase in indoleacetic acid formation in the concentrations used.

9. Effect of D-thyroxine and D-triiodothyronine on indoleacetic acid formation.

The D - forms of the thyroid hormones lack many of the biological properties associated with the endogenously produced L - forms. It was of interest, therefore, to ascertain whether this stereo-selectivity extended to the stimulation of indoleacetic acid production.

From Figure 16 it can be seen that no stereo-selectivity is evident. Both D-T_3 and D-T_4 were equally active at stimulating indoleacetic acid formation as their respective L-isomers.

10. Effect of lowered and increased aldehyde dehydrogenase activity upon the thyroid stimulated production of indoleacetic acid.

At this stage of the study, it was deemed important to determine whether the increased formation of indoleacetic acid by the thyroid hormones resulted from influences on the primary stage of oxidation

Figure 15. Effect of sodium iodide (NaI) and potassium iodide (KI) on indoleacetic acid (IAA) production from tryptamine.

Shown are percentage changes from control (C = control, no treatment = 100%) for two determinations at the concentrations shown. The substances or vehicle was added 15 minutes prior to the addition of tryptamine.

Whole rat brain homogenate (20 mg/ml). Reaction time = 20 minutes.

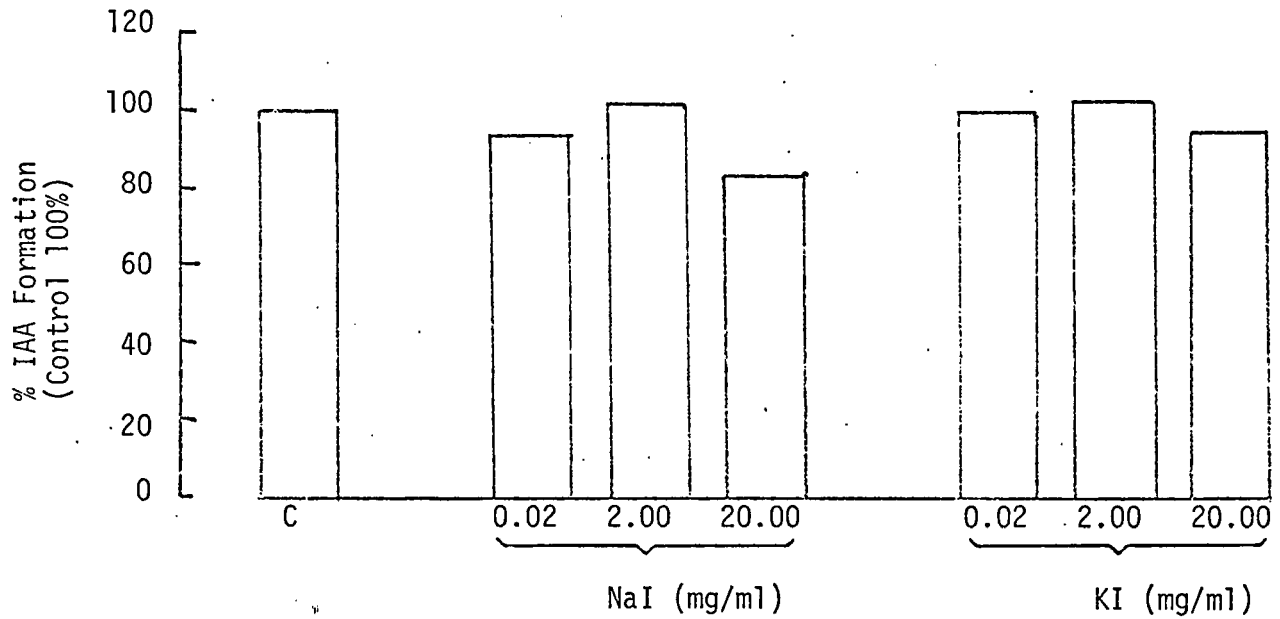
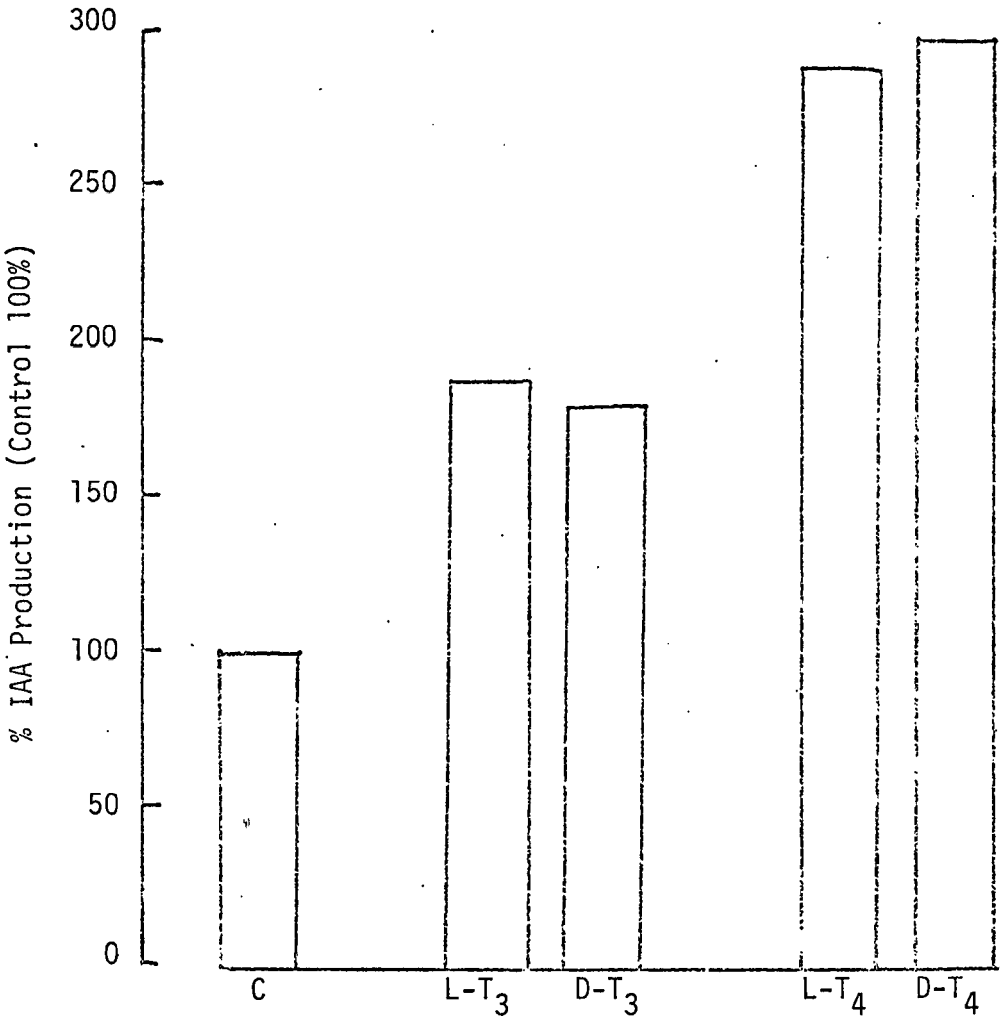

% IAA Formation
(Control 100%)
120
100
80
60
40
20
0
C
0.02
2.00
20.00
0.02
2.00
20.00
NaI (mg/ml)
KI (mg/ml)

Figure 16. Effect of D-thyroxine (D-T_4) and D-triiodothyronine (D-T_3) upon indoleacetic acid formation from tryptamine in whole rat brain homogenate.

Shown are percentage changes from control (C = control, no treatment = 100%) for at least two determinations. L-thyroxine = L-T_4 and L-triiodothyronine = L-T_3. All hormone concentrations = 10 μg/ml, and pre-incubation was made for 15 minutes prior to the addition of tryptamine.

Homogenate concentrations = 20 mg/ml and reaction time = 20 minutes.


% IAA Production (Control 100%)
300
250
200
150
100
50
0
C
L-T3
D-T3
L-T4
D-T4

(monoamine oxidase) or on the secondary oxidative process, mediated through aldehyde dehydrogenase. Although previous studies (Asaad et al., 1974; Asaad and Clarke, 1976) had shown that excess aldehyde dehydrogenase was present in assays of liver tissue (which contains a high endogenous content of these enzymes) this had not been shown for assays of brain homogenates.

A homogenate of several rat brains was prepared. Assays were made on aliquots in which no aldehyde dehydrogenase was added, on aliquots containing the usual amount of aldehyde dehydrogenase (0.4 ml, see Methods) and on samples containing twice this concentration. From Figure 17 it can be seen that both T_3 and T_4 gave rise to the usual increase in indoleacetic acid under normal assay conditions (addition of 1 x aldehyde dehydrogenase). In the absence of added aldehyde dehydrogenase, the production of indoleacetic acid in the control samples was reduced by about 80%, indicating an extremely low endogenous level of brain aldehyde dehydrogenase. Under these conditions, T_3 and T_4 caused about the same proportionate increase in indoleacetic acid as that seen under normal assay conditions. Doubling the concentration of aldehyde dehydrogenase failed to change markedly either the control value or the values obtained in the presence of the thyroid hormones from those obtained using only half the concentration of aldehyde dehydrogenase.

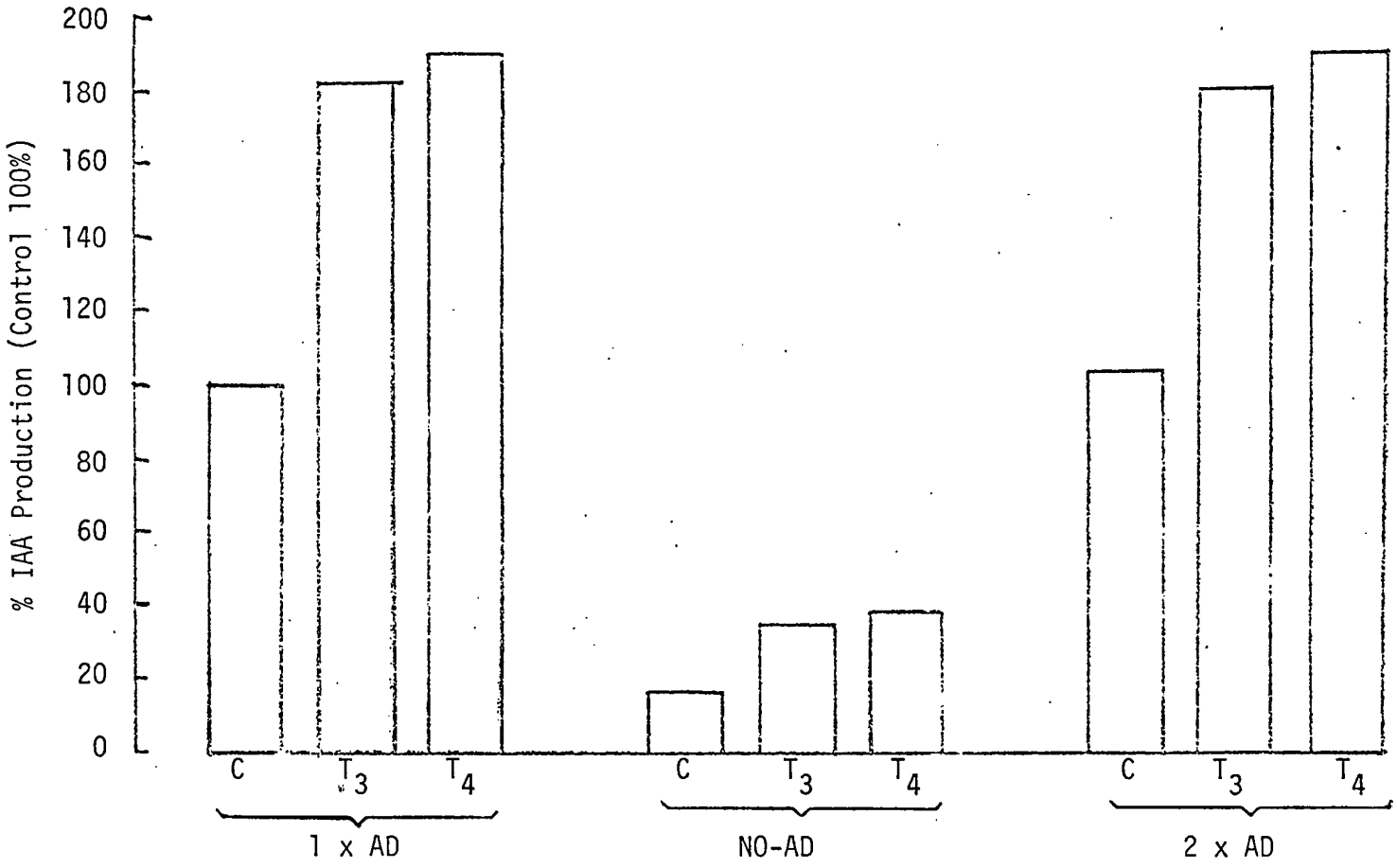
11. Effect of L-thyroxine (T_4) and L-triiodothyronine (T_3) on the formation of indoleacetic acid from indoleacetaldehyde.

Figure 17. Effect of altered aldehyde dehydrogenase concentrations upon the thyroid-stimulated production of indoleacetic acid (IAA).

Shown are percentage changes from the respective control values for at least two determinations. The hormones (10 μg/ml) or the vehicle were added 15 minutes prior to the tryptamine. T_3 - L-triiodothyronine, T_4 = L-thyroxine and AD = aldehyde dehydrogenase preparations (1 x AD = 0.4 ml, see Methods).

Control specific activities (μMoles IAA/g/hr) were: Control (1 x AD) 5.6; Control (no-AD) 0.9; Control (2 x AD) 5.8.

Whole rat brain homogenate (20 mg/ml). Reaction time = 20 minutes.


% IAA Production (Control 100%)
200
180
160
140
120
100
80
60
40
20
0
C
T3
T4
C
T3
T4
C
T3
T4
1 x AD
NO-AD
2 x AD

Although the previous experiment showed that the effect of the thyroid hormones was not mediated through the aldehyde dehydrogenase step, alternative possibilities, other than influences upon monoamine oxidase, were still feasible. For instance, the thyroid hormones might inhibit aldehyde reductases present within the tissue, thus shunting formed indoleacetaldehyde away from a reductive pathway (tryptophol formation) into the oxidative route. In order to test this and other possibilities regarding the deposition of the formed aldehyde, an experiment was made using indoleacetaldehyde itself.

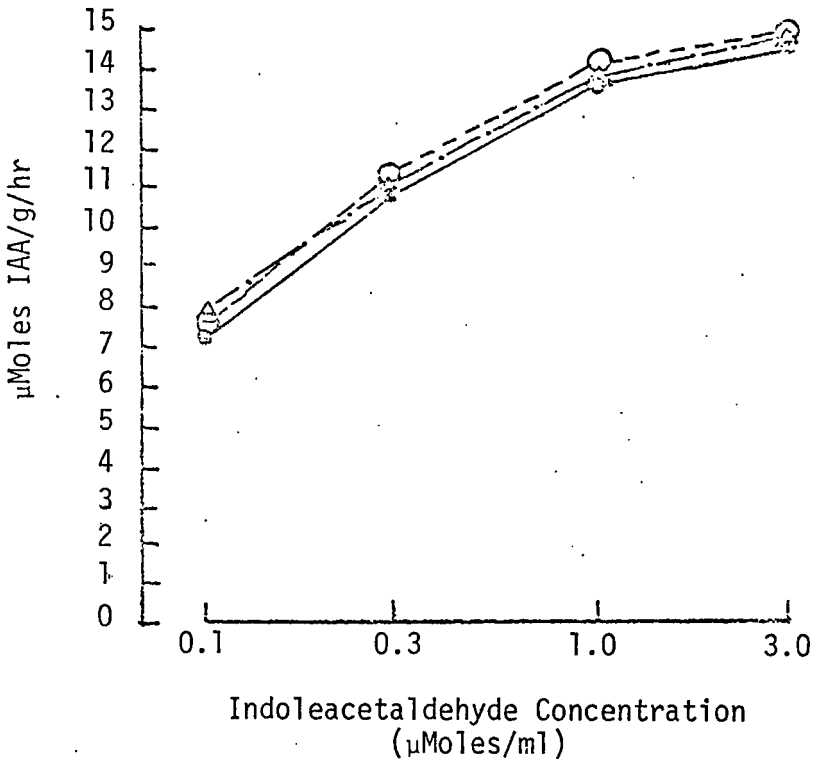
Figure 18 shows the effect of both T_3 and T_4 on the indoleacetic acid formation from indoleacetaldehyde. Over the range of aldehyde concentrations used, neither hormone enhanced indoleacetic acid production, either at submaximal or maximal substrate concentrations. The indoleacetaldehyde concentration of 0.1 μMoles is particularly important since this approximates to the amount of indoleacetaldehyde formed when tryptamine (14 μMoles) is used as the substrate.

12. Effect of L-thyroxine (T_4) and L-triiodothyronine (T_3) on the oxidation of kynuramine.

The previous experiment had indicated strongly that thyroid hormones stimulate indoleacetic acid production by influencing the activity of monoamine oxidase. Since monoamine oxidase exists in more than one molecular form, it was decided to determine whether the thyroid hormones would enhance the deamination of a different substrate (kynuramine).

Figure 18. Effect of L-thyroxine ( ) and L-triiodothyronine (o) (both 10 µg/ml) on the formation of indoleacetic acid from indoleacetaldehyde. (Control, no hormone = 0).

The indoleacetic acid formed is expressed in Moles IAA/g/hr Each value is the mean of at least three determinations. The hormones (10 µg/ml) or vehicle were added 15 minutes prior to the addition of the indoleacetaldehyde. Whole rat brain homogenate (20 mg/ml). Reaction time = 20 minutes.


15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
µMoles IAA/g/hr
0.1
0.3
1.0
3.0
Indoleacetaldehyde Concentration
(µMoles/ml)

When kynuramine was used as the substrate, neither T_3 nor T_4 enhanced the production of 4-hydroxyquinoline. This result is illustrated in Figure 19 using 6 mg/ml of brain tissue. This experiment was confirmed using different tissue concentrations, as shown in Figure 20. Unlike tryptamine, kynuramine exhibited a linear relationship between product and homogenate concentration (compare Figures 3 and 5).

13. Effect of tryptamine on kynuramine deamination and the influence of the thyroid hormones: Mixed substrate experiments.

The failure of the thyroid hormones to influence kynuramine oxidation might have been due to the fact that kynuramine was deaminated by a different molecular form of monoamine oxidase from that acting on tryptamine. In order to investigate this possibility, a mixed substrate experiment was performed using equi-molar concentrations of the two amines.

Figure 21 shows that tryptamine reduced the amount of 4-hydroxyquinoline formed from kynuramine at both low (2 mg/ml) and high (20 mg/ml) homogenate concentrations. Thus, both kynuramine and tryptamine interact with a common enzyme(s). However, this interaction was not influenced by the thyroid hormones, again revealing a substrate specific effect by these substances.

Figure 19. Effect of L-thyroxine (T_4) and L-triiodothyronine (T_3) (both 10 µg/ml) on the formation of 4-hydroxyquinoline from kynuramine.

The 4-hydroxyquinoline formed is expressed in relative intensity of fluorescence units (RIF).

Shown are mean values for at least two determinations. The hormones or vehicle were added 15 minutes prior to the addition of kynuramine. Whole rat brain homogenate (6 mg/ml). Reaction time = 30 minutes.

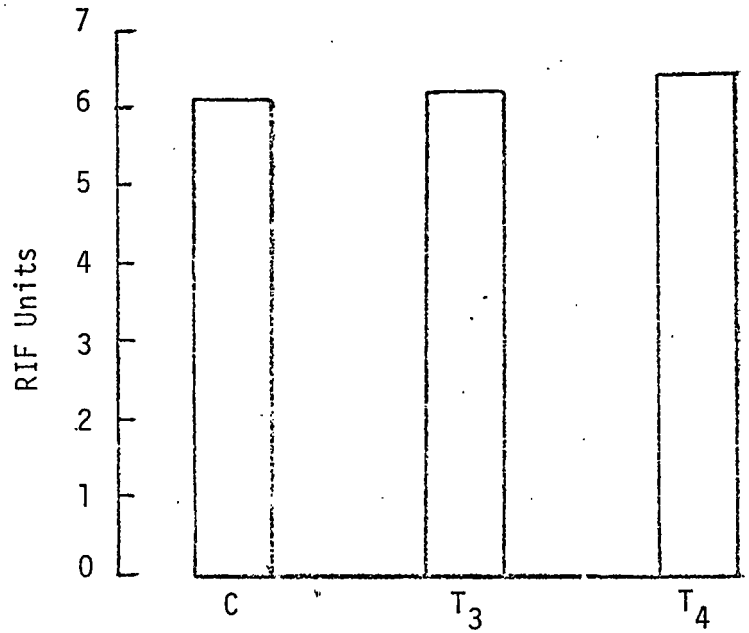

RIF Units
7
6
5
4
3
2
1
0
C
T_3
T_4

Figure 20. Effect of different tissue concentrations, L-thyroxine (Δ) and L-triiodothyronine (o)(both 10 μg/ml) on the formation of 4-hydroxyquinoline from kynuramine.

The 4-hydroxyquinoline formed is expressed in realtive intensity of fluorescence units (RIF). Each value is the mean of at least two determinations.

The hormones or vehicle were added 15 minutes prior to the addition of kynuramine. Whole rat brain homogenate. Reaction time = 30 minutes.

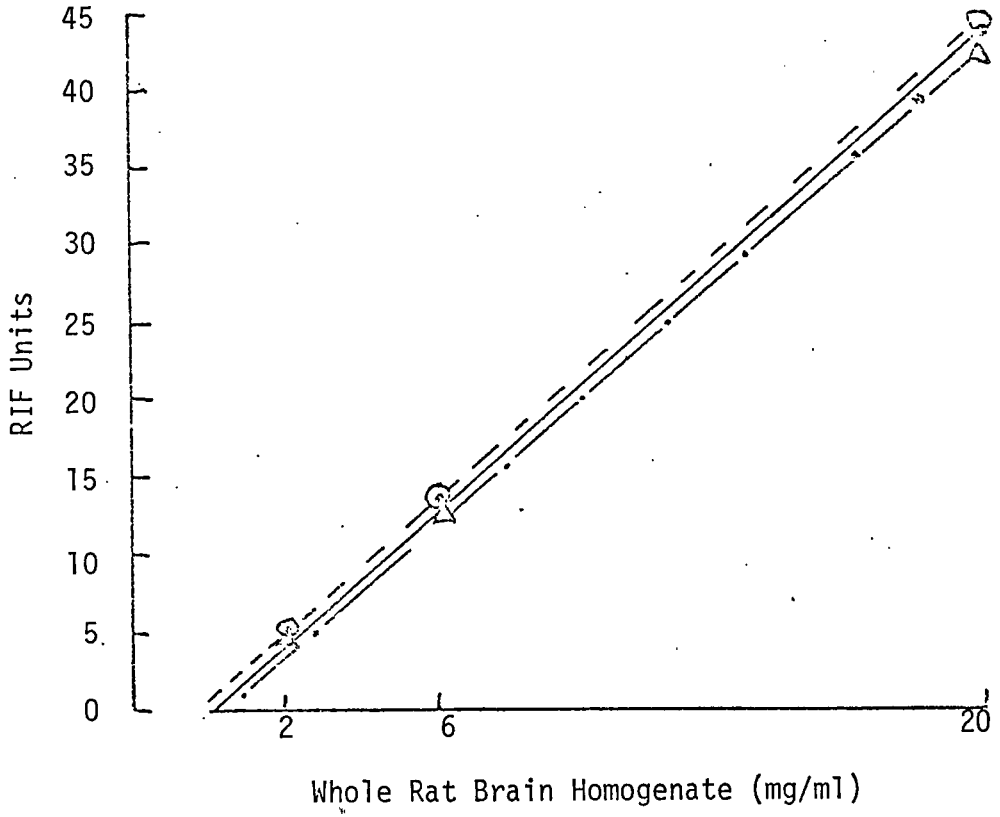
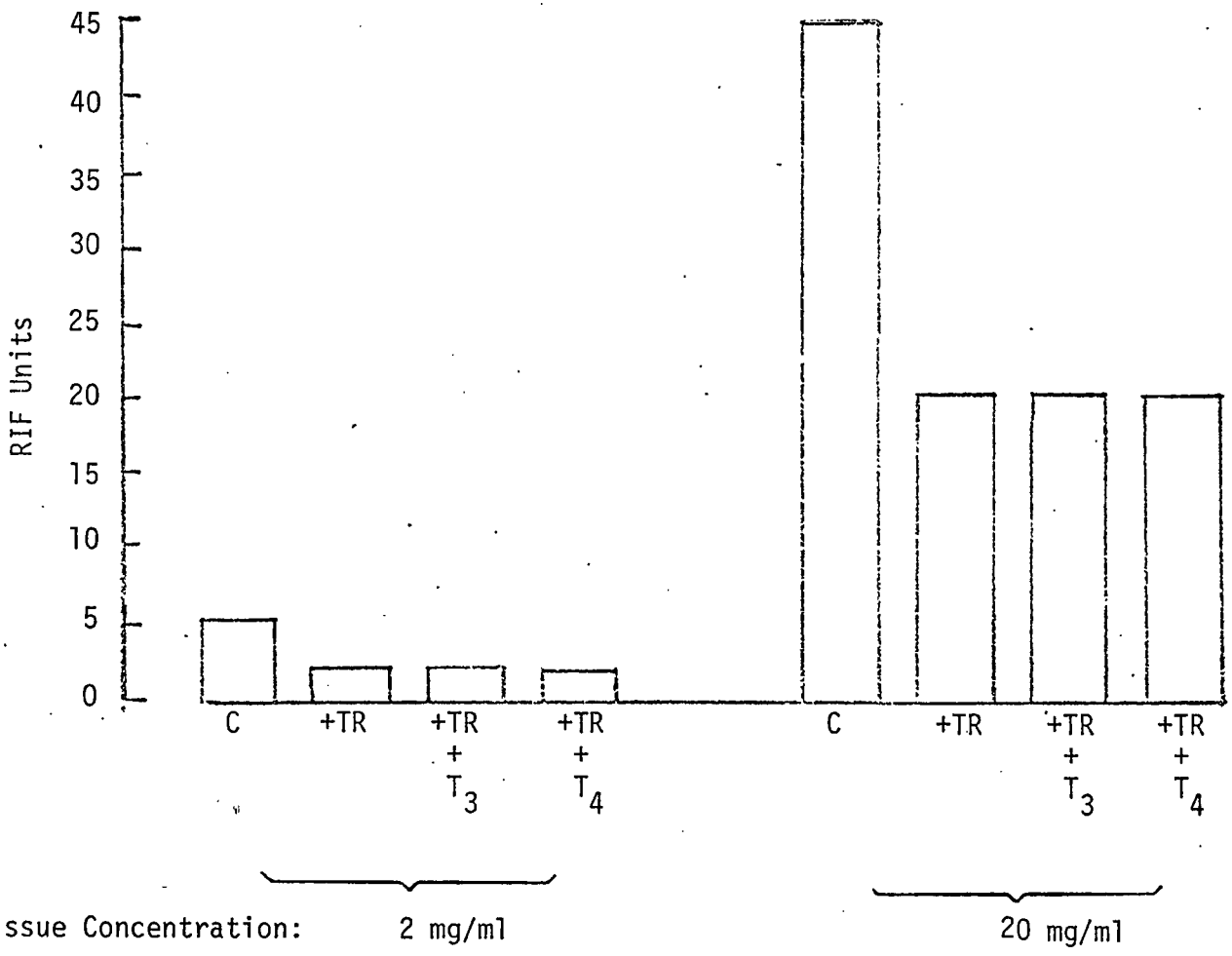

45
40
35
30
25
20
15
10
5
0
RIF Units
2
6
20
Whole Rat Brain Homogenate (mg/ml)

Figure 21. Mixed substrate experiments: the influence of tryptamine (TR), and tryptamine plus L-thyroxine (T_4) or L-triiodothyronine (T_3), on the formation of 4-hydroxyquinoline from kynuramine.

Two different concentrations of whole rat brain homogenate.

The 4-hydroxyquinoline formed is expressed in realtive intensity of fluorescence units (RIF). Each value is the mean of at least two determinations. C = control, no tryptamine. Reaction time = 30 minutes.


45
40
35
30
25
20
15
10
5
0
RIF Units
C
+TR
+TR + T_3
+TR + T_4
C
+TR
+TR + T_3
+TR + T_4
Tissue Concentration:
2 mg/ml
20 mg/ml

14. Effect of L-thyroxine (T_4) and L-triiodothyronine (T_3) on indoleacetic acid formation from tryptamine in the liver, kidney and heart of rats.

This experiment was made to determine whether the thyroid hormones exerted specific effects upon brain monoamine oxidase or whether the effect extended to the monoamine oxidase activity of other organs. The organ distribution of the various forms of monoamine oxidase is believed to vary and differing effects might be anticipated.

Table 2 shows the results obtained and these are compared to the specific activity and percentage changes obtained with T_3 and T_4 in the whole brain. The thyroid hormones increased indoleacetic acid production in all organs studied but the effect was most marked in the brain and liver compared with the kidney. In the heart, only a very marginal increase was found. The difference between the brain and kidney is especially interesting since the specific activity of monoamine oxidase in control homogenates was virtually identical. Overall, however, it can be seen that no correlation existed between the control specific activities of monoamine oxidase and the effect of the thyroid hormones.

Table 3 gives mean values of monoamine oxidase activity using kynuramine as the substrate. As described previously, kynuramine oxidation in brain is not changed by T_3 or T_4. This same situation is now

Table 2. Effect of L-thyroxine (T_4) and L-triiodothyronine (T_3) on indoleacetic acid (IAA) formation from tryptamine in the brain, liver, kidney and heart of rats.

All hormone concentrations = 10 g/ml, which was added 15 minutes prior to the addition of tryptamine.

Tissue concentrations were: Brain - 20 mg/ml
Liver - 10 mg/ml
Kidney - 20 mg/ml
Heart - 20 mg/ml

Shown are mean values ± SEM for (n) experiments. Reaction time = 20 minutes.

ORGAN	CONTROL SPECIFIC ACTIVITY µMOLES IAA/g/hr	% INCREASE IN IAA FORMATION + T_3	+ T_4
BRAIN	4.37 ± 0.22 (31)	+87.6 ± 5.5 (31)	+163.5 ± 14.1 (20)
LIVER	15.4 ± 2.0 (3)	+79.9 ± 15.9 (3)	+101.3 ± 6.2 (3)
KIDNEY	4.5 ± 0.7 (4)	+19.8 ± 7.1 (4)	+ 20.2 ± 4.0 (4)
HEART	7.1 ± 0.9 (4)	+ 9.3 ± 4.9 (3)	+ 10.4 ± 1.5 (3)

Table 3. Effect of L-thyroxine (T_4) and L-triiodothyronine (T_3) on 4-hydroxyquinoline (4OHQ) formation from kynuramine in the brain, kidney and liver of rats.

All hormone concentrations = 10 µg/ml, which was added 15 minutes prior to the addition of kynuarmine.

Tissue concentrations were: Brain, 6 mg/ml
Kidney, 6 mg/ml
Liver, 3 mg/ml

Reaction time = 30 minutes.

ORGAN	SPECIFIC ACTIVITY µMOLES 4-OHQ/g/hr		
	CONTROL	+ T_3	+ T_4
BRAIN	6.0	6.2	6.4
KIDNEY	2.7	2.7	2.7
LIVER	26.7	26.7	26.7

seen to apply in the kidney and liver. Again liver monoamine oxidase activity shows the highest specific activity, but in contrast to that seen with tryptamine, brain specific activity is approximately double that of the kidney.

15. Effect of L-thyroxine (T_4) and L-triiodothyronine (T_3) on brain mitochondrial monoamine oxidase.

The initial rationale of this study (pp. 54) stressed the importance of utilizing whole cell homogenates. The present experiment was undertaken to evaluate the contribution of the cellular constituents in relation to the effects observed with T_3 and T_4. In order to achieve this aim, the major cellular source of monoamine oxidase, the mitochondria, were separated by centrifugation, and the effects of T_3 and T_4, plus the removed components, were studied upon indoleacetic acid formation.

Mitochondria were prepared according to the flow diagram illustrated in Figure 22. Figure 23 shows that the whole cell homogenate (Panel A) gave rise to a typical increase in indoleacetic acid production and serves as a valid control for the remaining experiments. Panel B reveals that both T_3 and T_4 failed to increase indoleacetic acid production when washed mitochondria were used as the enzyme source. However, when mitochondria were homogenized in the retained supernatant (Figure 22, Supernatant II) the activity of the thyroid hormones was restored toward control levels (Panel A). It is pertinent to note that addition of the Supernatant

Figure 22. Flow diagram describing the preparation of mitochondrial monoamine oxidase in relation to the experiments illustrated in Figure 23.

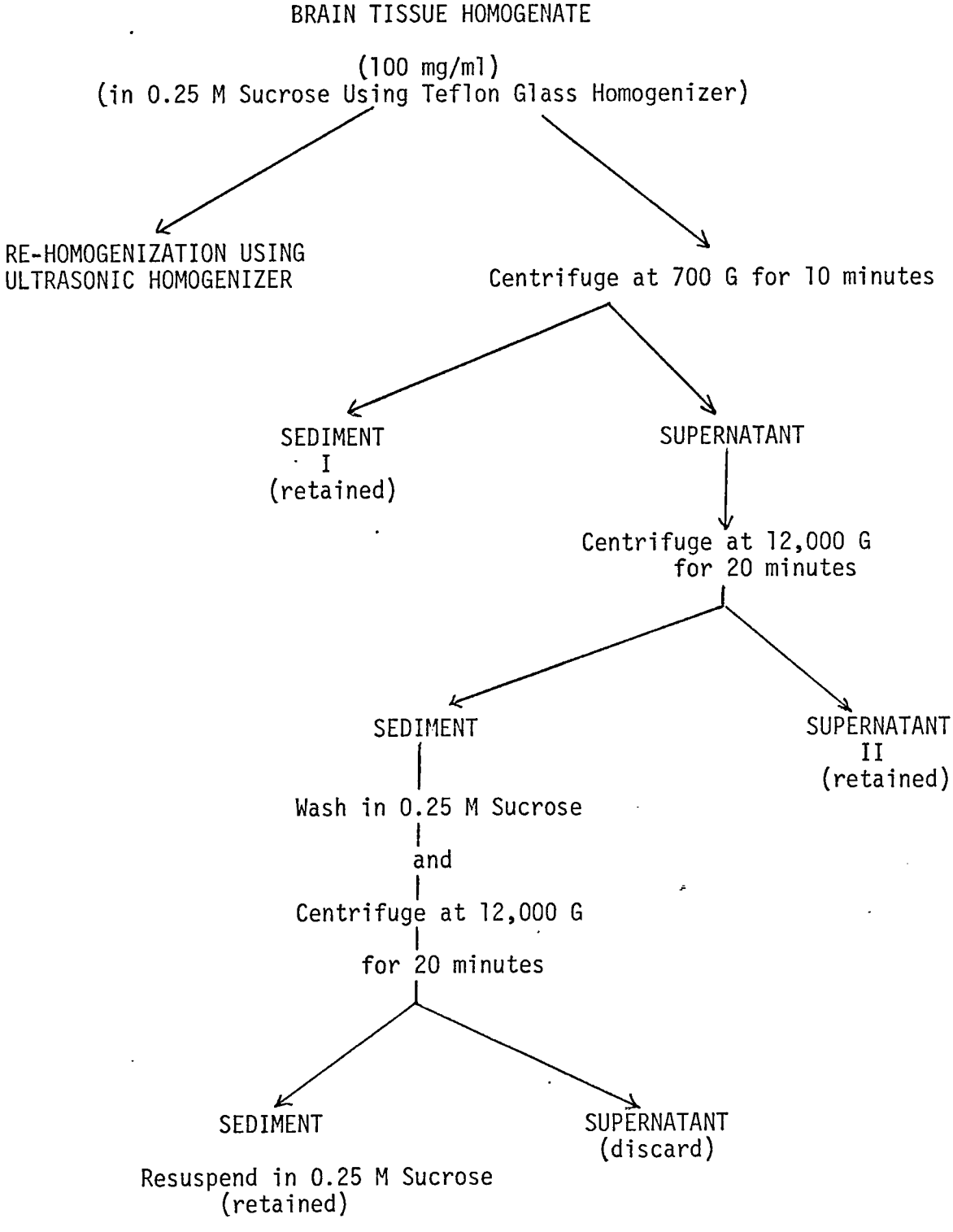

BRAIN TISSUE HOMOGENATE
(100 mg/ml)
(in 0.25 M Sucrose Using Teflon Glass Homogenizer)
RE-HOMOGENIZATION USING
ULTRASONIC HOMOGENIZER
Centrifuge at 700 G for 10 minutes
SEDIMENT
I
(retained)
SUPERNATANT
Centrifuge at 12,000 G
for 20 minutes
SEDIMENT
SUPERNATANT
II
(retained)
Wash in 0.25 M Sucrose
and
Centrifuge at 12,000 G
for 20 minutes
SEDIMENT
SUPERNATANT
(discard)
Resuspend in 0.25 M Sucrose
(retained)

Figure 23. Effect of L-thyroxine (T_4) and L-triiodothyronine (T_3) on indoleacetic acid (IAA) production from tryptamine using different cellular fractions of whole rat brain homogenate (see Figure 22).

Panel (A): Effect of T_3 and T_4 using whole rat brain homogenate. C = control, no hormone = 100%.

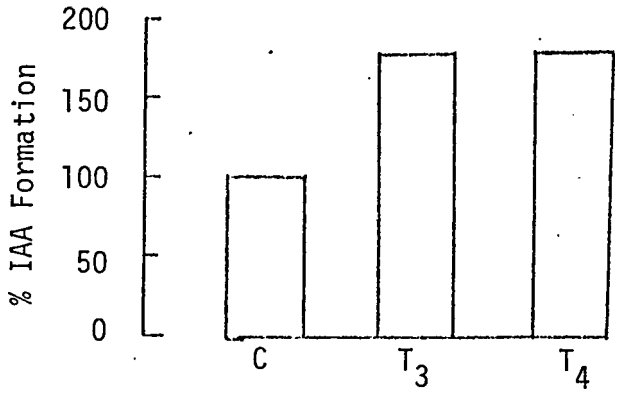
Panel (B): Effect of T_3 and T_4 using washed mitochondrial monoamine oxidase homogenized in 0.25 M sucrose. C_m = control, no hormone = 100%. NOTE: All other control values in Panels C and D are expressed as a percantage of C_m.

Panel (C): Effect of T_3 and T_4 using washed mitochondrial monoamine oxidase homogenized in supernatant II, such that the concentration was made equal to that used in Panel B. C_{ms} = control, no hormone.

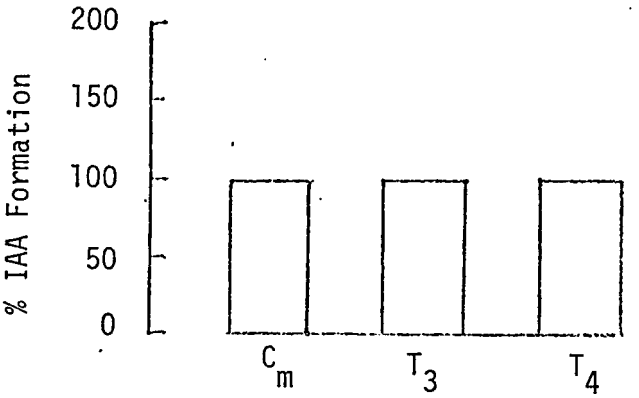
Panel (D): Effect of T_3 and T_4 using washed mitochondrial monoamine oxidase homogenized in supernatant II plus the pellet fraction I. The concentration of mitochondria was the same as that used in Panel (B). $C_{m,s,p}$ = control, no hormone.

Shown are mean values for at least two determinations. The hormones (10 g/ml) were pre-incubated with the enzyme for 15 minutes prior to the addition of tryptamine. Reaction time = 20 minutes.

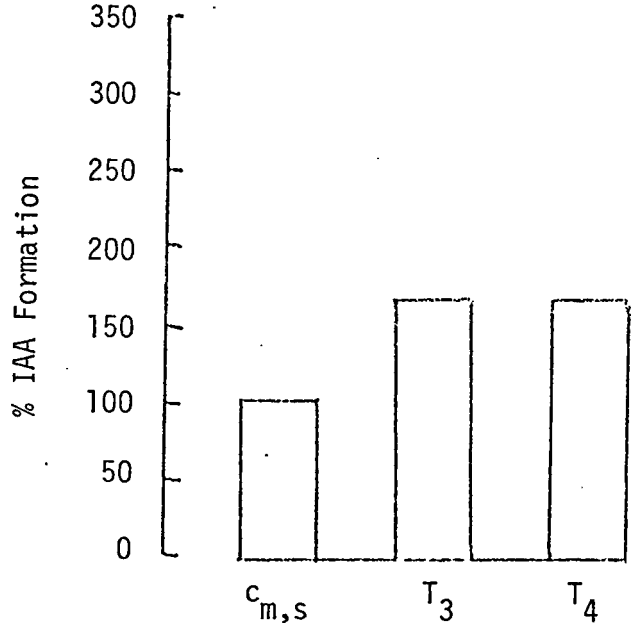
A


% IAA Formation
200
150
100
50
0
C
T_3
T_4

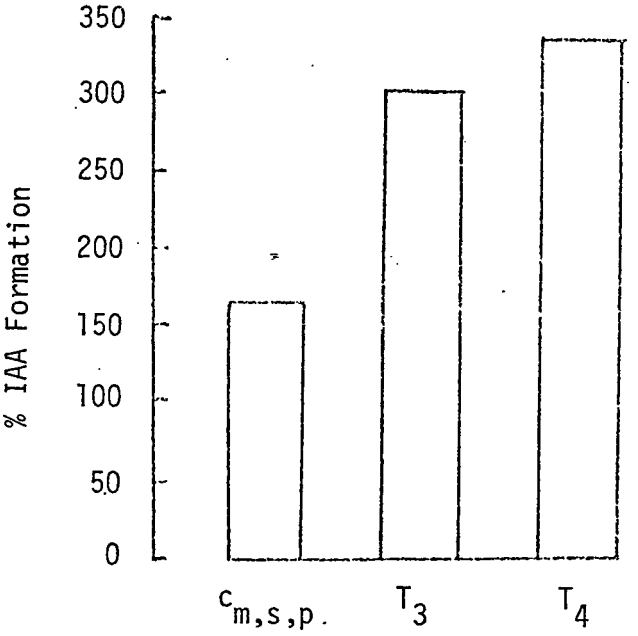
B


% IAA Formation
200
150
100
50
0
C_m
T_3
T_4

C


% IAA Formation
350
300
250
200
150
100
50
0
$c_{m,s}$
T_3
T_4

D


% IAA Formation
350
300
250
200
150
100
50
0
$c_{m,s,p}$
T_3
T_4

II to the mitochondria did not enhance or reduce monoamine oxidase activity from that obtained with mitochondria alone (compare $C_{m,s}$, Panel C with C_m, Panel B).

This situation did not hold true when both the supernatant II and pellet I were added back to the mitochondrial preparation (Panel D, $C_{m,s,p}$), indicating that the resultant increase in activity stemmed from additional monoamine oxidase residing in the pellet. However, under these conditions T_3 and T_4 again promoted a marked increase in indole-acetic acid production. The ratio of these increases to the control value ($C_{m,s,p}$) approximates closely to the same respective ratios derived from Panel A.

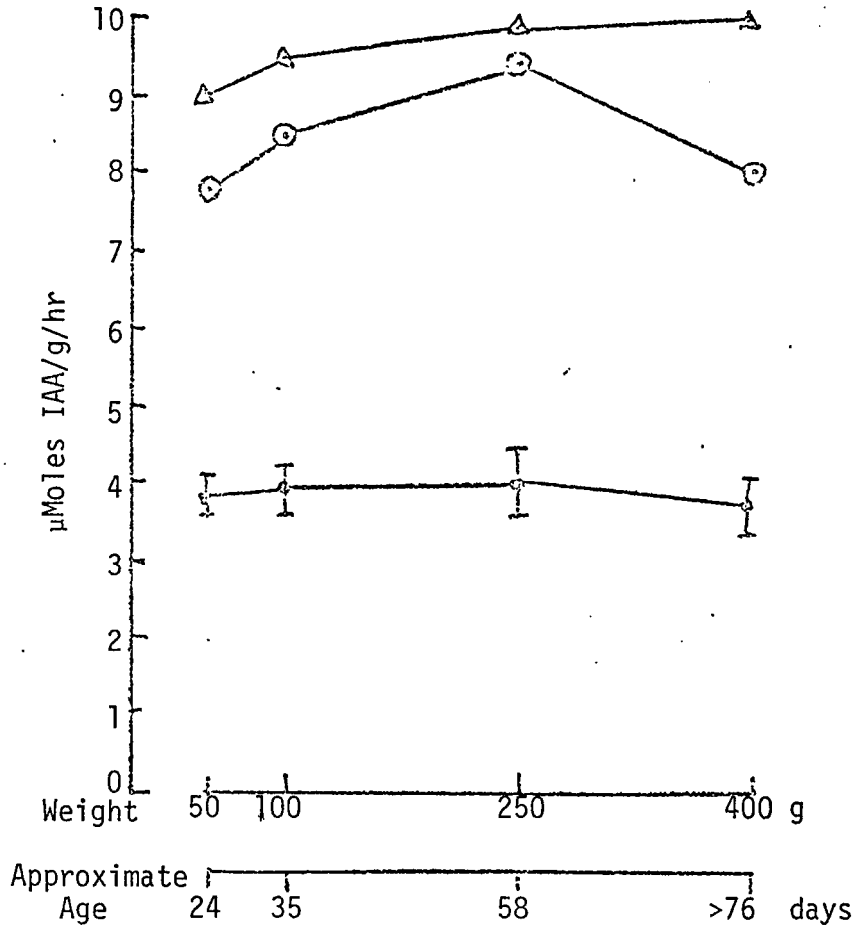
16. Effect of body weight (age) differences upon brain monoamine oxidase activity and the effects of L-thyroxine (T_4) and L-triiodothyrone (T_3).

The previous experiment had indicated the presence of a substance in the supernatant fraction of whole brain homogenates which is essential for the effects of T_3 and T_4 upon monoamine oxidase activity. Thus, the present experiment was undertaken to determine whether age differences influence the effectiveness of the thyroid hormones.

Four groups of rats, obtained at the same time from the same source, with a mean body weights of 50, 100, 250 and 400 g were used. Figure 24 shows that over the weight range studied, little or no change in

Figure 24. Effect of body weight (age differences) alone (●—●), and in conjunction with L-thyroxine (△—△) or L-triiodothyronine (○—○) on whole rat brain monoamine oxidase activity using tryptamine as the substrate.

Shown are mean values ± SEM for no treatment (controls) (n=4) and mean values for at least two determinations with the respective thyroid hormones. The hormones (10 μg/ml) were added 15 minutes prior to the addition of tryptamine. IAA = indoleacetic acid. Homogenate concentrations = 20 mg/ml and reaction time = 20 minutes.


µMoles IAA/g/hr
10
9
8
7
6
5
4
3
2
1
0
Weight
50
100
250
400 g
Approximate
Age
24
35
58
>76
days

monoamine oxidase activity occurred. Furthermore, the effect of T_3 and T_4 did not vary markedly, although a tendency toward a peak effect of T_3 is perhaps present in the 250 g body weight group.

V. DISCUSSION

Discussion 1.

The present study has revealed a new action of the thyroid hormones in that both L-thyroxine and L-triiodothyronine can increase indoleacetic acid formation from tryptamine using whole rat brain homogenate *in vitro*. The site of action appears to be mediated at the monoamine oxidase step since the hormones failed to enhance indoleacetic acid production from the aldehyde intermediate (Figure 18).

Following the deamination of tryptamine, formed indoleacetaldehyde may be reduced by aldehyde reductases (Tabakoff and Erwin, 1970; Tabakoff *et al.*, 1974; Deitrich and Erwin, 1975) to form tryptophol. This pathway will predominate if sufficient aldehyde dehydrogenase is lacking and/or if ethanol dehydrogenase is stimulated (Asaad *et al.*, 1974; Asaad and Clarke, 1976). Additionally, the high reactivity of biogenic aldehydes leads to significant non-specific protein binding (Deitrich and Erwin, 1975; Ris *et al.*, 1975). Thus, even though the assay system used was weighted to shunt indoleacetaldehyde through the oxidative route, the possibility existed that the thyroid hormones might either (1) divert metabolism away from tryptophol formation or (2) compete with non-specific binding sites to allow more indoleacetic acid production. The experiment utilizing indoleacetaldehyde as the substrate eliminates these possibilities. The product versus concentration curve was not affected by the thyroid homrones in a concentration (10 μg/ml) which produced marked increases in indoleacetic acid production when using tryptamine as the substrate. Furthermore, this same experiment precludes the notion that the thyroid hormones might have activated aldehyde dehydrogenase. That

excess aldehyde dehydrogenase activity was present in the standard reaction mixture was confirmed in Figure 17. The increase in formed indoleacetic acid in the presence of the thyroid hormones using 0.4 ml of the aldehyde dehydrogenase preparation was no greater than when this concentration was doubled. Thus, the experiment proves that secondary oxidation, by aldehyde dehydrogenase, was not rate-limiting. This possibility could well have explained the findings, since in the absence of exogenously added aldehyde dehydrogenase, the rate-limiting aspect of this enzyme system became apparent and indoleacetic acid formation was increased by the thyroid hormones (Figure 17). Furthermore, this same experiment indicates again that alternative metabolic routes are not an important factor since double the aldehyde dehydrogenase concentration did not give rise to additional indoleacetic acid from that obtained with the normal reaction mixture (0.4 ml aldehyde dehydrogenase). This experiment, and the study utilizing indoleacetaldehyde as the substrate, serves to pin-point the effect of the thyroid hormones at the level of monoamine oxidase. In fact, the exclusion of tryptophol formation as being a factor in the present experiments is consistent with the observations of Tabakoff and Erwin (1970) that indoleacetaldehyde, unlike certain other biogenic aldehydes, is only a poor substrate for rat brain aldehyde reductases. The possibility of activation of ethanol dehydrogenase is also remote, since unlike the liver (Detrich and Erwin, 1975) ethanol dehydrogenase activity of brain is stated to be virtually insignificant.

Further analysis of Figure 18 is worthy since it serves to support the quantitative measurements made using tryptamine as the substrate. In this sense, it is added validation of the experimental procedure and

findings. From the figure it can be seen that at the lowest concentration of indoleacetaldehyde, 7.3 μMoles indoleacetic acid/g/hr were formed. This value, although slightly higher, approximates the specific activity found using 14 μMoles of tryptamine and 20 mg/ml brain homogenate. At saturating concentrations of indoleacetaldehyde, 14.5 μMoles indoleacetic acid/g/hr were produced which is fully compatible with the thyroid-stimulated production of this substance using tryptamine as the substrate. These comparisons therefore indicate strongly that the use of the intermediate aldehyde has not involved a different or unexpected metabolic pathway from that normally utilized by endogenously formed aldehyde when utilizing tryptamine as the starting material.

Although it has been concluded that the thyroid hormones exert their effect at the monoamine oxidase step, this conclusion is only valid if an excess of substrate is shown to be present. Other metabolic routes for tryptamine exist, other than deamination. For instance, tryptamine is known to be N-methylated to N-methyl and N-dimethyl derivatives by brain tissue (Rosengarten et al., 1975) and can be metabolized to tryptolines (1,2,3,4-tetrahydro-beta-carbolines)(Mandel et al., 1974; Barchas et al., 1974; Wyatt et al., 1975). It was possible therefore, that the thyroid hormones might inhibit these metabolic routes thereby shunting more tryptamine through the monoamine oxidase pathway. Figure 7 shows clearly that this line of argument cannot be entertained. When the concentration of tryptamine was decreased by ten-fold, product formation was hardly affected, indicating both a marked excess of substrate and the insignificance of other metabolic routes under the conditions used. L-triiodothyronine produced a near parallel increase in indoleacetic acid formation providing

evidence for only one major interaction and again illustrating that substrate availability for monoamine oxidase was not a complicating factor.

Although the effect of the thyroid hormones can be placed at the level of monoamine oxidase, their influence upon the enzyme is not a simple one. This complexity relates to the fact that monoamine oxidase activity itself appears to be governed by endogenous modulating influences present in the whole rat brain homogenates. Figure 1 shows that as the concentration of whole rat brain homogenate is increased there is a progressive deviation from linearity with regard to product formation. As discussed above, this is not due to substrate insufficiency and this fact is further confirmed in Figure 6 where linearity was obtained for the rate of indoleacetic acid formation using the 20 mg/ml homogenate concentration. Furthermore, the nonlinearity of the relationship between homogenate concentration and product formation was not due to saturation of the spectrophotofluorimeter's ability to measure indoleacetic acid. This is clear from many experiments since much higher concentrations were measured in the presence of thyroid and by using external standards. When the thyroid hormones were pre-incubated with the homogenates, product formation was increased and a distinct tendency toward a more linear reaction resulted (Figure 5). From this figure, it can be seen that increased product formation was generally greatest in the presence of higher homogenate concentrations. A reasonable explanation for this phenomenon is that monoamine oxidase activity is being regulated by an endogenous substance or substances and that this regulatory inhibitory influence increases with increased homogenate concentration (Dixon and Webb, 1971).

The thyroid hormones, in the concentration used (10 µg/ml), appear to partially remove the inhibitory control leading to a more linear relationship due to an increased activity of monoamine oxidase and consequently, enhanced product formation.

For any given homogenate concentration the extent of the inhibitory influence would be expected to be constant if the inhibitor pre-existed in the brain. On the other hand, if the inhibitor substance(s) was endogenously manufactured during incubation at 37°C, then a progressive time-dependent increase in inhibitory influences would become apparent. The former condition is clearly the case under study, since the rate of formation of indoleacetic acid was linear with time (Figure 4 and 6). Furthermore, Figure 6 shows that both L-thyroxine and L-triiodothyronine maintained this linear relationship but allowed indoleacetic acid to be formed at a faster rate. Thus, the experimental data is fully consistent with the presence of a pre-formed endogenous inhibitor of monoamine oxidase in whole rat brain homogenates and with the notion that the thyroid hormones function to remove the inhibition. A direct action of the thyroid hormones upon monoamine oxidase itself is not tenable. If this were the case, then an increased monoamine oxidase activity would be expected at the low homogenate concentrations. Indeed, any increase here might be predicted to be greater than that higher homogenate concentrations since the ratio of hormone to enzyme would be highest.

The use of excess substrate was useful for determining the level at which the thyroid hormones influenced indoleacetic acid production, especially when a complex whole cell homogenate is under study. However, in vivo, enzymes are seldom called upon to function at maximal velocity.

Additionally, high substrate concentrations may be involved with interactions which are not seen at lower substrate concentrations. Thus, it was important to determine whether the thyroid hormones exerted the same effect on indoleacetic acid production at submaximal substrate concentrations. Figure 9 shows this to be the case for L-triiodothyronine with regard to the rate of indoleacetic acid formation using a 20 mg/ml homogenate and 0.07 μMoles of tryptamine. Therefore, under the conditions used, the ability of L-triiodothyronine to enhance indoleacetic acid production appears to be independent of substrate concentration.

This result promotes the possibility that the present findings with the thyroid hormones may have direct application under in vivo conditions. If the in vitro effect described in this thesis does represent a true physiological role, one would predict the effect of the thyroid hormones to be concentration related. Figures 10 and 11 show a definite concentration-related response for both L-triiodothyronine and L-thyroxine. However, differences in the concentration-effect relationships between the two hormones are apparent. L-triiodothyronine produced a greater percentage increase above control values than did L-thyroxine. Also, whereas 10 μg/ml of L-triiodothyronine gave approximately a half maximal response, the same concentration of L-thyroxine was maximal. This difference appears to be related to the specific activity of the homogenates used. Figure 12 shows that a correlation exists for L-thyroxine between the percentage change from control and the absolute specific activity of the homogenate. No such correlation was found for L-triiodothyronine. Thus, the relative potencies of the two hormones might well be expected to vary according to the specific activity of the homogenate to which L-thyroxine is added.

It should be noted that in the concentration-effect experiment for L-thyroxine (Figure 11) the specific activity of the control homogenate was 4.9 μMoles indoleacetic acid/g/hr. Thus, with the 10 μg/ml concentration of L-thyroxine, just over a 100% increase in indoleacetic acid would be predicted from the correlation curve. This in fact occurred, the increase being 110%. A greater effect than that actually obtained with the higher concentrations of L-thyroxine (30 and 100 μg/ml) might also have been expected, especially since the maximal capacity of the system to form indoleacetic acid is approximately 15 μMoles/g/hr (derived from Figure 18, using 20 mg/ml whole brain homogenate). However, the correlation data lead one to suspect that the mechanism underlying L-thyroxine's action on monoamine oxidase activity differs, at least in part, from that occurring with L-triiodothyronine. The nature of this difference is at present unknown. As detailed below, the only other difference noted between L-thyroxine and L-triiodothyronine was found in the pre-incubation study (Figure 14) where protracted pre-incubation failed to influence the effect of L-thyroxine, but diminished the effect of L-triiodothyronine.

In the present study the effect of the thyroid hormones was absolutely dependent upon pre-incubation of either L-thyroxine or L-triiodothyronine with the homogenate prior to the addition of tryptamine (Figures 13 and 14). This pre-requisite may be interpreted to represent the necessary inhibition of a substance or substances serving to inhibit monoamine oxidase activity (see previously pp. 118). One way by which this type of interaction may be explained is that monoamine oxidase is present in the form E-I, where E represents monoamine oxidase and I an inhibitor substance. Inhibition of I by the thyroid hormones would give rise to an increased MAO activity.

The concomitant addition of both tryptamine and thyroid failed to increase enzyme activity. However, the reason for this marked difference cannot be determined from the present experiments. One possibility is that the affinity of tryptamine for E greatly exceeds that of the thyroid hormones for I and that the resulting intermediate complex (tryptamine-E-I), which would be present in great excess, prevents thyroid intervention either through conformational changes or other means. It is clear from the pre-incubation experiments that tryptamine cannot reverse the effects of the hormones upon I, thus direct competition for the inhibitor seems to be an unlikely explanation.

The experiments in which the pre-incubation time was varied (Figure 14) were made to further characterize the nature of the interaction of the thyroid hormones with the inhibitor. The results show that pre-incubation at 4°C abolished the action of both L-thyroxine and L-triiodothyronine. The temperature dependence of the postulated interaction between the thyroid hormones and an inhibitor suggests that a metabolic process may be involved and/or that temperature dependent confirmational changes are required. Thus, the experimental results open up the possibility that the thyroid hormones might mediate their effects indirectly by an action on a factor or factors present in the homogenate to produce the actual supressor of the inhibitor.

Irrespective of whether the thyroid hormones act directly or indirectly upon the endogenous modulator of MAO, their effects are apparently reversible. This is clearly evident for L-triiodothyronine since the effectiveness of this hormone declined markedly after 30 and 45 minutes of pre-incubation. This result suggests the presence of on-going metabolism of the hormone since the decline was absolutely dependent upon the

presence of the homogenate. Further evidence for the reversibility of the interaction stems from the inability of the hormones to cause an enhanced effect on the activity of MAO with increasing pre-incubation times. Such a result suggests a rapid equilibrium of thyroid hormones at their active site and the lack of a progressive increase in binding.

It is now well documented that L-thyroxine and L-triiodothyronine undergo metabolism via deiodination (by a tissue deiodinase) while deamination and decarboxylation represents a minor route of metabolism (Robbins and Rall, 1967; Van Middlesworth, 1974). Van Middlesworth (1974) has indicated that only quantitative but not qualitative changes in the physiological effects of the thyroid hormones are associated with the above mentioned metabolic alterations. However, the possibility was entertained that the effect of L-thyroxine and L-triiodothyronine on MAO activity was mediated through a formed metabolite. This notion is supported by the fact that many investigators believe that all the physiological effects of L-thyroxine are mediated indirectly by way of formed L-triiodothyronine (Gross and LeBland, 1950; 1951; Gross, 1954; Gross and Pitt-rivers, 1954; Bravermann et al., 1970; Sterling et al., 1970; Schwartz et al., 1971; Oppenheimer et al., 1972). In turn, L-triiodothyronine is deiodinated to the inactive di- and mono-iodo derivatives. These metabolic tranformations suggested that the effects of L-thyroxine on the activation of MAO might be mediated through formed L-triiodothyronine and/or by way of iododination of the inhibitor. Iodination of the inhibitor could also be the means whereby L-triiodothyronine exerted its effects upon MAO. The temperature dependence of the effect of thyroid hoemones upon MAO and the shorter duration of action of L-triiodothyronine (Figure 14)

appeared consistent with these suggestions. However, the addition of sodium iodide or potassium iodide to the incubation mixture failed to mimic the effects of the thyroid hormones. Oppenheimer et al. (1972) reported that 6-n-propylthiouracil (PT) can reduce both the rate of conversion of L-thyroxine to L-triiodothyronine and the rate of L-triiodothyronine deiodination. They also showed that PT will antagonize the physiological effects of L-thyroxine while prolonging those of L-triiodothyronine. Previously, Braverman and Ingbar (1962) had demonstrated that PT (1 μMole/ml) can induce inhibitory effects upon tissue deiodinase in vitro, thus the same concentration of PT was utilized in the present experiments with the thyroid hormones. However, the studies revealed a potent monoamine oxidase inhibitory action in brain homogenates precluding its use as a tool in the evaluation of the role of thyroid hormone metabolism. By decreasing the concentration (0.1 μMoles, PT) and utilizing whole liver homogenates (because of higher inherent MAO activity), limited experimentation became possible (Table 1). However, PT failed to alter the effects of the thyroid hormones on monoamine oxidase activity while still reducing indoleacetic acid formation. The necessity of using a smaller concentration of PT than that used by Braverman and Ingbar (1962) opens up the possibility of insufficient deiodinase inhibition and thus definitive conclusions with regard to the data generated cannot be drawn. However, the finding that PT decreases indoleacetic acid production from tryptamine by rat liver and brain tissue raises an important question with regard to PT-induced hypothyroidism and monoamine oxidase activity. Lyles and Callingham (1974) reported a decrease in rat heart monoamine oxidase activity following six weeks of treatment

with 2-thiouracil. They explained this decrease upon the basis of thyroid hormone deficiency. The present findings throw some doubts upon this interpretation and may suggest a direct inhibitory effect on monoamine oxidase; a speculation that requires further investigation.

The role of decarboxylation and deamination in the thyroid-induced effects on monoamine oxidase were not investigated. Little is known about the deaminating enzyme, but it is clear from the present experiments that the thyroid hormones are not deaminated by monoamine oxidase. When either tryptamine or kynuramine were used as substrates, pre-incubation of low tissue homogenate concentrations with the thyroid hormones failed to affect product formation. These data are consistent with previous observations using other substrates that thyroid hormones are without direct effects upon monoamine oxidase in vitro (Zile and Lardy, 1969; Okamoto, 1971; Lyles and Callingham, 1974).

In order to further investigate the characteristics of the interaction between the thyroid hormones and MAO activity, an experiment was made utilizing the D-isomers of the hormones. In general, both enzymes and enzyme modulators often exhibit stereoselectivity and, in addition, the full biological spectrum of thyroid hormone activity is revealed only through the L-configuration (Strisower et al., 1968). However, the results depicted in Figure 16 clearly show that D-thyroxine and D-triiodothyronine are equally effective as their respective L-isomers at enhancing indoleacetic acid formation from tryptamine. Thus, if the thyroid hormones bind directly to the proposed inhibitor, it must be concluded that this process is not stereoselective.

Despite some controversy, immunological, electrophoretic and particularly substrate and inhibitor studies indicate that MAO exists in more

than one molecular form (for references, see Introduction #2). Thus, in order to obtain a more comprehensive assessment of the effects of the thyroid hormones on the activity of MAO, experiments were undertaken utilizing kynuramine as the substrate. Figure 20 shows an immediate distinction between kynuramine and tryptamine with regard to the relationship between product formed versus varying concentrations of whole rat brain homogenate. The curvi-linear relationship seen with tryptamine was absent with kynuramine. Instead, a linear relationship was obtained even though the experimental conditions were identical. This result in itself implies the absence of a modulating inhibitory substance and suggests differences in the deaminating enzymes for kynuramine compared with those involved with tryptamine deamination. This difference became clearly evident once again since neither L-thyroxine nor L-triiodothyronine modified 4-hydroxyquinoline production from kynuramine (Figure 19).

The above findings might be interpreted to mean that completely different forms of MAO are involved in kynuramine oxidation from the MAO concerned with tryptamine metabolism. However, this is clearly not the case. Mixed substrate experiments (Figure 21) revealed that tryptamine could inhibit 4-hydroxyquinoline production from kynuramine. However, most importantly, the thyroid hormones failed to potentiate this inhibitory effect. Thus, it appears that tryptamine and kynuramine can be oxidized by a common enzyme(s) but additionally, a different active site or enzyme(s) also metabolizes tryptamine. This tryptamine sensitive site or enzyme(s) can be concluded to be susceptible to inhibotor activity and consequently, explain the "specific" sensitivity of tryptamine deamination to the thyroid hormones. The hypothesis is represented diagrammatically

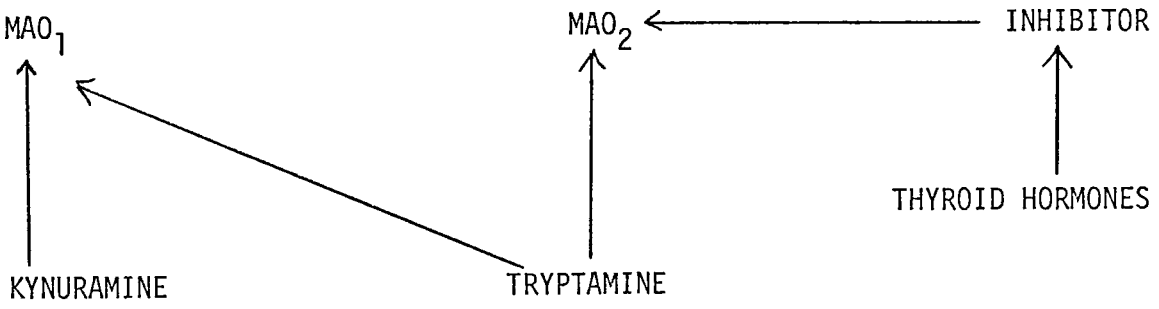
in Figure 25. The scheme presented is substantiated further by data indicating (1) the existence of more than one molecular form of MAO in the rat brain (Kim and D'Iorio, 1968; Youdim, 1974), (2) evidence for at least two catalytic sites on MAO (White and Wu, 1975; Edwards and Chang, 1975) and (3) the knowledge that tryptamine is a substrate for both Type A and Type B MAO (Neff and Yang, 1974). In this latter respect the enzyme specificity of kynuramine is unknown.

At this stage of the study it was important to determine whether the effects of the thyroid hormones were specific to whole brain MAO or whether similar observations could be made in other organs. Table 2 shows that the deamination of tryptamine was enhanced markedly by both L-thyroxine and L-triiodothyronine in rat liver, to a lesser extent in the kidney and to only a very minor extent in cardiac tissue. The increased activity in the liver is somewhat surprising since the result is diametrically opposite to all previous findings made with the thyroid hormones following in vivo administration (Spinks and Burn, 1952; Trendelenburg, 1953; Zile and Lardy, 1959; Zile, 1960; Novick, 1961). However, as reported previously (Moonat et al., 1975) the enhanced deamination in the kidney is in full accord with in vivo observations.

One major point to be made from these experiments is that the effect of the thyroid hormones, like that of many other hormonal secretions, is organ selective. This selectivity, in itself, tends to rule out the involvement of a non-specific effect due to an interaction with some generalized cellular component(s). However, it should be stressed that the present investigation has provided no evidence that the mechanism and characteristics of the induced increases in tryptamine deamination

Figure 25. A diagrammatic representation of the deamination of kynuramine and tryptamine in relation to inhibitor and thyroid hormone activity in rat brain homogenate.

MAO_1 and MAO_2 may represent different molecular forms of MAO or different catalytic sites on the same molecular form. Both kynuramine and tryptamine compete for MAO_1 but only tryptamine is oxidized by MAO_2. MAO_2 is normally suppressed by endogenous inhibitor activity, especially in "high" homogenate concentrations. The thyroid hormones act to remove inhibitor influences either by a direct or indirect mechanisms.


MAO1
MAO2
INHIBITOR
THYROID HORMONES
KYNURAMINE
TRYPTAMINE

parallel those described for whole brain homogenate. Nevertheless, assuming a common mechanism, the differential ability of the thyroid hormones to enhance tryptamine deamination in the brain, liver, kidney, and heart may be due to (1) different endogenous inhibitor concentrations, (2) different forms of tryptamine-sensitive MAO's in the various organs, (3) different routes of thyroid metabolism and (4) a varying availability of a substance upon which the thyroid hormones must act in order to effect a suppression of inhibitor activity.

As with brain homogenates, kynuramine metabolism was unaffected by the thyroid hormones in both liver and kidney. These observations support the proposal made above that the MAO's involved in tryptamine deamination are not identical to those oxidizing kynuramine.

Several investigators have shown that the specific activity of human blood serum MAO (McEwen and Harrison, 1965; Laurell et al., 1968; Tryding et al., 1969), human brain MAO (Robinson, 1975) and rat heart MAO (Horita and Lowe, 1972) are subject to changes with an increase in age. However, using tryptamine as the substrate, no evidence was obtained for such an effect in the brains of rats (Figure 24). Over a period of approximately 52 days the specific activity of MAO remained very constant. Similar results have been reported previously by Horita and Lowe (1972).

Furthermore, the present experiments also revealed that the effect of the thyroid hormones on MAO activity was seemingly unchanged. Thus, based upon the hypothesis proposed, it appears that the concentrations of the endogenous inhibitor, or factors which might determine the effect of the thyroid hormones on the inhibitor, likewise remain unaltered.

Strong evidence that the effects of the thyroid hormones are mediated on a factor or factors other than MAO itself, stemmed from the experiment

in which a crude washed mitochondrial source of the enzyme was utilized from whole rat brain (Figure 23). Under this experimental condition, both L-thyroxine and L-triiodothyronine failed to enhance indoleacetic acid formation from tryptamine. However, the "activating" property of the hormones could be restored by adding back certain of the centrifuged fractions to the prepared mitochondria. This result argues strongly in favor of the notion that an extra-mitochondrial factor is essential for the activity of the thyroid hormones on MAO. This factor may be the inhibitor itself, or a substance for thyroid-induced inhibitor suppression. The lack of evidence for MAO activity in the supernatant fraction is an important observation and is crucial to the conclusion drawn above. Evidence of MAO activity in this fraction could raise the possibility that the tryptamine-sensitive MAO might have been liberated from the mitochondria during the homogenization procedure. In fact, the cell membrane and nuclei fraction contained considerable MAO activity, presumably due to trapped mitochondria.

If in the above experiment the inhibitor substance was separated from mitochondrial MAO, then a loose association between enzyme and inhibitor must exist. Such a situation should lead to a relatively easy separation and characterization of this substance. On the other hand, indirect effects of the thyroid hormones on a tightly bound mitochondrial inhibitor-MAO complex is important to re-emphasize. Although pure speculation, such an effect might be connected with the known phosphodiesterase inhibiting properties of these hormones (Armstrong et al., 1974; Van Inwegen et al., 1975). At present the effects of the adenylyl cyclase-cyclic AMP system on MAO activity is unknown.

Discussion 2.

The activity and properties of an enzyme may vary according to its local environment. For instance, MAO is a mitochondrial enzyme and the procedures for solubilizing the enzyme are vigorous and harsh (Youdim and Sourkes, 1966; Tipton, 1968). Certain of the properties conveyed to MAO by the membrane environment are lost by such procedures (Houslay and Tipton, 1973). Multiple forms of MAO as separated by gel electrophoresis (Youdim et al., 1970) are attached to varying amounts of phospholipid membrane material (Tipton et al., 1972; Houslay and Tipton, 1973) which confers differential characteristic properties. Removal of the lipid components reduces enzyme heterogenicity to a single enzyme type (Houslay and Tipton, 1973). In the intact cell diffusion barriers and the structural organization of the subcellular organelles are determining factors for enzyme activity. Thus, the ultimate way to study enzymatic control mechanisms would be to employ intact cellular systems which preserve the structural integrity of an organ at the microscopical level together with preserving such factors as permeability barriers and metabolic and natural inhibitors.

In the present study an attempt was made to preserve as much as possible of the natural cellular environment by utilizing whole cell homogenates. However, as detailed above, the system is far removed from the complex integrated structural organization of the intact cell. However, this approach revealed a new action of the thyroid hormones which had previously been missed by the other investigators (Zile and Lardy, 1959; Okamoto, 1971; Lyles and Callingham, 1974). These investigators have claimed that thyroid hormones are without effect on MAO activity in vitro.

It is now apparent that the failure of the above investigators to detect the *in vitro* effects of thyroid hormones stems from one or more of the following factors:

1. The use of dilute enzyme preparations such that the effect of inhibitor activity was diluted out. Most enzyme activities are conventionally measured under conditions which ensure linearity with respect to both enzyme concentration and incubation time. The availability of radio-labelled substrates allows easy attainment of this experimental condition due to enhanced sensitivity and such methodology was employed by Lyles and Callingham (1974). However, as shown in the present study, under these conditions, thyroid influences upon the activity of MAO are non-existent.
2. Zile and Lardy (1959) and Okamoto (1971) used a mitochondrial source of MAO. Yet the present investigation, and the theoretical considerations given above, indicate the importance of the availability of other cellular components.
3. Okamoto (1971) gave no indication as to whether L-thyroxine was pre-incubated with the enzyme source. The present work shows that pre-incubation is an absolute necessity for demonstrating the *in vitro* effect of the thyroid hormones.
4. The specific organ under test and the specific substrate used are of utmost importance. Zile and Lardy (1959) and Okamoto (1971) conducted experiments on rat liver MAO using tyramine and benzylamine, respectively. Lyles and Callingham (1974) also used tyramine, but made experiments upon rat cardiac

tissue. From the results obtained in this thesis, no definitive effect was seen using tryptamine with heart homogenates, yet marked increases in indoleacetic acid production were obtained in conjunction with the thyroid hormones in the rat brain, liver and, to a lesser extent, the kidney. Conversely, kynuramine failed to reveal these effects.

In summary, the criteria for exposing the in vitro effect of the thyroid hormones on MAO activity have now been partially, if not completely, defined. Using tryptamine as the substrate the exposed criteria are:

1. Organ selectivity.
2. Pre-incubation of the hormones at 37°C with the homogenate prior to substrate addition.
3. The presence of an extra-mitochondrial cellular component in sufficiently high concentration.
4. Specificity with regard to the substrate used.

In accord with the observations of previous investigators (Zile and Hardy, 1969; Okamoto, 1971; Lyles and Callingham, 1974), the current study has shown that the effect of the thyroid hormones upon the activity of MAO is not mediated directly on the enzyme itself. From a theoretical point of view, Youdim et al. (1974) have considered that MAO activity might be modulated by endogenous inhibitors. However, until now, no experimental evidence has been derived to support this notion. Whether the currently proposed mechanism for the effects of the thyroid hormones upon the activity of MAO has physiological significane in vivo is unknown. Just as there are advantages to using broken cell preparations, the

disadvantages are also clear. It cannot be refuted that the present observations may be artifacts of this very procedure. However, they do raise the possibility that the in vivo actions of the thyroid hormones, with regard to MAO activity, may entail additional mechanisms to those exerted at the level of protein synthesis (Goridis and Neff, 1973; Lyles and Callingham, 1974).

An important consideration in this respect is the evidence derived in the present study that the effect of the thyroid hormones appear to be reversible in nature. Conventional techniques for disclosing in vivo activity on MAO mostly depend upon treatment of the animal with the hormones followed by tissue removal for in vitro assay. This technique demands homogenization and tissue dilution, thus favoring metabolism and/or dissociation of the hormone from its active site. Such an approach would not allow the disclosure of rapid initial effects of the hormones on the activity of MAO (such as reversible inhibitor suppression), while the promotion of enzyme synthesis, which requires a more protracted treatment schedule, would be easily detectable. The measurement of MAO activity in isolated perfused organs or in the whole animal are required to solve this problem. However, such approaches are also fraught with difficulties. For instance, the proportion of the total MAO responding to the effects of the thyroid hormones may be so small that enhanced metabolites emanating from this critical source become diluted out by the normal rate of product formation from the vastly greater concentration of unaffected enzyme. Furthermore, artificial presentation of the substrate (intravenous injection or via the perfusing fluid) may not gain access to the critical form of MAO; physiologically the substrate may be synthesized "next door" to the enzyme source.

Another factor of importance when considering the possible application of the present findings to *in vivo* states is the concentrations of the hormones used. The microgram concentrations employed in the present study exceeds the levels of circulating L-thyroxine and L-triiodothyronine found under normal physiological conditions. For instance, in normal human plasma the level of L-thyroxine falls between 0.033 and 0.066 µg/ml, while L-triiodothyronine ranges between 1.0 and 2.2 ng/ml (Sterling, 1974). Thus, based upon these levels, correlations between the observations made in the present study and postulates for a physiological control mechanism of the thyroid hormones on MAO activity would seem highly improbable. However, tissue concentrations of the hormones are the important parameter to consider and these are known to be exceedingly high in the liver and certain other organs (Cavalieri and Searle, 1966). Additionally, excessive concentrations of the hormones become available to tissues in hyperthyroid states. Such a situation is highly likely to quickly modify MAO activity as indicated by the work of Moonat *et al.* (1975) with regard to the rat kidney enzyme. The proposed inhibitor suppression mechanism by which the thyroid hormones modulate MAO activity would provide an ideal minute to minute control modulation, allowing rapid adjustments in enzymatic activity to meet the demands of the prevailing physiological situation. Since the proposed inhibitor apparently modifies only certain forms of MAO, specific rather than generalized effects could be obtained.

In the view of Klaiber *et al.* (1974) increased MAO activity decreases monoaminergic function while a decrease in MAO activity produces the opposite effect. Although somewhat simplistic, the concept may well

have important connotations. As pointed out in the Introduction #7, thyroid secretion also generally decreases sympathetic neuronal function whereas thyroid lack increases adrenergic activity. Thus, the thyroid hormones, which mimic many of the physiological effects of the catecholamines, may modulate sympathetic MAO activity so as to conserve neuronal function and prevent excessive organ and metabolic stimulation. In turn, it is now known that the sympathetic nervous system can play an important role in thyroid hormone secretion (Melander et al., 1974). Thus, a typical negative feed-back mechanism between the sympathetic nervous system and thyroid hormone secretion may be postulated. However, the validity of these speculations remains for further experimentation.

Finally, even if the data generated in this thesis is of only dubious applicability to physiological states, it has provided a stimulus for further research. Additionally, since only a portion of tryptamine deamination appeared to be affected while kynuramine oxidation remained unaltered, the research may have provided a novel in vitro technique for further discriminating between the various molecular forms of MAO.

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